Biochemistry of
Apoptosis Signal-regulating Kinase 1
Regulation and Hydrogen Peroxide
Signalling

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Abstract

All cells within an organism must respond to their environment. Responses are regulated by intracellular signalling pathways and signalling molecules. One such molecule is hydrogen peroxide (H$_2$O$_2$). H$_2$O$_2$ is required in many cell signalling pathways, however, too much H$_2$O$_2$ can be toxic. As a result, organisms have evolved various systems to sense, and respond to, H$_2$O$_2$.

How H$_2$O$_2$ can act as a signalling molecule is paradoxical, as it is rapidly metabolised to water by proteins called Peroxiredoxins (Prdxs). Prdxs—including mammalian Prdx1—are proposed to act as mediators of H$_2$O$_2$ signalling by oxidising protein targets—such as the protein kinase, apoptosis signal-regulating kinase 1 (ASK1)—via disulfide-relays. The overall contribution of Prdxs in H$_2$O$_2$ signalling is unknown, as there are few examples of Prdx disulfide-relays.

ASK1 is an apical protein kinase that is activated in response to stressors (including H$_2$O$_2$), ultimately regulating responses such as apoptosis and inflammation. ASK1 is a large (154 kDa) protein with extensive regulatory regions, both N- and C-terminal to a central kinase domain. Domains within the ASK1 N terminus are known to regulate ASK1, but the precise mechanisms remain unclear, as we lack molecular detail. The oxidoreductase protein, Thioredoxin 1 (Trx1), is purported to directly inhibit ASK1 by binding within an N-terminal regulatory region—described as the thioredoxin binding domain (TBD). The release of Trx1 from ASK1 is required for H$_2$O$_2$-induced activation of ASK1, but how this process is governed is inconclusive.

This Thesis explores various aspects of ASK1 regulation and H$_2$O$_2$ signalling using in vitro methods and proteomics. Recombinant human ASK1 protein constructs were used to investigate ASK1 regulation in vitro. Different domains within the N terminus
of ASK1 were seen to be able to both inhibit, and enhance ASK1 kinase activity. A model is proposed whereby conformational changes within the ASK1 TBD can regulate access of substrate proteins to the catalytic kinase domain. Once bound to ASK1, substrate proteins are proposed to undergo a conformational change, making them a better substrate for phosphorylation.

Using recombinant human Trx1 and ASK1, it was seen that Trx1 can interact with ASK1 \textit{in vitro}. Trx1 and ASK1 are able to form an inter-molecular disulfide bond, for which ASK1-Cys250—within the ASK1 TBD—is required. Surprisingly, Trx1 was not able to inhibit ASK1 \textit{in vitro}, suggesting that Trx1 is not a direct inhibitor of ASK1.

In order to examine the role of Prdx1 in H$_2$O$_2$ signalling, and identify potential Prdx1 signalling-partners other than ASK1, a pilot quantitative proteomics method was tested. Oxidised protein cysteine residues from cell lysates were labelled, and analysed using mass spectrometry. Whilst labelled peptides were detected, the method requires further work to investigate the full cellular contribution of Prdx1 in H$_2$O$_2$ signalling.

Overall, this work advances our knowledge of ASK1 regulation, highlighting mechanisms that are likely conserved throughout metazoa. The proteomics work provides the foundations for further studies, that will advance our understanding of the role of Prdx1 in H$_2$O$_2$ signalling.
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List of Abbreviations

λPP  Lambda phage protein phosphatase
3C  Human rhinovirus 3C-Protease
A_{280}  UV absorbance at 280 nm
ADB  Agarose dissolving buffer
ADP  Adenosine diphosphate
APS  Ammonium persulfate
ASK1–3  Apoptosis signal-regulating kinase 1–3 (common name)
ATP  Adenosine triphosphate
BCA  Bicinchoninic acid
BSA  Bovine serum albumin
C\textsubscript{P}  Peroxidatic cysteine
C\textsubscript{R}  Resolving cysteine
CC  Coiled-coil
Cdc25  Cell division cycle dual-specificity phosphatase 25
CI  Confidence interval
CID  Collision-induced dissociation
Cryo-EM  Cryo-electron microscopy
Cys  Cysteine
D\textsubscript{max}  Maximum dimension
DNA  Deoxyribonucleic acid
DTT  Dithiothreitol
dTTP  Deoxythymidine triphosphate
DUF  Domain of unknown function
DVD  Domain for versatile docking
ECL  Enhanced chemiluminescence
E. coli  Escherichia coli
EDTA  Ethylenediaminetetraacetic acid
EGF  Epidermal growth factor
ER  Endoplasmic reticulum
ERK  Extracellular signal-regulated kinases
FPLC  Fast protein liquid chromatography
FRET  Fluorescence resonance energy transfer
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
Gpx  Glutathione peroxidase
GSH  Glutathione
H\textsubscript{2}O\textsubscript{2}  Hydrogen peroxide
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
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<td>Small-angle X-ray scattering</td>
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<td>Standard deviation</td>
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<td>SDS polyacrylamide gel electrophoresis</td>
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<td>Signal transducer and activator of transcription 3</td>
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<tr>
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<td>TBS with Tween-20</td>
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<td>Tris(2-carboxyethyl)phosphine</td>
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<td>Trx1-lysozyme fusion protein</td>
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<tr>
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Chapter 1

Introduction

1.1 Overview

Cell signalling is a diverse process that requires accurate signal sensing and transduction. To facilitate cell signalling, numerous signalling molecules exist, including protein kinases—such as apoptosis signal-regulating kinase (ASK)1—and hydrogen peroxide (H$_2$O$_2$), both of which are explored within this Thesis. These areas have discrete underlying properties that require different approaches to study. As such, this Chapter introduces the general themes and concepts of protein kinases and H$_2$O$_2$ biology. Further background information will be provided in individual Chapters.

1.2 Introduction to cell signalling

Throughout all forms of life, cells within an organism must respond to a range of cues in order to maintain a healthy state. These cues may be extracellular in origin or be found within the cell itself (Figure 1.1). Responses to cell signalling events are varied. For example, a single-celled organism, such as a bacterium, needs to be able to identify potential new food sources in order to take advantage of them. By comparison, a multi-cellular organism, such as a human, may require a cell to die so the organism as a whole, can grow. Without an efficient signalling system, neither the single-cell or multi-celled organism can survive. Ensuring an appropriate response to any given
Figure 1.1: General cell signalling. A cell can respond to signals from its environment, but also within itself. Signalling pathways, such as a three-tiered protein kinase cascade can lead to a variety of responses.

signal requires an effective signal reception and transduction. In order to achieve these appropriate responses, a vast number of cellular signalling pathways have evolved.

Protein post-translational modification is a key aspect of cell signalling. Modification of proteins alters various aspects of a protein, such as activity and localisation. The sum output will be a change in state for the cell. Importantly, these modifications are usually reversible, thereby enabling the cell to switch off the signal. Competing on and off signals will determine the signal amplitude (how much), duration (how long) and frequency (how often). Signalling might be considered as digital (on or off) or analogue (a gradient). Consequently, cellular signalling pathways have numerous regulatory mechanisms to ensure that the correct response is achieved.

1.3 Protein kinase signalling

1.3.1 Phosphorylation

Post-translational modification of proteins is a fundamental aspect of cell signalling. In humans, phosphorylation is one of the most frequent forms of post-translational modification (Aebersold et al., 2018). Phosphorylation is the transfer of the $\gamma$-phosphate of ATP to a hydroxyl group on a substrate (Kornev and Taylor, 2015). Phosphorylation is a reversible process governed by kinases—enzymes that add phosphoryl groups—and
phosphatases—enzymes that remove phosphoryl groups (Reactions 1.1 and 1.2; Chen et al., 2017; Manning et al., 2002)

\[
\begin{align*}
\text{Substrate}^\text{OH} & + \text{ATP} \underset{\text{Kinase}}{\xrightarrow{\text{Mg}^{2+}}} \text{Substrate}^\text{O\text{-PO}_3^{2-}} + \text{ADP} \\
\text{Substrate}^\text{O\text{-PO}_3^{2-}} & + \text{H}_2\text{O} \underset{\text{Phosphatase}}{\xrightarrow{}} \text{Substrate}^\text{OH} + P_i
\end{align*}
\]

Protein phosphorylation can act as an allosteric regulator. That is to say that phosphorylation can induce a change in the overall three-dimensional shape of a protein, influencing its properties (Kornev and Taylor, 2015). For example, in mitogen activated protein kinase (MAPK) signalling, signal transduction occurs via sequential phosphorylation of several protein kinases (Figure 1.2a). Specifically, the MAPKs extracellular-regulated kinases (ERKs) are phosphorylated by the MAPK kinases (MAP2K), MAPK/ERK kinases (MEKs). MEKs are phosphorylated and activated by MAP2K kinases (MAP3K) RAFs. Phosphorylation of MEK and ERK induces conformational changes within each protein, thereby activating them (Lavoie and Therrien, 2015).

### 1.3.2 MAPK signalling

The human genome encodes for over 500 protein kinases (Manning et al., 2002). The remainder of this Thesis focuses on protein kinases within MAPK signalling pathways. MAPKs work in a cascade whereby each protein is phosphorylated and activated by the protein in the tier above (Figure 1.2a). In general, the cascade is controlled by activation of the apical MAP3K (Johnson and Lapadat, 2002).

MAPK signalling can broadly be divided depending upon which MAPK family is activated; the ERKs, the c-Jun N-terminal kinases (JNKs) and the p38 family (Johnson and Lapadat, 2002). In general, the ERKs regulate cell growth and proliferation whereas, the p38s and JNKs regulate stress responses, including apoptosis and inflammation (Figure 1.2b; Kyriakis and Avruch, 2012).

MAPKs and MAP3Ks are all serine-threonine protein kinases—they catalyse the phosphorylation of serine and threonine target residues (Kyriakis and Avruch, 2012). By comparison, MAP2Ks phosphorylate tyrosine and threonine residues (Dhanasekaran and Reddy,
Figure 1.2: MAPK Cascades. a) A MAPK cascade is activated by a signal, activating the MAP3K. The MAP3K phosphorylates (indicated by ‘P’) the MAP2K. The MAP2K phosphorylates the MAPK and finally, the MAPK phosphorylates a protein substrate eliciting a downstream response. b) Examples of MAPK pathways. One of the best known is the RAF-MEK-ERK pathway that is activated in response to growth factors such as platelet-derived growth factor (PDGF) or epidermal growth factor (EGF). The ASK-family of MAP3Ks (including ASK1, a focus of this Thesis), is activated in response to stressors including lipopolysaccharide (LPS), tumour necrosis factor-alpha (TNFα) and H₂O₂. Active ASKs activate MKK3 and 6, or MKK4 and 7, which in turn activate the p38 and JNK MAPK families, regulating responses such as apoptosis and inflammation Avruch (2007); Johnson and Lapadat (2002); Kyriakis and Avruch (2012).
MAP3Ks and MAP2Ks phosphorylate target residues within the activation loops of their respective substrate proteins (Piala et al., 2014).

All MAPKs, MAP2Ks and MAP3Ks have a core ~30 kDa protein kinase domain (Figure 1.3). The sizes of MAPKs and MAP3Ks is quite varied, with MAP3Ks being the most diverse in terms of size (from 40 to 180 kDa for MAP3Ks). The difference in size distribution highlights that MAP3Ks have diverse properties and need to be able to co-ordinate with further upstream signalling mechanisms. MAP2Ks, on the other hand, do not feature much beyond being a protein kinase, with a limited number of MAPK substrates. MAPKs are more divergent in size, highlighting their need to recognise a broader range of substrates than MAP2Ks.

Perhaps the best characterised MAPK pathway is the RAF-MEK-ERK pathway (Figure 1.2b). Within RAF, for example, regions outside the kinase domain are critical in the effective signal transduction. For example, active RAF signalling requires binding to the small GTPase, RAS, in order to correctly localise RAF to the plasma membrane (Lavoie and Therrien, 2015). Binding to RAS by RAF requires a RAS binding domain (RBD) within the N terminus of RAF (Lavoie and Therrien, 2015). For other MAP3Ks much less is known about regions beyond their kinase domains. For all MAP3Ks, the kinase domain represents far less than half of the total protein (Figure 1.3). It is more than likely that these regions have roles in modulating the overall signalling response. A focus of this Thesis is in investigating how regions within the MAP3K ASK1, can control protein kinase activity.

**Figure 1.3:** Sizes of human MAPKs, MAP2Ks and MAP3Ks. Each point represents one protein. The positions of the ASK and RAF MAP3Ks are indicated. The mean molecular weight of the core protein kinase domain of each group is shown. Sizes were calculated using Protparam (web.expasy.org/protparam; Gasteiger et al. 2005).
1.4 ASK1

1.4.1 ASK-family of MAP3Ks

The ASK-family of MAP3Ks are activators of the p38 and JNK MAPK pathways (Figure 1.2b; Nishida et al., 2017). There are three ASK proteins: ASK1 (also known as MAP3K5; Ichijo et al., 1997); ASK2 (also known as MAP3K6; Wang et al., 1998) and, ASK3 (also known as MAP3K15; Kaji et al., 2010).

As an upstream regulator of pathways connected to cellular responses such as apoptosis and inflammation, ASK proteins are important in various aspects of human health (Fujisawa, 2017; Kawarazaki et al., 2014; Matsuzawa, 2017a; Ryuno et al., 2017). For example, mutations within ASK1 have been reported in melanomas and ASK1 inhibitors have shown promise in treating gastric cancers (Hayakawa et al., 2011, 2012b; Prickett et al., 2014; Stark et al., 2012). More recently, ASK1 has garnered attention for its role in regulating inflammation within the context of nonalcoholic fatty liver disease (NAFLD; Fujisawa, 2017; Schuster et al., 2018). Elucidation of how ASK-type proteins are regulated is essential in understanding the underlying biochemistry of these diseases.

1.4.2 Architecture of ASK1

ASK1 (and all ASK-family proteins) has a conserved architecture of a central serine-threonine protein kinase domain and large N-terminal and C-terminal regulatory regions (Figure 1.4a). The N terminus of ASK1 contains a highly conserved (in metazoa) region, that in protein family (Pfam) database (pfam.xfam.org; Finn et al., 2016) is referred to as ‘domain of unknown function (DUF)4071’. In human ASK1, DUF4071 spans residue 165 to 545 (Figure 1.4a). The majority of ASK1, N-terminal to the kinase domain is predicted to be ordered, including a region denoted as the ‘thioredoxin binding domain’ (TBD) (Figure 1.4a,b). In contrast, the region C-terminal to the kinase domain is predicted to be much more disordered (Figure 1.4b), with a predicted coiled-coil and sterile alpha motif (SAM) domain (Schneider et al., 2013).

High-resolution structural data of ASK1 has also been invaluable in understanding the architecture of the protein. The crystal structure of the kinase domain was first solved in 2007 (Bunkoczi et al., 2007). The Mace laboratory recently published the first structure from within ASK1 DUF4071—residues 269–658 (Figure 1.4c; Weijman et al., 2017). The
1.4.3 Regulation of ASK1

Phosphorylation is a major regulatory mechanism for eukaryotic proteins. ASK1 is no exception to this and can be phosphorylated at various sites (see www.phosphosite.org (Hornbeck et al., 2015), Nishida et al. (2017) and, Betanzos et al. (2016)). Phosphorylation is associated with both activation and inhibition of ASK1.

Phosphorylation at Thr838 (located on the activation loop, within the kinase domain, Figure 1.4a), is regarded as representing active ASK1 (Tobiume et al., 2002). However,
Bunkoczi et al. (2007) showed that a Thr838Ala mutant of ASK1 was still an active protein kinase in vitro. But in cells, ASK1 Thr838Ala did not result in an active signalling response (Bunkoczi et al., 2007). Precisely why Thr838 phosphorylation is required is unclear, but possibly it is important in protein-recruitment (Bunkoczi et al., 2007).

C-terminal to the ASK1 kinase domain, Ser966 (Figure 1.4a) is another important phosphorylation site. Phosphorylated Ser966 can be bound, and inhibited, by 14-3-3 proteins (Cockrell et al., 2010; Goldman et al., 2004; Petrval ska et al., 2016; Zhang et al., 1999, 2003). The precise mechanism of how 14-3-3 proteins regulate ASK1 is not clear but biophysical evidence suggested that it may act as a steric block, preventing access to the kinase domain (Petrval ska et al., 2016). Stimulation of cells with either H2O2 or tumour necrosis factor-alpha (TNFα) can trigger dephosphorylation of ASK1, resulting in dissociation of 14-3-3 and active ASK1 signalling (Goldman et al., 2004; Zhang et al., 2003). Phosphorylation of ASK1 is a dynamic process that is important in both positively and negatively regulating signalling. However, phosphorylation at Thr838 and withdrawal of 14-3-3 proteins do not explicitly explain why these processes are able to modulate ASK1 signalling.

The regulation of ASK1 is heavily associated with the large protein complex—greater than 669 kDa in size—dubbed the ‘ASK1 signalosome’ (Noguchi et al., 2005). The composition of the signalosome is contingent on cellular conditions and can dictate overall ASK1 signalling (Nishida et al., 2017; Rusnak and Fu, 2017). Under resting conditions, the signalosome is said to contain ASK1, ASK2, and the oxidoreductase, Thioredoxin 1 (Trx1) (Fujino et al., 2007; Noguchi et al., 2005; Takeda et al., 2007). Stimulation of cells with TNFα or H2O2 triggers activation of ASK1 in a process that requires the association of ASK1 with TNF-receptor associated factors (TRAF)2 and 6 (Fujino et al., 2007; Nishitoh et al., 1998; Noguchi et al., 2005). TRAF recruitment requires the prior withdrawal of Trx1 (Fujino et al., 2007; Liu et al., 2000). Both Trx1 and TRAF association with ASK1 requires regions within the conserved N terminal region (i.e. within residues 88–658).

In 1998, Saitoh et al. proposed that Trx1 was a direct inhibitor of ASK1. ASK1 regulation by Trx1 represents a link between protein kinase signalling and cellular H2O2 sensing. When H2O2 levels become elevated, Trx1 was proposed to be directly oxidised by H2O2, resulting in dissociation from ASK1 and allowing active ASK1 signalling (Fujino et al., 2007; Liu et al., 2000; Saitoh et al., 1998). However, the precise nature of ASK1 regulation by Trx1 remains unclear, and there are opposing views on whether or not there is a covalent interaction between
ASK1 and Trx1 (Nadeau et al., 2007; Saitoh et al., 1998). Trx1 is thought to regulate ASK1 by binding to the ASK1-TBD, thereby preventing ASK1 oligomerisation (Fujino et al., 2007), however, the evidence is not conclusive. Further molecular detail into the interaction between Trx1 and ASK1 is required for a conclusive picture of ASK1 regulation by Trx1.

Chapter 3 will focus on how ASK1 is auto-regulated and the role the ASK1 N terminus plays. Chapter 4 moves onto applying how auto-regulatory mechanisms of ASK1 can be manipulated by Trx1 and investigate the nature of the interaction between ASK1 and Trx1. One reason why ASK1 regulation by Trx1 remains unclear is that the underlying biology and biochemistry of H$_2$O$_2$ have often been overlooked. Chapter 5 will focus on H$_2$O$_2$ and will explore mechanisms of H$_2$O$_2$ signalling.

1.5 H$_2$O$_2$ and signalling

Over the last 20 years, our view of H$_2$O$_2$ has undergone a paradigm shift. H$_2$O$_2$ is now regarded as an essential secondary messenger in many cell signalling pathways. For example, H$_2$O$_2$ is now known to be essential in platelet-derived growth factor (PDGF) signalling (Sundaresan et al., 1995). Typically, H$_2$O$_2$ leads to protein oxidation, a type of post-translational modification. Precisely why H$_2$O$_2$ is required in signalling is still being dissected, along with the mechanism(s) of H$_2$O$_2$ signalling. The following sections will provide an overview of oxidation and how proteins can be modified by oxidation. There will also be a brief commentary on how H$_2$O$_2$ is produced in mammalian cells and what defences exist to fight it giving rise to the paradoxical nature of H$_2$O$_2$ signalling.

1.5.1 What is oxidation?

Oxidation can be described as the loss of electrons from a species. The opposite of oxidation is reduction, the gain of electrons by a species (Halliwell and Gutteridge, 2007). These two terms are often expressed as a portmanteau, ‘redox’ (reduction, oxidation). A redox reaction requires the oxidation of one species and concomitantly the reduction of another (Halliwell and Gutteridge, 2007). The standard reduction potential ($E^\circ$) can be used to describe the ease of a species to take up electrons and is one of the thermodynamic parameters that govern a redox reaction (Poole, 2015). For example, cysteine residues within proteins can be in either a reduced or oxidised form and cysteine oxidation is critical in H$_2$O$_2$ signalling (see 1.5.5,
below). The reduction of a cysteine thiol reaction can be written as a redox-half equation (Reaction 1.3). The chemical reductant dithiothreitol (DTT) also exists in a reduced and oxidised form, so a redox-half equation can be written (Reaction 1.4). The overall redox reaction can be combined, with the protein becoming reduced, and DTT becoming oxidised (Reaction 1.5). Under standard conditions, the reaction will proceed spontaneously providing the difference between the individual standard reduction potentials ($\Delta E^{o'}$) is positive (Poole, 2015). For example (Reaction 1.5), DTT will be able to reduce a protein-disulfide, providing that $E^{o'} > -0.33$ V, for the protein-disulfide.

\[
\text{Protein} - \text{S}_2 + 2e^- + 2H^+ \rightleftharpoons \text{Protein} - (\text{SH})_2 \quad E^{o'} = x \text{ V} \quad (1.3)
\]

\[
\text{DTT} - \text{S}_2 + 2e^- + 2H^+ \rightleftharpoons \text{DTT} - (\text{SH})_2 \quad E^{o'} = -0.33 \text{ V}^1 \quad (1.4)
\]

\[
\text{Protein} - \text{S}_2 + \text{DTT} - (\text{SH})_2 \rightleftharpoons \text{Protein} - (\text{SH})_2 + \text{DTT} - \text{S}_2 \quad \Delta E^{o'} = x - -0.33 \text{ V} \quad (1.5)
\]

### 1.5.2 Toxicity of H\textsubscript{2}O\textsubscript{2}

One complication of H\textsubscript{2}O\textsubscript{2} signalling is that H\textsubscript{2}O\textsubscript{2} can be generated as a by-product of respiration (see below) and can be toxic. For example, the addition of between 1 and 100 µM H\textsubscript{2}O\textsubscript{2} to cells in culture is sufficient to induce cell death (Halliwell and Gutteridge, 2007). The potential danger of H\textsubscript{2}O\textsubscript{2} stems from it being a powerful oxidising agent (i.e. H\textsubscript{2}O\textsubscript{2} causes other species to lose electrons). However, the reality is that H\textsubscript{2}O\textsubscript{2} has relatively low reactivity towards most biomolecules within a healthy cell (see Chapter 5, (5.3.2), for more details; Winterbourn, 2015). On the other hand, when H\textsubscript{2}O\textsubscript{2} is able to react with free iron, for example, the Fenton reaction occurs (Reaction 1.6; D’Autréaux and Toledano, 2007). The Fenton reaction yields the very reactive hydroxyl radical ($^\cdot$OH) which can oxidise and damage DNA, lipids and other biomolecules (D’Autréaux and Toledano, 2007; Sies, 2017).

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + ^\cdot\text{OH} \quad (1.6)
\]

\[\text{Value from Cleland (1964)}\]
1.5.3 Defence from H$_2$O$_2$

To protect from any potential danger of H$_2$O$_2$, a large array of peroxidase proteins exist to break down H$_2$O$_2$. These include glutathione peroxidases (Gpxs) and peroxiredoxins (Prdxs), which use cysteine (or selenocysteine) based mechanisms and convert H$_2$O$_2$ to water (Flohé, 2015). More detail about defence from H$_2$O$_2$ is given in 5.3.2. In order to effectively break down H$_2$O$_2$, Gpxs require the small molecule glutathione (GSH), whilst Prdxs require the oxidoreductase protein Trx. GSH is subsequently replenished by glutathione reductase and Trx by Trx Reductase (TrxR) with the ultimate reducing power coming from nicotinamide adenine dinucleotide phosphate (NADPH; Hanschmann et al., 2013; Lu and Holmgren, 2014). Collectively these systems are referred to as the glutathione and thioredoxin systems (Hanschmann et al., 2013; Lu and Holmgren, 2014).

Gpxs and Prdxs are found in all domains of life. The human genome encodes for 8 Gpxs and 6 Prdxs (Brigelius-Flohé and Maiorino, 2013; Hanschmann et al., 2013), all having high reactivities with H$_2$O$_2$ (second order rate constants in the order of between $10^7$ and $10^9$ M$^{-1}$ s$^{-1}$ (Winterbourn and Hampton, 2008)). Additionally, these proteins are also very abundant, ~20 µM for Prdx2 (Winterbourn and Hampton, 2008). The question then arises of how is H$_2$O$_2$ signalling possible, given the large ordinance at a cell’s disposal to breakdown H$_2$O$_2$?

1.5.4 Where, how and why is H$_2$O$_2$ generated?

H$_2$O$_2$ is primarily generated from detoxification of the radical superoxide (O$_2^\bullet$) by superoxide dismutases (SODs) via a disproportionation$^2$ reaction (Reaction 1.7; Holmström and Finkel, 2014; Reczek and Chandel, 2015; Sies, 2014).

\[
\text{SOD} \quad 2\text{O}_2^\bullet + 2\text{H}^+ \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 + \text{O}_2 \quad (1.7)
\]

Superoxide can be produced by the electron transport chain within mitochondria, but also by a family of protein complexes named NADPH oxidases (NOXs; Murphy, 2009; Reczek and

$^2$Disproportionation refers to simultaneous reduction and oxidation of a species, yielding two different products.
**Figure 1.5:** Common cysteine oxidations. Structure and names of various oxidation states of cysteine (only the side chain is shown). The numbers refer to the oxidation status of the cysteine sulfur atom (Nagy and Winterbourn, 2010).

Chandel, 2015). NOXs are membrane-bound complexes that upon activation, use electrons from intracellular NADPH in order to reduce extracellular oxygen to superoxide (Winterbourn, 2008). Extracellular superoxide is converted to H$_2$O$_2$ by SOD3 (Trachootham et al., 2009). Extracellular H$_2$O$_2$ is able to diffuse across the plasma membrane in a concentration gradient dependent manner, but the process is also facilitated by membrane channels such as aquaporin-3 (Miller et al., 2010; Sies, 2017). The generation of superoxide (and consequently H$_2$O$_2$) by NOXs is important in bacterial defences (Cross and Segal, 2004; Nauseef, 2007), as well as in H$_2$O$_2$ signalling (Winterbourn, 2008; Winterbourn and Hampton, 2008).

### 1.5.5 Cysteine oxidation

The primary mode by which H$_2$O$_2$ signalling operates is via cysteine oxidation. Cysteine thiols can be oxidised to numerous forms, including disulfide bonds, sulfenic, sulfinic and sulfonic acids (Figure 1.5; Nagy and Winterbourn, 2010). Cysteines can become oxidised following reaction with, for example, H$_2$O$_2$ (Winterbourn and Hampton, 2008). Cysteine oxidation is required in the formation of disulfide bonds, serving a structural role (Azimi et al., 2011). Cysteine oxidation can also act analogously to phosphorylation, as an allosteric regulator of protein function (Azimi et al., 2011). Both disulfide bond formation and allosteric regulation are important in cysteine-dependent H$_2$O$_2$ signalling (see 1.5.6 and Chapter 5).

### 1.5.6 H$_2$O$_2$ as a signalling molecule

Many mammalian signalling pathways use H$_2$O$_2$ as a secondary messenger. For example, PDGF (Sundaresan et al., 1995), epidermal growth factor (EGF; Bae et al., 1997), and insulin (Mahadev et al., 2001) signalling all require the generation of H$_2$O$_2$ for effective signalling. Additionally, in pro-inflammatory signalling, TNF$\alpha$ can also stimulate the generation of H$_2$O$_2$.
(Kang et al., 1998; Kim et al., 2007; Meier et al., 1989). Activation of the relevant growth factor receptor results in the activation of NOXs in a mechanism that is thought to require phosphatidylinositol-4,5-bisphosphate 3-kinases (PI3Ks; Brandes et al., 2014; Holmström and Finkel, 2014; Park et al., 2004).

Precisely why $H_2O_2$ is required for effective signalling has not been fully deduced. One mechanism is that $H_2O_2$ inactivates protein phosphatases, thereby potentiating canonical receptor tyrosine kinase signalling (Holmström and Finkel, 2014; Truong and Carroll, 2012). For example, the active site cysteine residue in protein tyrosine phosphatase (PTP)1B is able to be oxidised (to either a sulfenic, sulfinic or sulfonic acid, see Figure 1.5), inactivating the protein (Lee et al., 1998; Meng et al., 2002; van Montfort et al., 2003). In contrast, the EGF receptor can have its kinase activity enhanced by sulphenylation of a cysteine residue within the active site (Paulsen et al., 2012).

TNFα signalling leads to the activation of JNK and p38 MAPKs (Kang et al., 2004), as well as active nuclear factor (NF)-κB (Latimer and Veal, 2016; Oliveira-Marques et al., 2009). Whilst TNFα can stimulate the generation of $H_2O_2$, the importance of $H_2O_2$ to TNFα-signalling remains unclear. ASK1 can be regarded as a link between TNFα and $H_2O_2$ signalling (Gotoh and Cooper, 1998). ASK1 activation following TNFα stimulation requires dissociation of Trx1 from ASK1 (see 1.4.3), which is thought to be stimulated by direct oxidation of Trx1 by $H_2O_2$ (Liu et al., 2000; Saitoh et al., 1998). However, as will be discussed in Chapter 5 (5.3.3), direct oxidation of proteins by $H_2O_2$ requires a protein to outcompete peroxidase proteins (Prdxs and Gpxs) for $H_2O_2$. The problem of $H_2O_2$ competition is best illustrated by comparing the rate constants for proteins reacting with $H_2O_2$. The second order rate constants for Prdxs reacting with $H_2O_2$ is in the order of $10^7 M^{-1} s^{-1}$, by comparison, Trx1 is $1.05 M^{-1} s^{-1}$ (Winterbourn and Hampton, 2008). The difference in the rate constants illustrates that Trx1 is unlikely to be directly oxidised by $H_2O_2$ in vivo. Consideration of kinetic and other parameters is essential in determining a feasible route for $H_2O_2$ to act as a signalling molecule.

### 1.5.7 Models of $H_2O_2$ signalling

Within cells, many peroxidase proteins exist to break down $H_2O_2$, which should prevent protein oxidation (see 1.5.3). However, as outlined in 1.5.6 above, $H_2O_2$ is an important messenger molecule in signalling pathways. How then can $H_2O_2$ act as a signalling molecule?
**Figure 1.6: Synopsis of H$_2$O$_2$ signalling models.**

H$_2$O$_2$ signalling is proposed to act following either the inactivation of Prdxs (left) or a disulfide-relay (right).

1) Production of H$_2$O$_2$ following a signal pathway activation. 2) Prdxs are inactivated by oxidation to sulfenic or sulfonic acids (see Figure 1.5). 3) H$_2$O$_2$ accumulates leading to target oxidation. 4) In the disulfide relay, a Prdx is oxidised (to a sulenic acid (shown) or disulfide). 5) Oxidised Prdx1 directly oxidises the target protein via an inter-molecular disulfide bond (Jarvis *et al.*, 2012; Wood *et al.*, 2003b).

For further details see 5.3.3.

if peroxidases will convert it into water?

Various models have been proposed to explain the paradox of H$_2$O$_2$ signalling (Figure 1.6, further details will be given in Chapter 5 (5.3.3)). One model focuses on how Prdxs are able to be inactivated. Inactivation can occur by oxidation of the active site cysteines to sulfenic or sulfonic acids (see 1.5.5) (further details are given in Chapter 5; Wood *et al.*, 2003b,c). Alternatively, Prdxs can also be inactivated by phosphorylation (Chang *et al.*, 2002; Rawat *et al.*, 2013; Woo *et al.*, 2010). Once inactivated, H$_2$O$_2$ is able to directly oxidise target proteins. One limitation of the inactivation model is that the Gpxs would still be present (Forman *et al.*, 2010). Lastly, the inactivation model does not explain how the accumulation of H$_2$O$_2$ leads to a specific response.

An alternative model (the disulfide-relay model) proposes that Prdxs themselves can act as direct mediators of H$_2$O$_2$ (Figure 1.6). Prdxs oxidised by H$_2$O$_2$ will in turn, oxidise a target protein via a disulfide-relay (Jarvis *et al.*, 2012). Under the disulfide-relay model, H$_2$O$_2$ signalling specificity can be explained, as it will be dependant upon Prdx protein-protein interactions.

Arguably, the best-known example of a signalling disulfide-relay is the yeast transcription factor, Yap1, which is oxidised in response to H$_2$O$_2$ by the yeast Prdx, Gpx3 (also known as Orp1; Delaunay *et al.*, 2000, 2002). Oxidation of Yap1 leads to the formation of disulfide bonds within Yap1 that promote translocation of the protein to the nucleus (Wood *et al.*, 2003a, 2004). A similar mechanism has been seen in a mammalian context with Prdx2 and
the transcription factor signal transducer and activator of transcription (STAT)3 (Sobotta et al., 2015). ASK1 and Prdx1 have also been proposed to form a disulfide relay (Jarvis et al., 2012).

It is not known how common the disulfide-relay mechanism is because there are only a few well-characterised examples. In order to determine how prevalent the mechanism is or whether the inactivation model is more likely, Chapter 5 will look into developing a methodology to determining the contribution of Prdx1 to protein oxidation and H$_2$O$_2$ signalling.

1.6 ASK1 and H$_2$O$_2$ signalling

Our understanding of H$_2$O$_2$ and its contribution to cellular signalling is ever increasing but questions still remain over the mechanism(s) of H$_2$O$_2$ signal transduction. ASK1 represents a link between H$_2$O$_2$ and protein kinase signalling, notable by activation of the p38 and JNK MAPKs (Gotoh and Cooper, 1998; Ichijo et al., 1997). Work from the Ledgerwood lab showed how Prdx1 is required for H$_2$O$_2$ induced p38 activation (Jarvis et al., 2012). Prdx1 was proposed to directly activate ASK1 in a disulfide-relay (Jarvis et al., 2012). Trx1 is regarded to be a negative regulator of ASK1 (Liu et al., 2000; Saitoh et al., 1998), connecting both positive and negative regulation of ASK1 to H$_2$O$_2$ metabolism. ASK1, Prdx1 and Trx1, therefore, represent a potential model system that can be exploited to further our understanding of H$_2$O$_2$ as a signalling molecule, and how it can alter protein activity.

1.6.1 Outstanding questions

Using ASK1, Trx1 and Prdx1 as a model system requires a detailed molecular understanding of the intrinsic regulatory mechanisms of ASK1. To date, the majority of studies on ASK1 have used either immunoprecipitated ASK1 from cells or the recombinant isolated ASK1 kinase domain. Whilst these methods have provided invaluable information into ASK1 regulation and association with other proteins, they do not allow us to understand how non-catalytic regions within ASK1 can directly regulate protein activity.

Specifically, it is not clear how the N-terminal region of ASK1 facilitates inhibition of ASK1 signalling—does the ASK1 N terminus directly regulate kinase activity or regulate protein-interactions? ASK1 is purported to be negatively regulated by Trx1, but precisely
how Trx1 regulates ASK1 is unclear. Elucidation of the mechanism of Trx1 inhibition would further inform as to the basis of ASK1 regulation and how H$_2$O$_2$ can regulate protein kinase signalling. Lastly, Prdx1 is proposed as a signalling protein, acting to transmit oxidative signals in order to regulate proteins. Jarvis et al. (2012) and Sobotta et al. (2015) used relatively targeted methods to identify ASK1 and STAT3 (respectively) as signalling substrates for Prdx1 and 2 (respectively). Developing a proteomic method to identify likely signalling targets of Prdx1, would help to clarify the true contribution of Prdx1 to H$_2$O$_2$ signalling at a cellular level.

### 1.7 Thesis aims

The aims of this Thesis are to:

**Aim 1** Characterise ASK1 auto-regulatory mechanisms;

**Aim 2** Characterise ASK1 regulation by Trx1;

**Aim 3** Carry out a pilot study to investigate the contribution of Prdx1 to H$_2$O$_2$ signalling.

Chapter 3 will use recombinant ASK1 protein constructs and biochemical analyses in order to investigate how ASK1 is self-regulated. It will be guided by structural and biophysical work from the Mace laboratory and use a novel approach for monitoring ASK1 kinase activity *in vitro*.

Chapter 4 will use recombinant ASK1 protein constructs to examine ASK1 regulation by Trx1 *in vitro*. The interaction between ASK1 and Trx1 will be probed with biochemical and biophysical methods.

Chapter 5 will look at whether a proteomics method is feasible for probing the contribution of Prdx1 to H$_2$O$_2$ signalling in mammalian cells. Chemical labelling and mass spectrometry will be used alongside a cell line that is deficient in Prdx1.
Chapter 2

Materials and Methods

2.1 General Software

This manuscript was written in \LaTeX\ using TEXstudio (texstudio.org) and the TEXLive distribution (www.tug.org/texlive). Mendeley Desktop (www.mendeley.com) was used for reference management. All figures were made using Inkscape: Open Source Scalable Vector Graphics Editor (inkscape.org). Quantification of gels was carried out using Image Studio Lite (LI-COR). Numeric processing was carried out using Microsoft Excel and graphic representation on GraphPad Prism. Molecular modelling was performed using both PyMol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) and UCSF Chimera (Pettersen et al., 2004). All other specific software packages are listed in relevant sections.

2.2 Reagents

Unless stated, all chemicals used were of analytical grade purchased from AppliChem, BioFroxx, Merck or Scharlab. Unless stated otherwise, all solutions were prepared using type I water (18.2 MΩ·cm) from either a Millipore Milli-Q or Elga PURELAB Chorus 1 system.
2.3 Cloning and mutagenesis

2.3.1 General cloning and mutagenesis information

Unless stated, all reagents for cloning were purchased from New England Biolabs. A C1000 Thermal Cycler (Bio-Rad) was used for all polymerase chain reactions (PCR) and molecular biology incubations unless stated otherwise. UltraPure water (Invitrogen) was used in all cloning and molecular biology.

All primer and oligonucleotide sequences can be found in Appendix A. All primers and oligonucleotides were purchased from either Sigma or Integrated DNA Technologies. Upon arrival primers were briefly centrifuged before the addition of UltraPure water to reconstitute primer to a final concentration of 100 µM. Primers were hydrated at room temperature for at least 10 minutes before vortexing and briefly centrifuging before being stored at −20 °C.

All cloning used chemically competent Escherichia coli MC1061 cells. MC1061 cells were grown in lysogeny broth (LB) containing 10 g sodium chloride (NaCl), 10 g peptone (from casein) and 5 g yeast extract made up to one litre with type I water. LB was sterilised by autoclaving at 121 °C, 100 kPa for 20 minutes.

2.3.2 Vectors

A list of all protein expression vectors used in this work can be found in Table 2.1

2.3.3 Miniprep isolation of plasmid DNA from MC1061 cells

Plasmids were isolated from 3 mL LB culture that had been grown from a single colony overnight with appropriate antibiotic (50 µg/mL for kanamycin, 100 µg/mL for ampicillin, 34 µg/mL for chloramphenicol). A Zippy plasmid miniprep kit (Zymo Research) was used for all plasmid mini-preps. All steps were performed at room temperature and all centrifugation steps were at 16,000 g unless stated otherwise.

Briefly, cells were transferred to a 1.7 mL tube and pelleted by centrifugation at 4,000 g for 2 minutes in two sequential rounds. The supernatant was discarded and the cell pellet was re-suspended in 500 µL of type I water. One hundred microlitres of
Table 2.1: Protein expression vectors

<table>
<thead>
<tr>
<th>Description</th>
<th>Expression system</th>
<th>Backbone</th>
<th>Antibiotic resistance</th>
<th>Cloning method</th>
<th>Insert sequencing primers</th>
<th>Original source</th>
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<tr>
<td>His&lt;sub&gt;6&lt;/sub&gt;-3C-X</td>
<td><em>E. coli</em></td>
<td>pET28</td>
<td>Kanamycin</td>
<td>LIC</td>
<td>T7/T7 Term</td>
<td>NKI</td>
</tr>
<tr>
<td>X-Lys-His&lt;sub&gt;6&lt;/sub&gt;</td>
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<td>pET29</td>
<td>Kanamycin</td>
<td>NcoI/XhoI</td>
<td>T7/T7 Term</td>
<td>Dr Peter Mace</td>
</tr>
<tr>
<td>His&lt;sub&gt;6&lt;/sub&gt;-MBP-3C-X</td>
<td><em>E. coli</em></td>
<td>pMAL</td>
<td>Ampicillin</td>
<td>LIC</td>
<td>MalE/T7 Term</td>
<td>Dr Peter Mace</td>
</tr>
<tr>
<td>His&lt;sub&gt;6&lt;/sub&gt;-3C-X (Trx1 co-expression)</td>
<td><em>E. coli</em></td>
<td>pET28</td>
<td>Kanamycin</td>
<td>LIC</td>
<td>DuetUp2/DuetDown</td>
<td>Dr Peter Mace</td>
</tr>
<tr>
<td>pACYC (No tag)</td>
<td><em>E. coli</em></td>
<td>pETDuet</td>
<td>Chloramphenicol</td>
<td>NcoI/XhoI</td>
<td>DuetUp1/DuetDown</td>
<td>Dr Peter Mace</td>
</tr>
<tr>
<td>His&lt;sub&gt;6&lt;/sub&gt;-3C-X (FB)</td>
<td>Insect</td>
<td>pFastBac</td>
<td>Ampicillin</td>
<td>LIC</td>
<td>Polyhedrin/SV40</td>
<td>Dr Peter Mace</td>
</tr>
</tbody>
</table>

His<sub>6</sub>—Hexahistidine tag; 3C—protease cleavage site; X—DNA sequence of the protein to be incorporated; MBP—maltose binding protein; Trx1—human Thioredoxin 1; LIC—ligation independent cloning; Lys-His<sub>6</sub>—C-terminal T4 lysozyme<sup>R12G,C54T,C97A,I137R</sup>. Sequencing primers—forward/reverse, sequences are given in Appendix Table A.1. The pET28, His<sub>6</sub>-3C-X vector was a gift from the Netherlands Cancer Institute Protein Facility (NKI).

Zyppy lysis buffer was added to the cell suspension and mixed by inversion at least five times, for no longer than two minutes. Zyppy Neutralisation buffer (350 µL) was added to each tube and mixed by inverting tubes at least five times. The lysate was clarified by centrifuging for two minutes at maximum speed. The supernatant was transferred to a fresh DNA-binding column in a collection tube and centrifuged for 30 seconds. Bound DNA was washed with 200 µL of endotoxin wash and 400 µL of DNA-wash buffer with centrifugation in-between each wash. Trace ethanol was removed by centrifuging DNA column for 2 minutes. The DNA-column was subsequently transferred to a fresh 1.7 mL microfuge tube and 40 µL of UltraPure water added directly to the membrane. DNA was eluted by centrifugation for 30 seconds and concentration quantified (2.3.4). Plasmids were stored at −20 °C.

2.3.4 DNA quantification

DNA concentration was quantified using a NanoDrop2000 (Thermo Scientific) to measure absorbance at 260 nm.
2.3.5 Agarose gel electrophoresis

Agarose gels were made in TAE buffer consisting of 40 mM tris(hydroxymethyl)aminomethane (Tris), 20 mM acetate and 1 mM ethylenediaminetetraacetic acid (EDTA).

Linearised vector and PCR products were resolved and visualised on agarose-TAE gels. Agarose-TAE gels (0.5–2% (w/v) agarose, HydraGene) were poured in ~1 cm thick slabs. Ethidium bromide was added fresh to molten gel at a final concentration of approximately 0.5–2.0 µg/mL. Samples were mixed with 6× agarose gel loading dye (Thermo Scientific) and loaded into wells. Gels were resolved by electrophoresis at 120 V for 25 minutes and then visualised on UV transilluminator.

2.3.6 DNA Gel extraction and purification

Linearised vector and PCR products were routinely purified by extraction following agarose gel electrophoresis using a ZymoClean gel recovery kit (Zymo Research). Briefly, bands of interest were excised from agarose gel and transferred to a fresh 1.7 mL tube. Agarose dissolving buffer (ADB) was added (≈500 µL) to the same tube and was incubated at 50 °C until agarose had melted. Melted agarose-gel was transferred to a DNA-binding column and centrifuged at 16,000 g for 30 seconds. Bound DNA was washed with an additional 200 µL ADB and centrifuged again at 16,000 g for 30 seconds. The column was washed twice with 200 µL ethanol-containing wash buffer before centrifugation for 30 seconds at 16,000 g. The column was dried by centrifugation at 16,000 g for 2 minutes to remove all ethanol. DNA was eluted by the addition of 14 µL of UltraPure water directly to the centre of the column. Columns were incubated at room temperature for 10 minutes. DNA was eluted with a final centrifugation at 16,000 g for 30 seconds. Eluted DNA was quantified spectrophotometrically using a NanoDrop (2.3.4). Eluted DNA was stored at −20 °C until use.

2.3.7 PCR of inserts

Inserts for ligation independent cloning (LIC) and ligation cloning utilised a PCR recipe consisting of a 25 µL reaction containing 5 µL Q5 buffer, 0.28 mM dNTPs, between
20 and 50 ng DNA template, 1 µM each primer and 0.5 units of Q5 DNA polymerase. UltraPure water was added to adjust final volume to 25 µL. The PCR reaction was incubated under the following conditions: 98 °C for 30 seconds, 55 °C for 30 seconds 72 °C for 2 minutes, 31 cycles of 98 °C for 10 seconds, 72 °C for 2 minutes, 72 °C for 2 minutes.

PCR products were resolved by electrophoresis agarose-TAE gels (2.3.5). DNA bands were visualised under UV and bands excised using a clean razor blade and transferred to a 1.7 mL microfuge tube. DNA was liberated from gel slabs using a DNA-agarose extraction kit (2.3.6).

2.3.8 Isolation of ASK1 cDNA

Full-length (2–1374) human ASK1 cDNA was isolated by sequential PCR using human cDNA as a template. Reaction and PCR conditions were the same as in 2.3.7. MegaMan cDNA (Agilent) was diluted 10-fold in UltraPure water and 0.25 µL of the diluted cDNA was used as a template. Primers in one reaction corresponded to ASK1 residues 2–335 and in a second reaction, residues 325–1374 (primer sequences are given as in A.2). These reactions were resolved on a 1% (w/v) agarose-TAE gel (2.3.5). A pipette tip was stabbed into each band and placed into a fresh PCR reaction with the same recipe as previous (2.3.7) with primers for ASK1 2-forward and ASK1 1374-reverse. The primers for ASK1 325-forward and 335-reverse overlapped completely, so for the first few stages of the PCR the previous bands acted as both template and primer. The final PCR product then contained LIC-compatible ASK1 2–1374 cDNA, which was processed and inserted into vectors as previous (2.3.10) before confirming by colony PCR and sequencing.
2.3.9 Restriction enzyme cloning of Trx1-Lysozyme fusion construct

2.3.9.1 Linearisation of lysozyme-His$_6$ fusion vector by $Xho$I and $Nde$I restriction enzymes

Cloning for Trx1-Lysozyme fusion construct utilised $Xho$I and $Nde$I sites within the X-T4 lysozyme-His$_6$ vector (Table 2.3.2). Two micrograms of lysozyme-His$_6$ fusion vector were incubated with 40 units of both $Xho$I and $Nde$I, 5 µL CutSmart buffer and the reaction was made to a final volume of 50 µL with UltraPure water. The vector was digested for 1.5 hours at 37 °C. Linearised vector was resolved by agarose-TAE electrophoresis (2.3.5) before gel purification and extraction (2.3.6).

2.3.9.2 PCR generation of ligation cloning inserts

The program and recipe for PCR of inserts for ligation cloning was identical to that as for all PCR-insert generation (2.3.7) with the exception that primers featured either a $Nde$I cut-site (5’-CATATG-3’) within the forward primer or $Xho$I cut-site (5’-CTCGAG-3’) within the reverse primer. Primers for ligation-based PCR can be found in Appendix A.3. Following PCR, inserts were resolved by TAE-agarose gel electrophoresis (2.3.5), and bands were excised and purified (2.3.6).

2.3.9.3 Restriction digestion of insert

Inserts were made compatible with vector by restriction digest with $Xho$I and $Nde$I. One microgram of gel-purified insert was incubated with 20 units of $Nde$I and $Xho$I and 2 µL of CutSmart buffer in a 20 µL reaction made up with UltraPure water. Digested insert was heat-inactivated at 65 °C for 20 minutes.

2.3.9.4 Ligation of insert and vector

Vector and insert were mixed together in a 1:3 (vector:insert) molar ratio. DNA ligation took place in a reaction containing 21 ng of insert DNA, 125 ng of vector, 1 µL of T4 DNA ligase buffer, 200 units of T4 DNA ligase and UltraPure water was added to 10
µL final volume. The ligation reaction took place at room temperature for 10 minutes. Three microlitres of the ligation reaction were used to transform competent MC1061 cells (2.3.11).

2.3.10 Ligation independent cloning (LIC)

2.3.10.1 PCR for generation of LIC inserts

All primers for LIC had previously been designed by Dr Peter Mace using the ProteinCCD tool (Mooij et al., 2009). LIC primers featured either an overhang of 5’-CAGGGACCCCGGT-3’ for forward primers or 5’-CGAGGAGAAGCCCGGTATA-3’ for reverse primers. The sequences for all LIC-primers can be found in Appendix A. PCR for LIC-inserts was identical to ligation-based cloning (2.3.7).

2.3.10.2 Linearisation of LIC-vectors by KpnI

All LIC-compatible vectors in this work featured a KpnI site for linearisation. Two micrograms of vector were incubated with 1 unit of KpnI-HF with 5 µL of CutSmart buffer in a 25 µL reaction made up with UltraPure water. The KpnI digestion was incubated at 37 °C for 2 hours. Linearised plasmid was separated and by TAE-agarose electrophoresis (2.3.5) before purification by gel-extraction (2.3.6).

2.3.10.3 T4 DNA polymerase treatment of vector

Approximately 800 ng of KpnI-cut vector was treated in a 30 µL reaction consisting of 3 µL NEB Buffer 2, 2.5 mM dTTP and 4.5 units of T4 DNA polymerase with the remaining volume made up with UltraPure water. T4 DNA polymerase treatment occurred in a thermal cycler at 25 °C for 30 minutes followed by heat-inactivation at 75 °C for 20 minutes. T4 DNA polymerase-treated vectors (denoted as ‘LIC-ready vector’) were stored at −20 °C before use.

2.3.10.4 T4 DNA polymerase treatment of insert

Between 100 and 200 ng of insert DNA are added to a 10 µL reaction containing 1 µL NEB Buffer 2, 2.5 mM dATP and 1.5 units of T4 DNA polymerase with the final
volume made up with UltraPure water. Where insert-DNA concentration was too low to quantify or yield was too low UltraPure water was omitted and the volume made up with as much insert-DNA as possible. Insert T4 DNA polymerase treatment was incubated identically to vector T4 DNA polymerase treatment; 25 °C for 30 minutes followed by heat-inactivation at 75 °C for 20 minutes. T4 DNA polymerase-treated inserts (denoted as ‘LIC-ready insert’) were stored at −20 °C before use.

2.3.10.5 LIC Combination

In a 1.7 mL microfuge tube, 1.5 µL of LIC-ready insert and 0.5 µL LIC-ready vector were combined and centrifuged briefly. Insert and vector were incubated room temperature for 5 minutes during which complementary ends anneal. The entire reaction was transformed into MC1061 cells (2.3.11).

2.3.11 DNA Transformation of E. coli

For each transformation, approximately 20–50 ng of DNA was incubated with 20 µL of chemically competent E. coli MC1061 cells. Cells were incubated on ice for 10 minutes, before being heat-shocked in a 42 °C water bath for 45 seconds. Cells were returned to ice for two minutes before the addition of 350 µL of sterile, room temperature LB. Cells were transferred to a 37 °C shaking incubator for 45 minutes for antibiotic resistance gene(s) to be expressed. Transformed cells were aseptically spread onto 15 mm Petri dishes (Thermo Scientific) with 0.5% (w/v) agar-LB medium supplemented with either ampicillin or kanamycin at 100 or 50 µg/mL respectively. Plated cells were placed in 37 °C incubator overnight.

2.3.12 Screening by colony PCR

LIC and ligation cloning were screened by colony PCR. Single colonies were picked using a sterile 20 µL tip and placed in a PCR-strip tube containing 10 µL of pre- aliquoted PCR master mix. Colony PCR master mix contained 1 µL ThermoPol Reaction Buffer, 0.5 units of Taq DNA polymerase, 0.7 mM dNTPs, 1 µM of forward and reverse primer (vector dependant, see 2.3.2) made up to 10 µL with UltraPure water. Picked colonies
were incubated in PCR master mix for between 1 and 5 minutes at room temperature before tip was removed. The reaction was incubated in thermal cycler under the following conditions; 95 °C for 2 minutes, followed by 33 rounds of 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 4 minutes, and a final step of 72 °C for 6 minutes.

The entire reaction was analysed by TAE-agarose electrophoresis (2.3.5). Colonies that had an insert of the correct size, as judged by the colony PCR, were used to inoculate 3 mL of sterile LB with the appropriate antibiotic and incubated overnight at 37 °C in a shaking incubator. Plasmids were extracted using a mini-prep kit (2.3.3).

2.3.13 Sequencing of plasmid DNA

Plasmid DNA was regularly sequenced by Sanger sequencing by either Genetic Analysis Services (University of Otago) or Massey Genome Service (Massey University) as per their recommended instructions. DNA sequencing was analysed using 4Peaks and EnzymeX (both www.nucleobytes.com) with comparison alignments against expected sequences made using ClustalOmega (Li et al., 2015).

2.3.14 Mutagenesis

Site-directed mutagenesis was carried out by QuickChange using overlapping primers (sequences can found in Appendix A) with deliberate 3’ overhangs (Liu and Naismith, 2008). The desired mutation was included in the overlapping portion.

QuickChange PCR was carried out in a 12.5 µL reaction containing 2.5 µL Q5 reaction buffer, 0.2 mM dNTP, 0.4 µM of each primer, 20 ng of template DNA. The reaction volume was made up using UltraPure water. QuickChange PCR reactions were incubated at 98 °C for 1 minute before 19 rounds of 98 °C for 30 seconds, 55 degrees for 30 seconds, 72 °C for 5 minutes, before a final step of 72 °C for 7 minutes.

Once the PCR was complete, 3 µL of the reaction was removed for a no-\(DpnI\) treated sample. Ten units of \(DpnI\) was added to the remainder (\(~9.5\) µL) of the reaction. \(DpnI\) digestion of host methylated DNA took place overnight (\(~16\) hours) at 37 °C. Four microlitres of the \(DpnI\)-treated reaction was used to transform 50 µL of MC1061 cells in the same manner as described in 2.3.11. Cells were plated onto agar plates and
allowed to grow overnight. Single colonies were picked with a sterile pipette tip and used to inoculate 3 mL of LB (with the appropriate antibiotic) for mini-prep isolation of plasmid DNA (2.3.3). Plasmids were screened by Sanger sequencing to confirm the inclusion of desired mutation (2.3.13).

2.3.15 Constructs used in this study

A list of all wild-type (WT) protein constructs is given in Table 2.2.

The majority of constructs used in this study were expressed in *E. coli* BL21(DE3) using a modified pET28 vector featuring a LIC-cloning site that introduced an N-terminal-His$_6$-tag and 3C-protease cleavage site (SAALEVLFQ-GPG, where 3C cuts at _).}

2.4 Polyacrylamide gel electrophoresis (SDS-PAGE)

2.4.1 Preparation of acrylamide gels for SDS-PAGE

All SDS-PAGE (except Phos-Tag SDS-PAGE) was conducted using hand-poured, 15-well, 1 mm × 80 mm × 100 mm, 10–20% acrylamide gradient gels with a discontinuous stacker.

Gradient gels were hand-poured using a Model 385 gradient former (Bio-Rad), P-1 peristaltic pump (Pharmacia Fine Chemicals) and SE215 10-gel multi-caster (Hoeffer) with polycarbonate sheets separating each gel. Table 2.3 gives the recipe used in gradient SDS-PAGE. Once poured, the gels were gently tapped to dislodge any trapped air, placed on a level surface and 150 µL of isopropanol was gently pipetted on top of each gel. Gels were left to cure for between 30 minutes and one hour. Once the resolving gel had set, isopropanol was poured off of gels and the stacking gel was poured on. The combs were inserted and the stacking gel was left to cure for an additional hour. Once the stacking gel had set the multi-caster was disassembled and gels stored in an airtight, humidified, box at 4 °C until use.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Residues</th>
<th>Tag(s)</th>
<th>Vector(^1)</th>
<th>Cloning Method</th>
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<tbody>
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<td>His(_6)-3C-X</td>
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</tbody>
</table>

All proteins sequences are human with the exception of T7 phage lysozyme and \(\lambda\) phage PP. Wild-type (WT) proteins were initially isolated and any mutations were derived via QuickChange (see section 2.3.14). 1) Refers to vector description in Table 2.3.2. λPP—Lambda phage protein phosphatase, QC—QuickChange, RE—Restriction enzyme.
Table 2.3: Gradient (10–20%) polyacrylamide 10-gel recipe

<table>
<thead>
<tr>
<th>Solution</th>
<th>Low (10%) resolving gel</th>
<th>High (20%) resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M Tris, pH 8.8 (mL):</td>
<td>7.5</td>
<td>7.5</td>
<td>0</td>
</tr>
<tr>
<td>0.5 M Tris, pH 6.8 (mL):</td>
<td>0</td>
<td>0</td>
<td>7.8</td>
</tr>
<tr>
<td>40% (w/v) Acrylamide (mL):</td>
<td>7.5</td>
<td>15</td>
<td>3.75</td>
</tr>
<tr>
<td>Type I water (mL):</td>
<td>14.5</td>
<td>1.82</td>
<td>18</td>
</tr>
<tr>
<td>10% (w/v) SDS (mL):</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>60% (v/v) Glycerol (mL):</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>0.5% (w/v) Bromophenol blue (µL):</td>
<td>0</td>
<td>210</td>
<td>10</td>
</tr>
<tr>
<td>TEMED (µL):</td>
<td>15</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>10% (w/v) APS (µL):</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
</tbody>
</table>

TEMED—N,N,N’,N’-tetramethylethane-1,2-diamine, APS—ammonium persulfide. TEMED was from Sigma, APS and 40% acrylamide/bis-acrylamide 37.5:1 was from Bio-Rad. TEMED and APS were added to gel solution immediately before pouring.

### 2.4.2 Preparation of samples for SDS-PAGE

Samples were mixed with either 2× or 4× SDS-PAGE sample buffer as required. Two times SDS-PAGE reducing sample buffer contained 120 mM Tris pH 6.8, 16% (v/v) glycerol, 100 mM DTT, 0.01% (w/v) bromophenol blue. Four times SDS-PAGE reducing sample buffer contained 240 mM Tris pH 6.8, 32% (v/v) glycerol, 200 mM DTT 0.02% (w/v) bromophenol blue. Non-reducing SDS-PAGE sample buffers were of an identical recipe except for the omission of DTT. Samples containing whole-cell mixtures were always boiled and briefly centrifuged before running. Samples for both Phos-Tag SDS-PAGE and non-reducing SDS-PAGE were never boiled. Samples were routinely stored at –20 °C, if not being processed immediately.

### 2.4.3 Running of SDS-PAGE

SDS-PAGE was run using a SE250 gel tank (Hoeffer). The back reservoir of the tank was filled with SDS-running buffer (consisting of 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS). The comb was gently removed and wells were rinsed with running buffer. For SDS-PAGE, 3.5 µL of Precision Plus Protein Standards (Bio-Rad) were loaded.
into one well. Samples to be analysed on the gel were loaded and gels were run with water-cooling at a constant voltage of 200 V for 55 minutes. Once gels had run, the gels were carefully removed from the apparatus. The wells were cut off and the gel was transferred to a container and rinsed briefly in distilled water.

2.4.4 Coomassie staining of SDS-PAGE

Gels were stained using Coomassie brilliant blue (R-250) in a solution of 10% (v/v) acetic acid, 50% (v/v) methanol, 0.25% (w/v) R-250, in distilled water. Gels were then placed on a ROCKit rocker (Select BioProducts) for between 10 and 30 minutes until even stain was achieved. Gels were subsequently destained by removing staining solution and rinsing with distilled water. Destaining solution (10% (v/v) acetic acid, 40% (v/v) methanol, in distilled water) was poured over to completely cover the gel. A paper towel was added to accelerate the destaining process. Gels were left to destain until a desired level of background was reached. Gels were subsequently imaged on a gel imaging system (GE Healthcare) using Epson Scan (Epson).

2.4.5 Phos-Tag SDS-PAGE

Phos-Tag SDS-PAGE used hand-poured, 15-well, 1 mm × 80 mm × 100 mm, 10% acrylamide gels, 20 µM Phos-Tag acrylamide, 100 µM MnCl₂ with a discontinuous stacking gel. Gels were cast in a 5-gel SE200 multi-caster (Hoeffer). The recipe for Phos-Tag polyacrylamide gels is given in Table 2.4.

Phos-Tag acrylamide (AAL-107, Wako-Chem) solution (5 mM) was prepared by dissolving 10 mg of Phos-Tag acrylamide in 100 µL analytical grade methanol. Once dissolved, 3.2 mL of type I water was added. The Phos-Tag acrylamide solution was aliquotted and stored at −20 °C in the dark. Once thawed, working aliquots were kept protected from light at 4 °C.

Gels were poured, tapped to dislodge trapped air and 150 µL of isopropanol was added to the top of each gel. Phos-Tag gels were covered with a cardboard box to protect from light and left to set for one hour. Once gels were set, the isopropanol was poured off and the tops of the gels were rinsed with distilled water. The resolving
Table 2.4: Phos-Tag (20 µM) SDS-PAGE (10%) 5-gel recipe

<table>
<thead>
<tr>
<th>Solution</th>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M Tris, pH 8.8 (mL):</td>
<td>6.25</td>
<td>0</td>
</tr>
<tr>
<td>0.5 M Tris, pH 6.8 (mL):</td>
<td>0</td>
<td>4.175</td>
</tr>
<tr>
<td>40% (w/v) acrylamide (mL):</td>
<td>6.25</td>
<td>2.075</td>
</tr>
<tr>
<td>Type I water (mL):</td>
<td>12.0</td>
<td>9.425</td>
</tr>
<tr>
<td>10% (w/v) SDS (µL):</td>
<td>250</td>
<td>167.5</td>
</tr>
<tr>
<td>5 mM Phos-Tag acrylamide (µL):</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10 mM MnCl₂·4H₂O (µL):</td>
<td>250</td>
<td>0</td>
</tr>
<tr>
<td>0.5% (w/v) Bromophenol blue (µL):</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>TEMED (µL):</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>10 % (w/v) APS (µL):</td>
<td>125</td>
<td>125</td>
</tr>
</tbody>
</table>

MnCl₂·4H₂O—manganese chloride tetrahydrate (Sigma). TEMED and APS were added to gel solution immediately before pouring.

gel solution was poured on top of the gels and combs were inserted. The gels were covered with the box and left to cure for an additional hour. Once fully set, the gels were disassembled and placed in an airtight, humidified, dark box and stored at 4 °C. Phos-Tag gels were used within two weeks.

The running (2.4.3) and staining (2.4.4) of Phos-Tag SDS-PAGE was as per conventional SDS-PAGE. Samples for analysis by Phos-Tag SDS-PAGE were never boiled and always frozen at −20 °C and thawed before analysis. Coomassie stained Phos-Tag acrylamide gels were imaged in the 700 nm channel of an Odyssey FC (LI-COR) and processed using Image Studio (LI-COR).

2.5 Western blot analysis

Gels for western blotting were run as for normal SDS-PAGE. With the exception that 4 µL of prestained Precision Plus Protein Standards (Bio-Rad) (which were already diluted four-fold in 4× reducing SDS-sample buffer) were loaded into one well.
2.5.1 Antibodies

Working dilutions and other details of primary and secondary antibodies used in this work are given in Tables 2.5 and 2.6, respectively.

**Table 2.5: Primary antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clonality</th>
<th>Species raised in</th>
<th>Working dilution</th>
<th>Source</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-p-p38</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>1 in 1,000</td>
<td>BD Biosciences</td>
<td>612288</td>
</tr>
<tr>
<td>α-p-MKK6</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>1 in 2,500</td>
<td>SantaCruz</td>
<td>sc-7994-R</td>
</tr>
<tr>
<td>α-ASK1</td>
<td>Monoclonal</td>
<td>Rabbit</td>
<td>1 in 1,000</td>
<td>Abcam</td>
<td>ab45178 [EP553Y]</td>
</tr>
<tr>
<td>α-Prdx1</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>1 in 2,000</td>
<td>Abcam</td>
<td>ab41906</td>
</tr>
<tr>
<td>α-Prdx1</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>1 in 1,000</td>
<td>Abcam</td>
<td>ab133524 [EPR6110]</td>
</tr>
<tr>
<td>α-Trx1</td>
<td>Polyclonal</td>
<td>Mouse</td>
<td>1 in 1,000</td>
<td>Abcam</td>
<td>ab18184</td>
</tr>
<tr>
<td>α-His6</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>1 in 1,000</td>
<td>IBA Lifescience</td>
<td>2-1509-001</td>
</tr>
<tr>
<td>α-α-Tubulin</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>1 in 10,000</td>
<td>Millipore</td>
<td>05-829 (clone DM1A)</td>
</tr>
</tbody>
</table>


**Table 2.6: Secondary antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clonality</th>
<th>Species raised in</th>
<th>Working dilution</th>
<th>Source</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-rabbit-680LT</td>
<td>Polyclonal</td>
<td>Goat</td>
<td>1 in 20,000</td>
<td>LI-COR</td>
<td>925-68021</td>
</tr>
<tr>
<td>α-rabbit-800CW</td>
<td>Polyclonal</td>
<td>Goat</td>
<td>1 in 20,000</td>
<td>LI-COR</td>
<td>925-32211</td>
</tr>
<tr>
<td>α-mouse-800CW</td>
<td>Polyclonal</td>
<td>Goat</td>
<td>1 in 20,000</td>
<td>LI-COR</td>
<td>925-32210</td>
</tr>
<tr>
<td>α-mouse-HRP</td>
<td>Polyclonal</td>
<td>Goat</td>
<td>1 in 20,000</td>
<td>BioRad</td>
<td>170-6516</td>
</tr>
<tr>
<td>α-rabbit-HRP</td>
<td>Polyclonal</td>
<td>Goat</td>
<td>1 in 20,000</td>
<td>BioRad</td>
<td>170-6515</td>
</tr>
</tbody>
</table>

HRP—horseradish peroxidase.
2.5.2 Wet transfer

For wet transfer, gels were run as in 2.4. Once run, gels were trimmed of stacking gel and any remaining bromophenol blue containing gel. The gel was soaked in ice-cold transfer buffer (25 mM Tris, 192 mM glycine, 10% (v/v) methanol) for 10 minutes. Nitrocellulose membrane (0.2 µm, GE Healthcare) was cut to size and soaked in the same buffer. Two pieces of 1.5 mm blotting paper (Whatman) were trimmed and soaked along with sponges. The transfer cassette was assembled from the bottom-up—sponge, two pieces of blotting paper, membrane, gel, two pieces of blotting and then a final sponge. At each layer, the transfer-sandwich was rolled to remove air. The gel sandwich was placed inside a plastic clamp and placed in a TE22 transfer tank (Hoeffer). The tank was filled with ice-cold transfer buffer and run with water cooling at 100 V, 300 mA for 2 hours.

2.5.3 Semi-dry transfer

Semi-dry transfer used a Trans-Blot Turbo Transfer system (Bio-Rad). Running and preparation of the gel was the same as for wet transfer (2.5.2). From the bottom-up, four pieces of pre-soaked blotting paper were topped with the membrane, the gel, and another four pieces of blotting paper. At each stage, the sandwich was rolled to exclude air. Semi-dry transfer was run at 2.5 A, 15 V for 30 minutes.

2.5.4 Ponceau S staining

After transfer to membrane, total protein was stained and visualised using Ponceau S. In brief, the membrane was placed in a clean container and submerged with Ponceau S staining solution (0.5% (w/v) Ponceau S (BDH Chemicals), 1% (v/v) acetic acid, in type 1 water). The membrane in Ponceau S solution was incubated on a rocker at room temperature for between 3 and 10 minutes before being rinsed three times in distilled water. The membrane was left in distilled water for a further 3 to 5 minutes to help ensure an even level of staining. The stained membrane was imaged in the 800 nm channel on an Odyssey FC (LI-COR) and Image Studio (LI-COR). Once imaged,
membranes were rinsed with distilled water before being rolled and placed inside a clean 50 mL tube.

2.5.5 Blocking and probing

Membranes already rolled in tubes were blocked in a solution of Tris-buffered saline (TBS; 20 mM Tris pH 7.4, 150 mM NaCl) containing 5% (w/v) bovine serum albumin (BSA; MP Biomedical). Membranes were blocked for one hour at room temperature on a SRT1 roller (Stuart Scientific).

Primary antibodies were diluted as appropriate (Table 2.5) in TBS with 0.1% (v/v) Tween-20 (Sigma) (TBST) with 1% (w/v) BSA. Membranes were incubated with primary antibodies overnight at 4 °C on a roller. Membranes were washed 4 × 5 minutes in TBST with rolling at room temperature. Secondary antibodies (see Table 2.6) were diluted in TBST with 1% BSA. Membranes were probed with LI-COR secondary antibodies were wrapped in foil to protect them from the light. Membranes were incubated with secondary antibodies for one hour at room temperature on a roller before being washed 4 × 5 minutes in TBST with rolling at room temperature.

2.5.6 Western blot imaging

Membranes with LI-COR secondary antibodies were imaged using appropriate channels on an Odyssey FC (LI-COR). HRP-conjugated secondary antibodies were detected using Clarity western ECL Substrate (Bio-Rad), as per manufacturer’s instructions. Briefly, the two solutions in the kit were mixed in a 1:1 ratio then applied over the membrane and left to incubate for one minute before imaging on an Odyssey FC (LI-COR).
2.6 Protein expression and purification

2.6.1 Transformations

Transformations for protein expression were identical to that for molecular cloning (2.3.11) with the exception that all transformations for protein expression utilised *E. coli* BL21(DE3). All protein expression used freshly transformed BL21(DE3) cells.

2.6.2 Inoculation, growth and induction of cultures

All colonies from one petri dish were suspended in 3 mL sterile LB (formulation in 2.3.1). One millilitre of cell suspension was used to inoculate one litre of sterile LB. Appropriate antibiotics were added to a final concentration of 50 µg/mL for kanamycin or 100 µg/L for ampicillin.

Inoculated cultures were transferred to a 37 °C incubator shaking at 200 rpm (Innova 4300, New Brunswick Scientific). After two hours growing the optical density (OD) of the culture was checked by aseptically aspirating one millilitre of culture and measuring the absorbance at 600 nm in a Ultrospec 2000 spectrophotometer (Pharmacia Biotech). When the OD$_{600}$ was between 0.6 and 0.8 the cultures were transferred to an 18 °C shaking incubator and left to acclimatise for 45 minutes. After cultures had cooled, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM to induce protein expression. Cultures were left to express proteins overnight (approximately 16 to 20 hours).

The next day, cultures were transferred to 1 L centrifuge bottles (Beckman Coulter) for the harvesting of cells. Cells were pelleted by centrifugation at 4,000 $g$ (JLA-9.100 rotor, Avanti JXN-26 centrifuge both Beckman Coulter), 4 °C for 30 minutes. The supernatant was poured off of cell pellets and cells were resuspended (10 mL per litre of culture) in freeze buffer (20 mM Tris pH 8.0, 300 mM NaCl). Resuspended cells were transferred to a 50 mL tube and stored at −20 °C before downstream processing. Protein expression cell pellets were always frozen before processing.
2.6.3 Bacterial cell lysis

For bacterial cell lysis, frozen cell pellets were thawed and standard-purification buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% (v/v) glycerol, 10% (w/v) sucrose, 10 mM imidazole) was added to a final approximate volume of 30 mL. Hen-egg lysozyme (Hampton Research) was added to the slurry at a final concentration of 166 µg per mL of cell suspension. Cells were incubated on ice for at least 15 minutes.

Following incubation with lysozyme, cells were disrupted by repeated rounds of sonication using a Sonifier Cell Disrupter (Bronson Sonic Power Co.), output 7, 70% amplitude for one minute pulsed-sonication in an ice-bath, one minute sitting on ice and a final minute of pulsed-sonication in ice-bath.

2.6.4 Clarification of bacterial homogenate

Following cell lysis, insoluble material was pelleted by centrifugation at 13,000 g, 4 °C, 30 minutes (JA-17 Rotor, Avanti JXN-26 centrifuge both Beckman Coulter). Five microlitres of cell homogenate was removed and separately centrifuged at 13,000 g, for 15 minutes, 4 °C (F45-30-11 rotor 5417R centrifuge, both Eppendorf) for an insoluble sample for SDS-PAGE analysis. Clarified lysate supernatant was carefully transferred to a clean 50 mL tube. Five microlitres of the supernatant was removed for a soluble sample for SDS-PAGE analysis.

2.6.5 HIS-Select nickel affinity purification

His6-tagged proteins were purified using HIS-Select nickel affinity gel (Sigma). Generally, one millilitre of HIS-Select resin-bed (pre-equilibrated in standard purification buffer) was used per one litre of E. coli culture. Proteins were allowed to bind to resin for 1 hour at 4 °C on a RATRSM7DC orbital rotator (Ratek) at 10 rpm. Resin was substantially washed in batch by three rounds of centrifugation at 3,500 g for 5 minutes, 4 °C (SX4400 rotor, Allegra X-30R centrifuge, both Beckman Coulter). After the initial pelleting of resin, 5 µL of supernatant was removed for a flow-through sample for analysis by SDS-PAGE. Pelleted resin was washed after each centrifugation step by
resuspension in between 30 and 40 mL of standard purification buffer. After the final wash, the resin was pelleted as previously, before being resuspended in 10 mL of standard purification buffer and transferred to a Poly-Prep Chromatography hand-column (Bio-Rad).

Proteins were eluted from resin using 300 mM imidazole diluted in standard purification buffer. Typically 500 µL of elution buffer was applied to resin and allowed to flow through the resin by gravity. This was repeated 4 times or until no further protein eluted from the resin. Protein elutions were measured spectrophotometrically at 280 nm to determine protein concentration. Protein containing elutions were combined.

Depending on the downstream application, proteins were either cleaved using 3C-protease (2.6.6), stored overnight at 4 °C before fast protein liquid chromatography (FPLC; 2.6.8), or aliquoted and frozen (2.6.12).

2.6.6 3C-protease cleavage of proteins

Where applicable, the N-terminal His<sub>6</sub>-tag was removed from proteins by recombinant 3C-protease (made by Sam Jamieson, Mace laboratory) in a 50:1 protein:protease ratio (by mass) with the addition of 2 mM DTT. Cleavage took place overnight (∼16 to 20 hours) at 4 °C.

2.6.7 Ultrafiltration concentration of proteins

Proteins were concentrated using appropriate molecular weight cut-off ultrafiltration centrifugal concentrators (Pall or Sartorius Stedim Biotech). Concentrators were used according to manufacturer instructions and were always pre-equilibrated in equivalent protein-containing buffer.

2.6.8 FPLC

All FPLC was performed using an ÄKTA Pure 150 Chromatography system (GE Healthcare). Buffers were always freshly filtered through a 0.2 µm cellulose acetate filter (Sartorius Stedim Biotech) before being degassed for at least 15 minutes.
2.6.9 Anion-exchange chromatography (IEX)

IEX was performed using a home-packed 2 mL column with SOURCE 15Q Resin (GE Healthcare). IEX was routinely carried out using a low-molarity salt buffer-‘A’ (10 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (pH 7.6), 10 mM NaCl) and a high molarity salt buffer-‘B’ (10 mM HEPES (pH 7.6), 1 M NaCl). Reductant (2 mM DTT) was added fresh to buffers when required. Proteins were diluted ten-fold in the low-molarity salt buffer-A in order to decrease the starting salt concentration before being loaded onto the column by way of a S9H sample pump (GE Healthcare). Once bound, the column was washed using 10 column-volumes of buffer-A. Proteins were eluted by a gradient of 0 to 50% buffer-B over 25 column volumes at a flow rate of 4 mL/min.

2.6.10 Size-exclusion chromatography (SEC)

SEC was routinely performed using either a Superdex 75 10/300 (S75) or Superdex 200 Increase 10/300 (S200I) (both GE Healthcare). Proteins were typically concentrated (2.6.7) to ∼0.5 mL before being centrifuged at 20,000 g 4 °C for 5 minutes to pellet any particulate or insoluble material. Samples were injected onto the column by way of a 2 mL sample loop. SEC was conducted at a flow rate of 0.8 mL/min (Superdex 75) or 0.5 mL/min (Superdex 200 Increase) in a buffer of 300 mM NaCl, 10 mM HEPES pH 7.6 with the addition of fresh reductant (2 mM DTT or 0.5 mM tris(2-carboxyethyl)phosphine (TCEP; Hampton Research), where appropriate.

2.6.11 Quantification of protein concentration

Proteins concentrations were quantified using a NanoDrop2000 and measuring their absorbance at 280 nm. Concentration (in mg/mL) was adjusted using the calculated extinction coefficients from the ProtParam web tool (web.expasy.org/protparam, Gasteiger et al., 2005).
2.6.12 Freezing of proteins

Proteins were routinely aliquoted into PCR-strip tubes (Axygen) in 30–200 µL volumes before being snap-frozen in liquid nitrogen and stored at −80 °C.

2.6.13 Purification of His\textsubscript{6}-ASK\textsubscript{1\textsubscript{88–941}}-StrepII from \textit{E. coli}

A C-terminal StrepII tagged ASK\textsubscript{1\textsubscript{88–941}} construct enabled more efficient purification compared to the equivalent construct without the C-terminal StrepII tag.

ASK\textsubscript{1\textsubscript{88–941}}-StrepII was co-expressed with untagged \lambda-phage protein phosphatase (APP) (2.2) to dephosphorylate potential ASK1 auto-phosphorylation during expression. Transformations were the same as in 2.3.11 but ~100 ng of each plasmid was used to transform 75 µL of BL21(DE3) cells and left to incubate on ice for at least 30 minutes. Transformation proceeded as previous with cells being plated on agar-LB with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol. Cultures were inoculated as for other constructs, with the exception of 50 µg/mL kanamycin and 34 µg/mL chloramphenicol both being added to the culture. Culture growth, induction by IPTG, and harvesting were the same as previous (2.6.2).

Lysis was similar as in 2.6.3 with the exception that the cell pellets were resuspended in strep-purification buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% (v/v) glycerol, 10% (w/v) sucrose). Cell homogenate was clarified as previous (2.6.4). Clarified lysates were transferred to 50 mL tubes. StrepTactin XT SuperFlow resin (IBA-Lifesciences), pre-equilibrated in strep-purification buffer was added to each tube for 1 mL of resin bed per 1 L of original culture. The tubes were placed on an orbital rotator for 1 hour at 4 °C. StrepTactin resin was washed in batch by three repeat rounds of pelleting resin at 3,500 g for 5 minutes, 4 °C. Pelleted resin was washed after each centrifugation step by resuspension in between 30 and 40 mL of strep-purification buffer. After the final wash, the resin was pelleted as previously, before being resuspended in 10 mL of strep-purification buffer and transferred to a Poly-Prep Chromatography hand-column. Proteins were eluted from resin in 4 × 500 µL fractions in a buffer consisting of 5 mM biotin (Sigma) dissolved in strep-purification buffer. Eluted fractions were pooled, DTT was added to 2 mM and 3C-protease added as previous (2.6.6).
Cleaved ASK1\textsubscript{88-941} proteins were concentrated to a total volume of less than 0.5 mL before SEC on S200I as described in 2.6.10. Eluted protein was aliquoted and snap-frozen (2.6.12) as soon as possible, before storage at \(-80\) °C.

### 2.6.14 Purification of full-length ASK1 from Sf\textsubscript{9} insect cells

All insect culture (bacmids, baculovirus production, cell culture, infection and harvesting) was performed by Abigail Burgess and Jack Curry (both Mace laboratory). In brief, Sf\textsubscript{9} cells were infected with an ASK1 2–1374 (full length) containing baculovirus and grown for three days before harvesting by centrifugation. Cell pellets were stored at \(-20\) °C before processing.

Sf\textsubscript{9} cell pellets (equivalent to 20 mL of culture) were thawed and resuspended in 30 mL of insect-purification buffer. DTT was added to a concentration of 2 mM as was 40 µL of P8340 protease inhibitor cocktail (Sigma) and 15 µg of DNaseI. Cells were immediately sonicated using a Sonifer Cell Disrupter, output 7, 70% amplitude, with pulsing, for 2 minutes, on ice. Lysed cells were clarified by centrifugation as in 2.6.4.

Clarified insect lysate was transferred to a 50 mL tube with 600 µL of HIS-Select resin bed (that had been pre-equilibrated in insect purification buffer). Protein was allowed to bind to resin at 4 °C on an orbital rotator for 1 hour. The resin was washed in batch with three rounds of sequential centrifugation (3,500 \(g\), 5 minutes, 4 °C). The supernatant was removed and resin resuspended in 30 mL of insect-purification buffer. After the final wash, the resin was resuspended in 5 mL of insect purification buffer and transferred to a hand-column. Resin was allowed to settle and protein was eluted with 500 mM imidazole diluted in insect-purification buffer.

Eluted protein was analysed for presence of full-length (2–1374) ASK1 by SDS-PAGE analysis (2.4). Protein containing fractions were aliquoted and snap-frozen (2.6.12) before being stored at \(-80\) °C. An example gel is given in Appendix A.2g, showing the 150 kDa full-length ASK1 with a partial degradation product (roughly \(\sim\)100 kDa in size).
2.6.15 Summary of protein purification methodologies by construct

Table 2.1 outlines which purification methodologies were applied to each protein construct. In general, all proteins were treated with 3C-protease to cleave the His$_6$-tag except where the presence of His$_6$-tag was required.

2.7 In vitro kinase assays

Purified recombinant human proteins were used in all kinase assays. Kinase dead MKK6$^{K82A}$, hereafter referred to as MKK6, was used as a substrate. Individual components of kinases assays were kept on ice until use where they were allowed to warm to room temperature. Kinase assays were carried out at room temperature in a buffer containing a final concentration of 10 mM HEPES (pH 7.6), 100 mM NaCl, 25 mM MgCl$_2$. Reactions were set up as a master mix containing buffer and, where appropriate, substrate and enzyme.

Reactions were always started with the addition of either 50 µM or 1 mM ATP. To facilitate simultaneous starting and sample taking reactions were set up in PCR strip tubes using a multichannel pipette. At each time point, 15 µL of each reaction was removed, simultaneously and then mixed directly into 5 µL of a 4× SDS-sample buffer (reducing) (2.4.2). Before the addition of ATP a sample was removed (denoted as ‘time 0’). ATP was added and the samples were mixed and briefly spun down and incubated for the desired length of time. Once all time-point samples were taken, samples were always frozen at −20 °C. Samples were thawed at room temperature before analysis by either Phos-Tag SDS-PAGE (2.4.5 and 2.7.1.1) or western blotting for p-MKK6 (2.5 and 2.7.1.2).
<table>
<thead>
<tr>
<th>Protein</th>
<th>Residues</th>
<th>Tag(s)</th>
<th>Expression system</th>
<th>Purification method(s)</th>
<th>Size (kDa)</th>
<th>Example purification</th>
</tr>
</thead>
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<tr>
<td>ASK1</td>
<td>88–266</td>
<td>N-terminal-His&lt;sub&gt;6&lt;/sub&gt;-3C</td>
<td><em>E. coli</em></td>
<td>Ni-A, SEC-75</td>
<td>20.3</td>
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<td><em>E. coli</em></td>
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<td></td>
<td>88–658</td>
<td>N-terminal-His&lt;sub&gt;6&lt;/sub&gt;-3C, C-terminal StrepII</td>
<td><em>E. coli</em></td>
<td>Ni-A, IEX, SEC-200</td>
<td>65.5</td>
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<tr>
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<td>269–658</td>
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<td><em>E. coli</em></td>
<td>Ni-A, IEX</td>
<td>66.3</td>
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<td>669–941</td>
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<td><em>E. coli</em></td>
<td>Ni-A, SEC-200</td>
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<td>C-terminal StrepII (Trx1 co-expression)&lt;sup&gt;3&lt;/sup&gt;</td>
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<td>Streptactin, SEC</td>
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<tr>
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<td>2–1374</td>
<td>N-terminal-His&lt;sub&gt;6&lt;/sub&gt;-3C</td>
<td>Insect</td>
<td>Ni-A</td>
<td>98.1</td>
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<td>MKK6</td>
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<td><em>E. coli</em></td>
<td>Ni-A, SEC-200</td>
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<td>Ni-A, SEC-200</td>
<td>11.8</td>
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<td><em>E. coli</em></td>
<td>Ni-A, IEX-200</td>
<td>11.7</td>
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<td>Ni-A</td>
<td>24.2</td>
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</table>

All proteins sequences are for human, with the exception of the T7 phage lysozyme and APP. WT and relevant mutant protein constructs had identical purification strategies. 1) Refers to 2.6. 2) Expected size is as calculated following cleavage (on constructs that were purified) of tag with 3C protease, note His<sub>6</sub>-Prdx1 was never cleaved. 3) Construct used for SAXS and had the following mutations: Cys206Ser, Cys225,226Ser (A C-terminal StrepII tag was also present, but this was not used for purification). Ni-A—HIS-Select nickel affinity; IEX—anion exchange chromatography; SEC-200/75—SEC on either S200I or S75 column; n/a—not applicable, construct was not purified; App. Fig.—Appendix Figure.
2.7.1 Data analysis of kinase assays

2.7.1.1 Phos-Tag SDS-PAGE kinase analysis

For kinase assays analysed by Phos-Tag SDS-PAGE, Coomassie stained gels were imaged in the 700-channel on an Odyssey FC (LI-COR). From each lane, bands representing either the phosphorylated protein or unphosphorylated parent were quantified using ImageStudio (LI-COR). The sum of the two bands was used to calculate the total MKK6. Phosphorylated MKK6 was calculated as a percentage of the total and converted to a concentration by multiplying by the concentration of MKK6 used in the assays (3 µM).

2.7.1.2 Western blot kinase assay analysis

For kinase assays analysed by western blot, blots were imaged on an Odyssey FC (LI-COR). For each lane, the phosphorylated MKK6 band was quantified using ImageStudio (LI-COR). Band intensity for each lane was normalised by expressing individual intensity as a percentage of the signal at the 10 minute time point.

2.8 Nickel affinity pull-down of ASK1 and Trx1 in vitro

Bl21(DE3) cells expressing His-tagged ASK constructs and untagged Trx1 were grown, induced and pelleted as before (2.6). Pellets (equivalent to 33 mL of culture) were thawed and resuspended in 1 mL standard-purification buffer before being incubated with 250 µg of hen-egg lysozyme and sonicated for one minute at 35% amplitude with pulsing on a Vibra Cell (Sonics). HIS-Select resin, pre-equilibrated in standard-purification buffer was added to the His6-tagged ASK1 constructs with 2 mM DTT. Proteins were allowed to bind the resin at 4 °C on a rotator for 30 minutes. Bound proteins on resin were subsequently washed with standard-purification buffer in three repeated rounds of centrifugation (4,000 g at 4 °C for 5 minutes). The resin was pelleted and resuspended in a buffer (containing 25 mM HEPES, pH 7.6, 150 mM
NaCl) whereupon it was divided into two tubes per ASK1 construct. Untagged Trx1 containing *E. coli* clarified lysate (100 µL) was added to the tubes and the volume was adjusted to 200 µL with buffer (25 mM HEPES (pH 7.6), 150 mM NaCl). Half of the tubes had DTT added to a final concentration of 2 mM. The tubes were placed on a rotator at 4 °C for 30 minutes before being centrifuged and washed three times with 25 mM HEPES, pH 7.6, 150 mM NaCl (either with or without 2 mM DTT) to remove unbound Trx1. The resin was pelleted, resuspended in 25 µL of 2 × SDS buffer before boiling and resolving by SDS-PAGE (2.4). Proteins were transferred to nitrocellulose (2.5.2) before probing using antibodies against His$_6$ and Trx1 (2.5).

### 2.9 Preparation of disulfide-linked ASK1-Trx1 complex for SAXS

Nickel affinity purified ASK1$_{88-658}^{C206S,C225,226S}$ with a C-terminal StepII tag and Trx1$_{C35S, C73S}$, C-terminal-Lys-His$_6$ was prepared as in 2.6 (referred to as ASK1 and Trx1-Lys for the remainder of this section). Both ASK1 and Trx1-Lys were reduced for 1 hour on ice with 2 mM DTT before either IEX for ASK1 (as per 2.6.9) or SEC for Trx1-Lys as per 2.6.10. ASK1 was aliquoted and snap-frozen (2.6.12) immediately post SEC. Trx1 was allowed to sit at 4 °C for 5 days before aliquoting and freezing.

From 6 L of *E. coli* culture, approximately 10 mg of purified ASK1 was produced versus 10 mg of Trx1-Lys from 1 L of culture. Proteins were thawed and mixed (at roughly equimolar amounts—approximately 10 mg of ASK1 and 5 mg of Trx1-Lys) in a total volume of 7.1 mL in a buffer containing 10 mM HEPES, pH 7.3, 300 mM NaCl. The proteins were incubated in a 37 °C water bath for 4 hours. After incubation, the proteins were alkylated by addition of iodoacetamide (IAM; Sigma) to a final concentration of 10 mM. Alkylation occurred in the dark at room temperature for 30 minutes. Proteins were concentrated by ultrafiltration (2.6.7). Concentrated proteins were separated by three rounds of SEC on a S200I column, running at a flow rate of 0.5 mL/minute in a buffer consisting of 10 mM HEPES (pH 7.6), 300 mM NaCl. Relevant fractions were pooled, concentrated by ultrafiltration (2.6.7), aliquoted and
2.10 SEC-Small-angle X-ray scattering

All SEC-small-angle X-ray scattering (SAXS) data collection was carried out at the Australian Synchrotron SAXS/WAXS beamline under the guidance of Associate Professor James Murphy (Walter and Eliza Hall Institute, Melbourne, Australia). Fifty microlitres of purified recombinant protein was injected onto an in-line Superdex 200 5/150 column (GE Healthcare) by way of an auto-sampler. Proteins were eluted from the column at 12 °C at a flow rate of 0.2 mL/min in a buffer consisting of 10 mM HEPES pH 7.5, 500 mM NaCl, 5% (v/v) glycerol. Proteins eluted from the column into a 1.5 mm glass-capillary positioned in the X-ray beam using co-flow to reduce radiation damage to the sample (Kirby et al., 2016).

Initial data reduction and background subtraction were performed by Associate Professor James Murphy (Walter and Eliza Hall Institute, Melbourne, Australia). Briefly, scattering profiles of samples were collected in 2-second exposures during the course of elution. 2D intensity plots were averaged radially and normalised to the sample transmission. Background scatter was assessed by Scatterbrain software (Stephen Mudie, Australian Synchrotron). The background scatter was determined by comparing the scattering profiles from before and after protein elution on SEC. Six profiles for ASK1_{88-658}C^{206S, C^{225,226S}}, six profiles for Trx1_{C^{35S}}-Lys and nine profiles for the ASK1-Trx1 complex were averaged and background-subtracted using Scatterbrain to produce the scatter patterns presented in this work.

2.10.1 Analysis of SAXS data

The majority of the software used for SAXS data analysis was carried out using the ATSAS package (Franke et al., 2017) following the instructions for each individual package. Pair-distance distribution function (P(r)) and the maximum dimension (D_{max}) were determined using GNOM and adjusted manually (Svergun, 1992). The radius of gyration (R_g) and was calculated by both Guinier analysis (within the program.
2.10.1.1 Estimation of molecular weight

Molecular weights were estimated using the SAXS Molecular Weight server (SAXS-MoW; saxs.ifsc.usp.br; Fischer et al. 2010) with \( q = 8/R_g \) used as the integration limit.

2.10.1.2 Rigid body modelling

Rigid body modelling was carried out using CORAL (Petoukhov et al., 2012) as per the default user instructions. More details are given in Chapter 4. For each species to be modelled a conditions file was written specifying the domains to model, extra residues (N- and C-terminal as well as inter-domain linkers). Parameters for the conditions file are given in Appendix A.6. ASK1\(_{88-266}\) was modelled using the previously published (Weijman et al., 2017) de novo constructed using the Robetta server (Kim et al., 2004).

For the ASK1-Trx1 complex species, a constraint was placed on the modelling based on the disulfide linkage whereby the alpha-carbon (\(\alpha C\)) atoms from the relevant cysteine residues was constrained to be no more than 10 Å away. This was based on observations by Schmidt et al. (2006) whereby the mean distance between the two \(\alpha C\) atoms in 6874 measured disulfide bonds was 5.63 Å. However, using a distance of less than 10 Å resulted in overlapping protein structures from CORAL modelling. CRYSOL (Svergun et al., 1995) was used to calculate theoretical scattering profiles of the models to compare the data and generate goodness of fit (represented by the chi, \(\chi\)) values.

2.11 Mammalian cell culture

All mammalian cell culture was carried out in a Class II biological safety cabinet. Cells were maintained in a 37 °C humidified incubator with 5% (v/v) CO\(_2\).
2.11.1 Cell counting

All cell counting was done using a TC10 automated cell counter and disposable counting slides (both Bio-Rad) as per the manufacturer’s instructions.

2.11.2 Culture of HAP1 cells

HAP1 WT cells (catalogue number: C859) and Prdx1 knock-out (KO) cells (catalogue number: HZGHC002675c008) were purchased from Horizon Discovery. Background information on HAP1 cells is given in 5.5.1. The Prdx1 KO cells contain a 172 base pair deletion in exon 4 of the \textit{PRDX1} gene. HAP1 cells were maintained in Iscove’s modified Dulbecco’s medium (IMDM) (Gibco and HyClone) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma and Gibco), 100 µg/mL streptomycin sulfate and 100 units/mL penicillin G (both Sigma).

Cells were passaged every 2 to 3 days by aspirating media from cells and washing with 5 mL (for a T75 flask) of with pre-warmed sterile phosphate buffered saline (PBS; one PBS tablet (Oxoid) was dissolved in 100 mL type I water and autoclaved at 121 \degree C, 100 kPa, for 20 minutes). PBS was aspirated and 3 mL of 0.05% (w/v) trypsin-EDTA (Gibco) diluted in sterile-PBS was added. The flask was returned to 37 \degree C incubator for 5 minutes before being firmly tapped to encourage detachment. Cells were resuspended and trypsin was neutralised by the addition of 5 mL of pre-warmed complete media. Cells were typically diluted 1 in 20 in fresh, pre-warmed, complete media and returned to a flask.

2.11.3 HAP1 p38 response to H\textsubscript{2}O\textsubscript{2} treatment

2.11.3.1 Suspension H\textsubscript{2}O\textsubscript{2} treatment of HAP1 cells

For H\textsubscript{2}O\textsubscript{2} treatment of HAP1 cells, cells were either treated in an adherent state, or in a suspension state post-trypsinising. For cells treated in a suspension state, cells were grown as in 2.11.2. On the day of the assay, cells were trypsinised (as in 2.11.2) and counted. Cells were plated in a 6-well plate (Corning) with $2 \times 10^6$ cells per well in 3 mL of complete pre-warmed media. Cells were allowed to rest for 30 minutes. After
resting, \text{H}_2\text{O}_2 (100 \mu\text{M} in \text{type I water, determined spectrophotometrically at } \Lambda_{280}\text{ nm using the absorbance coefficient for } \text{H}_2\text{O}_2 of 43.6 \text{ cm}^{-1}\text{ M}^{-1}; \text{Noble and Gibson 1970}) was added to the media in reverse time-course so each time point had a common end-point. Cells were returned to the incubator between treatments.

At the end-point, the 6-well plate was transferred to a cold-stage consisting of an aluminium sheet on ice. Ice-cold PBS containing bovine catalase (Sigma, 20 \mu\text{g/mL, referred to as PBS-catalase hereafter) was added (3 mL per well). Cells were transferred to 15 mL tubes containing 3 mL of PBS-catalase. Cells were pelleted at 800 g, 4 °C for 5 minutes. The supernatant was aspirated and cells were resuspended in 1 mL of ice-cold PBS-catalase and transferred to a 1.5 mL tube. Cells were pelleted by centrifugation (800 g, 4 °C for 5 minutes). The supernatant was aspirated and the cells were resuspended in 1 mL of ice-cold PBS and pelleted as before. The supernatant was aspirated and the cells were resuspended in 40 \mu\text{L of radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1\% (v/v) Triton X-100, 0.5\% (w/v) sodium deoxycholate, 0.1\% (w/v) SDS, 50 mM Tris (pH 8.0), 5 mM EDTA, 1 mM EGTA, cOmplete protease inhibitor (Roche), 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate). Cells in lysis buffer were incubated on ice for 20 minutes with periodic vortexing. The cell lysate was clarified by centrifugation (15,000 g, 4 °C, 15 minutes). Soluble material was aliquoted and snap-frozen in liquid nitrogen. Clarified lysate protein concentration was determined by bicinchoninic acid (BCA) assay (2.11.4) and between 20 to 30 \mu\text{g of lysate was analysed by western blot (2.5) following SDS-PAGE and wet-transfer to nitrocellulose.}

2.11.3.2 Adherent \text{H}_2\text{O}_2 treatment of HAP1 cells

For HAP1 cells treated in an adherent state, cells were grown as in 2.11.2. HAP1 cells were seeded in a 6-well plate at 1 \times 10^6 \text{ cells/mL in 3 mL of complete media. Twenty-four hours after plating, the media was aspirated and cells were washed with pre-warmed sterile PBS. Fresh pre-warmed complete media (2 mL) was added to the cells and the plate as returned to the incubator for cells to rest for 1 hour. \text{H}_2\text{O}_2 (100 \mu\text{M}) was added as in 2.11.3.1, above.
At the end point, 6-well plates were placed on a cold-stage and 3 mL of ice-cold PBS-catalase was added. The wells were aspirated and cells washed with an additional 3 mL of ice-cold PBS-catalase. The wells were aspirated and cells washed in 3 mL of ice-cold PBS. The wells were aspirated and 0.5 mL of ice-cold 10% (v/v) trichloroacetic acid (TCA: Sigma) was added to each well to lyse cells and precipitate proteins (Jarvis et al., 2012; Lee and Esselman, 2002). Plates were incubated on the cold stage for 10 minutes before cells were scraped off using either a 1 mL pipette tip or the plunger from a 1 mL syringe. The precipitated cell lysate was transferred to a 1.5 mL tube and the well and scraper were rinsed with an additional 0.5 mL of ice-cold 10% (v/v) TCA, which was transferred to the same tube. Precipitated material was pelleted by centrifugation (10,000 g, 10 minutes, 4 °C). After pelleting, the majority of the TCA was removed and approximately 100 µL was left. Leaving a small amount of TCA helped to fully resuspend the pellet. The pellet was resuspended in the remaining TCA before the addition of 1 mL of ice-cold acetone and centrifugation (10,000 g, 10 minutes, 4 °C). The supernatant was aspirated (taking care not to touch the pellet as it would block the pipette tip). The pellet was washed in acetone for a total of three washes. For each resuspension step, the pellet was originally resuspended in 200 µL of acetone, using a 200 µL pipette tip with the end cut off. An additional 500–800 µL of acetone was then washed. After the final wash, acetone was removed and the pellet left to dry in a 50 °C heat block for approximately 10–15 minutes until there was no longer a smell of acetone. Dried cell-lysate pellets were stored at −80 °C.

For analysis, the pellets were resuspended in a buffer containing 200 mM Tris (pH 8.5), 5 mM EDTA, 0.05% (w/v) SDS and 8 M urea (urea was always added fresh). To each dried pellet, 40 µL of buffer was added. Samples were vortexed for 5 minutes before sonication in a water bath for 10 minutes and a further 5 minutes vortexing. Insoluble material was removed by centrifugation (10,000 g, 4 °C, 5 minutes). Soluble material was transferred to a fresh tube. Protein quantification was determined by a BCA assay (2.11.4) and analysed by western blot (2.5).
2.11.4 Protein quantification by BCA assay

Soluble protein from mammalian cell lysate was routinely quantified by BCA assay (Thermo Scientific), as per kit instructions. In brief, a 0.5 mg/mL stock of BSA (determined spectrophotometrically based on a 0.5 mg/ml solution having an $A_{278}$ absorption value of 0.327 (Kirschenbaum, 1973)) was dissolved in type I water, stored at $-20$ °C. A working BSA protein standard was made by two-fold serial-dilution of the BSA stock in 1% (v/v) Triton-x-100. Protein samples were diluted (generally between 1 in 10 and 1 in 50) in 1% (v/v) Triton-x-100. A working BCA reagent was made by combining 50 parts of reagent A to 1 part reagent B. Enough working reagent was made for 200 µL per sample. Protein standards and samples were added to a 96-well plate (between 10 and 20 µL per well) in triplicate. A buffer blank matching the buffer the sample was in was also prepared and added to the 96-well plates before the addition of 200 µL (per well) of working BCA reagent. Samples and standards were set up in triplicate. The plate was covered with parafilm and incubated at 37 °C for 30 minutes before reading in a Multiskan GO (Thermo Scientific) plate reader at 562 nm. The BCA protein standard was used to generate a standard curve from which the sample protein concentration was determined.

2.12 ICAT-labelling of reversibly oxidised protein cysteines in HAP1 cells

ICAT-labelling was conducted following the instructions from the ICAT kit (Sciex, product number: 4339036) with amendments based on García-Santamarina et al. (2014) and Kumar et al. (2013b). The full method used is given below.

2.12.1 Cell treatment

HAP1 WT and Prdx1 KO cells were grown as in 2.11.2. 150 mm cell culture dishes (Corning and Falcon) were seeded with $1 \times 10^6$ cells/mL in 20 mL of complete media 24 hours prior to treatment. For each time point, one dish was prepared. Seeding at
this density ensured that cells were fully confluent on day of treatment. On the day of treatment (24 hours post-seeding), the media was aspirated and cells were washed with 10 mL of pre-warmed PBS. PBS was aspirated and replaced with 10 mL of pre-warmed complete media. Cells were returned to the 37 °C incubator and allowed to rest for 1 hour. A 2 mM stock of \( \text{H}_2\text{O}_2 \) was prepared (in type I water; the concentration determined as in 2.11.3.1). Cells were treated with 500 \( \mu \text{L} \) of the working \( \text{H}_2\text{O}_2 \) (100 \( \mu \text{M} \) final concentration) and returned to the 37 °C incubator for the duration of the assay. Samples were set up in reverse order so they would have a common endpoint but no more than three dishes were treated and harvested in one session. Untreated cells had an equal volume of type I water added to them and were immediately harvested.

2.12.2 TCA harvesting

After cell treatments, dishes were placed on a cold stage (comprised of an aluminium sheet on ice). Ten millilitres of ice-cold PBS—containing 20 \( \mu \text{g/mL} \) bovine liver catalase (Sigma)—was added to the dishes. Media and PBS-catalase were aspirated and replaced with an additional 10 mL of PBS-catalase. The PBS-catalase was aspirated and the cells were rinsed with an additional 10 mL of PBS. The PBS wash was aspirated before 10 mL of ice-cold 10% TCA (\( w/v \)) was added. Cells were allowed to lyse in the dish on the cold-stage for 15 minutes. Cells were carefully scraped with a cell scraper (Nunc). Cell homogenate was transferred to a 15 mL tube and precipitated material collected by spinning at 4,500 \( g \) for 10 minutes at 4 °C. All but 1 mL of the TCA-containing supernatant was aspirated from the pellet. The pellet was resuspended in the remaining supernatant and transferred to a 1.7 mL tube. The material was pelleted again by spinning at 10,000 \( g \), 4 °C, 5 minutes. The supernatant was removed and 500 \( \mu \text{L} \) of cold acetone was added. Using a 200 \( \mu \text{L} \) tip (with the end cut-off) the pellet was carefully resuspended in acetone to attempt to break it apart as much as possible. An additional 500 \( \mu \text{L} \) of cold acetone was added to the sample and it was vortexed for \( \sim 30 \) seconds to dislodge the pellet from the bottom of the tube. The material was centrifuged (10,000 \( g \), 4 °C, 5 minutes) and the supernatant removed. The cold acetone wash was repeated for a total of three washes. Residual acetone was
removed by placing tube with the lid open in a 50 °C heat-block for approximately 20 minutes until pellet was dry. Pellets were stored at −80 °C until further processing.

2.12.3 Resuspension and alkylation of reduced cysteine thiols

Pellets (from 2.12.2) were removed from −80 °C and centrifuged at 10,000 g for 5 minutes at room temperature to ensure all material was at the bottom of the tubes. The pelleted material was gently crushed to a dust-like appearance using a plastic pestle. A 5 × ICAT resuspension buffer (containing 1 M Tris (pH 8.0), 0.25% (w/v) SDS, 25 mM EDTA) was prepared and stored at 4 °C and diluted fresh with additional components when required. Fresh ICAT-alkylating buffer (8 M urea, 200 mM Tris (pH 8.0), 0.05% (w/v) SDS, 5 mM EDTA, 100 mM IAM) was prepared and kept protected from light. Five hundred microlitres of ICAT-alkylating buffer was added to each sample and an additional 500 µL was used to rinse any material off of the pestle into the same tube. Samples were kept protected from light before being vortexed for 5 minutes and sonicated in a water bath for 10 minutes before an additional 5 minutes of vortexing. Any insoluble material\(^1\) was pelleted by centrifugation at 10,000 g, 5 minutes, 4 °C and the supernatant was transferred to a fresh 1.7 mL tube. The samples were incubated at room temperature in the dark for an additional 30 minutes to ensure complete alkylation of protein thiols. Protein was subsequently precipitated by the addition of cold TCA to a final concentration of 10% (w/v). TCA-precipitation was allowed to incubate on ice for 10 minutes before being centrifuged (10,000 g, 4 °C, 5 minutes). The precipitated material was washed three times in cold acetone and dried, identical to as described above (2.12.2). The dried, alkylated material was stored at −80 °C.

2.12.4 Resuspension and reduction of oxidised cysteine thiols

Pellets (from 2.12.3) were resuspended in fresh ICAT-resuspension buffer (8 M urea, 200 mM Tris (pH 8.0), 0.05 % (w/v) SDS, 5 mM EDTA) identical to previous (2.12.3). A BCA assay (2.11.4) was performed to quantify the protein amount in each sample.

\(^1\)An insoluble, jelly-like pellet always formed at each resuspension step.
From a fully confluent 150 mm dish, between 0.5 and 2 mg of lysate was achieved. The relatively low-yield from was due to an insoluble pellet that formed after each resuspension step (see Footnote 1). Five hundred micrograms of material (as determined by BCA assay) was transferred to a 1.7 mL tube. The volume of each tube was adjusted to 384 µL with ICAT-resuspension buffer and 16 µL of 0.5 M TCEP was added (final concentration of 20 mM). Reduction of protein thiols by TCEP took place at 30 °C for 30 minutes.

### 2.12.5 ICAT-labelling and trypsin digestion

The ICAT reagents (from the ICAT kit) were allowed to warm to room temperature after being removed from −20 °C. The vials were briefly centrifuged before opening, whereupon each vial of ICAT reagent had 200 µL of 100% acetonitrile added. The ICAT-labelling reagent was vortexed for ~30 seconds before being spun down again. The alkylated and reduced cell lysates (from 2.12.4) to be labelled were briefly spun down.

The entire sample was transferred to the resuspended vial of ICAT-labelling reagent and volume adjusted with fresh ICAT-resuspension buffer for a final volume of 750 µL. This was then further vortexed for 30 seconds before being briefly spun down again. The tubes were wrapped in foil and placed in a shaking 37 °C incubator for 2 hours. Any unreacted ICAT reagent was quenched by the addition of 1 µL of 1 M DTT followed by incubation at room temperature for 5 minutes.

Relevant heavy- and light ICAT-labelled samples were combined into a fresh 15 mL tube. Samples were diluted to 10 mL with type I water in order lower urea concentration to below 1 M, and CaCl₂ was added to a final concentration of 10 mM. Trypsin (Promega) was resuspended in trypsin resuspension buffer (Promega) for a final concentration of 0.5 µg trypsin per 1 µL. Trypsin was added in a 1:20 protease:protein ratio—i.e. 50 µg trypsin per 1 mg of lysate. Tubes were wrapped in foil and lids were wrapped in parafilm. Samples were placed in a shaking 37 °C incubator overnight (for at least 16 hours).
2.12.6 Desalting of ICAT-labelled samples

Digested samples (from 2.12.5) were removed and allowed to cool to room temperature. The samples were acidified to between pH 2–3 with the addition of formic acid, this was checked by pipetting 10 µL of the sample onto a pH indicator strip. Approximately 300 µL of 100% (v/v) formic acid was sufficient for pH adjustment.

The samples were desalted using Oasis HLB Plus LP extraction cartridges (Waters). A 1 mL tip (with the end cut-off) was placed in the top of the cartridge and a pipette used to push the solutions through. The cartridges were conditioned with 5 mL of 100% (v/v) acetonitrile before being equilibrated with 5 mL of 5% (v/v) formic acid in type I water. The sample was slowly (dropwise) loaded into the cartridge. The cartridge was washed with 5% (v/v) formic acid in type I water. Samples were eluted from the columns by passing 5 mL of 70% (v/v) acetonitrile, 5% (v/v) formic acid in water through and collecting the elution. The samples were dried in a centrifugal vacuum concentrator (Savant) before storage at −80 °C.

2.12.7 Clean-up of ICAT-labelled samples using the ICAT-kit

ICAT-labelled samples were further processed as per the ICAT-kit instructions with the notable difference that in-house solutions were used and not those supplied with the kit (with the exception of the column storage buffers). The samples were each split into four parts for processing through a strong-cation exchange and biotin affinity column. The columns supplied with the ICAT kit required the use of a specialised adapter, which was borrowed from the Otago Centre for Proteome Research. A blunt, ø 0.7 mm injection port needle was used alongside 1 mL plastic syringes to inject samples and solutions. The needle was thoroughly washed with type I water between solution changes and sample changes.

The dried, desalted, peptides (from 2.12.6) were resuspended in 2 mL of cation-load buffer (10 mM KH₂PO₄, 25% (v/v) acetonitrile, pH 3.0). Tubes were vortexed for one minute, heated at 50 °C for 5 minutes, vortexed for one minute, sonicated in a water bath for one minute and vortexed for a further minute before being briefly centrifuged to bring everything to the bottom.
2.12.7.1 ICAT strong-cation exchange

The ICAT strong-cation exchange cartridge was washed with 2 mL of cation-clean buffer (10 mM KH$_2$PO$_4$, 25% (v/v) acetonitrile, 1 M KCl, pH 3.0). The column was equilibrated with 4 mL of cation-load buffer. One-quarter of the resuspended sample (~250 µL) was slowly applied (drop-wise) to the column. The column was washed with 1 mL cation-load buffer. Peptides were eluted drop-wise with 0.5 mL of cation-elute buffer (10 mM KH$_2$PO$_4$, 25% (v/v) acetonitrile, 350 mM KCl, pH 3.0). The column was cleaned with 2 mL of cation-clean buffer then the steps were repeated, from equilibration, until the entirety of one sample was processed. In between different samples, the column was washed with an additional 2 mL of cation-storage buffer (10 mM KH$_2$PO$_4$, 25% (v/v) acetonitrile, 0.1% (w/v) NaN$_3$, pH 3.0), after which all steps were repeated for the next sample, from the first cation-clean buffer wash. Eluted peptides were stored overnight at 4 °C.

2.12.7.2 ICAT biotin affinity

Eluted cation-exchange samples were pH adjusted with an equal volume of affinity-load buffer (20 mM NaH$_2$PO$_4$ 300 mM NaCl, pH 7.2)—i.e. 2 mL of total cation-exchange elution was neutralised with 2 mL of affinity-load buffer. The pH of the sample was checked with a pH indicator strip to check it was between pH 7.0–7.2. The affinity column was washed with 2 mL of affinity-elution solution (30 % acetonitrile (v/v) 0.4 % (v/v) TFA) before being equilibrated with 2 mL of affinity-load buffer. One-quarter of the sample (~1 mL) was loaded drop-wise. The column was washed with 500 µL of affinity-load buffer followed by 1 mL of affinity-wash(1) buffer (10 mM NaH$_2$PO$_4$ 150 mM NaCl, pH 7.2). The column was washed further with 1 mL of affinity-wash(2) buffer (50 mM NH$_4$HCO$_3$ 20% (v/v) methanol, pH 8.3) followed by 1 mL of type I water.

The sample was eluted drop-wise from the column with 800 µL of affinity-elution solution. The first 50 µL of this (2 drops) was discarded. For processing the remainder of the same sample, the process was repeated from the beginning. In between samples, the column was washed additionally with 2 mL of affinity-elution solution and 2 mL.
of affinity-storage buffer (20 mM NaH₂PO₄ 300 mM NaCl, 0.1% (w/v) NaN₃, pH 7.2) before repeating from the beginning for the next sample. Eluted peptides were dried in a SpeedVac centrifugal evaporator (Savant) before being stored at −80 °C.

2.12.7.3 Cleavage of biotin-tag on ICAT-labelled samples

The biotin tag on the ICAT-labelled peptides is cleaved to help the LC-MS/MS. Cleavage required two proprietary reagents (Reagent A and B) supplied with the ICAT kit. Cleavage reagent A and B were mixed in a 95:5 ratio. For cleavage, 150 µL of fresh working cleavage reagent was added to the dried affinity-eluted peptides. The samples were vortexed for one minute, sonicated in a water bath for one minute, before vortexing for a further minute. The tubes were placed in a 37 °C shaking incubator for 2 hours for cleavage to occur. Following cleavage, samples were allowed to cool to room temperature, briefly centrifuged before drying peptides in a centrifugal evaporator and stored at −80 °C.

2.12.7.4 Sep-Pak clean-up of ICAT-labelled samples post cleavage

Dried, cleaved, ICAT-labelled peptides (from 2.12.7.3) were further cleaned up by using SepPak single-use cartridges (Waters). Peptides were resuspended in 2 mL of 2% (v/v) acetonitrile, 0.2% (v/v) formic acid. The tubes were vortexed for one minute, heated at 50 °C for 5 minutes, vortexed for one minute, sonicated in a water bath for 1 minute, before a final minute of vortexing. Samples were briefly centrifuged to collect all solution at the bottom of the tube. A SepPak cartridge was conditioned with 5 mL 100% (v/v) methanol followed by 5 mL type I water. The column was washed with 5 mL of 70% (v/v) acetonitrile, 0.2% (v/v) formic acid, before being equilibrated with 5 mL of 2% (v/v) acetonitrile, 0.2% (v/v) formic acid. The sample was applied drop-wise to the cartridge and flow-through re-applied. Cartridges were washed with 5 mL of 2% (v/v) acetonitrile, 0.2% (v/v) formic acid. Bound peptides were eluted with 5 mL 70% (v/v) acetonitrile, 0.2% (v/v) formic acid. Eluted peptides were then evaporated to dryness in a centrifugal evaporator and stored at −80 °C until analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS).
2.12.7.5 LC-MS/MS analysis of ICAT-labelled peptides

All LC-MS/MS was performed by Dr Torsten Kleffmann and Dr Abhishek Kumar (Otago Centre for Protein Research). LC-MS/MS-ready, ICAT-labelled peptides (from 2.12.7.4) were resuspended in 20 µL of 2% (v/v) acetonitrile, 0.2% (v/v) formic acid. The tubes were vortexed for one minute, heated at 50 °C for 5 minutes, vortexed for one minute, sonicated in a water bath for 1 minute before a final minute of vortexing. Samples were briefly centrifuged to collect all solution at the bottom of the tube. Samples were transferred to a snap top auto-sampler vials (Thermo Scientific) for analysis. 

Resolublised samples were analysed, in duplicate, using an Ultimate 3,000 Nano-Flow LC System (Thermo Scientific) and LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific). The sample (5 µL) was injected into the LC-system, by way of an auto-sampler, and separated on an in-house packed emitter-tip column (ø 75 µm fused silica tubing containing C18 material, 8–9 cm in length). The sample was eluted from a gradient of 2% (v/v) acetonitrile, 0.2% (v/v) formic acid to 90% (v/v) acetonitrile, 0.2% (v/v) formic acid in water, over 105 minutes at a flow rate of 4 µL/min. Full MS (with a mass range between mass/charge (m/z) 300 and m/z 2000) was performed in the Orbitrap mass analyser with a resolution of 60,000 at m/z 400 and an automatic gain control (AGC) target of $1 \times 10^6$. The strongest ten signals were selected for collision-induced dissociation (CID)-MS/MS in the LTQ ion trap at a normalized collision energy of 35% using an AGC target of $2 \times 10^5$ and one microscan. Dynamic exclusion was enabled with two repeat counts during 90 seconds and an exclusion period of 120 seconds.

2.12.7.6 Analysis of ICAT-labelled peptides

MS spectrum data (Thermo Scientific RAW files) were analysed using Proteome Discover v2.4 (Thermo Scientific). The ICAT method enables the relative abundance of labelled-cysteine containing peptides to be determined, as pairs of heavy and light ICAT-labelled peptides have a mass difference of 9 Da. Abundance quantification uses the area of the precursor peaks of corresponding peptide pairs (labelled with either
the light or heavy ICAT reagent). Peptides and proteins were identified using Sequest (Eng et al., 1994), searching against the human SwissProt database (The UniProt Consortium, 2017) containing 20,188 entries (downloaded 03/01/2018). The search was for tryptic peptides with up to three missed cut-sites. The following dynamic modifications were allowed: oxidised methionine; deamidated asparagine, glutamate and arginine; acetylated N-termini. Cysteines were allowed to be modified with IAM, ICAT-light and ICAT-heavy. The precursor mass tolerance was 10 ppm and the maximum fragment mass tolerance was 0.4 Da. Proteome Discover calculates the confidence (a $q$ value) of the peptide based on an estimated false discovery rate of below 1% by the software. For all samples only proteins identified with a high confidence ($q$ value of 0.01) were used. The data was exported to Microsoft Excel for further processing (described in the relevant sections).
Chapter 3

Auto-regulation of ASK1

3.1 Overview

As a MAP3K, ASK1 lies atop of a signalling cascade that—in response to a range of cues—is essential in controlling diverse cellular responses. Accordingly, ASK1 regulation is multifaceted. In this Chapter, recombinant ASK1 is used to address the ability of ASK1 to regulate its own activity \textit{in vitro}. A kinase assay is developed with which to assess ASK1 activity. Domains, N-terminal to the ASK1 kinase domain, are seen to both inhibit, and enhance ASK1 kinase activity.

3.2 Publication outputs

3.3 Introduction

3.3.1 ASK1 auto-regulation

ASK1 is regulated by an array of different mechanisms. ASK1 also experiences a high degree of auto-regulation. For example, ASK1 signalling is not seen from cells over-expressing ASK1 in culture until they are stimulated with ligands such as TNFα (Gotoh and Cooper, 1998; Ichijo et al., 1997). Lack of activity from over-expressed ASK1 is indicative that ASK1 exhibits a high degree of self-regulation—namely auto-inhibition.

3.3.1.1 ASK1 auto-regulation by its N terminus

The N terminus (residues 1 to 658) of ASK1 is important for regulation and has dual roles in both inhibiting ASK1 and allowing activation. Many studies on ASK1 have reported that the ASK1 N terminus is important for ASK1 inhibition (Figure 3.1a). When over-expressed, ASK1 constructs lacking the N terminus (up to residue 649) and lacking the far N terminus (the first 277 residues) are intrinsically active (Fujino et al., 2007; Saitoh et al., 1998).

Precisely why the N terminus is inhibitory has not been completely deduced. The N terminus is purported to homo-oligomerise, which is required for TRAF6-induced ASK1 signalling (Figure 3.1b; Fujino et al., 2007; Wang et al., 2017). How oligomerisation occurs is not clear, but was thought to occur via a coiled-coil within the N terminus (Fujino et al., 2007). The coiled-coil was predicted to be contained within residues 297 to 324 within the conserved DUF4071 region (see 1.4.2; Fujino et al. 2007). However, the recent crystal structure from the Mace laboratory demonstrated that residues 269 to 658 of ASK1 instead formed a relatively compact arrangement putting doubt into how N-terminal oligomerisation is mediated (Obsil and Obsilova, 2017; Weijman et al., 2017).

The small oxidoreductase, Trx1, is purported to be able to block ASK1 N-terminal association, blocking activation (Figure 3.1b; Fujino et al., 2007; Saitoh et al., 1998). Trx1 inhibition of ASK1 is one of the most commonly cited mechanisms of how ASK1 is regulated and Chapter 4 will go into this in more detail. Trx1 mediated inhibition of
Figure 3.1: Regulatory mechanisms of ASK1. a) The N terminus of ASK1 (residues 1–658) is described as inhibitory to ASK1 signalling (Fujino et al., 2007; Saitoh et al., 1998), but the mechanism as to how is unknown. b) Under resting conditions, ASK1 can homo-oligomerise via its C-terminus (residues 950–1374) (Tobiume et al., 2002). When stimulated, the N terminus is thought to also homo-oligomerise in a manner that is regulated positively by TRAF proteins and negatively by Trx1 (Fujino et al., 2007; Noguchi et al., 2005; Saitoh et al., 1998; Tobiume et al., 2002). c) Oligomerised ASK1 is thought to trans-auto-phosphorylate to become fully active and initiate downstream signalling (Gotoh and Cooper, 1998; Tobiume et al., 2002).

ASK1 does not explain how over-expression of ASK1 is not sufficient to induce ASK1 signalling. That is to say, that over-expression will perturb the cellular molar ratio of ASK1 to Trx1. ASK1 auto-inhibition is, therefore, a critical regulatory mechanism of ASK1. Investigating how the N terminus can regulate ASK1, is one goal of this Thesis.

3.3.1.2 ASK1 auto-regulation by its C terminus

Like the N terminus, the C terminus (residues 950 to 1374) of ASK1 is also important in ASK1 regulation. Under resting conditions, ASK1 forms a homo-complex via its C terminus (Figure 3.1b; Tobiume et al., 2002). Following activation (such as following cellular stimulation with H$_2$O$_2$ or TNFα) a large (>669 kDa) complex forms—referred to as the ‘ASK1 signalosome’ (Noguchi et al., 2005). The signalosome involves recruitment of several other proteins, notably TRAF proteins (Nishida et al., 2017; Rusnak and Fu, 2017). Signalosome formation is a pre-requisite for subsequent active ASK1 signalling (Fujino et al., 2007; Noguchi et al., 2005; Tobiume et al., 2002). However, as
ASK1 signalling is dormant regardless of the in-cell oligomeric state, other regulatory mechanisms must be important.

To date, most studies on ASK1 have focused on truncation and deletion overexpression in a cell culture system. Whilst these studies have proved invaluable our understanding of ASK1 signalling, we have lacked a molecular understanding. To this end, it is crucial to develop tools to study ASK1 in vitro utilising kinase assays.

3.3.2 Types of in vitro kinase assay

The study of protein kinases requires an assay. Multiple types of kinase assay are available with their own advantages and limitations. Details of a few assays are given in Table 3.1 below, but it is not meant to provide a comprehensive guide.

Of the assay systems listed in Table 3.1, only two (western blotting and mass spectrometry) are able to be used with an unpurified sample. The majority are endpoint assays—the reaction must be stopped in order to measure the assay. Real-time assay—that continuously monitor the reaction (e.g., a fluorescence quenching assay)—are also available, but most require a peptide substrate (Eglen et al., 2008).

The use of in vitro kinase assays facilitates investigation into the nature and regulatory mechanisms of protein kinases, such as ASK1. This Chapter uses Phos-Tag SDS-PAGE and recombinant ASK1 constructs purified from E. coli.
Table 3.1: Examples of *in vitro* kinase assays

<table>
<thead>
<tr>
<th>Assay system</th>
<th>Description</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiolabelled-ATP</td>
<td>Incorporated radiolabelled $[^{32}\text{P}]$-ATP is measured by autoradiography or scintillation</td>
<td>High sensitivity, the ‘gold standard’ method</td>
<td>Hazardous</td>
</tr>
<tr>
<td>Western blot</td>
<td>Use antibodies against phosphorylated proteins.</td>
<td>Quite sensitive, can be specific to one proteoform</td>
<td>Requires reliable antibodies</td>
</tr>
<tr>
<td>Phos-Tag SDS-PAGE</td>
<td>SDS-PAGE-based. Phosphorylated proteins migrate at a slower rate. Total protein visualised via protein stain or western blot</td>
<td>Simple</td>
<td>Sensitivity is dependent upon protein detection method</td>
</tr>
<tr>
<td>ADP-Glo</td>
<td>Plate-based assay. ADP generated during kinase reaction is converted back to ATP, which is detected via a luciferase assay</td>
<td>High throughput</td>
<td>Any contaminant ATPase activity will be detected</td>
</tr>
<tr>
<td>Mass spectrometry</td>
<td>Direct detection of phosphorylated peptides/proteins by mass spectrometry</td>
<td>Incredibly sensitive, high specificity</td>
<td>Requires specialist equipment and knowledge, complex sample preparation</td>
</tr>
<tr>
<td>Alpha screen</td>
<td>Bead-based immunoassay. Phosphorylated protein complexes two beads. Singlet oxygen from one bead causes fluorescence at the second</td>
<td>High throughput</td>
<td>Requires reliable antibodies to conjugate to beads</td>
</tr>
<tr>
<td>Fluorescence quenching e.g., Trulight</td>
<td>Substrate peptide-based plate assay. Phosphorylated peptides bind to fluorescent beads, quenching fluorescence</td>
<td>High throughput, can measure activity in real-time</td>
<td>Requires peptide substrate</td>
</tr>
</tbody>
</table>

3.4 Aims

Aim 1  Design *in vitro* kinase assay for ASK1 activity

Aim 2  Assess ASK1 N-terminal auto-regulation *in vitro*

Aim 3  Identify residues within ASK1 important in ASK1 auto-regulation

3.5 Results

3.5.1 *In vitro* kinase assays used in this work

A reproducible assay system by which to measure protein activity has great potential for informing us how that protein works. With regards to ASK1 function, one accessible readout is measuring ASK1-catalysed phosphorylation of a substrate protein.

The majority of studies looking at *in vitro* activity of the ASK1 kinase domain (roughly residues 660–940) have used a peptide-based substrate (for example, Bunkoczi et al., 2007). However, this approach loses the detail of how the enzyme (ASK1) and substrate (MAP2K) may interact, and what non-catalytic properties of ASK1 may also govern the reaction.

Multiple forms of kinase assay were outlined within 3.3.2. This work uses *in vitro* kinase assays that have a kinase (two different recombinant ASK1 constructs) phosphorylating recombinant human MKK6. MKK6K82A, ‘kinase dead’ (referred to hereafter as MKK6) is used as a substrate in all assays, as it is unable to auto-phosphorylate (Figure 3.3 and 3.5). All assays are subsequently analysed by either western blot or Phos-Tag SDS-PAGE (Figure 3.2).

Phos-Tag SDS-PAGE uses a compound, (Phos-Tag™ from Wako Chem), that is immobilised within the acrylamide matrix. The Phos-Tag reagent has an affinity for phosphoryl groups and so slows the migration of phosphorylated proteins—relative to their unphosphorylated form (Figure 3.2a, Kinoshita et al., 2009). For example, when MKK6 is incubated with, and without the kinase domain of ASK1 (ASK1669–941) for 30 minutes at room temperature there is an observable shift of MKK6 when analysed by Phos-Tag SDS-PAGE (Figure 3.2b). Similarly, phosphorylated MKK6 (p-MKK6) can
Figure 3.2: **Illustration of in vitro kinase assays used in this work.** a) Cartoon demonstrating the Phos-Tag SDS-PAGE principle. b) Illustrative example where a substrate (in this case MKK6) was incubated with and without a protein kinase (in this case ASK1\textsubscript{669–941}). c) Illustrative example showing detection of phosphorylated substrate (p-MKK6) by either Phos-Tag SDS-PAGE or western blot.

be visualised by western blot, for example in Figure 3.2c, where MKK6 was incubated with catalytically active ASK1 for up to 10 minutes and analysed by both Phos-Tag SDS-PAGE and western blot.

### 3.5.2 The N terminus of ASK1 is auto-inhibitory in *cis*

Most studies using recombinant ASK1 activity have focused solely on the isolated kinase domain. We were able to express in *E. coli* and purify a longer protein construct containing the majority of the ASK1 N terminus and the kinase domain (residues 88 to 941; Figure 3.3a, see Methods for purification details). These two protein constructs (ASK1\textsubscript{669–941}, and ASK1\textsubscript{88–941}) enabled a direct comparison of protein kinase activity in
in order to assess what effect, if any, the N terminus may play in ASK1 regulation.

An in vitro kinase assay was performed with different concentrations of either ASK1\textsubscript{669–941} or ASK1\textsubscript{88–941} and 3 µM MKK6. MKK6 phosphorylation (visible by the upwards shift on Phos-Tag SDS-PAGE) after 5 minutes with 0.1 µM ASK1\textsubscript{669–941} (Figure 3.3b,c). Resolving the same samples on SDS-PAGE shows that there are no contaminants within the assay so the shift seen on Phos-Tag SDS-PAGE does indeed come from phosphorylation (Figure 3.3b). Without the addition of either ASK1 kinase construct there is no appearance of p-MKK6 showing that the phosphorylation is catalysed by ASK1 (Figure 3.3b, c).

No p-MKK6 was seen when incubated with either ASK1\textsubscript{669–941} or ASK1\textsubscript{88–941} at 0.01 µM for 10 minutes (Appendix Figure B.1). Conversely, 0.1 µM ASK1\textsubscript{669–941} (but not ASK1\textsubscript{88–941}), was sufficient to have detectable amounts of p-MKK6 after 5 minutes (Figure 3.3b,c). When the concentration was increased to 1 µM, there was a complete conversion of MKK6 to the phosphorylated form by ASK1\textsubscript{669–941} within 5 minutes. By comparison, 1 µM of ASK1\textsubscript{88–941} showed similar levels of activity as 0.1 µM ASK1\textsubscript{669–941} (Figure 3.3), showing that ASK1\textsubscript{669–941} is ∼10-fold more active than ASK1\textsubscript{88–941}.

One caveat to these results is that to minimise auto-phosphorylation during expression, ASK1\textsubscript{88–941} was co-expressed with lambda protein phosphatase (λPP), whereas ASK1\textsubscript{669–941} was not. As auto-phosphorylation is a well-studied mechanism of protein kinase regulation it is possible, therefore, that the differences between ASK1\textsubscript{669–941} and ASK1\textsubscript{88–941} are due to phosphorylation status. However, the comparison between ASK1\textsubscript{88–941} that was or was not co-expressed with λPP showed little difference between respective activities (Appendix Figure B.2). Taken together, these results suggest that ASK1\textsubscript{669–941} is at least 10-fold more active than ASK1\textsubscript{88–941} and that the N terminus of ASK1 is somehow negatively regulating ASK1 kinase activity.

SAXS data from the Mace laboratory yielded further insight into how the ASK1 N-terminal may operate to regulate the kinase domain (Weijman et al., 2017). A background to SAXS data will be provided in Chapter 4. Briefly, SAXS is a biophysical technique where the scattering of X-rays by a molecule in solution is measured (Putnam et al., 2007). SAXS data is useful to construct a model of a multi-domain protein when only the structure of individual domains are known (Putnam et al., 2007).
Figure 3.3: The N terminus of ASK1 is inhibitory to ASK1 kinase activity. In vitro kinase assay of ASK1 phosphorylating MKK6. a) Schematic showing which protein constructs are used in this assay. b) In vitro kinase assay with 3 µM MKK6 and a range of ASK1 concentrations. The assay was conducted at room temperature with 2 mM DTT. ATP was added (50 µM) to start the reaction. A Phos-Tag SDS-PAGE gel (top) and SDS-PAGE gel (bottom) are shown to indicate the shift from phosphorylation. Note that no protein marker is used in these gels so the shift is relative to the band position at 0 minutes. All proteins are visualised with Coomassie. c) Quantification of two separate experiments. The phosphorylated and unphosphorylated bands were quantified using ImageStudio. The percentage of total phosphorylated MKK6 was then converted to concentration based on 3 µM MKK6 being used in the assay. Error bars represent standard error of the mean (SEM). Gels used in quantification are shown in Appendix Figure B.1.
Rigid body modelling (using a program called BUNCH) of scattering data from ASK1<sub>88-941</sub> and ASK1<sub>88-658</sub> (a construct consisting of the TBD, the 7× TPR and PH domains on a single polypeptide) showed distinct conformational differences between the relative orientation of the ASK1-TBD within the N terminus (Figure 3.4; Weijman et al., 2017). Within the context of ASK1<sub>88-941</sub>, the TBD points towards the kinase domain. By comparison, when ASK1<sub>88-658</sub> is analysed, the TBD points away. A ‘flip’ of the ASK1-TBD could, therefore, be sufficient to limit the availability of binding proteins to the rest of ASK1, thereby altering the protein’s activity.

![Figure 3.4](image_url)

**Figure 3.4:** *N-terminal regulation of ASK1 modelled using SAXS.* a) Schematic of the ASK1 constructs in this Figure. b) and c) SAXS BUNCH model from Weijman et al. (2017) for either ASK1<sub>88-658</sub> (b) or ASK1<sub>88-941</sub> (c). The locations of the proposed substrate docking site (F623 and D632; 3.5.4.2; Figure 3.8) is indicated along with W476 (from 3.5.4.1).
3.5.3 ASK1 kinase activity is not inhibited by N-terminal regions when added in *trans*

In order to investigate how the N terminus of ASK1 might be regulating the ASK1 kinase domain a slightly different approach was used. If the regulation was a direct effect between the N terminus and the kinase domain then the addition of the N terminus in *trans* (i.e. on separate polypeptide chains) would inhibit the activity of ASK1$_{669-941}$.

To test whether the ASK1 N terminus is inhibitory when added in *trans*, an *in vitro* kinase assay was carried out (Figure 3.5). The assay contained ASK1$_{669-941}$ (0.01 µM) and isolated regulatory regions—either ASK1$_{88-658}$ or ASK1$_{269-658}$—were added at concentrations 100-fold above (1 µM) or equal (0.01 µM) to the kinase domain concentration. Due to the similar size of MKK6 and ASK1$_{269-658}$ (~37 and 45 kDa, respectively) Phos-Tag SDS-PAGE was not a suitable way of analysing the assay (as p-MKK6 runs at a similar size to ASK1$_{269-658}$) so western blotting (for p-MKK6) had to be used instead.

No change in MKK6 phosphorylation was observed from the addition of either ASK1$_{88-658}$ or ASK1$_{269-658}$ (Figure 3.5). The lack of inhibition from the addition of N-terminal constructs suggests that the N terminus does not modulate the ASK1 kinase domain directly. An alternative hypothesis is that the N terminus can somehow modulate the substrate (MKK6).

3.5.3.1 Phosphorylation of MKK6 can be enhanced by ASK1$_{269-658}$

No effect on MKK6 phosphorylation by ASK1$_{669-941}$ was seen when the N-terminal constructs were added to either 100-fold above or equal to the kinase concentration. In order to determine if there was any effect on the substrate (MKK6), the same approach was used; an *in vitro* kinase assay with 0.01 µM ASK1$_{669-941}$ and adding in N-terminal ASK1 constructs (ASK1$_{269-658}$ or ASK1$_{88-658}$) to amounts 10-fold below (0.3 µM) or above (30 µM), relative to the concentration of MKK6 (3 µM; Figure 3.6).

Addition of up to 30 µM ASK1$_{88-658}$ had no or little effect on MKK6 phosphorylation (Figure 3.6b). Interestingly, the addition of 30 µM ASK1$_{269-658}$ did not cause inhibition
**Figure 3.5:** Addition N-terminal ASK1 regions (up to 1 µM) in trans does not alter kinase activity. a) Schematic showing which regions of ASK1 and other proteins are used in this assay. b) Kinase assay conducted with 3 µM MKK6 and 0.01 µM ASK1669–941 performed at room temperature. Both ASK1269–658 and ASK188–658 were added to the assay with concentration up to 1 µM. The assay was started by the addition of 50 µM ATP. This assay was analysed by western blot against p-MKK6 with Ponceau S stained membrane shown below for loading control. c) Quantification of bands based on three separate experiments. Quantification is relative to p-MKK6 at 10 minutes for the band with no additional regulatory domain. Error bars represent SEM. Blots used in quantification are shown in Appendix Figure B.3.
**Figure 3.6:** Addition of excess ASK1<sub>269–658</sub> enhances phosphorylation of MKK6. In vitro kinase assay of ASK1 phosphorylating MKK6. a) Schematic indicating the protein constructs in this assay. b-c) In vitro kinase assay using ASK1<sub>669–941</sub> adding in increasing amounts of either ASK1<sub>88–658</sub> (b) or ASK1<sub>269–658</sub> (c). Assays were conducted with 3 μM MKK6, 2 mM DTT and 0.01 μM ASK1<sub>669–941</sub> performed at room temperature. Assays were started by the addition of 50 μM ATP. Assays were analysed by western blot against p-MKK6 with Ponceau S stained membrane shown below for loading control. d) Quantification of three separate experiments. Quantification is relative to p-MKK6 at 10 minutes for the band with no additional regulatory domain. Error bars represent SEM. Blots used in quantification are shown in Appendix Figure B.4. Note—for b) all lanes were from the same blot but it was loaded in the wrong order, hence it has been cropped here for the purposes of clarity.
of kinase activity but actually enhanced phosphorylation by up to 4-fold (Figure 3.6c,d). Conducting a similar assay, but adding in the ASK1$_{88-266}$ (the ASK1-TBD) up to 30 µM also had no effect on MKK6 phosphorylation (Appendix Figure B.5).

MEKK1 (another MAP3K) has a similar kinase domain to ASK1 (Appendix Figure B.6). Conducting a similar assay with a protein construct of rat MEKK1 (rMEKK1) kinase domain (residues 1160–1493), adding in up to 30 µM MKK6 did not cause enhancement (Appendix Figure B.6). However, the rMEKK1 construct used has a short N-terminal extension, so it is not directly comparable. Whilst it should be treated with caution, no enhancement with rMEKK1 kinase perhaps indicates that ASK1$_{269-658}$ is a regulatory domain that works in synergy with other regions of ASK1 rather than in isolation. Overall, these results suggest that ASK1$_{269-658}$ acts as a recruitment site for the substrate (MKK6), and that it might be a regulatory mechanism that is unique to the ASK family of MAP3Ks.

3.5.4 Mutations within ASK1$_{269-658}$ can alter the activity of ASK1$_{88-941}$

The crystal structure of ASK1$_{269-658}$, from the Mace laboratory (Weijman et al., 2017), enabled further investigation into how ASK1$_{269-658}$ regulates ASK1$_{88-941}$. ASK1$_{269-658}$ is comprised of an extended bundle of helices (7×TPR) and a cryptic PH domain (Weijman et al., 2017).

Given that ASK1$_{269-658}$ is able to enhance phosphorylation of MKK6 (3.5.3.1) it is hypothesised that there is a substrate recruitment site within the region. It was not clear, however, whether ASK1$_{269-658}$ exhibited any flexibility, which would regulate ASK1 activity by bringing distal N-terminal domains (such as the TBD) close to the kinase domain. By looking at conserved residues within ASK1$_{269-658}$ and mutating them we investigated whether there was flexibility within ASK1$_{269-658}$ and what residue(s) may be important in both auto-inhibition and substrate docking. Mutations within ASK1$_{269-658}$ were cloned and mutant ASK1$_{88-941}$ proteins were purified (by Sam Jamieson, Mace laboratory). The mutations were all at residues conserved throughout metazoa (see Appendix Figure B.7).
3.5.4.1 ASK1_{269–658} is a rigid domain

If ASK1_{269–658} were flexible—and its main role was to bring ASK1_{88–266} into close proximity to the kinase domain—then mutations within the 7×TPR could increase the activity of ASK1_{88–941}. Arg395, at the centre of the 7×TPR (Figure 3.7a,b) was mutated to Glu—under the hypothesis that if ASK1_{269–658} were flexible, then Arg395Glu would increase kinase activity. However, ASK1_{88–941}^{R395E} had a decrease in kinase activity (Figure 3.7c,d), suggesting that ASK1_{269–658} is a rigid domain.

A further pair of mutations within the 7×TPR were made: Arg322Glu and Trp509Glu (Figure 3.7a,b). If there were any flexibility within the 7×TPR, then Arg322Glu would disrupt the protein, whereas Trp509Glu would likely stabilise the protein (by forming a salt bridge with Arg322), thereby decreasing activity. However, neither of these mutations had any effect on kinase activity (Figure 3.7c,d), again suggesting that ASK1_{269–658} is a rigid domain.

Lastly, to investigate whether the 7×TPR may facilitate stabilising of the ASK1-TBD, Trp476 was mutated (Figure 3.7a,b). Trp476 is a conserved (Appendix Figure B.7), surface exposed residue. We therefore, hypothesised if Trp476 was important in ASK1 auto-regulation via influencing the relative position of the TBD. Mutating Trp476 to Ser should not destabilise the ASK1 protein but if it were important in stabilising the ASK1-TBD, then mutating it would increase kinase activity. Indeed, an increase in kinase activity is what was seen (Figure 3.7c,d). Overall, the functional data from mutations suggest that ASK1_{269–658} is a rigid domain, consistent with SAXS data (Weijman et al., 2017).
Figure 3.7: *ASK1*<sub>269-658</sub> is a rigid domain. *In vitro* kinase assay of ASK1 phosphorylating MKK6. a) Schematic showing protein constructs used in this assay and mutations. b) Crystal structure of *ASK1*<sub>269-658</sub> showing the position of mutations within the 7×TPR (PDB ID: 5ULM; Weijman et al., 2017). c) Kinase assay conducted with 3 µM MKK6 and 1 µM ASK1<sub>88-941</sub> and 2 mM DTT, performed at room temperature. 50 µM ATP was added to start the reaction d) Quantification of c) based on three separate experiments (Note: R322E and the 30 minute time points were not used in the quantification experiments). MKK6 and p-MKK6 bands were quantified using ImageStudio. The percentage of total phosphorylated MKK6 was then converted to µM based on 3 µM MKK6 being used in the assay. Error bars represent SEM. A gel showing the purified ASK1<sub>88-941</sub> proteins and gels used for quantification values can be found in Appendix Figure B.8.
3.5.4.2 The ASK1 PH domain may be important for substrate docking

As ASK1\textsubscript{269-658} increased activity of ASK1\textsubscript{669-941} when added in \textit{trans} (3.5.3.1), it suggested that there is a substrate docking site within ASK1\textsubscript{269-658}. In order to investigate this, a further two mutations within the context of ASK1\textsubscript{88-941} were also made at conserved, exposed residues within the PH domain—Phe623Glu, Asp632Arg (Figure 3.8a,b). The hypothesis was that if the PH domain acted as a substrate docking site, mutating Phe623 and Asp632 would cause a decrease in protein activity. Both mutations resulted in approximately a 2-fold decrease in activity of ASK1\textsubscript{88-941}, compared to WT (Figure 3.8c,d). An illustration of how the PH domain may serve to regulate ASK1 activity was shown previously in Figure 3.4. Under resting conditions, the TBD can prevent access of the substrate (MKK6) to the recruitment patch on the PH domain. When activated, the TBD is re-positioned, allowing MKK6 to bind and be phosphorylated by the kinase domain.

Overall, the results in this Chapter demonstrate that the N terminus of ASK1 is inhibitory but that ASK1\textsubscript{269-658} can enhance activity when added in \textit{trans}. Additionally, mutations at conserved residues within the 7\texttimes TPR and PH domains (contained within ASK1\textsubscript{269-658}) can have inhibitory effects, but also enhance activity. Altogether the results presented in this Chapter demonstrate an important role for ASK1\textsubscript{269-658} in ASK1 auto-regulation.
**Figure 3.8:** The ASK1 PH domain is a substrate docking site. In vitro kinase assay of ASK1 phosphorylating MKK6. 

a) Schematic showing protein constructs used in this assay and mutations.

b) Crystal structure of ASK1_{269–658} showing the position of mutations within the PH domain (PDB ID: 5ULM; Weijman et al., 2017).

c) Kinase assay conducted with 3 µM MKK6 and 1 µM ASK1_{88–941} and 2 mM DTT, performed at room temperature. 50 µM ATP was added to start the reaction.

d) Quantification of c) based on three separate experiments (Note: the WT sample shown is reproduced from Figure 3.7 and the 30 minute time points were not used in the quantification experiments). Error bars represent SEM. Quantification was identical as described in Figure 3.7. A gel showing the purified ASK1_{88–941} proteins and gels used for quantification values can be found in Appendix Figure B.8.
3.6 Discussion

3.6.1 General comments on \textit{in vitro} assays in this work

The work in this Chapter has looked into auto-regulatory mechanisms of ASK1. In order to examine ASK1 activity, an \textit{in vitro} assay system was developed. The assay system used both recombinant protein substrates (MKK6) and enzymes (ASK1\textsubscript{669–941} and ASK1\textsubscript{88–941}). The assays have been visualised on gels using both Phos-Tag SDS-PAGE and western blotting. Both methods of visualisation were relatively simple and reproducible.

The advantage of Phos-Tag SDS-PAGE, as it has been used in this work, is that analysis is straightforward. To analyse the assay all that is required is SDS-PAGE and Coomassie staining. By comparison, western blotting has several extra steps introducing additional chance for variation. For example, the efficiency of transfer may differ from blot to blot, or even within one blot (MacPhee, 2010). A linear response range—i.e. that the signal is directly proportional to the amount of protein on the blot—is also critical in accurate western blots (MacPhee, 2010).

One limitation of Phos-Tag SDS-PAGE is sensitivity. For example, with Phos-Tag SDS-PAGE assays 0.1 µM ASK1\textsubscript{669–941} was used in an assay (e.g., Figure 3.3). Conversely, the western blot method had increased sensitivity so 0.01 µM ASK1\textsubscript{669–941} was used (e.g., Figures 3.5 and 3.6). Lower sensitivity is not actually inbuilt to Phos-Tag SDS-PAGE and more associated with the use of Coomassie for visualisation of protein. If a more sensitive method—such as silver staining—was used there would be increased detection sensitivity (Chevalier, 2010). However, the advantage of using Coomassie is it is very simple and fast, hence why it was used for this work. Western blotting also proved invaluable for the experiments where ASK1\textsubscript{269–658} was added into the assays in \textit{trans} (for example, Figures 3.5 and 3.6). ASK1\textsubscript{269–658} and MKK6 have similar molecular weights (45 and 37.8 kDa, respectively). On Phos-Tag SDS-PAGE (as it was used in this work) ASK1\textsubscript{269–658} and MKK6 (unphosphorylated and phosphorylated forms) overlapped one another. Therefore, western blotting was used to analyse the assays instead.
The major benefit from Phos-Tag SDS-PAGE is that the method enables detection of both the phosphorylated and unphosphorylated forms of a protein. Therefore, it is possible to quantify the total fraction (and thereby an indication of the total amount) of phosphorylation (as in Figures 3.7 and 3.8). Conversely, by western blot, one cannot say how much of a protein is modified, but only how much relative to the control (as in Figure 3.6). Accordingly, by using Phos-Tag SDS-PAGE we were able to measure phosphorylated MKK6 and the total protein from the same method of visualisation (Coomassie staining). Determining both constraints enabled us to quantify the total proportion of MKK6 that was phosphorylated and to convert it to molarity based on the concentration of MKK6 used in the assays (as in Figures 3.3, 3.7 and 3.8). Quantification offered a rapid way to compare the activities of different protein constructs or different assay conditions. Overall, the assays work well, and the use of a full-length substrate provided a useful assay for investigating ASK1 activity and regulation.

3.6.2 Inhibition of ASK1 by the ASK1 N terminus (residues 88–658)

Comparison of ASK1\textsubscript{669–941} and ASK1\textsubscript{88–941} showed that ASK1\textsubscript{669–941} is approximately 10-fold more active than ASK1\textsubscript{88–941} (Figure 3.3). The N terminus of ASK1 has long been known to facilitate inhibition. For example, Saitoh \textit{et al.} (1998) reported that an N-terminal truncation (i.e. missing the first 648 residues) of ASK1 (that was over-expressed in cells and immunoprecipitated) had \~{}10-fold more activity compared to WT. Additionally, over-expression of N-terminally truncated ASK1 is sufficient to induce apoptosis indicating that it has a major role in regulating ASK1 activity (Hatai \textit{et al.}, 2000; Saitoh \textit{et al.}, 1998).

Dimerisation is common to many protein kinases and is known to be an important mode of regulation (Marianayagam \textit{et al.}, 2004). For example, EGFR and RAF1 dimerise before becoming fully active (Lavoie and Therrien, 2015; Lemmon \textit{et al.}, 2014). Synthetic dimerisation of ASK1 is sufficient to activate ASK1 in HEK293 cells (Gotoh and Cooper, 1998). The ASK1 kinase domain is known to be a dimer in solution (Appendix Figure A.2f and Bunkoczi \textit{et al.}, 2007). Conversely, ASK1\textsubscript{88–941} is monomeric in
solution (Appendix Figure A.2g and Weijman et al. 2017). The N terminus of ASK1, therefore, may play a role in regulation via interference with dimerisation of the kinase domain.

The reported dissociation constant ($K_d$) of the ASK1 kinase domain homodimer is 0.2 µM (Bunkoczi et al., 2007). In the in vitro kinase assays analysed by Phos-Tag SDS-PAGE (such as in Figure 3.3), 0.1 µM ASK1$_{669-941}$ was used. Under these conditions, it would be expected that less than half of ASK1$_{669-941}$ would be dimeric. Analysing the effect of dimerisation on enzyme kinetics would help to delineate what role dimerisation plays in regulating ASK1 kinase activity.

Lastly, N-terminal mediated inhibition of ASK1 is canonically explained via Trx1 binding and stopping N-terminal oligomerisation of ASK1 (see 3.3.1). The work presented here demonstrates that the N terminus is sufficient to inhibit ASK1 kinase activity, providing it is on the same polypeptide (3.5.2). Trx1, therefore, may regulate ASK1 via altering N-terminal inhibition, instead of acting as a direct inhibitor. Chapter 4 will explore Trx1 regulation of ASK1 in greater depth.

### 3.6.3 Addition of ASK1$_{269-658}$ in trans can enhance phosphorylation of MKK6

Addition of different ASK1 N-terminal constructs to in vitro kinase assays had intriguing results. Addition of either ASK1$_{269-658}$ or ASK1$_{88-658}$ at concentrations equal to, or exceeding the kinase, but under the concentration of the substrate had no effect (Figure 3.5). Conversely, when ASK1$_{269-658}$ was added in trans, at concentrations exceeding that of the substrate, there was an enhancement of kinase activity (Figure 3.6).

One explanation for this effect is that ASK1$_{269-658}$ acts as a docking site for the substrate. Comparison of the available crystal structures of full-length MKK6 reveals that the N-terminal lobe and αC-helix can undergo a conformational shift of approximately 18 Å, depending upon the state of the protein (Appendix Figure B.9 and Weijman et al. 2017).

Within MKK6, Ser207 and Thr211 (the two residues that are required to be phosphorylated for the protein to become active) are buried in the unphosphorylated form
of the protein (Appendix Figure B.9; Matsumoto et al., 2012; Weijman et al., 2017). MKK6 can, therefore, undergo a large conformational change and such a change is required for phosphorylation to occur. ASK1\textsubscript{269-658} could promote phosphorylation of MKK6 by inducing a conformational change. A conformational change would subsequently expose the key phosphorylation targets and augment the suitability for MKK6 to be a substrate for ASK1\textsubscript{669-941}.

3.6.3.1 Mutations within ASK1\textsubscript{269-658} can promote and inhibit ASK1 kinase activity

The high degree of conservation of ASK1\textsubscript{269-658} between ASK1, 2 and 3 (Appendix Figure B.7), and the recent crystal structure of ASK1\textsubscript{269-658} (PDB ID: 5ULM; Weijman et al., 2017) enabled structure-guided mutational and functional analysis (3.5.4). Mutations within ASK1\textsubscript{269-658} (Figure 3.7, alongside SAXS data (Weijman et al., 2017)) showed that region is not flexible. Mutations at conserved, surface-exposed residues can alter activity, indicating that there are interactions with MKK6 that promote binding (Figure 3.8). The mutation and functional assays helped to develop a model for ASK1 auto-regulation.

3.6.4 Model of ASK1 auto-regulation and scaffolding

Based upon the results in this Chapter, it is proposed that ASK1\textsubscript{269-658} within ASK1 acts as a scaffold to recruit MAP2Ks and promote their phosphorylation.

3.6.4.1 MAP2K recruitment

The N-terminal extensions of MKKs are well-known to be important in binding to respective MAPKs via kinase interaction motifs (KIMs), also known as D-motifs (Peti and Page, 2013). D-motifs—alongside other MAPK-binding proteins (Peti and Page, 2013)—are responsible for high-affinity binding and specificity between MAPKs and MAP2Ks (Bardwell et al., 2009; Ho et al., 2003, 2006). MKK7, for instance, contains three D-motifs giving it a high affinity for JNK-MAPKs (Ho et al., 2006; Kragelj et al., 2015). By comparison, recruitment of MAP2Ks by MAP3Ks is more obscure.
The C terminus of several MAP2Ks (including MKK6) has been reported to contain a ‘domain for versatile docking’ (DVD) motif that is integral to MAP2K-MAP3K recruitment (Avruch, 2007; Takekawa et al., 2005). Whilst a DVD-motif within MKK6 has been shown to be necessary for interacting with the ASK1 kinase domain (Takekawa et al., 2005) there are many exceptions to DVD-mediated MAP2K-MAP3K interaction mechanisms (Gloeckner et al., 2009; Tatebayashi et al., 2003, 2006; Wengier et al., 2018).

3.6.4.2 Signalling scaffolds

Scaffolding proteins represent another mechanism by which MAP2Ks and MAP3Ks are able to co-localise. Scaffold proteins are common regulatory proteins in signalling systems. Two of the best-known examples are that of Kinase Suppressor of Ras (KSR)1 and the analogous yeast protein, Ste5 (Chol et al., 1994; Good et al., 2011; Scott and Pawson, 2009; Therrien et al., 1996).

Several scaffolding proteins have been described for ASK1 including the protein kinase LRRK2 (Yoon et al., 2017), the phosphatase DUSP22 (Ju et al., 2016) and the zinc finger protein ZNF622 (also known as ZPR9; Seong et al., 2011). Several other scaffold proteins are highlighted in Rusnak and Fu (2017), Nishida et al. (2017) and, Weijman et al. (2017).

Scaffolding proteins are able to act as communication hubs within a cell by facilitating co-localisation of functionally connected proteins. Ste5 contains various domains that help it associate to the plasma membrane, and co-localise a MAPK cascade consequently enhancing the signalling output (Good et al., 2009, 2011; McKay et al., 2009). Ste5 has been shown to confer a conformational change within the yeast MAPK, Fus3 (Good et al., 2009). Fus3 binds to a von-Willebrand Type-A (VWA) domain within Ste5, making Fus3 a better substrate for the yeast MAP2K, Ste7. (Good et al., 2009). In this manner the ASK1 PH domain could serve analogously to the Ste5 VWA domain. Moreover, Ste5 also regulates the overall MAP3K pathway by directly inhibiting Fus3 until the pathway becomes activated (Zalatan et al., 2012). Consequently, ASK1 may not only work as to prime MKK6 for phosphorylation, but the ASK1-TBD would
Figure 3.9: Model of ASK1 auto-regulation. Under resting conditions, access to the ASK1 kinase domain is limited by the TBD. Conformational change of the TBD position (a ‘swing’), allows MKK6 to be recruited to the ASK1 PH domain, causing a conformational change within MKK6, making it a better substrate for phosphorylation. MKK6 is phosphorylated by the ASK1 kinase domain. Phosphorylated MKK6 can continue with downstream signalling. Control over ASK1 and the TBD is frequently associated with Trx1 and H$_2$O$_2$ (redox regulators, explored in Chapters 4 and 5), but the mechanism that governs this is unclear and will be explored in Chapter 4.

regulate access of MKK6, helping to prevent unsolicited activation of the pathway.

3.6.4.3 Model of ASK1 auto-regulation and scaffolding

The ability of ASK1$_{269-658}$ to enhance MKK6 phosphorylation in trans merits it being more than just a recruitment site (such as DVD-motifs). In this manner, the PH domain within ASK1$_{269-658}$ is akin the yeast Ste5 scaffold protein. Therefore, it is proposed that ASK1$_{269-658}$ is a scaffolding-subunit within ASK1, able to promote docking of MKK6 and induce a conformational change that primes MKK6 to be phosphorylated (Figure 3.9).

Under the proposed model, the ASK1-TBD (ASK1$_{88-266}$) can inhibit ASK1 kinase activity by restricting access to the substrate docking site. When the ASK1-TBD shifts from the auto-inhibitory position, MKK6 can bind, inducing a conformational change to allow for phosphorylation by ASK1. Phosphorylated MKK6 is then active and able to transmit the signal downstream. Such a model of MAP2K recruitment and priming by a MAP3K has not previously been described.
The proposed model is compatible with previous models of ASK1 regulation where that N-terminal oligomerisation (or inhibition) is regulated by Trx1 (see 3.3.1). One caveat is that the observations made in this Chapter do not suggest that N-terminal oligomerisation is required for ASK1 regulation. However, as this Chapter only uses constructs lacking the C-terminal portion of ASK1 (which is critical to ASK1 oligomerisation within cells) there is scope for N-terminal oligomerisation playing a role in the context of full-length ASK1. Nonetheless, control of the ASK1-TBD by cellular stimuli, in particular, oxidation by H$_2$O$_2$ and reduction by Trx1 (Fujino et al., 2007; Saitoh et al., 1998) provides a mechanism to modulate the N terminus of ASK1, thereby controlling overall ASK1 activity (discussed further in Chapter 4).

3.7 Conclusions

This Chapter has looked into how the protein kinase, ASK1, regulates itself in vitro. Understanding ASK1 auto-regulation is fundamental in understanding how ASK1 signalling is regulated within cells. Kinase assays have been used to analyse ASK1 activity in vitro. The assays have shown that the N terminus (residues 88–658) of ASK1 is auto-inhibitory to ASK1 kinase activity when on the same polypeptide chain. Curiously, the addition of ASK1$_{269-658}$ in trans to the kinase assays enhanced phosphorylation of M KK6 by up to 4-fold. When conserved, surfaced exposed residues within ASK1$_{269-658}$ are mutated (such as Phe623 and Asp632), there is a decrease in ASK1 kinase activity.

Taken together, and in conjunction with biophysical work from our group, the work in this Chapter suggests that the N terminus of ASK1 is both auto-inhibitory but also contains a substrate docking site within a PH domain. It is proposed that the PH domain acts as a scaffolding protein. Under this model the PH domain not only serves to recruit the substrate but also induce a conformational change within M KK6, making it a better substrate. Moreover, biophysical data from the Mace laboratory suggests that the ASK1-TBD can sit in different positions. The TBD therefore, is able to control access to the docking site, controlling overall ASK1 activity.

Altogether this Chapter proposes that the N terminus facilitates both auto-inhibition of ASK1, but also substrate docking and priming for signalling via a mechanism that
is hitherto unseen in any other MAP3K. This Chapter highlights that non-catalytic regions of signalling proteins merit investigation as much, as catalytic domains. Further work into the ASK1 TBD will increase our overall understanding of ASK1 regulation.
Chapter 4

ASK1 regulation by Trx1

4.1 Overview

ASK1 is purported to directly be inhibited by Trx1, with dissociation of Trx1 from ASK1 being required for ASK1 to become active. However, the nature of the interaction between ASK1 and Trx1 is ambiguous. Within this Chapter, the interaction between ASK1 and Trx1 is examined in vitro. Trx1 can bind to ASK1 in vitro. ASK1-Cys250 is seen to be necessary, and sufficient for ASK1 and Trx1 to form an inter-molecular disulfide bond. SAXS data is used to derive a model of how Trx1 may modulate ASK1. However, functional data reveals that Trx1 does not directly inhibit ASK1 kinase activity in vitro.

4.2 Introduction

ASK1 is well known for becoming activated in response to cellular stressors, such as H$_2$O$_2$. Chapter 3, outlined possible mechanisms of ASK1 auto-regulation. Modulation of ASK1 auto-regulation provides a basis for how ASK1 activity can be altered in response to H$_2$O$_2$. The oxidoreductase, Trx1, is commonly associated with H$_2$O$_2$-induced ASK1 signalling. Precisely how ASK1 is regulated by Trx1 remains somewhat unclear because a defined molecular picture of the interaction is lacking. Overall building upon the ASK1-Trx1 picture not only helps to further our understanding of ASK1 regulation
but also informs us fundamentally how $H_2O_2$ can regulate proteins within a cell.

### 4.2.1 General Trx substrate recognition

Trx is a 12 kDa protein found in all domains of life. Humans have two Trxs: Trx1, which is primarily cytosolic; and Trx2, which is mitochondrial (Lu and Holmgren, 2014). All Trxs have a conserved overall structure and dithiol containing active site motif of Cys-Gly-Pro-Cys (Figure 4.1a). Within a cell, Trxs have important roles in reducing oxidised protein cysteine residues, becoming oxidised during the process. Trx is subsequently reduced itself by TrxR with the ultimate reducing power coming from NADPH (Figure 4.1; Collet and Messens 2010; Lu and Holmgren 2014).

The cellular role of Trxs as reducers of proteins is well understood. On the other hand, Trx substrate specificity is less clear. In mammals, Trx1 has a wide range of substrates, including ASK1 (for a review see Matsuzawa 2017b and Lee et al. 2013). One of the best-known targets of Trx1 is the cytosolic peroxidase proteins, Prdxs, where Trx1 is required for effective cellular protection from $H_2O_2$ (Chae et al., 1994, 1999). Trx1 is also important in preventing the formation of non-native disulfide bonds that can occur within cells (Linke and Jakob, 2003). Perhaps because of the need to be able to act on any cytosolic or nucleic disulfide bond, there has been no universal motif or sequence that Trx1 (or any other Trx) recognises. One recent study postulated that Trx-substrate recognition is driven not by a sequence specificity, but by entropy (Palde and Carroll, 2015). That is to say, thermodynamically, the reduction reaction is governed by Gibbs free energy—$\Delta G=\Delta H-T\Delta S$ (where $G$ is Gibbs free energy, $H$ is enthalpy, $T$ is temperature and $S$ is entropy)—and the reaction will proceed spontaneously providing $\Delta G < 0$ (Voet and Voet, 2011). Palde and Carroll (2015) argue that in the reduction of disulfide substrates by Trx, entropy is the biggest contributor (i.e. $T\Delta S > \Delta H$) to favourable thermodynamics and therefore driving the reaction to completion (Voet and Voet, 2011). In more simplistic terms it could be thought of as a disulfide-bond representing a more stable conformer of a protein, thus increasing order and decreasing entropy. Therefore breaking the disulfide bond will allow a change in protein conformation, thereby decreasing order and increasing entropy.
Figure 4.1: Thioredoxin, structure and mechanism. a) Crystal structure of reduced human Trx1 (PDB ID: 1ERT; Weichsel et al. 1996) rendered in PyMol. The active site cysteines (Cys32 and Cys365) are indicated as sticks. b) Schematic of Trx-mediated reduction of proteins. Proteins can become oxidised (from a reaction with H₂O₂, for example) leading to the formation of a disulfide bond. Reduced Trx can reduce the protein target—via an intermediate disulfide-linked species (Wynn et al., 1995). Oxidised Trx (with a Cys32-Cys35, intra-molecular disulfide bond) is subsequently reduced by thioredoxin reductase (TrxR) and NADPH.

In addition to entropy, protein-protein interactions also contribute in Trx substrate recognition. Structural studies by Palde and Carroll (2015) on E. coli Trx and a substrate—phosphoadenosine phosphosulfate reductase (PAPR)—highlighted residues within PAPR that interact with Trx. However, the binding interactions—modelled as favourable changes in enthalpy (ΔH)—whilst a contributor, were never able to out-compete the entropic factor (Palde and Carroll, 2015). Therefore the conclusion was reached that Trx substrate specificity is primarily driven by entropy (Palde and Carroll, 2015).

4.2.2 ASK1 regulation by Trx1

Originally, Trx1 was identified as an interactor with ASK1 via a yeast two-hybrid screen (Saitoh et al., 1998). Saitoh et al. (1998) found that Trx1 interacted with the N-terminal portion of ASK1 (residues 1 to 648), with later work identifying residues 46 to 277 being the core ‘TBD’ (thioredoxin binding domain; Fujino et al. 2007). Saitoh et al. (1998) proposed that the interaction between ASK1 and Trx1 was dependent upon the redox status of Trx1. That is to say, reduced Trx1 associates with ASK1 and...
oxidation of Trx1 leads to the dissociation of Trx1 from ASK1, which can be regarded as the ‘Trx1 oxidation model’ (Figure 4.2a; Saitoh et al., 1998). Under the Trx1 oxidation mechanism, the oxidoreductase ability of Trx1 is not involved in regulating ASK1, only the physical association between the two proteins is required.

In contrast, in 2007, Nadeau et al. proposed the activity of Trx1 is required. Trx1 was proposed to maintain ASK1 in a reduced state in a model referred to here as the ‘ASK1 oxidation model’ (Figure 4.2b). Oxidation of ASK1 results in reduction of ASK1 by Trx1, and subsequent oxidation and withdrawal of Trx1 (Figure 4.2b; Nadeau et al., 2009). Nadeau et al. (2007) was not the first study to propose that ASK1 was actually a reductive-target of Trx1. Liu and Min (2002) observed that a mutant of Trx1 (with either Cys32 or Cys35 mutated to a serine) could not disassociate from ASK1. Reduction of ASK1 by Trx1 was further developed by mutational studies, changing some of the 23 cysteine residues within ASK1 to alanine (Nadeau et al., 2007). Several cysteine residues (Figure 4.2c) were implicated as being important. Later work by Nadeau et al. (2009) indicated that three cysteine residues within ASK1 were the most important, with Cys250 being the principal site for Trx1 to act upon (Figure 4.2c). Lastly, Nadeau et al. (2007) also suggested that an unknown component (referred to as ‘Factor X’) was also important for modulating ASK1 activity, but the nature of this component remains undetermined.

4.2.2.1 Why is ASK1-Trx1 inhibition important?

Currently, ASK1-Trx1 represents a signalling nexus that links the redox status of a cell with cellular events such as inflammation and apoptosis (Fujisawa, 2017; Hayakawa et al., 2012a). As inflammation and cell death have widespread implications for human health and disease (Hayakawa et al., 2012a; Matsuzawa, 2017a; Ryuno et al., 2017), understanding Trx1 regulation of ASK1 has translational benefits. For instance, the nature of ASK1 regulation by Trx1 and \( \text{H}_2\text{O}_2 \) can provide a basis for targeted drug development to regulate ASK1. The ASK1 inhibitor selonsertib is currently undergoing clinical trials for several hepatic diseases including NAFLD (Fujisawa, 2017; Loomba et al., 2018). However, selonsertib, alongside many other ASK1-inhibitors, targets the
Figure 4.2: Models for ASK1 regulation by Trx1 and ASK1 cysteine residues. a) Trx1 oxidation model. Trx1 binds to the N terminus of ASK1 preventing N-terminal oligomerisation. Oxidation of Trx1 (such as by H$_2$O$_2$ ‘†’) leads to dissociation of Trx1 and an active ASK1 complex. b) ASK1 oxidation mechanism Trx1 binds to the N terminus of ASK1, maintaining ASK1 in a reduced state (including the formation of a mixed-species disulfide, see Figure 4.1). Oxidation of ASK1 (such as by H$_2$O$_2$ ‘†’) can lead to intra-molecular or inter-molecular disulfide bonds within ASK1 (indicated by ‘?’). The oxidised ASK1 complex can actively signal. c) Scale schematic of ASK1 indicating the location of each of all 23 cysteine residues. The indicated residues on the bottom of the schematic are residues implicated by Nadeau et al. 2007 and Nadeau et al. 2009.
ASK1 kinase domain (Lovering et al., 2018). As Trx1 is proposed to regulate ASK1 via its N terminus, there is a greater potential to identify additional drug target sites that would offer increased ASK1-specificity and expand our fundamental understanding of ASK1 regulation.

4.2.3 ASK1 and oxidation

The two models for Trx1-mediated regulation of ASK1 outlined in 4.2.2 (Figure 4.2) highlight that protein oxidation is required to alleviate Trx1 inhibition, allowing ASK1 signalling. Within the ASK1-Trx1 models, exactly how either ASK1 or Trx1 become oxidised has not been fully explained. As introduced in Chapter 1 (and will be discussed in more depth in Chapter 5), \( \text{H}_2\text{O}_2 \) is an unlikely protein oxidant in vivo. The family of peroxidase proteins, Prdxs (peroxiredoxins), are both very abundant and have high reactivities with \( \text{H}_2\text{O}_2 \) (second-order rate constant on the order of \( 1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \); Winterbourn and Hampton 2008). By comparison, Trx1 has a fairly low reactivity with \( \text{H}_2\text{O}_2 \) (second-order rate constant of \( 1.05 \text{ M}^{-1} \text{ s}^{-1} \); Winterbourn and Hampton 2008) and therefore \( \text{H}_2\text{O}_2 \) will react preferentially with Prdxs. Consequently, Trx1, will not be able to react with \( \text{H}_2\text{O}_2 \) within the cell. Likewise, whilst there has been no published characterisation of the reactivity of ASK1 and \( \text{H}_2\text{O}_2 \), ASK1 would require an extraordinary high reactivity be able to out-compete the specialist peroxidases (such as Prdxs) for \( \text{H}_2\text{O}_2 \).

To overcome the Prdx problem, work from the Ledgerwood laboratory (Jarvis et al., 2012) has suggested that Prdxs can act as conductors of \( \text{H}_2\text{O}_2 \) signalling, transmitting oxidation via disulfide bonds (see Chapter 5 for more detail). Specifically, Jarvis et al. (2012) postulate that Prdx1 can directly oxidise ASK1 via transient inter-molecular disulfide bonds. However, understanding how ASK1 might become oxidised does not inform as to why oxidation is able to alter ASK1 signalling. As regulation of ASK1 by oxidation and Trx1 are intrinsically linked, understanding how Trx1 can regulate ASK1 will inform on how oxidation can also regulate ASK1. Structural and biophysical information, such as from SAXS, can help us to greater understand ASK1 regulation.
4.2.4 Overview of SAXS

In this Chapter, SAXS is used to gain information on the interaction between ASK1 and Trx1. A brief overview of SAXS is given below to help better interpret SAXS data.

Biological SAXS (small angle X-ray scattering) is a technique that measures the ability of a molecule, in solution, to scatter X-rays. Scattering is proportional to the size and concentration of the molecule in question. SAXS gives low-resolution data (in the range of 10–20 Å) that can be used to inform us of the oligomeric and quaternary states of proteins (Korasick and Tanner, 2018; Mertens and Svergun, 2010). In this way, when coupled with a high-resolution structural data (such as from X-ray crystallography), SAXS data gives a powerful way for understanding how a protein behaves in-solution. As a molecule in solution can adopt multiple orientations, SAXS data represents the average of all molecule orientations (in contrast to X-ray crystallography where the molecule analysed is very ordered).

SAXS scattering data is recorded as a factor of the momentum transfer vector, \( q \) which is related to both the angle of scattering and beamline wavelength (Figure 4.3 and Table 4.1). As scattering occurs in a circular pattern, \( q \) is averaged across the circle and hence is often plotted as radial \( q \) (Figure 4.3a,b). From SAXS data, a number of structural parameters can be determined (see Table 4.1 and Figure 4.3). The ‘radius of gyration’ (\( R_g \)), is one parameter which represents the mean distance from the centre of mass of a molecule to the perimeter. The scattering data can be transformed to represent the total of inter-atomic vectors—i.e. the sum of distances between every atom and every other atom within the molecule. The output, referred to as the \( P(r) \) can inform of the \( R_g \) and also represents a histogram of the vectors, providing an impression of the overall shape of a molecule. \( P(r) \) analysis generally requires a defined value for the maximum dimension (\( D_{\text{max}} \)) of a given molecule. The value of \( D_{\text{max}} \) can be estimated by programs (such as GNOM), but can also be adjusted manually, meaning care is needed not to over or under-estimate the true value of \( D_{\text{max}} \).

SAXS data can be used to build three-dimensional representations of the molecule in question. Rigid-body modelling is one such method that can be used to model a multi-domain protein where there are high-resolution structures for individual domains,
but not the molecule as a whole (Putnam et al., 2007). The model output can then be used to generate a theoretical scattering plot which enables comparisons to the raw data for user assessment. Such modelling can provide invaluable information to better understand the overall arrangement of a molecule. However, dueful consideration must be used so as to not over-interpret the data. Furthermore, additional experimental approaches should be used to validate any conclusions (Jacques and Trewhella, 2010).

**Figure 4.3: Illustration of SAXS.** a) Beamline set up for SEC-SAXS. A solution exiting the chromatography column enters a capillary and is irradiated with X-rays. The X-ray scattering is detected. The momentum transfer vector, $q$ is indicated with the dotted line representing the circular scatter pattern. b) Example of a scatter plot indicating the Guinier region. c) Pairwise distance distribution illustrating how a globular protein with a single domain might be projected versus a two-domain, globular protein. The maximum distance ($D_{\text{max}}$) is indicated. See Table 4.1 for definitions.
### Table 4.1: Definition of SAXS terms

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Intensity of scattering, has arbitrary units</td>
</tr>
<tr>
<td>q</td>
<td>Also called s, has units of Å(^{-1}). It is the momentum transfer vector which is related to the angle of scattering (2(\theta)) and the wavelength ((\lambda)) as follows: (q=(4\pi \cdot \sin \theta)/\lambda)</td>
</tr>
<tr>
<td>Guinier</td>
<td>Region of scattering at low (q) that is used for data quality control and to calculate (R_g) and I(0). Values should agree with P(r)</td>
</tr>
<tr>
<td>P(r)</td>
<td>Pair-distance distribution function. Fourier transformation of SAXS curve into real space. Produces a histogram of inter-atomic vectors. Used to calculate (R_g) and I(0), the values should agree with the Guinier region. Uses all data points but requires a value for (D_{max})</td>
</tr>
<tr>
<td>(R_g)</td>
<td>Radius of gyration, has units of Å. The mean distance from the centre of mass for a given molecule</td>
</tr>
<tr>
<td>I(0)</td>
<td>The forward scattering at (q=0), has units of cm(^{-1}). It is proportional to molecular mass and protein concentration</td>
</tr>
<tr>
<td>(D_{max})</td>
<td>Maximum dimension, has units of Å. The longest dimension for the measured molecule</td>
</tr>
</tbody>
</table>

See Figure 4.3, also. Useful references: Jacques et al. 2012; Jacques and Trewhella 2010; Kikhney and Svergun 2015; Korasick and Tanner 2018; Liu and Zwart 2012; Mertens and Svergun 2010; Putnam et al. 2007; Rambo and Tainer 2011.

### 4.3 Aims

**Aim 1**
Characterise ASK1 and Trx1 binding and identify key cysteine residue(s)

**Aim 2**
Generate an in solution model of ASK1 and Trx1

**Aim 3**
Determine the effect of Trx1 and \(H_2O_2\) on ASK1 kinase activity *in vitro*
4.4 Results

4.4.1 Trx1 and ASK1 interact in vitro

Whilst a lot is known about ASK1 signalling and how Trx1 can modulate it, the majority of data has come from cellular over-expression and immunoprecipitation based methods. Accordingly, there is limited data from a molecular level informing us into how ASK1 and Trx1 interact. The recombinant system used in Chapter 3 was used to better understand the interaction between Trx1 and isolated regions of ASK1 in vitro.

A pull-down assay was initially performed to investigate ASK1-Trx1 binding in vitro (Figure 4.4). His\textsubscript{6}-tagged ASK1 constructs (bound on HIS-Select nickel affinity resin) were mixed with an E. coli clarified lysate containing untagged, human Trx1\textsuperscript{WT,1}. The resin was washed and the resultant bound proteins were analysed by western blot (Figure 4.4). Due to the fact that the association between ASK1 and Trx1 is regarded as being redox-regulated (Saitoh et al., 1998), the assay was washed either under non-reducing conditions (no DTT), or reducing conditions (with 2 mM DTT).

Four ASK1 constructs (ASK\textsubscript{88–266}, ASK\textsubscript{88–338}, ASK\textsubscript{88–658} and, ASK\textsubscript{88–941}) were tested and all were able to pull-down Trx1 under non-reducing conditions (Figure 4.4c, Figure 4.4: ASK1 and Trx1 interact in vitro. Pull-down of untagged Trx1\textsuperscript{WT} (see Footnote 1) by His\textsubscript{6}-tagged ASK1 constructs. a) Protein constructs used in this assay. b) Illustration of pull down. ASK1 proteins on HIS-Select resin (bait) are incubated with a clarified E. coli lysate containing untagged human Trx1\textsuperscript{WT} (prey). The resin was washed and analysed by western blot. c) Western blot of pull-down with anti-Trx1 in green and anti-His\textsubscript{6} in red. Ponceau S stained membrane is shown below for total protein. The individual channels and a Coomassie stained SDS-PAGE gel are shown in the Appendix Figure C.1.

\footnote{1}{For the pull-down assay (Figure 4.4), WT Trx1 was used. Trx1 is also able to form a disulfide-linked homodimer via Cys73 (Weichsel et al., 1996). However, the significance of Cys73 disulfide-linked dimers, and whether they form in vivo, is unclear (Campos-Acevedo et al., 2017; Gronenborn et al., 1999). To stop Cys73 disulfide bonds forming, Cys73 was mutated to serine. All further experiments in this Chapter used Trx1\textsuperscript{C73S}, which is referred to as Trx1 hereafter.}
Figure 4.4: \textit{ASK1} and \textit{Trx1} interact in vitro. Figure legend on previous page.
The two shorter ASK1 constructs (ASK1\textsubscript{88-266} and ASK1\textsubscript{88-338}) were able to pull down a greater amount of Trx1 suggesting that these shorter constructs have a higher affinity for Trx1.

When performed under reducing conditions (2 mM DTT was included in binding and wash buffer), there was a decrease in the amount of Trx1 pulled-down by ASK1 (Figure 4.4c, right). Moreover, under reducing conditions, Trx1 was only detected via antibody against Trx1. Indeed, when stained with Coomassie there is no noticeable Trx1 band over the background (Appendix Figure C.1) Additionally, when looking at the individual channels for α-Trx1, there appears to be non-specific binding to the resin. Nonetheless, the western blot shows Trx1 remaining bound to ASK1\textsubscript{88-266}, ASK1\textsubscript{88-338}, and ASK1\textsubscript{88-941}, but not ASK1\textsubscript{88-658}.

One caveat of the approach used for the pull-down is that it is purely qualitative. The different sizes of the ASK1 constructs used will result in different molar amounts of each construct bound on the resin (as binding is relative to protein mass). Therefore the stoichiometry of resin:ASK1:Trx1 is not consistent between each ASK1 construct.

Overall, the pull-down demonstrates ASK1-Trx1 binding to all tested ASK1 constructs under non-reducing conditions. However, ASK1-Trx1 binding is less definite when performed under reducing conditions. The stark difference between the level of Trx1 pulled-down by the shorter ASK1 constructs suggested that the interaction between ASK1 and Trx1 is (at least in part) sensitive to the redox status of one or both of the proteins.

### 4.4.2 ASK1 can form a disulfide-linked species with Trx1

ASK1 regulation by Trx1 is viewed as being chiefly controlled by reduction and oxidation (Saitoh \textit{et al.}, 1998). There have been contrasting reports suggesting that Trx1 oxidation is key to regulation vs ASK1 being the target of oxidation (see 4.2.2; Nadeau \textit{et al.}, 2007; Saitoh \textit{et al.}, 1998).

The previous pull-down experiment above (4.4.1) indicated that the recombinant ASK1 used in this work may also associate with Trx1 in a redox-sensitive manner. To investigate this, ASK1 was incubated with purified Trx1 (see Methods, 2.6.15)
under non-reducing conditions. A ‘kinetic trap’ mutant of Trx1 was used (Trx1\textsuperscript{C35S}). Trx1\textsuperscript{C35S} is able to interact with disulfide bonds within protein substrates forming a mixed-species disulfide via Trx1-Cys32 (see Figure 4.1). In the absence of the second cysteine residue (Cys35), Trx1\textsuperscript{C35S} is unable to break the inter-molecular disulfide bond, forming a covalently-linked species (Schwertassek \textit{et al.}, 2007).

Following incubation and analysis by SDS-PAGE, a band at the approximate molecular weight of ASK1\textsubscript{88–658} plus Trx1 appeared (\(\sim\)75 kDa), but only when incubated with Trx1\textsuperscript{C35S} (Figure 4.5b, top). The 75 kDa band was no longer present when the same samples were treated with DTT (Figure 4.5b, bottom). The disappearance of the 75 kDa band is indicative of a disulfide bond between two species. The molecular weight of the DTT-sensitive species suggested that ASK1\textsubscript{88–658} and Trx1\textsuperscript{C35S} are able to form a complex with 1:1 stoichiometry containing an inter-molecular disulfide bond. A similar assay using ASK1\textsubscript{88–338} also formed a DTT sensitive species, suggesting that the cysteine residue(s) that are critical in the formation of this species are located within ASK1\textsubscript{88–338} (Appendix Figure C.2).

Curiously, incubation of ASK1\textsubscript{88–658} on its own did not result in the formation of any DTT-sensitive species (Figure 4.5b, left). For Trx1 to form a mixed-species with ASK1 it suggests that ASK1 contains an intra-molecular disulfide bond or other cysteine oxidation product (discussed in 4.5.2.1).

The observation of ASK1 forming an inter-molecular disulfide bond with Trx1 recapitulates the observations by Nadeau \textit{et al.} (2007). The observations by Nadeau \textit{et al.} (2007) were based upon an over-expression and immunoprecipitation methodology. The demonstration of similar results within this work—using purified, recombinant proteins—demonstrates that ASK1 (residues 88–338) and Trx1 alone are sufficient to form a covalent complex. The next question asked was which cysteine residue(s) within ASK1 were responsible for the inter-molecular disulfide bond with Trx1.
Figure 4.5: ASK1 forms an inter-molecular disulfide bond with Trx1. a) Schematic of protein constructs in this assay. All proteins used for non-reducing experiments were purified under reducing conditions then had a final SEC step without any reductant, after which they were snap-frozen as soon as they had eluted from the column. b) ASK188–658 (20 µM) was incubated with equimolar amounts of either Trx1 or Trx1C35S (in a buffer containing 10 mM HEPES, pH 7.6, 300 mM NaCl). The proteins were incubated for the times indicated at 4 °C. At each time point (every 24 hours), a sample was removed and placed into a non-reducing SDS-PAGE buffer, containing 10 mM IAM (to alkylate cysteine thiols and stop potential disulfide-shuffling) and incubated in the dark for 30 minutes at room temperature. Samples were resolved by non-reducing and reducing SDS-PAGE (for reducing gels, the samples were incubated with 100 mM DTT for 5 minutes at RT before loading). Proteins were visualised with Coomassie. Trx1 alone gels are given in Appendix Figure C.4.
4.4.3 ASK1-Cys250 is essential for ASK1 inter-molecular disulfide bond with Trx1

Full-length, human ASK1 contains 23 cysteine residues (Figure 4.6a). N-terminal to the kinase domain (up to residue 669), there are 16 cysteine residues. Given that ASK1\textsubscript{88–338} is sufficient to form an inter-molecular disulfide bond (4.4.2) and ASK1\textsubscript{88–266} and ASK1\textsubscript{88–338} have apparent higher binding affinities for Trx1 (4.4.1), it suggests that the cysteine residue(s) required are within residues 88 to 338. Preliminary mass spectrometry data (Jamieson and Mace, unpublished) indicated that Cys206, Cys225 or 226 and, Cys250 were the most likely candidates for forming the mixed-species disulfide with Trx1. Therefore, these residues were mutated (to serine) and the mutant ASK1 proteins were incubated with Trx1 in a similar manner to 4.4.2.

ASK1\textsubscript{88–658WT}, ASK1\textsubscript{88–658Cys206Ser} and ASK1\textsubscript{88–658Cys225,226Ser} were all able to form a DTT-sensitive species when incubated with Trx1\textsuperscript{C35S} (Figure 4.6b). Western blotting confirmed that the 75 kDa molecular weight species did contain Trx1 (Figure 4.6b). ASK1\textsubscript{88–658C250S} did not form a 75 kDa species, indicating that ASK1 Cys250 is required for the formation of a mixed-disulfide with Trx1. When analysed in an identical manner as in Figure 4.5, ASK1\textsubscript{88–658C250S} also did not form a mixed-species disulfide (Appendix Figure C.4). Lastly, a variant of ASK1\textsubscript{88–266} that only has Cys250 (all other cysteines are mutated to serine) is still able to form a mixed-disulfide species with ASK1, demonstrating that Cys250 is sufficient to form the species (Appendix Figure C.5).

During the course of this Thesis, Kylarova et al. (2016) also reported that ASK1 was able to form an inter-molecular disulfide bond with Trx1 in vitro. Kylarova et al. (2016) incubated recombinant ASK1 (residues 88 to 302) with equimolar amounts of recombinant Trx1 (Cys35Ser, Cys73Ser) and a 5-fold molar excess of H\textsubscript{2}O\textsubscript{2} for 15 minutes and analysed the resulting species by non-reducing SDS-PAGE. Kylarova et al. (2016) used mass spectrometry to determine that ASK1-Cys200 was in the mixed-species disulfide with Trx1-Cys32. However, they did not carry out any validation (such as using a Cys200 mutant). When ASK1\textsubscript{88–658C200S} was incubated with Trx1\textsuperscript{C35S}, as in Figure 4.5, a mixed-disulfide species was still able to form (Appendix Figure C.4).
Nadeau *et al.* (2007) originally described that multiple cysteine residues were able to form disulfide-linked species, so it is feasible that multiple cysteine residues within ASK1 will be able to form a disulfide-linked species with Trx1. However, in this Chapter, it is shown that Cys250 is sufficient and necessary for the formation of a mixed species disulfide bond between ASK1 and Trx1 (Figure 4.6, Appendix Figure C.4 and, C.5).
Figure 4.6: ASK1-Cys250 is essential in forming inter-molecular disulfide bond with Trx1.

a) Schematic of protein constructs used in this assay showing the location of all cysteine residues in ASK1 and the residues mutated in this assay. b) ASK188–658 (2 µM) was incubated with either Trx1 or Trx1\textsubscript{C35S} (20 µM), in PBS (pH 7.4) for 16 hours at 4 °C. Samples were blocked with 10 mM IAM in SDS sample buffer and subjected to non-reducing SDS-PAGE and western blot (against Trx1) and reducing SDS-PAGE. Full gels and blots are shown in Appendix Figure C.3. Identical results were obtained when 20 µM H\textsubscript{2}O\textsubscript{2} was added to the assay (Appendix Figure C.3).
4.4.4 Biophysical analysis of ASK1 and Trx1

4.4.4.1 Trx1-fusion proteins are able to form a mixed-species disulfide with ASK1

The desired method to study ASK1 and Trx1 by SAXS was via a stable covalent-complex. To this end, and because previous work had indicated that a disulfide-linked species forms between ASK1$_{88-658}$ and Trx1$^{C35S}$ (4.4.2), a disulfide-linked species was determined to be the best candidate for analysis by SAXS. However, the relatively small size difference between ASK1$_{88-658}$ and ASK1$_{88-658}$-Trx1 (around 65 and 75 kDa, respectively) made it challenging to isolate a native ASK1-Trx1 complex. To overcome this limitation, two new Trx1 containing fusion-protein constructs were designed, cloned, expressed and purified. The first construct was an N-terminal fusion with *E. coli* MBP, the second construct was a C-terminal fusion T7 phage lysozyme (referred to as MBP-Trx1$^{C35S}$ and Trx1$^{C35S}$-Lys hereafter). Both of these fusion constructs contained no additional cysteine residues (the amino acid sequences are given in Appendix Figure C.10) helping to ensure only the desired disulfide-bond with ASK1 would form. Additionally, a triple cysteine mutant of ASK1$_{88-658}$ was also used (ASK1$_{88-658}^{C206S, C225,226S}$).

Both Trx1$^{C35S}$-Lys and MBP-Trx1$^{C35S}$ were able to form an inter-molecular disulfide bond with ASK1$_{88-658}^{C206S, C225,226S}$ (Figure 4.7 for Trx1$^{C35S}$-Lys and Appendix Figure C.6 for MBP-Trx1$^{C35S}$). When analysed by SEC, both species eluted at the predicted molecular weight (before the 150 kDa marker; Figure 4.8 for Trx1-Lys and Appendix Figure C.7 for Trx1-MBP). However, when analysed by non-reducing SDS-PAGE, the species ran larger than expected, around the 250 kDa Mw marker for MBP-Trx1$^{C35S}$ and 150 kDa for Trx1$^{C35S}$-Lys (Appendix Figure C.6). However, when the ASK1-MBP-Trx1$^{C35S}$ species was treated with 3C-protease (as MBP-Trx1$^{C35S}$ contains a 3C-cleavage site between MBP and Trx1) the expected size for the complex (∼75 kDa) was seen on non-reducing SDS-PAGE (Appendix Figure C.7).

Whilst both Trx1-fusion proteins were able to form a disulfide-linked species with ASK1$_{88-658}$, the overall yield from using Trx1$^{C35S}$-Lys was greater. Additionally, the similar molecular weights of MBP-Trx1$^{C35S}$ and ASK1$_{88-658}$ (Appendix Figure C.6) meant that there would be difficulty in isolating the two individual proteins from a
Figure 4.7: Trx1-fusion proteins form a mixed-species disulfide with ASK1. a) Schematic showing constructs used. C-terminal T7-Lysozyme fused to Trx1^{C35S}, described in the Figure as Trx1-Lys. An ASK1 cysteine mutant (ASK1\textsubscript{88–658}^{C206S, C225,226S}) was also used (referred to as ASK1 in the remainder of this legend). b) Trx1-Lys was purified by nickel-affinity before being reduced, on ice, for 1 hour with 2 mM DTT and resolved by non-reducing SEC. ASK1 was purified by nickel affinity chromatography and immediately snap-frozen, whilst Trx1-Lys was stored at 4 °C for 4 days before being snap-frozen. Thawed ASK1 and Trx1-Lys were combined (in a buffer containing 10 mM HEPES (pH 7.6), 300 mM NaCl) and at 37 °C with samples taken at the indicated times. At the endpoint, samples were alkylated in non-reducing SDS sample buffer (50 mM IAM, 30 minutes at room temperature, in the dark) before analysis by SDS-PAGE. All gels were stained with Coomassie. For reducing gels, samples were incubated with 100 mM DTT for 5 minutes at room temperature.
single size-exclusion run. For these reasons, and due to impending synchrotron time, Trx1\textsuperscript{C35S}-Lys was taken forwards in order to isolate a stable disulfide-linked species with ASK1.

4.4.4.2 Preparation of a disulfide-linked ASK1-Trx1 for SAXS analysis

Following on from the preliminary work using Trx1-fusion proteins (4.4.4.1), a large-scale preparation of a disulfide-linked ASK1-Trx1 species was produced. Full details are given in the methods (2.9). Briefly, ASK1\textsubscript{88-658}\textsuperscript{C206S, C225,226S} and Trx1\textsuperscript{C35S}-Lys were incubated together at 37°C for 4 hours. After incubation, the sample was alkylated with IAM to block any potential disulfide-rearrangement. The entire sample was concentrated and individual species were separated via SEC (Figure 4.8). Performing incubations with ASK1\textsubscript{88-658}\textsuperscript{WT} yielded a similar complex (Appendix Figure C.8). By comparison, incubation of ASK1\textsubscript{88-658}\textsuperscript{C250S} with Trx1\textsuperscript{C35S}-Lys resulted in no complex (Appendix Figure C.8). These observations meant that a stable disulfide species had been isolated via Cys250 of ASK1 and Cys32 of Trx1.
Figure 4.8: Preparation of ASK1-Trx1-Lys species for SAXS. a) Schematic showing constructs used. Trx1$^{C53S, C73S}$, C-terminally fused to T7-Lysozyme, described as Trx1-Lys. An ASK1 cysteine mutant (ASK1$^{88-658 C206S, C225,226S}$), was also used (referred to as ASK1 in the remainder of this legend). For details on complex formation see methods (2.9). Briefly, purified ASK1 and Trx1-Lys were mixed and incubated at 37 °C. Pre- and post-incubation samples are shown on b) as input ‘pre’ and ‘post’. The sample was alkylated with IAM before resolving by SEC (c). SEC fractions were analysed by SDS-PAGE (b), and relevant fractions combined. Sample outputs are shown in (b). Arrowheads below (c) indicate SEC molecular weight standards. A reducing gel of these samples is shown in Appendix Figure C.8.
4.4.5 SEC-SAXS analysis of ASK1-Trx1 complex

The ASK1-Trx1 disulfide linked-complex from 4.4.4.2 was analysed by SEC-SAXS at the Australian Synchrotron on the SAXS/WAXS beamline. Primary data reduction and processing were performed by Associate Professor James Murphy (Walter and Eliza Hall Institute, Melbourne, Australia). Data collection and analysis statistics are shown in Table 4.2. Figure 4.9 shows the $R_g$ from both Guinier analysis and P(r) analysis, as well as the P(r) distribution curves. The scattering plots of the data are shown (along with fits of rigid-body modelling) in Figure 4.10 (the scattering data on their own can also be found in Appendix Figure C.9).

Guinier and P(r) analysis both give an approximation of the same structural values (namely I(0) and $R_g$). In general, the values from either analysis should agree with one another (Jacques et al., 2012). Overall the values for the analysed species show good agreement (Table 4.2 and Figure 4.9). However, the $R_g$ value for ASK1$_{88-658}$C$_{206S},C_{225,226S}$ differs by 1 Å. Differences between the P(r) and Guinier analyses can indicate aggregation within the sample (Putnam et al., 2007). However, the Guinier plot (Figure 4.10a-c) indicates that there is minimum contribution from aggregates to the scattering data.

The experimental molecular weight (as calculated by SAXS-MoW (Fischer et al., 2010), see Table 4.2) is within 5% of the predicted molecular weight for Trx1-Lys (predicted: 31.58 kDa, experimental: 30.01 kDa). However, for ASK1$_{88-658}$ alone and the ASK1-Trx1-Lys complex the values differ more from the predicted values (33.4 and 20.6% differences, respectively). Nonetheless, the sum of the determined molecular weights for the individual ASK1 and Trx1-Lys species (88.4 and 30.0 kDa, respectively) agree with the observed molecular weight for the ASK1-Trx1 complex (118.0 kDa), agreeing with a 1:1 (ASK1:Trx1) stoichiometry for the complex.
Table 4.2: SAXS data collection and analysis statistics

<table>
<thead>
<tr>
<th>Variable</th>
<th>ASK1&lt;sub&gt;88–658&lt;/sub&gt; (alone)</th>
<th>Trx1-Lys (alone)</th>
<th>ASK1-Trx1-Lys (complex)</th>
</tr>
</thead>
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<tr>
<td><strong>Data collection parameters</strong></td>
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<td></td>
</tr>
<tr>
<td>Instrument</td>
<td>Australian Synchrotron, SAXS/WAXS beamline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beam geometry</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Wavelength (Å)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Exposure time (s)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (K)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>q range (Å&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>0.0057–0.334</td>
<td>0.0106–0.625</td>
<td>0.0057–0.334</td>
</tr>
<tr>
<td>Protein concentration&lt;sup&gt;1&lt;/sup&gt;</td>
<td>50 µL of 12.5 mg/mL</td>
<td>50 µL of 7.5 mg/mL</td>
<td>50 µL of 8.5 mg/mL</td>
</tr>
<tr>
<td><strong>Structural parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I(0) (cm&lt;sup&gt;−1&lt;/sup&gt;) from P(r)</td>
<td>0.0692 ± 0.0002</td>
<td>0.0240 ± 0.0001</td>
<td>0.0497 ± 0.0002</td>
</tr>
<tr>
<td>R&lt;sub&gt;g&lt;/sub&gt; (Å), from P(r)</td>
<td>36.54 ± 0.12</td>
<td>24.00 ± 0.11</td>
<td>43.86 ± 0.18</td>
</tr>
<tr>
<td>D&lt;sub&gt;max&lt;/sub&gt; (Å)</td>
<td>120</td>
<td>70</td>
<td>135</td>
</tr>
<tr>
<td>I(0) (cm&lt;sup&gt;−1&lt;/sup&gt;) from Guinier</td>
<td>0.0690 ± 0.0002</td>
<td>0.0240 ± 0.0001</td>
<td>0.0500 ± 0.0003</td>
</tr>
<tr>
<td>R&lt;sub&gt;g&lt;/sub&gt; (Å), from Guinier</td>
<td>35.64 ± 0.18</td>
<td>23.98 ± 0.17</td>
<td>43.26 ± 0.35</td>
</tr>
<tr>
<td>Molecular mass (kDa), predicted</td>
<td>66.25</td>
<td>31.58</td>
<td>97.82</td>
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<tr>
<td>Molecular mass (kDa), experimental</td>
<td>88.35</td>
<td>30.01</td>
<td>118.00</td>
</tr>
</tbody>
</table>

| **Software used** |                               |                  |                        |
| Primary data reduction | Scatterbrain (Australian Synchrotron) |                  |                        |
| Data processing | PRIMUS, GNOM |                  |                        |
| Molecular weight estimation | SAXS MoW |                  |                        |
| Computation of model intensities | CRYSOL |                  |                        |
| Rigid-body modelling | CORAL |                  |                        |
| 3D graphical representations | PyMol |                  |                        |

<sup>1</sup>—The indicated volume and concentration of protein was analysed via inline SEC. ASK1<sub>88–658</sub> (alone)—ASK1<sub>88–658</sub><sup>C206S,C225,226S</sup>, Trx1-Lys (alone)—Trx1<sup>C155S</sup> with C-terminal lysozyme, ASK1-Trx1-Lys (complex)—disulfide-linked species of the aforementioned ASK1 and Trx1-Lys proteins. Full protein sequences are given in Appendix Figure C.10. For a definition of terms see Table 4.1.
4.4.5.1 ASK1-Trx1 complex is relatively compact

The main conclusion from the SAXS data is that, overall, the ASK1-Trx1 complex has a relatively compact arrangement. The argument for compactness comes from a comparison of the $R_g$ and $D_{\text{max}}$ values, as well as the overall $P(r)$ distribution curves (Table 4.2 and Figure 4.9). The $R_g$ value represents the mean distance from the centre of mass of a molecule and gives an estimation of the molecule size in solution (Putnam et al., 2007). 

ASK1$_{88-658}^{\text{C206S, C225,226S}}$ has an $R_g$ value of $\sim$36 Å, and Trx1$_{\text{C35S-Lys}}$ $\sim$24 Å. The ASK1-Trx1 complex, by comparison, has an $R_g$ value of $\sim$43.6 Å, which is smaller than the sum of the two individual components.

The $P(r)$ curve indicates the distribution of inter-atomic vectors, i.e. the sum of distances between any two atoms within a molecule. The shape of the curve represents, to some degree, the overall topology of a molecule in solution (Putnam et al., 2007). The $D_{\text{max}}$ represents the point where $P(r)$=0 and is the maximum dimension for a given molecule (as illustrated by Figure 4.3c).

![Figure 4.9: Comparison of $R_g$ values and $P(r)$ curves.](image)

**Figure 4.9:** Comparison of $R_g$ values and $P(r)$ curves. Graphical representation of (a) $R_g$ values and (b) normalised $P(r)$ distribution from $P(r)$ and Guinier analysis (Table 4.2). ASK1 alone—ASK1$_{88-658}^{\text{C206S,C225,226S}}$, Trx1-Lys—Trx1$_{\text{C35S-Lys}}$ with C-terminal lysozyme, ASK1-Trx1-Lys—disulfide-linked species of the aforementioned ASK1 and Trx1-Lys proteins. Normalised $P(r)$ curves were generated by dividing the raw $P(r)$ value by the maximum $P(r)$ value for a given curve. Raw $P(r)$ curves are shown in Appendix Figure C.9d.

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$D_{\text{max}}$ values for the individual ASK1 and Trx1-Lys species and the complex again shows the same result as comparing the $R_g$ values. The complex P(r) curve is not drastically different from the curve of ASK1 alone (Figure 4.9b). Additionally, the sum of the individual $D_{\text{max}}$ values (70 and 120 Å) is less than that for the complex (135 Å). However, the $D_{\text{max}}$ value can be adjusted by the user and should not be taken at face value. Nonetheless, overall the $R_g$ values and P(r) distributions support the notion that Trx1-Lys forms a compact unit with ASK1, which in turn suggests a change in conformation of ASK1. Modelling of the complex and individual species can better represent changes in conformation.

4.4.5.2 Rigid-body modelling of ASK1-Trx1 complex

SAXS data can be used to model a multi-domain protein when only structures of individual domains are known (Putnam et al., 2007). For modelling the SAXS data presented in 4.4.5, the program ‘complexes with random loops’ (CORAL, part of the ATSAS package; Franke et al. 2017; Petoukhov et al. 2012) was used. The program CRYSOL (also part of the ATSAS package; Franke et al. 2017; Svergun et al. 1995) was used to generate theoretical scattering curves against which the goodness of fits for the models—denoted by a chi ($\chi$) value—is produced. In general, the lower the $\chi$ value, the better the data agrees with the model. However, it should be noted that the $\chi$ value is relative for a given data set, and therefore should not be used to compare models for different data sets.

There is no current structure of the ASK1-TBD (88–269)$^2$. Consequently, to model ASK1$_{88-658}$, the recent crystal structure of ASK1$_{269-658}$ (PDB ID: 5ULM; Weijman et al. 2017) along with a de novo model for ASK1$_{88-266}$ was used. The de novo ASK1$_{88-266}$ was generated using the Robetta server (Kim et al., 2004). It is the same de novo model as used in Weijman et al. (2017). The model depicts a globular protein with an $\alpha$-$\beta$-sandwich (a core of $\beta$-sheets surrounded by $\alpha$-helices, Appendix Figure C.11). Five models were generated by the Robetta server (Kim et al., 2004) and a comparison of all is shown in Appendix Figure C.11. For modelling using CORAL, the same

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$^2$Numerous attempts were made towards crystallising N-terminal ASK1 constructs (e.g., 88–266, 88–338 and 88–658, all with and without Trx1). However, no ASK1-containing crystals formed.
Robetta model was used in this work as in Weijman et al. (2017). In this fashion, there was some degree of freedom within where the ASK1-TBD could be placed by CORAL relative to ASK1\textsubscript{269–658}.

For the ASK1-Trx1-Lys complex, ASK1\textsubscript{88–658} was allowed to be modelled as above (i.e. allowing a positional change for the ASK1-TBD). Trx1-Lys was modelled independently using the crystal structure of Trx1\textsuperscript{C35S} in a mixed-disulfide with TrxR1 (PDB ID: 3QFB; Fritz-Wolf et al. 2011) and T4 lysozyme (PDB ID: 2I2L; Vetter et al. 1996). The model output was then used as a rigid molecule in combination with ASK1\textsubscript{269–658} and the ASK1-TBD model. The disulfide-linkage between ASK1 and Trx1-Lys (Cys250–Cys32) enabled the use of a distance restriction, where αC atoms from each cysteine residue within the disulfide bond should be no more than 10 Å apart. This distance was based on observations by Schmidt et al. (2006), who analysed over 6,000 disulfide bonds from the PDB and saw that the distance between the Cα from each cysteine residue (within the disulfide bond) was between 5.61 and 5.65 Å. A distance of 10 Å was used to prevent CORAL placing two molecules in overlapping space.

4.4.5.3 Proposed model for Trx1 mediated inhibition of ASK1

The models generated using CORAL showed a reasonable agreement with the experimental data (Figure 4.10). Comparison with the CORAL models shown in this Chapter with the previous BUNCH models (from Weijman et al. 2017 and presented in Chapter 3) highlights two possible conformations for the ASK1-TBD which can explain how ASK1 activity may be controlled (Figure 4.11).
Figure 4.10: CORAL rigid-body modelling of SAXS data. Figure legend on next page.
**Figure 4.10:** CORAL rigid-body modelling of SAXS data. a) ASK1\textsubscript{88-658}C\textsubscript{206S}, C\textsubscript{225,226S} (ASK1) alone. i. CORAL model. The fit of the model to experimental data was calculated using CRYSTOL ii. \(q\) vs Log(I) and iii. Guinier region (\(q^2\) vs Ln(I)). The agreement of the model to experimental data is shown by the \(\chi\) value. b) ASK1-Trx\textsubscript{1C\textsuperscript{35S}}-Lys disulfide-linked complex. i., ii., and iii. are as in a). The red patch within the PH domain and blue patch within the 7×TPR denote the sites mutated in Chapter 3 (see Figure 3.8). c) Agreement between the Trx1-Lys (alone) CORAL model that is used to generate b). i. and ii. are theoretical scattering and Guinier regions for the model compared to the experimental data generated using CRYSTOL. Models were rendered in PyMol.

**Figure 4.11:** Comparison of CORAL and BUNCH SAXS models. Overlay of the ASK1-TBD positions in the CORAL models from Figure 4.10 and BUNCH models from Weijman \textit{et al.} (2017) (shown in Chapter 3, Figure 3.4) illustrating the proposed ‘open’ (a) and ‘closed’ (b) forms. Positions are relative to ASK1\textsubscript{269-658} (1) depending upon whether they are from the BUNCH models (from Weijman \textit{et al.}, 2017) or CORAL models from this work (note—lysozyme from the ASK1-Trx1-Lys complex is omitted for clarity). c) Overlay of the CORAL models in this work (Figure 4.10) illustrating the relative change in position of the TBD. Models were rendered in PyMol.
The model (Figure 4.12) proposes that the TBD-switching mechanism (proposed in Chapter 3) can be modulated by Trx1. Under this model, the disulfide-linked complex represents an inhibitory, ‘closed’ state whereby MKK6 recruitment to the PH domain within ASK1_{269-658} is blocked by the TBD. Consequently, the release of Trx1 from the TBD would allow the TBD to change to the ‘open’ form, permitting MKK6 recruitment and downstream signalling. The proposed model would mean that addition of Trx1 to \textit{in vitro} kinase assays would result in inhibition of ASK1 and addition of the ‘kinetic trap’ version of Trx1 (Trx1\textsuperscript{C35S}) will further increase inhibition of ASK1\textsubscript{88-941}. 
Figure 4.12: Model of ASK1 regulation by Trx1. 

a) Inactive ASK1. i. The ASK1-TBD can block recruitment of MKK6 to the ASK1-PH domain. ii. Trx1 maintains the ASK1-TBD in a reduced state, whilst also preventing MKK6 to the ASK1-PH domain.

b) Active ASK1. i. When H₂O₂ levels become elevated, ASK1 becomes oxidised, and the TBD becomes ‘locked’ in a more rigid conformation, allowing MKK6 to be recruited and phosphorylated. ii. Trx1 can reduce ASK1, returning ASK1 to an inactive state.
4.4.6 Functional evaluation of ASK1 regulation by Trx1 *in vitro*

In order to test the proposed model for Trx1-mediated inhibition of ASK1, *in vitro* kinase assays were used, performed essentially identical to as in Chapter 3, but with the notable difference that all proteins used in the assay were reduced with DTT, before a final SEC step under non-reducing conditions. The proteins were aliquoted and snap-frozen as soon as they eluted from the SEC column. This step was used in order to minimise potential non-specific oxidation.

Many assays were performed and the consistent result was that Trx1 does not alter ASK1 kinase activity. Whilst these assays were being carried out, full-length, recombinant, ASK1 (ASK1_{2–1374}) expressed and purified from insect cells became available (see Methods, 2.6.14). Trx1 also did not alter ASK1_{2–1374} kinase activity. However, there was one exception where Trx1 did inhibit ASK1 kinase activity. Addition of both Trx1 and H\textsubscript{2}O\textsubscript{2} resulted in inhibition of MKK6 phosphorylation (4.4.7.2). Table 4.3 provides a summary of performed assays.

<table>
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<tr>
<th>ASK1 construct</th>
<th>Description (addition of:)</th>
<th>Change in activity?</th>
<th>Figure</th>
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</thead>
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<td>88–941</td>
<td>DTT</td>
<td>No</td>
<td>App. Fig. C.12</td>
</tr>
<tr>
<td>88–941</td>
<td>Trx1 (reducing)</td>
<td>No</td>
<td>App. Fig. C.13</td>
</tr>
<tr>
<td>88–941</td>
<td>Trx1 (non-reducing)</td>
<td>No</td>
<td>Figure 4.13</td>
</tr>
<tr>
<td>Full-length</td>
<td>Trx1 (reducing)</td>
<td>No</td>
<td>App. Fig. C.15</td>
</tr>
<tr>
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<td>Trx1 (non-reducing)</td>
<td>No</td>
<td>Figure 4.14</td>
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<tr>
<td>88–941</td>
<td>H\textsubscript{2}O\textsubscript{2}</td>
<td>No</td>
<td>Figure 4.15</td>
</tr>
<tr>
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</table>

App. Fig.—Appendix Figure. Reducing—2 mM DTT in assay. Non-reducing—no DTT. See specific Figures for details.
4.4.6.1 Trx1 does not inhibit ASK1<sub>88-941</sub> under reducing or non-reducing conditions

Initially, effects from non-specific protein oxidation—that could have occurred during protein preparation—were assessed. DTT was added into kinase assays (from 0 to 100 mM) performed with two different (2 and 6 µM) concentrations of ASK1<sub>88-941</sub>. No change (increase or decrease) in kinase activity was detected (Appendix Figure C.12). Consequently, it could be assumed that any potential effect on activity from addition of Trx1 would be because of a specific Trx1-ASK1 interaction rather than the oxidoreductase activity of Trx1 (which would be mimicked by DTT).

Next, Trx1 was added to the kinase assay (with ASK1<sub>88-941</sub>) under both non-reducing (Figure 4.13) and reducing (Appendix Figure C.13) conditions. Neither condition affected the rate of MKK6 phosphorylation. Under non-reducing conditions, an ASK1-Trx1 disulfide-linked species still formed as previously (Figure 4.13d). In order to try to promote the formation of a disulfide-linked species, ASK1<sub>88-941</sub> and Trx1 were incubated together overnight at 4 °C. The longer incubation of 1 µM ASK1<sub>88-941</sub> (in the presence of 100 µM Trx1<sup>C35S</sup>) resulted in the majority of ASK1 forming a disulfide-linked species with Trx1<sup>C35S</sup> (Appendix Figure C.14). However, despite the increased amount of the disulfide-linked ASK1-Trx1 species, no effect on kinase activity was seen.

Figure 4.13: Trx1 does not inhibit ASK1<sub>88-941</sub> activity in vitro under non-reducing conditions. a) Schematic of proteins in this assay. b) Flow diagram showing order of incubations. c) Trx1 and d) Trx1<sup>C35S</sup> were incubated (at the concentrations indicated) with ASK1<sub>88-941</sub> (1 µM) as indicated. ATP (50 µM) was added to start the reaction. At each time point, a sample was removed and added to a non-reducing SDS-PAGE buffer (containing 10 mM IAM). Samples were resolved by either Phos-Tag SDS-PAGE (for which sample was reduced with the addition of 100 mM DTT for 5 minutes) or non-reducing SDS-PAGE. Proteins were visualised with Coomassie.
**Figure 4.13:** Trx1 does not inhibit ASK1<sub>88-941</sub> activity in vitro under non-reducing conditions. Figure legend on previous page.
4.4.6.2 Full-length ASK1 is not inhibited by Trx1 *in vitro*

As previously mentioned, full-length recombinant ASK1 (ASK1$_{2–1374}$) from insect culture (Sf9 cells) became available during the course of this Thesis. The cultures were supplied by Abigail Burgess and Jack Curry (both Mace laboratory) and subsequently purified via nickel affinity chromatography (Figure 4.14c). The purified ASK1$_{2–1374}$ was active and could phosphorylate MKK6 *in vitro* (Figure 4.14d). It should be noted, however, that the full-length recombinant ASK1 prepared in this manner always had either contaminant proteins and/or degradation products present (Figure 4.14c). Therefore, it is not conclusive whether the apparent kinase activity is from true full-length ASK1 or from a degradative species.

ASK1$_{2–1374}$ was incubated with Trx1 under non-reducing conditions and subjected to an *in vitro* kinase assay (Figure 4.14). Phos-Tag SDS-PAGE, as well as western blotting for p-MKK6, were used to monitor MKK6 phosphorylation in case the increased sensitivity of western blotting would reveal a more subtle change. In agreement with the Phos-Tag SDS-PAGE analysis, no change in activity was detected by western blot (Figure 4.14c).

Overall, the addition of Trx1 did not alter ASK1 activity under reducing or non-reducing conditions. All of the conditions tested thus far can be considered ‘reductive’ as they have contained DTT (a chemical reductant), Trx1 (normally a cellular reductant), or both DTT and Trx1. To further assess the implications of a lack of change of activity, the effect of ‘oxidising’ agents on ASK1 was next considered.
Figure 4.14: *Trx1 does not inhibit full-length ASK1* in vitro. a) Schematic of proteins in this assay. b) Flow-diagram showing order of addition of proteins. c) SDS-PAGE gel showing elution from nickel affinity purification of ASK1_{2-1374} from Sf9 cells. The arrowhead indicates the position of ASK1_{2-1374}. d) *In vitro* kinase assay conducted under non-reducing conditions (no DTT) with addition of indicated amounts of Trx1 or Trx1_{C35S}. The assay was started with addition of 1 mM ATP. Note—the actual concentration of ASK1_{2-1374} in the assay was not quantifiable by A_{280 nm} but based on the band from purification (c), it was estimated to be at 0.1–0.5 μM. The assay was analysed by both Phos-Tag SDS-PAGE and western blot (for p-MKK6), both under reducing conditions. An equivalent assay under reducing conditions (2 mM DTT) was also performed with identical results (Appendix Figure C.15).
4.4.7 Functional evaluation of ASK1-Trx1 under oxidising conditions

4.4.7.1 Addition of H$_2$O$_2$ or Prdx1 does not change ASK1 activity in vitro

Treatment of many human cell lines with H$_2$O$_2$ is sufficient to activate ASK1 and downstream signalling pathways (Jarvis et al., 2012; Nadeau et al., 2009, 2007; Saitoh et al., 1998). In combination with these observations—and owing to the lack of inhibition from addition of Trx1 (or DTT)—H$_2$O$_2$ was added to in vitro kinase assays. Addition of up to 10 µM H$_2$O$_2$ (to ASK1$_{88-941}$) resulted in no discernible change in ASK1 kinase activity (Figure 4.15).

Figure 4.15: ASK1 kinase activity is not changed by H$_2$O$_2$. a) Schematic of proteins in this assay. b) Flow diagram showing how proteins were incubated. c) ASK1$_{88-941}$ (1 µM) was incubated with up to a 10-fold molar excess of H$_2$O$_2$ for 30 minutes at room temperature before being added to MKK6 (3 µM) and the kinase assay starting. The reaction was started with the addition of 50 µM ATP. The assay was analysed with Phos-Tag SDS-PAGE and proteins were visualised with Coomassie.
The thiol peroxidase protein, Prdx1, has been postulated to be able to oxidise ASK1 in human U937 cells, thereby altering ASK1 activity (Jarvis et al., 2012). To this end, recombinant human Prdx1 was added to *in vitro* kinase assays with both ASK1\textsubscript{88-941} and ASK1\textsubscript{12-1374}. Prdxs are well-known for being sensitive to oxidation *in vitro*, causing Prdx1 to run as a disulfide-linked dimer on non-reducing SDS-PAGE (Winterbourn and Peskin, 2016). To compensate, the Prdx1 used in assays was first reduced with DTT, before subsequent desalting through a spin-column (Appendix Figure C.16a). Under these conditions, reduced, monomeric Prdx1 could be maintained and was sensitive to \( \text{H}_2\text{O}_2 \) induced dimerisation (Appendix Figure C.16b). When Prdx1 was added (either 1 or 10 \( \mu \text{M} \)) to an *in vitro* kinase assay with ASK1\textsubscript{88-941} and \( \text{H}_2\text{O}_2 \) was added (10 \( \mu \text{M} \)), there was no change in MKK6 phosphorylation (Figure 4.16). A similar experiment was also performed using ASK1\textsubscript{12-1374} and Prdx1 with no detectable change in the appearance of p-MKK6 (Appendix Figure C.17).

Overall, the addition of \( \text{H}_2\text{O}_2 \) did not change the rate of MKK6 phosphorylation by ASK1. As ASK1 regulation by \( \text{H}_2\text{O}_2 \) and Trx1 *in vivo* can be regarded as being opposites, with \( \text{H}_2\text{O}_2 \) activating and Trx1 inhibiting ASK1, a final set of assays was performed using both \( \text{H}_2\text{O}_2 \) and Trx1.
Figure 4.16: ASK1 kinase activity is not changed by Prdx1. a) Schematic of proteins in this assay. b) Flow diagram showing the order proteins and H₂O₂ were combined. c) Recombinant, human Prdx1 was reduced and desalted before being added (at the concentrations indicated) to ASK188-941 (1 µM) and H₂O₂ (either 0, 1 or 10 µM). The proteins were incubated together for 15 minutes at room temperature before MKK6 was added. The reaction was started with the addition of 50 µM ATP and analysed via Phos-Tag SDS-PAGE. Proteins were visualised with Coomassie. The reduction and desalting conditions for Prdx1 were sufficient to produce a predominately monomeric species (on non-reducing SDS-PAGE that dimerised in response to treatment with H₂O₂ (Appendix C.16).
4.4.7.2 Addition of H\textsubscript{2}O\textsubscript{2} and Trx1 results in inhibition of MKK6 phosphorylation

As a final attempt to see whether any reductive or oxidative treatment could alter MKK6 phosphorylation by ASK1, both H\textsubscript{2}O\textsubscript{2} and Trx1 were included in the kinase assay system. ASK1\textsubscript{88–941}, Trx1 (Trx1 or Trx1\textsuperscript{C35S}) and H\textsubscript{2}O\textsubscript{2} (up to 100 µM\textsuperscript{footnote 3}) were incubated together overnight (Figure 4.17a,b). The incubated proteins were then used in an \textit{in vitro} kinase assay with MKK6. No change in activity was seen with either ASK1 alone (Appendix Figure C.18) or with Trx1 (Figure 4.17c). In contrast, with Trx1\textsuperscript{C35S} and 100 µM H\textsubscript{2}O\textsubscript{2}, there was a decrease in the appearance of p-MKK6 (Figure 4.17d).

A follow-up assay (Figure 4.18) was performed to better understand what the precise cause of MKK6 phosphorylation inhibition was. The previous assay (as in Figure 4.17) used a combination of ASK1, Trx1 and H\textsubscript{2}O\textsubscript{2} that were incubated together for 15 minutes before addition of MKK6. The kinase assay performed used the same stopping method as in Chapter 3—a reducing (including DTT) SDS-PAGE buffer. Therefore, any information into the possible oxidation status of MKK6 was lost.

\textsuperscript{3}Note—the concentration of H\textsubscript{2}O\textsubscript{2} used in Figure 4.17 is 10-fold higher than the previous assays (4.4.7.1).
Figure 4.17: Inhibition of M KK6 phosphorylation following incubation with Trx1 and H$_2$O$_2$. Figure legend on next page.
**Figure 4.17:** Inhibition of MKK6 phosphorylation following incubation with Trx1 and \(H_2O_2\). a) Schematic of proteins in this assay. b) Flow diagram showing the order of addition of proteins and incubations. c) and d) ASK1\(_{88-941}\) (1 \(\mu\)M) was incubated with either 100 \(\mu\)M of Trx1 (c) or Trx1\(^{C35S}\) (d) in addition to the indicated concentration of \(H_2O_2\) (see Footnote 3). ASK1, Trx1 and \(H_2O_2\) were incubated together for 15 minutes at room temperature, before which a sample was taken for analysis by non-reducing SDS-PAGE (ii). The remainder had MKK6 added (3 \(\mu\)M) and an *in vitro* kinase assay was performed. ATP (50 \(\mu\)M) was added to start the reaction. The assay was monitored by Phos-Tag SDS-PAGE. All gels (i and ii) were stained with Coomassie. For non-reducing SDS-PAGE (i), the sample was alkylated with 10 mM IAM. The arrowheads under d)ii. indicate decreased levels of p-MKK6. An equivalent assay was also performed without the addition of any Trx1, with no change in ASK1 activity detected (Appendix Figure C.18).

The follow-up assay deviated in two ways. Firstly, the pre-incubation was omitted. Instead ASK1, Trx1 and MKK6 were combined before the simultaneous addition of \(H_2O_2\) (100 \(\mu\)M) and ATP (Figure 4.18b). Secondly, the kinase reaction was stopped by placing the sample into a non-reducing sample buffer, supplemented with IAM (to stop potential thiol-rearrangements). The results of performing the kinase assay this way broadly mimicked those seen previously in Figure 4.17. The addition of \(H_2O_2\) to only ASK1\(_{88-941}\) and MKK6 did not inhibit MKK6 phosphorylation, neither did the addition of Trx1 (Figure 4.18c i.). By comparison, the addition of both \(H_2O_2\) and Trx1\(^{C35S}\) resulted in less p-MKK6 forming (compared to the same assay set-up without \(H_2O_2\), Figure 4.18c i.). When the same samples were resolved by non-reducing SDS-PAGE (Figure 4.18c ii.), several bands appeared when both Trx1\(^{C35S}\) and \(H_2O_2\) were added. Most notably, the parent MKK6 band decreased and bands of an approximate molecular weight of MKK6 in a disulfide-linked state with Trx1 appeared (which was not present under any other condition). The occurrence of Trx1-MKK6 species likely means that the observed inhibition of MKK6 phosphorylation (as in Figure 4.17) is not due to modulation of ASK1, but oxidation from \(H_2O_2\) followed with reduction by Trx1\(^{C35S}\), which was unable to detach from MKK6. The implication of MKK6-Trx1 disulfide-linked species formation will be discussed in 4.5.4.
Figure 4.18: Formation of M KK6-Trx1 species causes inhibition of M KK6 phosphorylation. a) Schematic showing which proteins are used in this assay. b) Flow diagram illustrating the order proteins were combined. c) Kinase assay with ASK188–941 (1 μM) alone or with 100 μM Trx1 or Trx1C35S. The proteins were mixed with ATP (50 μM) and H2O2 (either none or 100 μM) were simultaneously added before incubation at room temperature. i. Samples were taken at the times indicated for the kinase assay resolved by Phos-Tag SDS-PAGE. ii. A separate sample was taken at 10 minutes and alkylated with 10 mM IAM for non-reducing SDS-PAGE.
4.5 Discussion

4.5.1 ASK1 and Trx1 interact in vitro

To date, the majority of published studies on ASK1 and Trx1 have been from co-immunoprecipitation following overexpression in cells. Across multiple studies, ASK1 and Trx1 (both endogenously and when over-expressed) have been seen to associate via pull-downs (Cho et al., 2012; Fujino et al., 2007; Liu et al., 2000; Liu and Min, 2002; Nadeau et al., 2009, 2007; Saitoh et al., 1998). Two notable reports have instead used a recombinant system which also showed an interaction between the ASK1-TBD and Trx1 (Kosek et al., 2014; Kylarova et al., 2016).

In this work, an interaction between ASK1 and Trx1 is seen (Figure 4.4). The interaction is stronger with shorter constructs of ASK1, agreeing with the notion that residues 88–266 represent (or form the majority of) the core binding region for Trx1 by ASK1. The fact that less Trx1 is pulled down by the longer ASK1 constructs (88–658 and 88–941) suggests that the interaction between ASK1 and Trx1 is antagonised by regions outside the core binding domain.

Kosek et al. (2014) reported the binding affinity ($K_d$) for ASK1$_{88-302}$ and Trx1 as 0.3 µM. The observations in this work that longer ASK1 constructs (88–658 and 88–941) appear to have a decreased affinity for ASK1 (Figure 4.4), means that the $K_d$ reported by Kosek et al. (2014) should be interpreted with the understanding that it may not accurately reflect the true ASK1-Trx1 interaction in vivo. When the ASK1-Trx1 pull-down was performed under reducing conditions (with 2 mM DTT), a decreased amount of Trx1 was pulled-down by all but the longest (88–941) ASK1 construct (Figure 4.4). This result is in contrast to the observation by Kosek et al. (2014), where their observed $K_d$ of 0.3 µM was under reducing conditions (2 mM β-mercaptoethanol). Kosek et al. (2014) also used oxidised Trx1 (where Trx1 was incubated with a 100-fold molar excess of H$_2$O$_2$ for 15 minutes), which caused the $K_d$ to increase to 6 µM, indicating a weaker affinity. When Kosek et al. (2014) mutated both Trx1 active site cysteine residues to serine (Trx1$_{C32S,C35S}$), the $K_d$ increased to 900 µM. It is clear from the results by Kosek et al. (2014) (and a follow-up study by
Kylarova et al. 2016), that the association between ASK1 and Trx1 is stronger under reducing conditions. Therefore, without further follow-up work, the differences seen in this work of a pull-down under reducing conditions (Figure 4.4) should be interpreted with caution. It is clear, however, that the redox-status of cysteine residues, within both Trx1 and ASK1, is important to the ASK1-Trx1 interaction.

4.5.2 Importance of Cys250 in ASK1 for ASK1 interaction with Trx1

There are two proposed models for the interaction between ASK1 and Trx1 (see 4.2.2 and Figure 4.2). One model purports that the oxidoreductase activity of Trx1 is not required, instead, oxidation of Trx1 causes Trx1 to disassociate from ASK1. The other model states that Trx1 maintains ASK1 in a reduced state and therefore ASK1 oxidation can regulate ASK1 activity.

In this work, ASK1 and Trx1 were seen to interact in vitro allowing the formation of an inter-molecular disulfide bond between ASK1 and Trx1 (Figure 4.6). Moreover, when ASK1-Cys250 was mutated to Ser, the disulfide-linked species did not form (Figure 4.6 and Appendix Figure C.4c). The necessity of Cys250 was previously reported by Nadeau et al. (2009), whereby Cys250 was described as being necessary for downstream signalling from ASK1, but not for activation (i.e. phosphorylation at ASK1 T838, see 1.4.3) of ASK1. On the contrary, Kylarova et al. (2016) described ASK1-Cys200 as being able to form a mixed-species disulfide with Trx1. However, Kylarova et al. (2016) did not validate the role of ASK1-Cys200 in the ASK1-Trx1 interaction. In this work, when ASK1C200S was incubated with Trx1C35S, a mixed-disulfide species between ASK1 and Trx1 still formed (Appendix Figure C.4d). Lastly, a variant of ASK188–266 that only has Cys250 (the other 6 cysteine residues in the construct were mutated to serine), was still able to form a mixed-species disulfide with Trx1 (Appendix Figure C.5). The more important question, perhaps, is not which cysteine residue(s) are important but rather how does Trx1 form a mixed-species disulfide with ASK1?
4.5.2.1 Mechanism of ASK1-Trx1 mixed-species disulfide bond formation

Trx1, and other Trxs, have essential roles in all known forms of life. The primary role of Trxs—as part of the ‘thioredoxin system’—is in the maintenance of the oxidation status of a cell by reducing disulfide bonds (Linke and Jakob, 2003). The mechanism occurs via two cysteines in the active site of Trx. One cysteine (Cys32 in human Trx1) can attack a disulfide bond, forming an inter-molecular disulfide with the protein target and Trx itself (Wynn et al., 1995). The second cysteine in the active site (Cys35 in human Trx1) breaks the mixed-species disulfide, releasing oxidised Trx (containing an intra-molecular disulfide bond between the two active site cysteines) and the reduced substrate (see 4.2.1 and Figure 4.1b).

Following the system outlined above, ASK1 should contain an intra-molecular disulfide-bond for Trx1 to break, allowing the inter-molecular disulfide bond to form. However, as an ASK1 construct with only ASK1-Cys250 is still able to form a mixed-species disulfide (Appendix Figure C.5) it suggests that perhaps a disulfide-bond within ASK1 is not required. Trx-mediated reduction of non-disulfide, oxidised cysteines (namely sulfenic acid and sulfenyl amide) has previously been reported in vitro (Figure 4.19; Kim and Kim, 2008; Schwertassek et al., 2014).

Sulfenic acid and sulfenyl amide require only a single cysteine residue and therefore make an enticing hypothesis for how ASK1 and Trx1 might form a disulfide-linked species. Experimental evidence to investigate whether ASK1-Cys250 can form a sulfenic acid or sulfenyl amide could come from chemical labelling and mass spectrometry. For example, the chemical 5,5-dimethylcyclohexane-1,3-dione, better known as dimedone, has been used to identify sulfenic acids both from live cells and in vitro (Nelson et al., 2010; Reddie et al., 2008). More recently, it has been suggested that dimedone is not specific for sulfenic acids but rather sulfenyl amide, (Forman et al., 2017). However, as sulfenic acid is able to act as a precursor to the formation of sulfenyl amides, labelling with dimedone would prove invaluable regardless of the exact species it reacts with (Defelipe et al., 2015; Forman et al., 2017; Salmeen et al., 2003; van Montfort et al., 2003).

Lastly, as a point of evidence in support of ASK1-Cys250 being important, the
Figure 4.19: Reduction of sulfenic acid and sulfenyl amides by Trx. Depicted is a cysteine residue in a polypeptide chain. Shown is the cysteine side chain (SH) and the amide (NH) from the adjacent C-terminal amino acid. 1) Cysteine thiols can be oxidised to sulfenic acid by H$_2$O$_2$. 2) Sulfenic acids can condense on the amide from the polypeptide backbone to form a cyclic sulfenyl amide. 3) Reduced Trxs (Trx$^{\text{Red}}$)—and other thiol-containing reductants, such as GSH)—are proposed to reduce both sulfenyl amides and sulfenic acids via the formation of a mixed-species disulfide. 4) The second active site cysteine within Trx can break the intermediate yielding oxidised Trx (Trx$^{\text{Ox}}$) and the reduced cysteine thiol.

The formation of a sulfenic acid or sulfenyl amide would require Cys250 to be relatively reactive. One aspect that controls the reactivity of cysteine residues within proteins is the deprotonation of the thiol group, to form a thiolate (R-S$^-$), which depends on the pKa of the side chain (Poole, 2015). Cysteines at the N terminus of a helix are more prone to being reactive owing to the electron withdrawing effect of the helix (Iqbalsyah et al., 2006; Kortemme and Creighton, 1995; Paulsen and Carroll, 2013). Secondary structure predictions for ASK1 show that Cys250 is predicted to be N-terminal to a helix (Appendix Figure C.19), meaning it would be more likely to deprotonate. Other
cysteine residues within the ASK1-TBD are not predicted to occur N-terminal to a helix (Appendix Figure C.19). A high-resolution structure would be vital to confirm the secondary structural prediction. During this Thesis, attempts were made to crystallise the ASK1-TBD, but no ASK1-containing crystals formed. Potential strategies for achieving a structure of full-length ASK1 will be outlined in the final Discussion (6.3.1).

4.5.3 Observation of multiple Trx1 species on non-reducing SDS-PAGE

In this work, use of the ‘kinetic trap mutant’ version of Trx1 (Trx1$^{C35S}$) facilitated the formation of several Trx1-containing species (for example in Figure 4.7c and Appendix Figure C.4b). These bands are likely the result of oxidation of Trx1 cysteine residues. Human Trx1 contains five cysteine residues—at positions 32, 35, 62, 69 and 73 (Figure 4.20a).

Trx1 Cys32 and Cys35 form the active site of the protein. Cys73, for example, is well-noted as being able to form a disulfide-linked homo-dimer, which is why Trx1-Cys73 is generally mutated to a serine (Weichsel et al., 1996). Nitrosylation of Cys62 and Cys73 is proposed to have a regulatory role for Trx1 (Haendeler et al., 2002; Hashemy and Holmgren, 2008). Moreover, Cys62 and Cys69 have also been proposed to form an additional intra-molecular disulfide bond within Trx1 (Cheng et al., 2011; Watson et al., 2003). Intra-molecular disulfide bonds are well-known to increase the mobility of a denatured protein on SDS-PAGE (Pollitt and Zalkin, 1983; Wells and Powers, 1986). Cys62 has also been reported to form inter-molecular disulfide bonds, forming homo-dimers (Hall et al., 2010; Weichsel et al., 2010). All of these modifications can result in multiple Trx1-containing species that have different mobilities on non-reducing SDS-PAGE.

A combination of all of the aforementioned disulfide bonds is likely the cause of the multiple Trx1 bands, which possible specific species indicated in Figure 4.20. Additionally, the samples were alkylated (with IAM) before running on SDS-PAGE. The additional mass from alkylation could result in different sized products depending on the relative amount of alkylation that has occurred. The multiple Trx1-containing
Figure 4.20: Multiple Trx1 species. a) Location of cysteine residues on reduced Trx1 (PDB ID: 1ERT; Weichsel et al. 1996) and a ‘fully-oxidised’ Trx1 (PDB ID: 5DQY; Hwang et al. 2015). The oxidised form depicts intra-molecular Cys32-Cys35 and Cys62-Cys69 disulfide bonds. Structures were rendered in UCSF Chimera (Pettersen et al., 2004). b–d) Examples of multiple Trx1-species on non-reducing SDS-PAGE seen in this work. Postulated explanations are annotated. C62-C69—intra-molecular disulfide bond between Cys62 and Cy69; Dimer ‡—unknown disulfide-linked dimer; R—reducing (+DTT), NR—non-reducing (−DTT). See Figure sources for details on individual experiments. b) from Appendix Figure C.4b; c) from Figure 4.7c; d) from Figure 4.17di.

bands only occur with the Cys35Ser mutant, and under conditions of long incubations, or exposure to relatively high concentrations of H₂O₂, so are unlikely to be of any true biological importance. In the context of this work, the multiple species do raise a point of concern for the SAXS-analysis, as it will mean that the samples analysed were a mixture of oxidised (containing an intra-molecular disulfide bond) and reduced Trx1. Whilst it will not affect the quality of data, it will mean that the data is representative of a heterogeneous population making accurate interpretation harder. To avoid this in the future, the use of a Trx1 variant with all non-active site cysteines mutated to
serine (i.e. Trx1\(^{C35S,C62S,C69S,C73S}\)) should be considered.

### 4.5.4 \(\text{H}_2\text{O}_2\) and Trx1 mediated inhibition of MKK6 phosphorylation

In addition to the multiple Trx1-only containing disulfide-linked species, the use of Trx1\(^{C35S}\) and \(\text{H}_2\text{O}_2\) also caused the formation of Trx1-MKK6 disulfide-linked species (Figure 4.18). The occurrence of Trx1-MKK6 species likely means that the observed inhibition of MKK6 phosphorylation (as in Figure 4.17) is not due to alteration of ASK1, but oxidation from \(\text{H}_2\text{O}_2\) followed with reduction by Trx1\(^{C35S}\), which was unable to detach from MKK6. MKK6 has previously described as being sensitive to oxidation \textit{in vitro} (Diao et al., 2010; Sturchler et al., 2010). Human MKK6 contains 6 cysteine residues, including one cysteine, Cys216 on the activation loop—near Ser207 and Thr211—the residues that are phosphorylated by ASK1, Appendix Figure C.20). A formation of a Trx1-linked species at Cys216 therefore, would provide enough of a steric hindrance to block access of ASK1 to MKK6-Ser207/Thr211, thereby stopping phosphorylation without direct inhibition of ASK1 itself. Alternatively, Trx1-linkage at other cysteine residues could also promote an unfavourable MKK6-conformation for ASK1 to phosphorylate.

Ultimately, it is difficult to argue that the observed inhibition is anything other than an \textit{in vitro} artefact given that the inhibition was only seen from dual treatment with Trx1\(^{C35S}\) and higher levels of \(\text{H}_2\text{O}_2\). Future work should either use enzymatic (such as catalase) or physical (such as desalting) removal of excess \(\text{H}_2\text{O}_2\) prior to the addition of MKK6. Alternatively, mutagenesis of MKK6 to substitute the cysteine residues is also possible.

### 4.5.5 In-solution SAXS model of ASK1 and Trx1

Use of a Trx1-lysozyme fusion protein construct enabled the isolation of a disulfide-linked ASK1-Trx1 species. The isolated species was analysed using SAXS to determine structural parameters and rigid body modelling to illustrate how the species may be arranged (4.4.5). As previously discussed (4.5.3), the data needs to be interpreted in
light of a likely heterogeneous population of oxidised and reduced (relative to Trx1-Cys62 and Cys69) Trx1. Notwithstanding, the model can be of use to inform as to the relative arrangement of ASK1 and Trx1. From the structural parameters—$R_g$ and $P(r)$ (Figure 4.9)—it can be argued that the ASK1-Trx1 complex has a fairly compact arrangement (for instance the sum of the $R_g$ values for ASK1 and Trx1-Lys alone is greater than actually observed for the ASK1-Trx1 complex). The compact arrangement for the ASK1-Trx1 disulfide-linked complex was subsequently visualised by rigid body modelling.

4.5.5.1 Comparison with published ASK1-Trx1 SAXS models

A SAXS model of ASK1 in complex with Trx1 was previously reported by Kosek et al. (2014). As in the work presented in this Chapter, Kosek et al. (2014) used a de novo model of the ASK1-TBD (corresponding to residues 88–302). The overall ASK1-TBD model used by Kosek et al. (2014) is broadly similar to the de novo model used in this work (4.4.5, and in Weijman et al., 2017)—a globular protein with an $\alpha$-$\beta$-sandwich. Kosek et al. (2014) proposed, based on SAXS and other biophysical analyses, that the ASK1-TBD is a fairly rigid domain, and does not drastically change in shape upon binding with Trx1 (forming a non-covalent complex). Furthermore, the complex is proposed to be fairly compact. However, the model presented by Kosek et al. (2014) also assumes that ASK1 contains a coiled-coil within its N terminus (residues 272 to 302). From the crystal structure of ASK1$_{269-658}$ it is clear that within there is no coiled-coil and instead this portion of ASK1 has a closely-packed arrangement (Weijman et al., 2017).

On the whole, the model proposed by (Kosek et al., 2014) is useful as it analysed the more isolated ASK1-TBD as opposed to the longer N-terminal region used in this work (ASK1$_{88-658}$). The data agree with the TBD being a relatively compact, globular domain (as assumed by the Robetta models; 4.4.5.2 and Appendix Figure C.11). Beyond this, it is difficult to extrapolate. Kylarova et al. (2016) refined the model with their observations that Cys200 can form a disulfide bond, therefore, adjusting their de novo model so that ASK1-Cys200 and Trx1-Cys32 are relatively close. As they saw an
ASK1-Cys200–Trx1-Cys32 linkage, the model proposed by Kylarova et al. (2016) places Cys250 on a loop far away from the active site of Trx1. Ultimately, the low-resolution data that SAXS is capable of is useful for informing of the general relative arrangement of ASK1 and Trx1. Understanding how and why specific residues within either protein can control the protein-protein interaction requires higher resolution data from methods such as X-ray crystallography and this will be explored further in the final Discussion (6.3.1).

4.5.6 Trx1 does not inhibit ASK1 in vitro

Within cells, Trx1 has long been regarded as a direct inhibitor of ASK1 signalling (Saitoh et al., 1998). The general viewpoint has been that Trx1 associates with the N terminus of ASK1, blocking ASK1 N-terminal oligomerisation. Disassociation of Trx1 is required to allow for the formation of a fully active ASK1 complex (see 4.2.2; Fujino et al., 2007; Saitoh et al., 1998). Exactly how Trx1 inhibits ASK1 has remained elusive (see 4.2.2) hence in this work a biophysical model of ASK1-Trx1 was developed (4.4.5) and in vitro kinase assays were performed (4.4.6).

Whilst in vitro kinase assays have been performed on ASK1 previously, the approach used has generally been to over-express and immunoprecipitate ASK1 from cells. For example, Saitoh et al. (1998) over-expressed ASK1 in HEK293 cells, immunoprecipitated ASK1 and used the subsequent material in an in vitro kinase assay to monitor ASK1 activity directly. From this approach, Saitoh et al. (1998) reported roughly two-fold less ASK1 activity when ASK1 was co-expressed with Trx1 and then ASK1 was immunoprecipitated. One major caveat of this approach is that other molecules may be co-purified and it is impossible to know with any certainty what they may be. Co-purification of non-ASK1 molecules is even more likely in light of observations that when purified in this manner, ASK1 forms a large complex (Noguchi et al., 2005). Additionally, Saitoh et al. (1998) produced ASK1 via an in vitro (eukaryotic cell-free) translation system alongside recombinant GST-tagged Trx1 from E. coli. However, Saitoh et al. (1998) did not show any kinase assays with the in vitro translated ASK1, nor did they add any form of Trx1 directly to their kinase assays with immunoprecip-
itated ASK1.

The assays presented in this Chapter (4.4.6) have used recombinant ASK1, substrate and regulatory proteins, purified to homogeneity from *E. coli*. Throughout the *in vitro* kinase assays performed addition of Trx1 has shown no inhibition regardless of the conditions tried. These assays have included up to a 100-fold molar excess of Trx1. Based on these observations it has to be assumed that either Trx1 does not inhibit ASK1 directly or the system used in this work is not complete enough to emulate the exact conditions required for Trx1-inhibition.

Saitoh *et al.* (1998) show quite conclusively that the association between ASK1 and Trx1 can be modulated by, for example, treating cells with H2O2. However, the assumption is made that Trx1 no longer associates with ASK1 because Trx1 becomes oxidised, yet the actual oxidation status of Trx1 following these treatments is never directly assessed. Moreover, whilst Saitoh *et al.* (1998) do state that the oxidation mechanism is unclear and is a proposed hypothesis, it is widely accepted in the literature. The work done by Nadeau *et al.* (2009) goes some-way in improving the model, with the inclusion that an unknown contributor (dubbed “Factor X”) is likely required for Trx1 to control ASK1 signalling. However, the missing element suggested by Nadeau *et al.* (2009) is generally omitted and the model of direct Trx1-inhibition of ASK1 still predominates in the literature.

Jarvis *et al.* (2012) proposed that Prdx1 activates ASK1 via an inter-molecular disulfide bond. In this fashion, Prdx1 may act as the “Factor X”. However, in this work, the addition of Prdx1 to kinase assays did not change the activity of ASK1 (Figure 4.16 and Appendix Figure C.17). Whilst not conclusive, it does imply that oxidative regulation of ASK1 requires more than the presence of Trx1, H2O2 or Prdx1.

One aspect that is not recapitulated by the methodology used in this work is ASK1 N-terminal homo-oligomerisation. Of the constructs used in this work, only the ASK1 kinase domain (ASK1_{669-941}) behaves as a dimer in solution (Appendix Figure A.2f). ASK1_{88-266} is monomeric, as is ASK1_{88-658}, and ASK1_{88-941} (Appendix Figure A.2b,d, Table 4.2; Bunkoczi *et al.*, 2007; Kosek *et al.*, 2014; Kylarova *et al.*, 2016; Weijman *et al.*, 2017). Whether Trx1 would inhibit an oligomeric form of ASK1 *in vitro* remains to be seen. ASK1_{2-1374} (as used in 4.4.6.2) will likely be oligomeric owing to the
presence of the C-terminal coiled-coil and SAM domain (Trevelyan, 2017). However, experiments using ASK1$_{2-1374}$ purified from insect cells also did not show any inhibition by Trx1 (4.4.6.2). On the other hand, the ASK1$_{2-1374}$ species used exhibited many degradation products (or other contaminants) and therefore there can be no certainty that the activity seen is from ASK1$_{2-1374}$. Further experiments with a homogeneous ASK1$_{2-1374}$ sample is required to say for certain. Use of full-length ASK1 would also be very telling for the true oligomeric nature of ASK1. As was discussed in Chapter 3 (3.6.2), dimerisation, and further oligomerisation are also viewed as important regulatory mechanisms of ASK1. It follows, therefore, that Trx1 regulation may only occur on an oligomeric form of ASK1.

As mentioned in the introduction (4.2.1), Palde and Carroll (2015) proposed that the biggest driver towards *E. coli* Trx substrate recognition is entropy. Palde and Carroll (2015) used isothermal titration calorimetry (ITC) to reach their conclusions. Similar ITC-based experiments between ASK1 and Trx1 would help towards investigating what, thermodynamically speaking, drives ASK1 and human Trx1 association. Such ITC-based experiments would thoroughly benefit from a high-resolution structure of ASK1 and Trx1 in order to probe the interaction interface with mutagenesis. Moreover, experiments such as these would also have broader conclusions as to the nature of human Trx1 substrate recognition.

Altogether, this Chapter highlights that ASK1 regulation by Trx1 requires more than an interaction between the two proteins. It is highly probable that other regulatory mechanisms of ASK1 signalling are required in order for Trx1 to have a regulatory role.

### 4.6 Conclusions

The work in this Chapter has shown that ASK1 and Trx1 do interact *in vitro*. Moreover, residues within the N terminus of ASK1 (88–266) are sufficient for binding between ASK1 and Trx1. Trx1 can form a disulfide-linked species with ASK1 forming a 1:1 complex. ASK1-Cys250 is necessary and sufficient for formation of the disulfide-linked species. The precise mechanism leading to the formation of the species is unclear. The
formation of a disulfide-linked ASK1-Trx1 species allowed for the isolation of a stable protein complex to be analysed via SAXS. Rigid body modelling of the SAXS data indicated that Trx1 could influence the relative conformation of the ASK1 TBD, regulating overall ASK1 activity. However, functional analysis has shown that ASK1_{88-941} is not inhibited by Trx1 under any condition tested. Altogether, further work is required to understand the true nature of Trx1 regulation of ASK1 and identify missing regulatory features of Trx1-regulated ASK1 signalling.
Chapter 5

A pilot study into the contribution of Prdx1 to H$_2$O$_2$ signalling

5.1 Overview

H$_2$O$_2$ is a molecule that is required in many cell signalling pathways, but how H$_2$O$_2$ signalling is possible is paradoxical, as \textit{in vivo}, H$_2$O$_2$ will be rapidly converted to water by Prdxs. Prdxs, such as Prdx1, are proposed to facilitate H$_2$O$_2$ signalling by forming inter-molecular disulfide bonds with signalling-substrates, such as ASK1. However, it is unclear how universal disulfide bond transmission is, as there are limited examples. The focus of this Chapter is to test a redox proteomics approach for investigating the hypothesis that Prdx1 is a \textit{bona fide} signalling protein. A pilot study is performed using isotopic-labelling of cysteine residues from a cell lysate. Whilst the method is able to identify differentially oxidised proteins, it is not sufficiently optimised to address the hypothesis.

5.2 Publication outputs

5.3 Introduction

5.3.1 Background on H$_2$O$_2$ signalling

Since the discovery of H$_2$O$_2$ in the early 1800s, our view of H$_2$O$_2$ has progressed from it being a dangerous, reactive molecule that has to be controlled, to being essential for healthy homoeostasis (Holmström and Finkel, 2014; Ledgerwood et al., 2017; Reczek and Chandel, 2015; Sies et al., 2017). The discovery that growth factor signalling could stimulate the production of H$_2$O$_2$ by NOXs represented a paradigm shift in our fundamental understanding of H$_2$O$_2$ biology (Bae et al., 1997, 2000; Lassègue et al., 2001; Park et al., 2004; Sundaresan et al., 1995). When stimulated, growth factor receptors (such as the PDGF receptor) not only initiate canonical receptor tyrosine kinase signalling, but there is also activation of NOXs (Holmström and Finkel, 2014). NOXs produce superoxide (extracellularly) which is subsequently converted (by SODs, see 1.5.4) to H$_2$O$_2$ (Winterbourn, 2008). The nascent H$_2$O$_2$ can subsequently freely diffuse across the plasma membrane. Whilst the generation of H$_2$O$_2$ is quite well understood, how and why H$_2$O$_2$ is then important for downstream receptor signalling is more enigmatic.

Our understanding of precisely how such a small and simple molecule could influence the cellular environment continues to gather interest. Much of this interest is because eukaryotes express an armament of proteins named Prdxs (Peroxiredoxins) whose chief role is to break down H$_2$O$_2$ to water.

5.3.2 Peroxiredoxins

Prdxs are abundant proteins found in every domain of life. Within mammalian cells, peroxiredoxins (and Gpxs) make up the majority of our anti-peroxide defences\(^1\) (Cox et al., 2010; Rhee et al., 2012; Winterbourn, 2008).

Mammals have 6 Prdxs: Prdx1–4 are very similar and use a two cysteine (2-Cys)

\(^1\)Catalase is an abundant peroxidase protein with a very fast reaction rate with H$_2$O$_2$ (see Table 5.1). However, catalase is primarily in peroxisomes and so does not greatly contribute to the breakdown of cytosolic H$_2$O$_2$ (Schrader and Fahimi, 2006).
### Table 5.1: Second-order rate constants and concentrations of proteins and molecules reacting with H$_2$O$_2$

<table>
<thead>
<tr>
<th>Protein Type</th>
<th>Approximate rate constant (M$^{-1}$ s$^{-1}$)</th>
<th>Approximate cellular concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antioxidant proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peroxiredoxins</td>
<td>$1-4 \times 10^7$ $^a$</td>
<td>20 $^a$</td>
</tr>
<tr>
<td>Glutathione peroxidases</td>
<td>$6 \times 10^7$ $^b$</td>
<td>2 $^a$</td>
</tr>
<tr>
<td>Catalase</td>
<td>$2-3 \times 10^7$ $^{a,b}$</td>
<td>1,000 $^{e,h}$</td>
</tr>
<tr>
<td>Thioredoxin 1</td>
<td>1.05 $^a$</td>
<td>1–10 $^c$</td>
</tr>
<tr>
<td><strong>Antioxidant small molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione (reduced)</td>
<td>0.89 $^b$</td>
<td>2,000 $^a$</td>
</tr>
<tr>
<td>Cysteine</td>
<td>2.9 $^a$</td>
<td>30–200 $^f$</td>
</tr>
<tr>
<td>N-acetylcysteine</td>
<td>0.16 $^a$</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Non-antioxidant proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTPs</td>
<td>9–20 $^{b,e}$</td>
<td>0.01–0.1 $^{a,e}$</td>
</tr>
<tr>
<td>PTEN</td>
<td>14 $^c$</td>
<td>&lt; 0.002 $^g$</td>
</tr>
<tr>
<td>KEAP1</td>
<td>140 $^d$</td>
<td>1 $^e$</td>
</tr>
<tr>
<td>GAPDH</td>
<td>100–500 $^{a,b}$</td>
<td>50 $^a$</td>
</tr>
<tr>
<td>Cdc25s</td>
<td>120–164 $^b$</td>
<td>0.1 $^a$</td>
</tr>
</tbody>
</table>

PTPs—Protein tyrosine phosphatases, PTEN—Phosphatase and tensin homologue, KEAP1—Kelch-like ECH-associated protein 1, GAPDH—Glyceraldehyde 3-phosphate dehydrogenase, Cdc25s—Cell division cycle 25 dual-specificity phosphatases. References: $^a$—Winterbourn and Hampton (2008); $^b$—Stone (2004); $^c$—Holmgren and Luthman (1978) and Schwertassek et al. (2014); $^d$—Marinho et al. (2014); $^e$—Antunes and Brito (2017); $^f$—Tian et al. (2014); $^g$—Johnston and Raines (2015). $^h$—Catalase, though abundant, is primarily located within peroxisomes (see Footnote 1).
mechanism (see Figure 5.1a) whereas Prdx5 uses an atypical 2-Cys mechanism and Prdx6 uses a 1-Cys mechanism (Nelson et al., 2011). The main difference between Prdx1–4 is in their primary sub-cellular locations: Prdx1 and 2 are cytosolic; Prdx3 is mitochondrial and, Prdx4 is in the ER. All Prdxs have high rate constants (in the order of $10^7 \text{M}^{-1} \text{s}^{-1}$) with H$_2$O$_2$ (Table 5.1; Winterbourn and Hampton 2008).

Eukaryotic 2-Cys Prdxs exhibit a paradoxical characteristic whereby they can be inactivated by their substrate, H$_2$O$_2$ (Rabilloud et al., 2002; Rhee et al., 2012). Inactivation occurs via ‘over-oxidation’ (also referred to as hyper-oxidation) of the peroxidatic cysteine (C$_P$) residue (Figure 5.1a). Over-oxidised Prdx involves the formation of a sulfinic (R–SO$_2$H) or sulfonic (R–SO$_3$H) acid at the peroxidatic cysteine. Whereas the sulfenic acid and disulfide bond forms are reversible via the Trx/TrxR system (Figure 5.1a), the sulfinic acid form requires an ATP dependent process with the protein sulfiredoxin (Biteau et al., 2003; Woo et al., 2003). By comparison, there is no known process by which the sulfonic acid form can be reduced.

**Figure 5.1:** Prdx mechanism, structure, and comparison of Prdx1 and 2. a) Peroxidase cycle of eukaryotic 2-Cys Prdxs. 1: The peroxidatic cysteine (C$_P$, drawn as the more reactive thiolate, S$^-$) reacts with H$_2$O$_2$ forming a sulfenic acid (R-SOH). 2: The resolving cysteine (C$_P$, on the second subunit) and sulfenic-C$_P$ condense to form a disulfide bond. 3: The Prdx is regenerated via the Trx/TrxR/NADPH system. 4) When concentrations of H$_2$O$_2$ are high, the peroxidatic cysteine can become over-oxidised to sulfinic (R–SO$_2$H) or sulfonic (R–SO$_3$H) acids which is irreversible by the Trx/TrxR system (Karplus, 2015) (see 5.3.2). b) Structure of human Prdx1 showing the peroxidatic cysteine (Cys52) and resolving cysteine (Cys172). Left: reduced (PDB ID: 1QQ2; Hirotsu et al. 1999), right: oxidised showing the protein surface (PDB ID: 2RII; Jönsson et al. 2008). The inset shows the inter-subunit disulfide bond. c) Amino acid alignment of Prdx1 and 2 showing 77.4% identity. The position of the peroxidatic and resolving cysteines are indicated. *—identical, :—strongly similar, .—weakly similar. The alignment was generated using ClustalOmega (Li et al., 2015).
Figure 5.1: Prdx mechanism, structure, and comparison of Prdx1 and 2. Figure legend on previous page.
5.3.2.1 Why do mammals have two cytosolic Prdxs?

The existence of both Prdx1 and 2 has continued to gather interest amongst researchers. Are the two proteins redundant, or do they each serve a different process? The primary sequence is very similar but there is approximately a 20% difference in the primary amino acid sequence (Figure 5.1c). Evidence that Prdx1 and 2 are not functionally redundant can come from kinetic data. For example, Lee et al. (2007) compared the relative reactivity of Prdx1 and 2, finding that Prdx2 was approximately 3-fold more active than Prdx1. More recently, Carvalho et al. (2017) published a second-order rate constant for Prdx1 with \( \text{H}_2\text{O}_2 \) as \( 3.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \), which is higher than the reported value for Prdx2—\( 1.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \) (Peskin et al., 2007). A side-by-side determination of rate constants would be required for a more accurate comparison. Nonetheless, Lee et al. (2007) also observed that Prdx1 was markedly more sensitive to over-oxidation than Prdx2, with Prdx1 being over-oxidised by exposure to 0.2 mM \( \text{H}_2\text{O}_2 \), whereas Prdx2 was not over-oxidised until exposure to 1 mM \( \text{H}_2\text{O}_2 \). Other differences between Prdx1 and 2 have been seen in their interactions with other proteins. For example, Prdx1 can bind to the protein kinase MST1, but Prdx2 cannot (Morinaka et al., 2011). Additionally, Prdx1-KO (knock-out) and Prdx2-KO mouse models have different cancer phenotypes (Hampton et al., 2017), suggesting the two proteins serve different purposes. Overall, the differences between Prdx1 and 2 (and other Prdxs) may be important in regards to \( \text{H}_2\text{O}_2 \) signalling.

5.3.3 Models of \( \text{H}_2\text{O}_2 \) signalling

As eluded to in 5.3.1, \( \text{H}_2\text{O}_2 \) signalling is somewhat paradoxical. \( \text{H}_2\text{O}_2 \) is a reactive molecule capable of damage and thus is kept under tight control by various peroxidases. However, \( \text{H}_2\text{O}_2 \) production is required for many cell signalling events. How then is \( \text{H}_2\text{O}_2 \) able to react with any targets and how does it do so with any specificity?

Several models (Figure 5.2) have been postulated to explain the discrepancy:

- Direct oxidation
- The floodgate model
5.3.3.1 Direct oxidation as a model for H$_2$O$_2$ signalling

Direct oxidation is the simplest explanation for how H$_2$O$_2$ can modulate protein signalling (Figure 5.2a). H$_2$O$_2$ reacts with cysteine residues in a protein, altering that protein’s activity.

Specificity from direct oxidation can be achieved owing to the chemistry of cysteine. The most reactive form of cysteine comes from the deprotonated thiolate (Reaction 5.1). The degree of deprotonation is dependent upon the p$K_a$ of cysteine thiol which is influenced by the localised environment of the cysteine residue (Bulaj et al., 1998; Poole, 2015). The p$K_a$ of a thiol in free cysteine is $\sim$8.3 meaning it will be protonated at physiological pH ($\sim$7.4). Conversely, the peroxidatic cysteines of human Prdxs have a p$K_a$ of $\sim$5–6 meaning the thiolate is more likely to form, making the cysteine more reactive (Winterbourn and Hampton, 2008). Therefore, a cysteine residue’s milieu can influence the thiol p$K_a$, making it more (or less) likely to react with H$_2$O$_2$.

\[
\text{Cys} - \text{SH} \rightleftharpoons \text{Cys} - \text{S}^- + \text{H}^+ \quad (5.1)
\]

The notion of direct oxidation is a simple idea, H$_2$O$_2$ produced on demand (e.g., from NOXs, see 5.3.1), can find and react with cysteine residues that are ‘ready’ (i.e. thiolates). Additionally, H$_2$O$_2$ being a small molecule will allow it to permeate into regions of proteins and other macromolecules. The major problem with this simple model of direct oxidation is it cannot explain how H$_2$O$_2$ could escape being converted to water by abundant and fast peroxidases. Peroxidases, such as Prdx1, would out-compete any non-peroxidase protein (see Table 5.1). Even non-peroxidase proteins (such as GAPDH or Cdc25 phosphatases, Table 5.1) that have a relatively fast rate constant ($\sim$100–500 M$^{-1}$ s$^{-1}$) with H$_2$O$_2$ are several orders of magnitude below that of Prdxs (second-order rate constants of $\sim$10$^7$ M$^{-1}$ s$^{-1}$). One model put forward to address the shortfall of direct H$_2$O$_2$ is the inactivation of Prdxs by H$_2$O$_2$—‘the floodgate model’.
Figure 5.2: $H_2O_2$ signalling models. 

a) Direct oxidation model. $H_2O_2$ reacts with protein targets, oxidising cysteine thiols (“Cys–$S_{ox}$”). The oxidised and reduced protein will have a different output from either a direct effect on protein activity or a change in localisation, stability or interaction partners. b) The floodgate model. High concentrations of $H_2O_2$ will over-oxidise and inactivate Prdxs (see 5.3.2). Inactive Prdxs will no longer be able to break down $H_2O_2$ allowing $H_2O_2$ to oxidise protein targets as in a). c) The disulfide relay model. 1) Prdxs act as sensors of $H_2O_2$ becoming oxidised during the breakdown (see Figure 5.1a). 2) Oxidised Prdx (either as a disulfide or a sulfenic acid) will oxidise a protein target forming an intermediate mixed-species disulfide bond. 3) The mixed-species disulfide bond collapses resulting in an oxidised protein target (likely containing a disulfide bond) and reduced Prdx.
5.3.3.2 The floodgate model of H\textsubscript{2}O\textsubscript{2} signalling

Eukaryotic Prdxs are able to be inactivated by H\textsubscript{2}O\textsubscript{2} via over-oxidation of the peroxidatic cysteine residues (see 5.3.2 and Figure 5.1a). The phenomenon of over-oxidation has been used to explain how H\textsubscript{2}O\textsubscript{2} could react with non-peroxidase thiols (Wood \textit{et al.}, 2003a). When the concentration of H\textsubscript{2}O\textsubscript{2} is high enough, over-oxidation of Prdxs occurs allowing H\textsubscript{2}O\textsubscript{2} to signal to targets as in the direct oxidation model above (Figure 5.2a,b). Furthermore, phosphorylation has also been seen as a way of inactivating Prdx1, which would also facilitate the floodgate model (Woo \textit{et al.}, 2010).

Whilst the floodgate model does explain how H\textsubscript{2}O\textsubscript{2} may be able to overcome Prdxs it does have two problems. Firstly, even if Prdxs were inactivated, other peroxidases (namely glutathione peroxidases, Gpxs) are still quite abundant and have high rate constants with H\textsubscript{2}O\textsubscript{2} (Table 5.1). Secondly, there is the problem of H\textsubscript{2}O\textsubscript{2} specificity. Even if a target cysteine thiol had a relatively low pK\textsubscript{a}, H\textsubscript{2}O\textsubscript{2} cannot delineate between different targets therefore not allowing for signalling specificity. The disulfide relay model attempts to overcome the limitations above by viewing Prdxs not simply as peroxidases, but as playing an active role in signal transduction.

5.3.3.3 Disulfide relay of H\textsubscript{2}O\textsubscript{2} signalling

The disulfide relay is another model for H\textsubscript{2}O\textsubscript{2} signalling. In this model, peroxiredoxins will ‘sense’ H\textsubscript{2}O\textsubscript{2}, becoming oxidised, and in turn oxidise a signalling substrate (Figure 5.2c). This model explains how there can be specificity within the system and how abundant and fast peroxidase do not compromise the system. Specificity is allowed by protein-protein interactions by the Prdx, and the peroxidase activity is a requirement (Rhee \textit{et al.}, 2018).

The biggest drawback of the disulfide relay model of H\textsubscript{2}O\textsubscript{2} signalling is that whereas there are many proteins known to be modulated by oxidation (e.g., the non-peroxidase proteins in Table 5.1), there is only a handful of disulfide-relay examples from yeast and mammalian systems.

Arguably, the best-characterised relay, comes from yeast, between the transcription factor, Yap1, and the peroxiredoxin, Gpx3 (also called Orp1) (Marinho \textit{et al.}, 2014).
Gpx3 becomes oxidised in response to H$_2$O$_2$. Oxidised Gpx3 oxidises Yap1 via the formation of a transient mixed-species disulfide bond. Gpx3 disassociates from Yap1, leaving a disulfide bond within Yap1. The nascent Yap1 disulfide bond causes a change in the protein, secluding a nuclear export sequence allowing the protein to localise to the nucleus and initiate transcription (Delaunay et al., 2000, 2002; Wood et al., 2004, 2003b). Examples from mammalian systems include Prdx1 and ASK1, and Prdx2 and the transcription factor signal transducer and activator of transcription (STAT)3 (Jarvis et al., 2012; Sobotta et al., 2015).

Further evidence that Prdxs have a role in signalling comes from a yeast transcription study. Saccharomyces cerevisiae that had all Prdxs and Gpxs genetically deleted were unable to transcriptionally respond to H$_2$O$_2$ treatment (Fomenko et al., 2011). WT yeast under the same conditions had ~1500 genes that were differentially regulated by H$_2$O$_2$ treatment (Fomenko et al., 2011). The absence of a transcriptional response without expression of Prdxs and Gpxs is strongly indicative that, in S. cerevisiae at least, Prdxs/Gpxs are responsible for signalling from H$_2$O$_2$ to transcription factors.

The requirement of Prdxs and Gpxs for H$_2$O$_2$ transcriptional response in yeast would not be compatible with the floodgate model pointing towards the disulfide relay model. Examples such as the Gpx3-Yap1 relay provide evidence towards the disulfide-relay, however, without more examples and a broader study from a mammalian setting it is difficult to assess whether a disulfide-relay is the major signalling mechanism or simply one part of many.

5.3.4 Prdx1 as a disulfide-relay partner

Previous work from the Ledgerwood laboratory showed that Prdx1 and the protein kinase ASK1 were involved in a disulfide-relay (Jarvis et al., 2012). When treated with exogenous H$_2$O$_2$, U937 cells responded with the phosphorylation of p38 MAPK. Knock-down of Prdx1 ablated the p38 response. Interestingly, over-expression of Prdx2 in U937 cells also inhibited the H$_2$O$_2$-p38 response suggesting that the two cytosolic Prdxs have contrasting roles.

Studies such as by Jarvis et al. (2012) provide great power in terms of our mecha-
nistic understanding. Very recently, Stöcker et al. (2018a) published a study suggesting that Prdxs do contribute to $\text{H}_2\text{O}_2$ signalling and this will be expanded upon in the final Discussion (6.4). However, more examples and other evidence from a more system-wide approach is required to inform us into the greater role a disulfide-relay model plays in $\text{H}_2\text{O}_2$ signalling.

5.3.5 Redox proteomics

A holistic study of cellular proteins—proteomics—allows for a picture of a cell on the whole rather than a brief snapshot. Such a method is useful for questions such as investigating the signalling role of Prdx1, as it does not assume what may or not be targets.

One method that has been devised for redox proteomics is isotope-coded affinity tag (ICAT) (for a more comprehensive view of the redox proteomics landscape, see Bach et al. 2013 and Yang et al. 2016). ICAT was originally developed as a tool for quantitative proteomics (Shiio and Aebersold, 2006). The ICAT reagent features an IAM-based thiol-modifying group, an isotopic tag and an acid-cleavable biotin moiety (Figure 5.3a; Hägglund et al. 2008; Shiio and Aebersold 2006). The original use of ICAT is based on the principle that despite only $\sim 25\%$ of human tryptic peptides contain cysteines, this pool will represent over 95% of the human proteome (Shiio and Aebersold, 2006). Therefore, by labelling peptides from two different samples (one with the light reagent and the other with heavy) and analysing it by LC-MS/MS, a ratio can be calculated for the relative abundance of proteins from the two samples.

Redox proteomics using ICAT takes advantage of the thiol-reactive group in order to label cysteines (Leichert et al., 2008). Sequential labelling, reduction and alkylation then informs us not of the abundance of a protein, but rather its oxidation status. This Chapter seeks to address whether an ICAT-proteomics approach would work and whether a cell line that is deficient for Prdx1 has a different protein oxidation profile to the WT cell line. An ICAT-based method is used for several reasons. Firstly, the ICAT method is a kit-based system lowering the methodological development time. Secondly, the ICAT method uses a biotin-enrichment step which allows for enrichment
of lower abundant ICAT-labelled proteins.

The ICAT method can be used in a monoplexed (Figure 5.3b) or duplexed approach (Figure 5.3c). For the monoplexed version, two samples are combined with analysis of either oxidised or reduced cysteines (Figure 5.3b). By comparison, the duplexed approach enables the user to label an entire proteome (reduced and oxidised cysteines) from one sample Figure 5.3c).

There are advantages and disadvantages for opting for a monoplexed or duplex approach. The duplexing approach captures a broader image (reduced and oxidised) of one cellular population. By contrast, the monoplexed version allows for the comparison between two cell treatments, decreasing the overall monetary cost of the total experiment. Additionally, the monoplexed version can label either reduced protein thiols or reversibly oxidised thiols, depending upon the aim of the experiment. Lastly, as the duplexed approach compares proteins within the same sample there is consistency for the total protein amount. By comparison the monoplexed version is dependant upon accurate protein quantification for the individual populations to be analysed.

Figure 5.3: Overview of ICAT redox proteomic experiments. a) The ICAT reagent consists of the thiol-reactive component, based on IAM (iodoacetamide), an isotopic tag containing either 9× Carbon-12 (\(^{12}\text{C}\), light) or Carbon-13 (\(^{13}\text{C}\), heavy) and a biotin moiety. The biotin group enables enrichment and is cleavable under acidic conditions (Hägglund et al., 2008; Shiio and Aebersold, 2006). b) Monoplexed version. Cells from two different conditions processed in tandem. The cells are lysed and proteins are denatured. The proteins will contain a mixture of reduced cysteines and oxidised cysteines. Reduced cysteines are alkylated with IAM. Reversibly oxidised cysteines (such as disulfide bonds and sulfenic acids) are reduced with TCEP. Liberated reduced cysteine thiols are labelled with either the light (ICAT\(_L\)) or heavy (ICAT\(_H\)) ICAT reagent. The two samples are combined and digested with trypsin. Digested peptides are enriched using a biotin affinity column. The samples are cleaned up and analysed by LC-MS/MS. The order of alkylation and ICAT-labelling can be inverted to analyse reduced cysteine thiols. c) Duplexed version. As with the monoplexed version except only one condition is analysed and the sample is sequentially labelled with the light then heavy ICAT reagent.
Figure 5.3: Overview of ICAT redox proteomic experiments. Figure legend on previous page.
5.3.6 Redox proteomics to investigate a signalling role for Prdx1

This Chapter will look into whether an ICAT-proteomics approach can be used to investigate the role of Prdx1 in H$_2$O$_2$ signalling. The overall hypothesis is presented in Figure 5.4. If Prdx1 does not contribute to H$_2$O$_2$ metabolism, then Prdx1 KO cells should show no change in overall protein oxidation. In contrast, if Prdx1 inactivation is required for signalling (the floodgate model), the loss of Prdx1 should lead to an increase overall protein oxidation. On the other hand, if Prdx1 actively transmits a signalling from H$_2$O$_2$ (the disulfide-relay model), the loss of Prdx1 will result in an overall decrease in protein oxidation.

**Figure 5.4:** Hypotheses of consequences of Prdx1 KO on protein oxidation. In the direct oxidation model, Prdx1 would play no major role in altering a protein’s oxidation status, therefore there will be no change in protein oxidation. In the floodgate model, the loss of Prdx1 will lower the required H$_2$O$_2$ concentration to elicit a response, thus protein oxidation will increase. In the disulfide relay model, Prdx1 is actively oxidising proteins so the loss of Prdx1 will result in decreased protein oxidation.
5.4 Aims

Aim 1  Characterise HAP1 p38 response to H$_2$O$_2$

Aim 2  Perform a pilot proteomic study to determine if WT and Prdx1 KO HAP1 cells are similar

Aim 3  Determine the feasibility of a larger ICAT based study to investigate the role of Prdx1 in response to H$_2$O$_2$

5.5 Results

5.5.1 Prdx1 is required for p38 activation in HAP1 cells following H$_2$O$_2$ treatment

5.5.1.1 HAP1 cells respond to H$_2$O$_2$ treatment with p38 phosphorylation

In order to carry out a proteomic method, a cell line is required to generate a sample for analysis. In this work, the human HAP1 cell line was chosen. HAP1 cells are a near-haploid cell line, originally derived from the chronic myelogenous leukaemia cell line—KBM-7 (Carette et al., 2011; Essletzbichler et al., 2014). Initially, we wanted to investigate how HAP1 cells respond to H$_2$O$_2$ by monitoring phosphorylation of the stress-responsive MAPK, p38.

Treatment of HAP1 cells with 100 µM H$_2$O$_2$ produced a similar p38 response as previously reported in U937 cells (Figure 5.5a). There is a fast initial response (at 2.5–5 minutes), with an increase in p-p38. Over time, p-p38 decreases and by 30 minutes is no longer present.

5.5.1.2 HAP1 Prdx1 KO cells do not express Prdx1

Jarvis et al. (2012) reported that knock-down of Prdx1 in U937 cells ablated the H$_2$O$_2$ response. In order to look further into the role Prdx1 may play in such a response, HAP1 cells that were deficient for Prdx1 (Prdx1 KO) were purchased (for further
Figure 5.5: H₂O₂ induces p38 activation in HAP1 cells. a) Flow diagram of the assay in this Figure. b) HAP1 cells (2 x 10⁶ cells in 3 mL, in a suspension state, 30 minutes post-trypsinising see Methods (2.11.3.1) for details) were treated with H₂O₂ (100 µM) for the times indicated. The cells were lysed in RIPA buffer and subjected to western blot. Phosphorylated-p38 (p-p38) was probed for (α-p-p38) and visualised with ECL. As a loading control, α-tubulin was probed for (α-α-tubulin). Note—a blot for total p38 (the alpha isoform) is shown in Appendix Figure D.2a, but the antibody used resulted in unspecific bands so α-tubulin was the only loading control used. Performing a similar assay on adherent cells, lysed using 10% (w/v) TCA gave similar results (Figure 5.6).

details see Methods (2.11.2)). The absence of Prdx1 expression at a protein level was confirmed by western blot (Figure 5.6b).

5.5.1.3 Prdx1 is required for H₂O₂ induced p38 response in HAP1 cells

Next, WT and Prdx1 KO HAP1 cells were treated with H₂O₂ before analysing p38 phosphorylation. For these assays, the cells were grown and treated in an adherent state and lysed using TCA. Phosphorylated p38 was visualised by western blot. For the WT HAP1 cells the same trend was seen in the fast response in p38 phosphorylation (2.5 minutes) but by 30 minutes post-treatment p-p38 is no longer detected (Figure 5.6). By comparison, the Prdx1 KO cells showed a lower response (Figure 5.6) indicating that, like U937 cells (Jarvis et al., 2012), HAP1 cells require Prdx1 to activate the p38 pathway in response to H₂O₂. In order to validate that Prdx1 was essential in the p38 response, attempts at reintroducing Prdx1 into Prdx1 KO HAP1
Figure 5.6: Prdx1 is required for H$_2$O$_2$ induced p38 response in HAP1 cells. a) Flow diagram of the assay in this Figure. b) HAP1 cells (seeded 24-hours previously at $1 \times 10^6$ cells in 3 mL) were treated with 100 µM H$_2$O$_2$ (or an equivalent volume of water for 0 minutes) for the times indicated before lysis with TCA and subsequently western blotting for p-p38 (see methods for details). Prdx1 and α-tubulin were subsequently probed against and visualised. c) Quantification of three experimental replicates. Bands (both the higher and lower p-p38 bands) were quantified using Image Studio and normalised to tubulin and the WT p-p38 band at 2.5 minutes. Error bars represent SEM. Full blots are given in Appendix Figure D.1.

cells were made. However, attempts at transient over-expression of Prdx1 failed to result in Prdx1 expression levels that were comparable with endogenous WT HAP1 cells (Appendix Figure D.2b). Nonetheless, Prdx1 has previously been reported to be integral to exogenous H$_2$O$_2$ activated p38 (Conway and Kinter, 2006; Jarvis et al., 2012). Observations such as these lend support to the notion that Prdx1 (and other mammalian Prdxs) may be more than a peroxidase protein (see 5.3.3). However, the hypothesis has been hampered by a limited number of identified signalling-substrates for mammalian Prdxs. In order to further investigate what role Prdx1 may have as a signalling protein, we moved onto the pilot redox proteomics study.
5.5.2 Using ICAT redox proteomics to investigate the role of Prdx1 in cellular thiol status—a pilot study

The main aim of this Chapter is to determine the feasibility of an ICAT-based proteomics experiment for investigating the role Prdx1 may have as a signalling protein. To achieve this aim a pilot ICAT study was performed using a monoplexed ICAT method (as illustrated in Figure 5.3a), based on a protocol by García-Santamarina et al. (2014) for analysing a yeast thiol proteome. The method, as trialled in this Thesis, has several key features:

1. TCA (trichloroacetic acid) is used to protonate cysteine thiols, keeping them reduced and less sensitive to oxidation during sample preparation.

2. IAM (iodoacetamide) is used to alkylate reduced thiols, preventing oxidation during sample preparation.

3. TCEP (tris(2-carboxyethyl)phosphine) is used to reduce any previously-oxidised thiols enabling labelling of only thiols that were oxidised.


An outline of the approach used is shown in Figure 5.7, the full experimental method is given in the Methods (2.12). An in-gel fluorescence method (similar to that of Baty et al. 2002) was used to confirm that reduced cysteine thiols could be blocked and reversibly oxidised thiols could be labelled (Appendix Figure D.3).

Observations from the Ledgerwood laboratory showed that Prdx1 KO HAP1 cells have similar characteristics (such as cell proliferation and H2O2-induced apoptosis) to WT HAP1 cell line (Carrad, 2017). With no overt differences in phenotypes and, as re-introduction of Prdx1 had proved ineffective (see 5.5.1.3 and Appendix Figure D.2b), it was decided to directly compare the HAP1 WT and Prdx1 KO cell lines.

This was a pilot study to determine whether the method works as intended and, more importantly, whether the HAP1 WT and Prdx1 KO cells have comparable basal levels of protein oxidation. As such the initial pilot consists of a direct comparison between the WT and Prdx1 KO HAP1 cells in an unperturbed state. This pilot was
Figure 5.7: ICAT pilot study overview. WT and Prdx1 KO HAP1 cells were grown and processed using a monoplexed ICAT method (see Figure 5.3a) where both samples were alkylated with IAM. The samples were subsequently reduced before labelling with the ICAT reagent. WT cells were labelled with the light ICAT reagent (ICAT<sub>L</sub>) and Prdx1 KO cells were labelled with the heavy ICAT reagent (ICAT<sub>H</sub>). The data is expressed as a ratio of heavy over light (Prdx1 KO/WT) indicating the relative abundance of a given protein modification in the starting material.

carried out three times (three experimental replicates). An additional preliminary experiment was also performed, treating both WT and Prdx1 KO HAP1 cells with H<sub>2</sub>O<sub>2</sub> and comparing them to the respective untreated cell line.

5.5.2.1 Evaluation of the ICAT pilot study

The pilot study (a direct comparison of WT and Prdx1 KO HAP1 cells as in illustrated in Figure 5.7) was completed three times (for three experimental replicates). Within these, the samples were analysed by LC-MS/MS either once (for replicate 1) or in duplicate (for replicates 2 and 3).

The data output is expressed in terms of an ‘ICAT ratio’. The ratio is calculated by the Proteome Discoverer software (see Methods (2.12), for details), which calculates the abundances of light and heavy ICAT-labelled peptides relative to the entire peptide pool for a given protein. The ICAT ratio displayed in this pilot study is expressed in terms of heavy ICAT (peptides from Prdx1 KO cells) divided by light ICAT (peptides from the WT cells). The data can also be expressed as a binomial log (Log<sub>2</sub>). The advantage of Log<sub>2</sub> is that the data, when displayed graphically, is symmetrical about 0. The data is also referred to in the ratio form as the less transformed state can make
it easier to understand that there was $x$-fold more or less of one peptide in one sample than the other.

The evaluation of the pilot study is divided into the following areas:

1. Coverage—how many proteins (and peptides) were identified?
2. What is the ICAT-labelling efficiency (out of total detected proteins)?
3. How close are the duplicate LC-MS/MS runs on the same sample(s)?
4. Do WT and Prdx1 KO HAP1 cells have a similar basal oxidation level?

### 5.5.2.2 Protein and peptide coverage

Table 5.2 shows the break down for how many peptides were identified in the ICAT pilot study. Between 2000 and 3500 individual peptides were identified per LC-MS run representing between 224 and 559 proteins (with either more than one peptide or identified in two LC-MS/MS runs, Figure 5.8a). Only a relatively small proportion of these proteins were labelled with the ICAT reagent ($\sim 40\%$) in replicate 1, and $\sim 10\%$ in replicates 2 and 3, Figure 5.8a and c. A number of proteins were only found either labelled as heavy or light only. As such, an ICAT ratio cannot be calculated for these proteins. Whilst incomplete labelling could indicate that for a certain protein it was only found in an oxidised state in either the WT or Prdx1 KO cell line, there was inconsistency in this (e.g., even within a technical replicate some proteins, such as calnexin (see Appendix Table D.1), had a ratio of 0.93 in one LC-MS/MS run but was only found in the heavy channel for the duplicate run$^2$.

---

$^2$Missing light or heavy channels. Proteome Discovery the ICAT ratio by taking the sum of all labelled-peptides (for a given protein) as representing 100%. The specific abundance for a heavy or light labelled peptide is set relative to this 100% value. The ratio is determined by the division of one channel over the other. Therefore, if a protein is only identified in one channel (heavy or light) the ratio will be either 100 or 0.01 (1/100).
Table 5.2: Identified peptide numbers for the ICAT pilot study (comparison of HAP1 WT and Prdx1 KO cells)

<table>
<thead>
<tr>
<th></th>
<th>Rep 1</th>
<th>Rep 2</th>
<th>Rep 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
<td>Run 2</td>
<td>Run 1</td>
</tr>
<tr>
<td>All peptides</td>
<td>2574</td>
<td>3473</td>
<td>3417</td>
</tr>
<tr>
<td>ICAT-labelled peptides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>490</td>
<td>199</td>
<td>207</td>
</tr>
<tr>
<td>Heavy</td>
<td>427</td>
<td>136</td>
<td>136</td>
</tr>
<tr>
<td>Total (H+L)</td>
<td>917</td>
<td>335</td>
<td>343</td>
</tr>
<tr>
<td>Total ICAT-labelled (% of all peptides)</td>
<td>35.63</td>
<td>9.65</td>
<td>10.04</td>
</tr>
</tbody>
</table>

Rep—Experimental replicate.

The duplicate LC-MS runs for replicates 2 and 3 showed reasonable replication of one another (ranging between 0 and 0.56 SD units) with the mean and median SD being 0.09 and 0.06 respectively (Figure 5.8c). All of the ICAT ratio values (regardless of SD variation) were taken forward as long as they were found in both of the duplicate LC-MS/MS runs. In total this meant that there were 90 proteins from replicate 1, 55 from replicate 2 and 46 from replicate 3.

5.5.2.3 Enrichment of ICAT-labelled proteins

The ICAT reagent features a biotin tag for enrichment purposes. Even though the biotin-enrichment was used (see Figure 5.3 and Methods; 2.12) there is a remarkably high number of non ICAT-labelled peptides identified in the LC-MS (Table 5.2 and Figure 5.8). More concerning is that the number of ICAT-labelled peptides (and proteins) decreases between replicate 1 and replicates 2 and 3 (Table 5.2 and Figure 5.8). Concomitant to the overall decrease in ICAT-labelling, there is also a decrease in the enrichment of ICAT-labelled peptides (Table 5.2 and Figure 5.8). The first replicate,
Figure 5.8: *Pilot ICAT study: replicate protein numbers.* a) Venn diagrams for the three experimental replicates (Rep 1, 2 and 3). Only proteins that were identified with high confidence by Proteome Discoverer were used. The top panel shows the total number of proteins identified in each run and either how many had more than 1 peptide identified (Rep 1) or the overlap between the duplicate LC-MS runs for replicates 2 and 3. The bottom panel shows how many peptides were identified being ICAT-labelled. Numbers in parentheses indicate additional proteins that were identified as ICAT-labelled, but only in either the heavy or light channel. b) Percentage of identified protein that were labelled by the ICAT reagent. The total value is as per the eligible identified proteins—more than 1 peptide for replicate 1 or found in both LC-MS/MS runs for replicates 2 and 3. c) Standard deviation (SD) of ICAT-ratios for the two LC-MS runs for replicates 2 and 3.
35% of all peptides were ICAT-labelled, but in the subsequent replicates only 9 to 14% were labelled (Table 5.2). The method used was the same, but there was a temporal gap of approximately 3 months between replicate 1 and replicates 2 and 3. The assumption for loss of efficiency (in labelling and enrichment) is due to reduced performance in the streptavidin column (that comes with the ICAT kit) or degradation of the ICAT reagent.

5.5.2.4 Comparison of WT and Prdx1 HAP1 cell basal oxidation levels

Across the three experimental replicates (comparing WT and Prdx1 KO HAP1 cells), a total of 23 proteins were identified in all three replicates (of these, 4 were incompletely labelled, see Footnote 2). Thirty eight proteins were identified as ICAT-labelled in both heavy and light channels and found in at least two replicates (Figure 5.9a, c and Table 5.3). Several proteins were identified labelled only in the heavy or light channel. As such a true ratio is not generated therefore only proteins that have a ‘complete’ ratio are shown (incompletely labelled proteins are given in Appendix Table D.1).

A histogram can be used to look at the data as a whole, showing the spread of ICAT ratios. Looking at the data as a whole illustrates whether the WT and Prdx1 KO are similar, independent of individual proteins. Figure 5.9b shows a histogram as both a ratio and binomial log (Log₂) of the ratio.
Figure 5.9: Distribution of protein ICAT ratios from untreated WT and Prdx1 KO HAP1 cells. HAP1 cells were lysed and ICAT-labelled as per in Figure 5.3. a) Venn diagram showing from the three experimental replicates which identified proteins were also found in the other replicates (reps). A protein that was identified in at least two of these replicates was carried forwards. Numbers in parenthesis represent proteins that were identified, but only as labelled with either the light or heavy ICAT reagent. b) and c) Histograms of ICAT ratios (b) or Log2(ratio) (c). The grey line represents a non-linear regression fit of the distribution data generated using GraphPad Prism (outputs can be found in Appendix Table D.6), with an R\textsuperscript{2} value representing the fit of the curve to the data. Median and mean positions of the data are indicated. The bin widths were 0.1 for b) and 0.2 for c).
The spread of the data (assessed by both the raw ratios and as Log$_2$) shows a near-normal distribution (Figure 5.9b). A normal distribution indicates there is not a large difference between either the WT or Prdx1 KO HAP1 cells.

When looking at the individual values assigned to each identified protein (for the 37 proteins that were labelled with both heavy and light reagent), the average ratios for all proteins falls between 0.53 and 1.53 (Figure 5.10 and Table 5.3). The data is also shown graphically as a ratio with SEM or as a Log$_2$ scale with error bars representing a 95% confidence interval. By showing the data in both layouts the spread of the replicates can be seen with the confidence intervals, but the differences between the mean values are not scaled down by the error bars.

Three proteins are statistically different from a ratio value of 1 (or Log$_2$ value of 0): IGF2R, CCDC58 and COXB6B1 (Figure 5.10). The remaining 34 proteins (92%) do not significantly differ from a ratio value of 1 (or Log$_2$ value of 0). However, the spread of the experimental replicates appears to be quite large (especially when looking at the error in terms of a confidence interval, Figure 5.10).

Taken together, the data does provide a starting point that shows there is no difference in the basal oxidation between the WT or Prdx1 KO HAP1 cells. A more thorough evaluation can be found in the Discussion (5.6.2).
Figure 5.10: ICAT-labelled proteins from untreated WT and Prdx1 KO HAP1 cells. Figure legend on next page.
**Figure 5.10:** ICAT-labelled proteins from untreated WT and Prdx1 KO HAP1 cells. Each graph is displaying the same data ranged by the mean for each protein. Only proteins that were identified in two or more replicates with a complete ICAT ratio (WT/Prdx1 KO) are plotted. Different symbols represent each experimental replicate (rep). Top: ICAT ratios are plotted and error bars represent SEM. Arbitrary lines have been drawn representing an ICAT ratio of 0.93 and 1.07. Bottom: Log2 scale of the ICAT ratio (WT/Prdx1 KO). Error bars represent a 95% confidence interval. Arbitrary lines have been drawn for a Log2 ratio value of 0.1 or -0.1 (equivalent to the lines in the top graph). * represents statistical significant ($P < 0.05$) difference from an ICAT ratio of 1 or a Log2 value of 0 as determined by a one-sample t-test. This data is also displayed in Table 5.3.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Accession code</th>
<th>ICAT Ratio (WT/Prdx1 KO)</th>
<th>Cysteine labelled</th>
<th>Known to form disulfide? (partner)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNPY2</td>
<td>Protein canopy homolog 2</td>
<td>Q9Y2B0</td>
<td>0.16 0.77 0.65</td>
<td>0.53</td>
<td>31 Yes (164)</td>
</tr>
<tr>
<td>IGF2R</td>
<td>Cation-independent mannose-6-phosphate receptor</td>
<td>P11717</td>
<td>0.60 0.62</td>
<td>0.61</td>
<td>731 Yes (760)</td>
</tr>
<tr>
<td>NPC2</td>
<td>Epididymal secretory protein E1</td>
<td>P61916</td>
<td>0.51 0.74</td>
<td>0.63</td>
<td>74 No</td>
</tr>
<tr>
<td>CCDC58</td>
<td>Coiled-coil domain-containing protein 58</td>
<td>Q4VC31</td>
<td>0.63 0.63</td>
<td>0.63</td>
<td>53 Yes (93)</td>
</tr>
</tbody>
</table>

*Continued on next page*
Table 5.3: Identified ICAT-labelled proteins for WT vs Prdx1 KO HAP1 cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Accession code</th>
<th>ICAT Ratio (WT/Prdx1 KO)</th>
<th>Cysteine labelled</th>
<th>Known to form disulfide? (partner)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rep 1</td>
<td>Rep 2</td>
<td>Rep 3</td>
</tr>
<tr>
<td>FAM136A</td>
<td>Protein FAM136A</td>
<td>Q96C01</td>
<td>0.25</td>
<td>1.11</td>
<td>0.74</td>
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<tr>
<td>NDUFA8</td>
<td>NADH dehydrogenase 1 alpha subcomplex subunit 8</td>
<td>P51970</td>
<td>0.25</td>
<td>1.20</td>
<td>0.72</td>
</tr>
<tr>
<td>TIMM13</td>
<td>Mitochondrial import inner membrane translocase subunit Tim13</td>
<td>Q9Y5L4</td>
<td>0.17</td>
<td>1.30</td>
<td>0.81</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
<td>P14174</td>
<td>0.74</td>
<td>0.74</td>
<td>0.77</td>
</tr>
<tr>
<td>TXNDC5</td>
<td>Thioredoxin domain-containing protein 5</td>
<td>Q8NBS9</td>
<td>0.99</td>
<td>0.71</td>
<td>0.69</td>
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<tr>
<td>SOD1</td>
<td>Superoxide dismutase</td>
<td>P00441</td>
<td>0.75</td>
<td>0.94</td>
<td>0.78</td>
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<td>BSG</td>
<td>Basigin</td>
<td>P35613</td>
<td>0.75</td>
<td>0.93</td>
<td>0.84</td>
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<td>FKBP10</td>
<td>Peptidyl-prolyl cis-trans isomerase FKBP10</td>
<td>Q96AY3</td>
<td>0.66</td>
<td>1.09</td>
<td>0.88</td>
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<tr>
<td>GLG1</td>
<td>Golgi apparatus protein 1</td>
<td>Q92896</td>
<td>0.70</td>
<td>1.09</td>
<td>0.93</td>
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<tr>
<td>ACTB</td>
<td>Actin, cytoplasmic 1</td>
<td>P60709</td>
<td>1.08</td>
<td>0.76</td>
<td>0.92</td>
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<tr>
<td>NPTN</td>
<td>Neuroplastin</td>
<td>Q9Y639</td>
<td>0.78</td>
<td>1.06</td>
<td>0.92</td>
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<tr>
<td>CHCHD6</td>
<td>MICOS complex subunit MIC25</td>
<td>Q9BRQ6</td>
<td>1.06</td>
<td>0.80</td>
<td>0.93</td>
</tr>
<tr>
<td>SDF2L1</td>
<td>Stromal cell-derived factor 2-like protein 1</td>
<td>Q9HCN8</td>
<td>0.90</td>
<td>0.98</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Continued on next page
Table 5.3: Identified ICAT-labelled proteins for WT vs Prdx1 KO HAP1 cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Accession code</th>
<th>ICAT Ratio (WT/Prdx1 KO)</th>
<th>Cysteine labelled</th>
<th>Known to form disulfide? (partner)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHCHD3</td>
<td>MICOS complex subunit MIC19</td>
<td>Q9NX63</td>
<td>1.35 0.73 0.74 0.94</td>
<td>183 193</td>
<td>Yes (214)  Yes (204)</td>
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<tr>
<td>PPIA</td>
<td>Peptidyl-prolyl cis-trans isomerase A</td>
<td>P62937</td>
<td>1.26 0.69 0.90 0.95</td>
<td>62</td>
<td>No</td>
</tr>
<tr>
<td>ERP44</td>
<td>Endoplasmic reticulum resident protein 44</td>
<td>Q9BS26</td>
<td>1.17 0.93 0.81 0.97</td>
<td>189 301 318 318</td>
<td>Yes (241)  Yes (318)  Yes (301)</td>
</tr>
<tr>
<td>PRKCSH</td>
<td>Glucosidase 2 subunit beta</td>
<td>P14314</td>
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<td>471</td>
<td>No</td>
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<tr>
<td>TIMM10</td>
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<td>P62072</td>
<td>1.14 0.82 0.98</td>
<td>29 50</td>
<td>Yes (54)  Yes (33)</td>
</tr>
<tr>
<td>CD59</td>
<td>CD59 glycoprotein</td>
<td>P13987</td>
<td>1.13 0.85 0.99</td>
<td>70 92</td>
<td>Yes (88)  Yes (42)</td>
</tr>
<tr>
<td>AK2</td>
<td>Adenylate kinase 2</td>
<td>P54819</td>
<td>1.16 1.11 0.70 0.99</td>
<td>42 92</td>
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<td>SDF2</td>
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<td>No  No</td>
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<td>LMAN2</td>
<td>Vesicular integral-membrane protein VIP36</td>
<td>Q12907</td>
<td>1.11 0.91 0.98 1.00</td>
<td>202 239</td>
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<td>CD276</td>
<td>CD276 antigen</td>
<td>Q5ZPR3</td>
<td>0.75 1.32 1.03</td>
<td>383</td>
<td>Yes (438)  122/340a Yes (50/340)</td>
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<td>CTSB</td>
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<td>1.01 1.16 1.09</td>
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<td>CALR</td>
<td>Calreticulin</td>
<td>P27797</td>
<td>0.91 1.41 1.11</td>
<td>105 218</td>
<td>Yes (137)  No</td>
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<tr>
<td>AHSG</td>
<td>Alpha-2-HS-glycoprotein</td>
<td>P02765</td>
<td>1.05 0.81 1.50 1.12</td>
<td>132 219</td>
<td>Yes (114)  Yes (208)</td>
</tr>
</tbody>
</table>

*Continued on next page*
## Table 5.3: Identified ICAT-labelled proteins for WT vs Prdx1 KO HAP1 cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Accession code</th>
<th>ICAT Ratio (WT/Prdx1 KO)</th>
<th>Cysteine labelled</th>
<th>Known to form disulfide? (partner)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rep 1</td>
<td>Rep 2</td>
<td>Rep 3</td>
<td>Mean</td>
</tr>
<tr>
<td>ERGIC1</td>
<td>Endoplasmic reticulum-Golgi intermediate compartment protein 1</td>
<td>Q969X5</td>
<td>1.19</td>
<td>1.06</td>
<td>1.12</td>
</tr>
<tr>
<td>NDUFB7</td>
<td>NADH dehydrogenase 1 beta subcomplex subunit 7</td>
<td>P17568</td>
<td>1.49</td>
<td>0.85</td>
<td>1.17</td>
</tr>
<tr>
<td>LTF</td>
<td>Lactotransferrin</td>
<td>P02788</td>
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<td>0.79</td>
<td>1.18</td>
</tr>
<tr>
<td>GPC4</td>
<td>Glypican-4</td>
<td>O75487</td>
<td>1.09</td>
<td>1.60</td>
<td>1.17</td>
</tr>
<tr>
<td>PRDX4</td>
<td>Peroxiredoxin-4</td>
<td>Q13162</td>
<td>1.71</td>
<td>1.25</td>
<td>1.23</td>
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<tr>
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<td>P13674</td>
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<td>COX6B1</td>
<td>Cytochrome c oxidase subunit 6B1</td>
<td>P14854</td>
<td>1.58</td>
<td>1.51</td>
<td>1.55</td>
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</tbody>
</table>

This data is plotted graphically in Figure 5.10. Proteins shown here were detected in both heavy and light channels for at least two experimental replicates. Proteins are arranged by ascending mean ICAT ratio. The table was annotated manually using UniProt (www.uniprot.org; The UniProt Consortium, 2017) as a reference. 

- a—There is an identical peptide repeat within the protein so either cysteine could have been identified; 
- b—Cys51 of Prdx4 is in the N-terminal signal peptide.
5.5.3 Preliminary data into H$_2$O$_2$ response for WT and Prdx1 KO HAP1 cells

The previous ICAT pilot study (the comparison between WT and Prdx1 KO HAP1 cells) was focused on establishing whether the WT and Prdx1 KO HAP1 cell lines would be directly comparable. Whilst not conclusive, there was some evidence to suggest that the two cell lines have similar basal levels of protein oxidation. Following on from this a preliminary study of two single experiments was conducted, looking at how WT and Prdx1 KO HAP1 cells respond to H$_2$O$_2$ treatment (Figure 5.11). The same ICAT-labelling strategy was employed as for the untreated direct cell comparison. The difference being here that either cell line was treated with 100 µM H$_2$O$_2$ for 2.5 minutes (this treatment had previously shown to induce p38-MAPK activation, see 5.6). In addition to the overall Chapter aim of determining the feasibility of using ICAT to investigate H$_2$O$_2$ signalling, the aim of these preliminary experiments was to investigate the hypothesis that Prdx1 contributes to H$_2$O$_2$ signalling.

**Figure 5.11: Overview of H$_2$O$_2$ treated HAP1 ICAT experiments.** HAP1 cells (seeded 24-hours previously at $1 \times 10^6$ cells per mL in 20 mL)—WT in a) or Prdx1 KO in b)—were grown and either treated with 100 µM H$_2$O$_2$ (for 2.5 minutes) or an equivalent volume of water (untreated). The cells were processed as previously (as outlined in Figure 5.3) and analysed by LC-MS/MS. The data is expressed as an ICAT ratio of +H$_2$O$_2$ over untreated.
Figure 5.12: ICAT ratio distribution for H$_2$O$_2$ treated WT and Prdx1 KO HAP1 cells.

HAP1 cells treated with H$_2$O$_2$ compared to untreated cells (refer to Figure 5.11 for an overview of the experiment). Shown are histograms of Log$_2$(ICAT ratio) frequencies with 0.2 bin widths. Curves are a non-linear regression fit (calculated using GraphPad Prism) of the distribution data (the output from GraphPad Prism is shown in Appendix Table D.6). The mean and median ICAT ratios for the data as a whole is indicated. a) HAP1 WT cells treated with 100 µM H$_2$O$_2$ for 2.5 minutes compared to untreated WT cells. b) Prdx1 KO HAP1 cells treated with 100 µM H$_2$O$_2$ for 2.5 minutes compared to untreated Prdx1 KO cells.

Each of these experiments (comparison of H$_2$O$_2$ treated and untreated WT or Prdx1 KO cells) was only carried out once, so is shown for purely demonstrative purposes. The majority of the data is shown in Appendix Figure D.5 and Appendix Tables D.2–D.5.

The labelling efficiency and replication of the duplicate LC-MS runs were similar to that seen in the untreated pilot study (Appendix Figure D.4). Overall, proteins from both WT and Prdx1 KO cells had a higher mean and median ICAT ratio than compared untreated pilot (Figure 5.12). The Prdx1 KO cells had a slightly higher average ICAT ratio overall. In terms of the distribution, whilst the WT cells had a normal distribution (Figure 5.12a) the Prdx1 KO cells are less normally distributed (Figure 5.12b).

A total of 38 proteins were labelled (with both the heavy and light ICAT reagent) in both the treated/untreated WT and Prdx1 HAP1 cells (Figures 5.12 and 5.13).

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Figure 5.13: Comparison of $\text{H}_2\text{O}_2$ treated WT and Prdx1 KO HAP1 cells. a) Venn diagram showing overlap of identified ICAT-labelled proteins in the two $\text{H}_2\text{O}_2$ treatment experiments (as outlined in Figure 5.11). Numbers in parenthesis indicate proteins that were identified but only in either the light or heavy ICAT channels. b) Heat-map of the ICAT-labelled proteins that were found in both experiments (the overlap in a) Proteins that have different oxidation levels are indicated (1—P4HA1, 2—CMC2, 3—CNPY2), but as these experiments was only conducted once they are only indicated for illustrative purposes.

Comparing the proteins that were in each experiment, there are a few (such as P4HA1, CMC2, CNPY2), appear to behave differently between the two cell lines (Figure 5.13b). As these experiments only have a single replicate these subtle differences should be treated with caution, but they do suggest that the two cell lines have a different response to $\text{H}_2\text{O}_2$.

5.5.4 Comparison of all ICAT experiments: sub-cellular localisation and cysteine origins

As a final evaluation of the ICAT method used in this work, the sub-cellular localisation and cysteine origins of ICAT-labelled proteins were analysed. All data (from the WT-Prdx1 KO comparison and $\text{H}_2\text{O}_2$ treated WT or Prdx1 KO cells) were combined for this purpose in order to determine what proteins were being represented in the data set. The individual data sets are also shown separately in Appendix Figure D.6.

Looking at which cysteine within the identified protein was labelled (across all data sets), the majority (around 60%) are cysteine residues that are known (or predicted...
Figure 5.14: *ICAT-labelled cysteine origins and protein localisation.* Qualitative comparison of all ICAT experiments (as illustrated in Figures 5.7 and 5.11) a) Proportion of ICAT-labelled proteins identified where the labelled cysteine residue is predicted or known to be in a structural disulfide bond (as per UniProt annotations) for all ICAT experiments. The individual experimental breakdown is given in Appendix Figure D.6. b) Sub-cellular origins of ICAT-labelled proteins for all ICAT experiments, manually annotation as according to UniProt (www.uniprot.org; The UniProt Consortium, 2017).

ER—endoplasmic reticulum to be) in disulfide bonds (Table 5.3, Figure 5.14b). Disulfide bonds represent one form of cysteine oxidation, therefore it was expected that disulfide-containing proteins would be identified. That more than half of all proteins identified would come from disulfide-bonds was unexpected, but was consistently seen across the three experiments performed (Appendix Figure D.6a).

When the sub-cellular origins of the proteins were looked at, the majority of proteins originated from the ER/Golgi, mitochondria or the plasma membrane (Figure 5.14b). Moreover, only a relatively small fraction (about 10%) were cytosolic in origin. Given the majority of proteins identify could form a disulfide bond (Figure 5.14a) it follows that the proteins should originate from these locations, as they are more oxidative cellular environments (Cook and Hogg, 2013). The implication of disulfide bond enrichment and sub-cellular location will be explored in the discussion (5.6.2.4)
5.6 Discussion

This Chapter looks at the possible role Prdxs have beyond being a cysteine-peroxidase—e.g., Prdxs using H$_2$O$_2$ to oxidise specific targets thus altering downstream signalling (Figure 5.3.3). A redox proteomics approach was outlined that could provide experimental evidence, for or against, the hypothesis that mammalian Prdxs are *bona fide* mammalian signalling proteins.

5.6.1 Prdx1 is required for H$_2$O$_2$-induced p38 response

Phosphorylated p38 is frequently used to monitor oxidative stress in cells. Addition of exogenous H$_2$O$_2$ to media is also very commonplace as it is simple and affordable. Whilst the physiological relevance of a bolus H$_2$O$_2$ treatment can be debated, H$_2$O$_2$ is able to cross the plasma membrane (Antunes and Cadenas, 2000; Winterbourn, 2008). NOX complexes that are known to generate H$_2$O$_2$ (via superoxide, see 1.5.4) do so on the extracellular side of the cell (Holmström and Finkel, 2014). Addition of exogenous H$_2$O$_2$ can, therefore, be thought of as a user-controlled way of emulating the activation of NOXs.

HAP1 cells responded with p38 phosphorylation when treated with H$_2$O$_2$ (Figures 5.5 and 5.6). Conversely, in HAP1 cells without Prdx1 there was an ablated p38 response upon H$_2$O$_2$ treatment (Figure 5.6). The Prdx1 KO HAP1 cells were developed using CRISPR/Cas9 which is known to have off-target effects (Zhang *et al.*, 2015). In order to rule out that loss of Prdx1 is the reason behind the ablated p38 H$_2$O$_2$ response, ideally, Prdx1 would be re-introduced to the cells.

Unfortunately, difficulty in transient transfection of the HAP1 cells meant that Prdx1 was never re-introduced to anything near endogenous levels (Appendix Figure D.2b). However, the Prdx1-required p38 response emulates the finding by Jarvis *et al.* (2012) where U937 cells also required Prdx1 to respond to H$_2$O$_2$. Thus, whilst there are possible caveats, the broad conclusion is HAP1 cells do respond to exogenous H$_2$O$_2$ treatment and that Prdx1 is essential for the H$_2$O$_2$ response. Jarvis *et al.* (2012) saw that Prdx1 was able to form a transient, inter-molecular disulfide bond with ASK1. In this manner, it has been postulated that Prdx1 is required for H$_2$O$_2$
induced activation of ASK1 via a disulfide-relay (Jarvis et al., 2012). As ASK1 is the only (purportedly) known Prdx1 disulfide-relay signalling partner, more examples are required in order to fully appreciate the signalling role of Prdx1 and other Prdxs in H₂O₂ signalling.

5.6.2 ICAT proteomics to investigate the role of Prdx1 as a signalling protein

The main aim of this Chapter was to perform a pilot study in order to determine whether the ICAT method would be a suitable approach for investigating the contribution of Prdx1 to protein oxidation. This work was designed to feed into the hypothesis that Prdx1 acts as a signalling protein, and identify potential signalling-substrates of Prdx1. The ICAT method—as deployed in this Chapter—used the ICAT method to label oxidised cysteine residues within a cell lysate.

5.6.2.1 How different does a ratio need to be?

One concept that needs to be addressed for analysing a differential analysis is how different do two samples need to be (i.e. the ICAT ratio) for the samples to be considered discrete. Several of the studies using an ICAT-based method to look at redox-proteomics have a ratio between 1.2 and 1.5 as ‘different’ from their respective controls, for example, García-Santamarina et al. (2011); Hägglund et al. (2008) and Leichert and Jakob (2004). However, the ratio readouts from these studies cannot necessarily be directly compared owing to key experimental differences.

Figure 5.10 gave an arbitrary cut-off value (roughly a 10% change—an ICAT ratio value between 0.93 and 1.07 or Log₂ value range of -0.1 and 0.1) that is used to indicate a range for an ‘unchanged’ value. Whether a 10% change or an ICAT ratio of between 1.5 and 2 are biologically relevant is another question. For example, in protein kinase signalling, a residue on a protein may need to be phosphorylated for a pathway to be activated. However, less than 5% of the total protein in question may actually be phosphorylated, which may be sufficient to activate a downstream pathway (Jensen, 2006). Accordingly, a relatively small change (~5%) in the total oxidation status of a
protein pool may be sufficient to alter cellular signalling. Furthermore, it is also possible that multiple proteins within a pathway could be oxidised, having a synergistic effect on the signalling system.

Follow up validation experiments are critical to accurately evaluate ICAT-based results. For example, if known protein kinases were identified as differentially oxidised between WT and Prdx1 KO HAP1 cells, genetic editing could be used to mutate the ICAT-labelled cysteine for the protein in question. The Prdx1-dependant effects on oxidation on protein signalling could then be assessed by western blotting for a phospho-target (akin to the p38 phosphorylation monitoring in this work, 5.5.1). Alternatively, targeted MS-based experiments, such as by selected reaction monitoring (SRM), could be used (Held et al., 2010). SRM is a quantitative method where only pre-determined peptide masses are analysed by the mass spectrometer (Picotti and Aebersold, 2012). However, for an SRM method to be used, the precise cysteine modification would need to be determined. There are over a dozen different known forms of cysteine oxidation so determining a modification is not a trivial task (Devarie Baez et al., 2015). Lastly, whilst SRM would confirm the oxidation of a target protein, it does not confirm it being biologically relevant to cellular signalling so other methods would still be required.

5.6.2.2 WT and Prdx1 have equivalent basal oxidation levels

In this work, WT and Prdx1 KO HAP1 cells were directly compared with the ICAT method. A lysate from each cell type was labelled with one of the ICAT reagents and the samples were combined for analysis by LC-MS/MS (5.5.2 and Figure 5.7). The overall question for this pilot study being ‘do Prdx1 KO cells have a different basal oxidation state from WT’?

Broadly, the data collected can be used to argue that the two cell lines do not differ from one another, agreeing with the observations from within the Ledgerwood lab (Carrad, 2017). The distribution of the identified ICAT-labelled peptides (Figure 5.9) and only three proteins having an ICAT ratio that is statistically different from 1 (Figure 5.10) indicates that there are no major differences between the cell lines and that they can be considered to be equivalent. There are a few caveats to this conclusion
which will be outlined shortly.

It may be surprising that a complete loss of Prdx1 from the HAP1 cells does not cause an overall increase in protein oxidation. After all, Prdx1 is an abundant cytosolic peroxidase (see 5.3.2). However, Prdx1 is not the only cytosolic Prdx, with Prdx2 also being cytosolic, alongside Gpxs (Winterbourn, 2018). Furthermore, an absence in increased protein oxidation also supports the notion that proteins have a relatively low reactivity with H₂O₂ (see 5.3.3 and Table 5.1) and, therefore, will not become oxidised under basal conditions.

There are two main problems with the broad conclusion that the WT and Prdx1 KO cell lines do not have different basal oxidation statuses. Firstly, only a relatively small number of proteins were identified (37; Figure 5.9) in at least two experimental replicates. Therefore, the question can be raised as to whether the analysed pool is representative of the proteome and cellular state. Secondly, the lack of statistical significance could be representing the true state—that both cell lines have similar distributions of oxidised proteins. Alternatively, it could be viewed that the variation between individual experimental replicates is too great, as could be concluded from the 95% confidence intervals (Figure 5.10, bottom). However, the wider error bars (from either SEM and 95% confidence intervals) are also due to most of the analysed proteins only being detected in two replicates. Further work could not only increase the overall proteome coverage (helping with the first problem) and give greater statistical power.

Other problems with any potential conclusions from the ICAT data could come from potential off-target effects of the CRISPR/Cas9 KO system (as mentioned in 5.6.1) used to generate the Prdx1 KO HAP1 cells. Re-introduction of Prdx1 may alleviate any potential off-target effects of the KO system (as discussed in 5.6.1), however, the method of re-introduction needs to be carefully determined. Transient transfection to a level that is similar to the endogenous protein could be effective, depending on how robust the transfection system used is. By comparison, re-introduction by a lentiviral vector would achieve a stable system, however, the random nature of lentiviral genome-integration could result in similar problems (Sakuma et al., 2012).

A more effective control would be a comparison between WT and Prdx1 KO, and, WT and a Prdx1-overexpression system. An alternative approach would be to genet-
ically edit Prdx1. An strategy similar to that of Gaudelli et al. (2017) could be used make an active site mutant—Prdx1C52S, C173S—that is unable to break down H2O2. This method has reportedly far fewer off-target effects. The other advantage of mutating Prdx1 compared to deleting it is any non-peroxidase function will be preserved.

The ICAT proteomic method used in this thesis uses an equal amount of a cell lysate determined by a BCA assay (or similar). Whilst this way of controlling protein levels has been used in several redox-proteomic studies (e.g., Araki et al., 2016; Kumar et al., 2013a), and does ensure that the total protein levels used are comparable it does not mean that individual proteins will be expressed at similar levels. The ICAT method, as used in this work, examines changes in the labelling of cysteine-containing proteins. Therefore, if a protein is 2-fold increased in one cell-line (relative to control) the ICAT ratio will also show a 2-fold difference despite there not necessarily being any difference in the actual oxidation status of the labelled cysteine residue. Genes that are known to be under transcriptional control by redox-regulated transcription factors (such as NRF2) may possibly be expressed at different levels in Prdx1 KO cells (Marinho et al., 2014). A better way to compensate for differential protein expression would be to perform quantitative proteomics alongside the quantitative ICAT redox proteomics. Quantitative proteomic methods, such as isobaric tag for relative and absolute quantification (iTRAQ) or dimethyl labelling have been used for this purpose (for example, Fu et al. 2009 and García-Santamarina et al. 2014). Whilst additional quantitative methods would make the ICAT method used in this work more robust, there would also be the added cost of reagents and time to perform and analyse the second labelling method.

5.6.2.3 HAP1 cellular response to H2O2 treatment

In addition to the pilot study, comparing WT and Prdx1 KO HAP1 cells, HAP1 cells (WT and Prdx1) were also treated with H2O2 and analysed (relative to a vehicle control, 5.5.3 and Figure 5.11). One of the advantages of looking at changes in response to H2O2 is that as a cell line is essentially compared to itself, the problem of differing protein levels between cell lines (as discussed above, 5.6.2.2) is alleviated.
In this work, when HAP1 cells were treated with H$_2$O$_2$ (5.5.3), there were differences (between WT and Prdx1 KO cells) in the ICAT ratio distribution (Figure 5.12). Whilst the number of proteins common to the two cell lines was still relatively low (42; Figure 5.13), a small pool is more informative for inferring about cellular response to H$_2$O$_2$. Experimental replication of H$_2$O$_2$ treated cells and ICAT-labelling would be essential in order to draw any specific conclusions. Additionally, any results would also require validation (as discussed previously, 5.6.2.1) to draw a strong biological conclusion.

### 5.6.2.4 Enrichment of disulfide-bond proteins by the ICAT method

An advantage of using a MS-based method is that in addition to differential analysis, information is also gained about the protein itself, namely the residue that is labelled. The majority (∼60%) of proteins that were identified as ICAT-labelled in this study contained, or are predicted to contain, a structural disulfide bond (5.5.4). The method used in this work is primarily based on a protocol by García-Santamarina et al. (2014). The method is designed to label only oxidised cysteine residues (see Figure 5.3). Given the method is designed to label oxidised cysteine residues, and disulfide bonds will form a portion of that pool, it is to be expected that disulfide bond containing proteins were identified. Additionally, cysteine residues involved in structural disulfide bonds are unlikely to have a great change in their own oxidation status.

García-Santamarina et al. (2011) used the oxidised cysteine labelling method to analyse a yeast (*Schizosaccharomyces pombe*) proteome and the response to treatment with 0.2 mM H$_2$O$_2$. García-Santamarina et al. (2011) divided their data into structural disulfide bonds (that have a constant ICAT ratio upon H$_2$O$_2$ treatment), and redox-sensitive disulfide bonds (that have an ICAT ratio that changes upon H$_2$O$_2$ treatment, and this will be further discussed below). For the pilot study in this work (the comparison between basal WT and Prdx1 KO HAP1 cells, 5.7) seeing a high degree of labelling of known (or predicted) structural disulfide bond containing-proteins was, therefore, anticipated. In the same vein, the sub-cellular localisation also fits with these results. The majority of labelled proteins originated from the ER, Golgi, mitochondria.
or plasma membrane (Figure 5.5.4). In eukaryotes, disulfide bonds are formed within the ER, Golgi apparatus or the mitochondrial intermembrane space (Cook and Hogg, 2013). Therefore it fits for the identified proteins to originate within these locations.

In this work, HAP1 cells were also treated with H$_2$O$_2$ and analysed via the ICAT-method (5.5.3). Whilst only one single experiment was conducted, there were few proteins that had an ICAT ratio above 1.5 (5 and 6 proteins for WT and Prdx1 KO HAP1 cells, respectively; Appendix Figure D.5 and Appendix Tables D.2 and D.4). By comparison, García-Santamarina et al. (2011) detected over 40 proteins that had an ICAT ratio of 1.5 from H$_2$O$_2$ treated _S. pombe_ cells. García-Santamarina et al. (2011) based their observations on 1195 identified peptides, but there is no comment as to how many proteins this represents or how many proteins did not have an ICAT ratio of over 1.5. However, from their responses, García-Santamarina et al. (2011) do see an increase in proteins that would be expected to be oxidised under H$_2$O$_2$ treatment, such as the yeast Prdx, Tpx1, and yeast Trx1. Overall, whilst García-Santamarina et al. (2011) do not identify an especially large number of differentially oxidised proteins there is still 10-fold more than was detected in this work.

### 5.6.2.5 Closing remarks regarding ICAT experiments

The observation that Prdx1 is required for p38 phosphorylation in response to H$_2$O$_2$ as seen by Jarvis _et al._ (2012) has been replicated here in the HAP1 cell line. These experiments highlight that Prdx1 is important in translating an oxidative signal to a protein kinase cascade. An ICAT proteomics approach may be appropriate to investigating the role of Prdx1 as a signalling protein, but further optimisation will be required to ensure reliable results. For example, a cytosolic sub-cellular fraction could be isolated in order to minimise the structural disulfide-bond rich cellular components (such as the ER). However, this makes it more difficult for accurate comparisons between two different conditions (as it increases the number of handling steps for the proteome to be labelled). Secondly, it would also be incompatible with the TCA lysis method and an alternative thiol-preservation step would be required. However, these approaches would not help with the relatively low number of proteins identified nor
the poor biotin-enrichment (see Figure 5.8b). The use of a different enrichment step, such as loose streptavidin-conjugated resin, may be useful. Other methods could be used to address the role that Prdx1 plays in cell signalling and a few examples will be outlined in the final Discussion (Chapter 6).

5.6.3 Conclusions

The work in this Chapter has shown that human cell HAP1 cell line responds to H$_2$O$_2$ treatment. Upon addition of bolus H$_2$O$_2$ to cells, phosphorylated p38 can be detected by western blot. Prdx1 is essential for this p38 response, as has been seen previously in the U937 cell line (Jarvis et al., 2012). In order to determine whether Prdx1 was acting as a signalling protein under these conditions, a redox-proteomics method was established. The proteomics method used the ICAT reagent to isotopically label oxidised protein thiols so two cell states can be directly compared. A pilot study was carried out to determine whether HAP1 cells that have had Prdx1 genetically deleted exhibit a different basal oxidation level. Whilst the ICAT method successfully labelled and identified proteins from WT and Prdx1 KO HAP1 cells, there was a poor coverage of the overall proteome. The low coverage means further work is required to draw any biological conclusion about the basal oxidation status of WT and Prdx1 HAP1 cells and contribution of Prdx1 in H$_2$O$_2$ signalling.
Chapter 6

Discussion

6.1 Overview

This Thesis has explored regulatory mechanisms of ASK1 and the general principle of H$_2$O$_2$ signalling using *in vitro* and proteomic methods. This Chapter serves to connect the individual results Chapters, putting them into the perspective of the wider biological field. Outstanding questions will be raised and possible methods for addressing them will be highlighted.

6.2 Summary of results Chapters

The overall aims of this Thesis were to investigate the regulatory mechanisms of ASK1 and assess whether a proteomics method would be feasible for exploring the role of Prdx1 in H$_2$O$_2$ signalling.

Chapter 3 used recombinant ASK1 protein and *in vitro* kinase assays to look at ASK1 auto-regulation. The ASK1 N terminus was seen to be auto-inhibitory on ASK1 kinase activity. The ASK1 PH domain, within the N terminus, was postulated to act as a scaffold by recruiting MKK6, priming it for phosphorylation.

Chapter 4 used recombinant ASK1, Trx1 and SAXS data to investigate ASK1 regulation by Trx1. Trx1 was found able to bind to ASK1. Trx1 and ASK1 could form an inter-molecular disulfide bond for which ASK1-Cys250 was required. Somewhat
surprisingly, no Trx1 mediated inhibition of ASK1 kinase activity was observed \textit{in vitro}.

Chapter 5 was a pilot study in order to determine whether a redox proteomics method would be suitable for understanding the role Prdx1 plays in H$_2$O$_2$ signalling. The ICAT method was used in conjunction with a Prdx1 KO cell line. The data suggested, that there are no differences in basal protein oxidation levels between WT and Prdx1 KO cell lines. Preliminary data indicates that WT and Prdx1 KO HAP1 cells do respond differently to H$_2$O$_2$. However, further work is required to identify a greater number of proteins.

6.3 ASK1 regulation

In cells, ASK1 is activated in response to a myriad of factors, including H$_2$O$_2$, but little is known about how this process is governed at a molecular level. MAP3Ks contain large extra-kinase regions (see Figure 1.3 on page 5), but the role of these regions in regulating protein catalytic activity often goes overlooked. Concordantly, as a MAP3K, ASK1 contains a serine/threonine protein kinase domain, and large regulatory regions, both N- and C-terminal to the kinase domain (see Figure 1.4 on page 7). The work within this Thesis has allowed for an \textit{in vitro} investigation into N terminal regulation of ASK1, to a level hitherto not possible.

6.3.1 Auto-regulation of ASK1

In Chapter 3, it was seen that the N terminus (residues 88–658) of ASK1 is directly inhibitory on ASK1 kinase activity. A model was proposed that the ASK1 PH domain, N terminal to the kinase domain, could act as a docking site for MKK6. Overall control of access to the PH domain could be regulated by the TBD (see Figure 3.9 on page 82). This model is attractive, as it allows for the TBD to be the redox sensor of ASK1, regulating ASK1 in response to H$_2$O$_2$.

The high degree of conservation within ASK1$_{269-658}$ (see Appendix Figure B.7; Weijman \textit{et al.}, 2017), means it is probable that the model will also be true for both
ASK2 and ASK3. Replicating the work within Chapter 3 with the other ASKs would be needed to confirm whether the model is true for all ASKs. It remains to be seen whether other MAP2Ks would behave similarly to MKK6. Extending the work within Chapter 3 with other MAP2Ks (e.g., MKK3, 4 and 7), would help to inform how universal the proposed model is. Lastly, in order to confirm an affinity between the non-catalytic regions of ASK1 and MAP2Ks, it would be worthwhile to characterise binding affinities—by methods such as such as ITC or surface plasmon resonance (SPR)—between ASK1 domains and different MAP2Ks.

The model of TBD-mediated regulation of ASK1 is based on comparisons of SAXS data from two protein constructs: ASK1\textsubscript{88-658} and ASK1\textsubscript{88-941}. However, in light of the observations in Chapter 4 that ASK1\textsubscript{88-941} is not inhibited by Trx1 (despite ASK1 and Trx1 being able to interact), it is worth reconsidering the model. The model for ASK1 auto-inhibition was based on rigid-body modelling of the constitutive domains within ASK1\textsubscript{88-658} and ASK1\textsubscript{88-941}. However, the direct comparison of ASK1\textsubscript{88-658} and ASK1\textsubscript{88-941} rigid-body models cannot account for any potential flexibility between the ASK1 kinase domain and ASK1\textsubscript{269-658}. The relative positions of ASK1\textsubscript{269-658} and the ASK1-TBD will be quite constrained, by comparison, there will be a greater degree of freedom in the position of the kinase domain (there is a linker, 16 amino acids in length, between ASK1\textsubscript{269-658} and the kinase domain). Whilst ensemble rigid-body modelling of SAXS data could be used to give a series of most probable conformers (Tria \textit{et al.}, 2015), kinetic data may be more informative.

Fluorescence (or Förster) resonance energy transfer (FRET) could be used to understand how dynamic the constitutive domains within ASK1\textsubscript{88-941} are. FRET is a technique that allows for the inferences of distances (~20–60 Å) between two fluorophores: a donor and acceptor (Ma \textit{et al.}, 2014). When excited, a donor will transfer energy to the acceptor causing the acceptor molecule to fluoresce (Ma \textit{et al.}, 2014). A donor-acceptor pair could be covalently conjugated to the N- and C-termini of ASK1\textsubscript{88-941}, and the FRET signal measured under different conditions, such as when MKK6 or Trx1 are present. A FRET approach would help to show how dynamic ASK1\textsubscript{88-941} is, as movements at either the N or C terminus of the construct could alter the FRET signal. Correctly interpreting any FRET signal from ASK1\textsubscript{88-941} would benefit from
higher-resolution structural data on which to model any perceived movement.

To date, there are published crystal structures of the ASK1 kinase domain (Bunkoczi et al., 2007) and ASK1269–658 (Weijman et al., 2017). During this Thesis, attempts were made to crystallise various N-terminal ASK1 constructs (e.g., 88–266 and 88–658) to no avail. Other efforts within the Mace laboratory have been made at crystallising ASK188-941, also with no success. Other ASK1 constructs, such as full-length ASK1 (ASK12–1374) from Sf9 cells could be more useful. As ASK12–1374 is expected to form a large oligomer (Federspiel et al., 2016; Noguchi et al., 2005), it may be possible to use cryo-electron microscopy (cryo-EM) to determine a structure for ASK1. Cryo-EM requires relatively small amounts (∼5 µL) of low concentration (less than 2 mg/mL) protein (Murata and Wolf, 2018). Critically, cryo-EM does not require a crystal, yet can be used to achieve a high-resolution (below 4 Å) structure (Cheng, 2015). Applying cryo-EM to ASK12–1374 would give a detailed picture of how domains within ASK1 are arranged, and how they interact with each other in the context of the full-length protein. It would then be beneficial to revisit the ASK1 constructs used in this Thesis (88–266, 88–658 and 88–941), and re-evaluate the data in light of a cryo-EM structure.

6.3.2 ASK1 N-terminal mutations in health and disease

The importance of the N-terminal domains in ASK1 regulation can be further illustrated by looking at known mutations within the MAP3K5 gene. The cBioPortal for Cancer Genomics (www.cbioportal.org; Cerami et al., 2012) lists ∼600 missense mutations within the MAP3K5 gene. Within N-terminal ASK1 domains, two mutations stand out in context of this work: Arg395Gln and Arg256Cys.

In Chapter 3, Arg395 was mutated to Glu to investigate whether ASK1269–658 was a flexible or rigid domain. If there were flexibility, Arg395Glu would increase activity, whereas if it were a rigid domain, Arg395Glu would decrease activity (3.5.4). ASK188–941R395E was approximately 50% less active than WT (3.5.4.1). An Arg395Gln mutation will likely not be as detrimental as Arg395Glu, but based on this work it goes towards explaining how mutations within ASK1269–658 could be pathogenic.

Prickett et al. (2014) identified ASK1 Arg256Cys mutations in human melanomas.
When ASK1\textsuperscript{R256C} was over-expressed in HEK293T cells, Prickett et al. (2014) observed lower levels of p38 and JNK phosphorylation (Prickett et al., 2014). Prickett et al. (2014) saw, by co-immunoprecipitation, that ASK1\textsuperscript{R256C} associated with Trx1 to a greater degree than ASK1\textsuperscript{WT}. Based on these observations, Prickett et al. (2014) hypothesised that ASK1\textsuperscript{R256C} had increased inhibition by Trx1, resulting in decreased p38 and JNK activation and lower levels of cell death, which would contribute to cancer progression. However, the work in Chapter 4 argues that association between ASK1 and Trx1 is not sufficient to directly alter ASK1 kinase activity \textit{in vitro}, bringing in to question the true nature of the association between ASK1 and Trx1.

6.3.3 Is Trx1 a \textit{bona fide} inhibitor of ASK1?

In Chapter 4, the interaction between Trx1 and ASK1 was investigated. Trx1 is generally regarded to bind to the ASK1-TBD, directly inhibiting ASK1 kinase activity (Fujino \textit{et al.}, 2007; Saitoh \textit{et al.}, 1998). However, there are conflicting opinions as to whether Trx1 only binds to ASK1 or whether the oxidoreductase activity of Trx1 (on ASK1) is required (see Figure 4.2 on page 89; Nadeau \textit{et al.}, 2009, 2007; Saitoh \textit{et al.}, 1998). Whilst ASK1 and Trx1 were seen to interact \textit{in vitro} (agreeing with observations by Kosek \textit{et al.} (2014) and Kylarova \textit{et al.} (2016)), Trx1 mediated inhibition of ASK1 kinase activity was not observed. Lack of ASK1 inhibition by Trx1 was rather surprising given the prevalence of the direct-binding and inhibitor model in the literature. It is worth considering what is known about Trx1 interactions \textit{in vivo}.

The ubiquity of Trx (and Trx like proteins) has led to much interest in identifying interacting partners and substrates of Trxs in different systems. To this end studies have been conducted on identifying Trx targets from bacteria (Arts \textit{et al.}, 2016; Kumar \textit{et al.}, 2004), yeast (Vignols \textit{et al.}, 2005), plants (Hägglund \textit{et al.}, 2008; Maeda \textit{et al.}, 2005; Yano \textit{et al.}, 2001), mammalian cells (Benhar \textit{et al.}, 2010; Engelhard \textit{et al.}, 2011; Nakao \textit{et al.}, 2015; Schwertassek \textit{et al.}, 2007) and mouse tissue (Booze \textit{et al.}, 2016; Fu \textit{et al.}, 2009). These studies have used various approaches, including methodologies similar to the ICAT method that was deployed in Chapter 5.

Focusing on studies that have looked at Trx1 substrates and interacting partners
from mammalian material (in particular: Benhar et al. (2010); Booze et al. (2016); Fu et al. (2009) and Nakao et al. (2015)), ASK1 has not been reported as either a substrate nor interactor with Trx1. However, one of the limitations of studies such as these (that use shotgun-MS based methods) is that the relative abundances of proteins may skew the results. For example, highly abundant proteins, such as Prdxs and protein translation factors, are frequently reported as Trx1 interacting partners, making it difficult to identify lower-abundant proteins, including ASK1 (Benhar et al., 2010; Booze et al., 2016; Fu et al., 2009; Mellacheruvu et al., 2013).

Most of the studies reporting an association between ASK1 and Trx1 demonstrate the association by co-immunoprecipitation from cell over-expression systems. Federspiel et al. (2016) used a targeted (SRM) mass spectrometry method to look at ASK1 interacting partners. When ASK1 was over-expressed in HEK293, less than 0.5% of ASK1 was associated with Trx1. When Federspiel et al. (2016) looked instead at endogenous levels, 5% of ASK1 was associated with Trx1 and did decrease when they treated cells with 4-hydroxynonenal (a lipid electrophile that can be used to activate JNKs and ASK1 (Federspiel et al., 2016; Soh et al., 2000)). By comparison, 70–80% of endogenous ASK1 was found to associate with ASK2 and ~40% with 14-3-3 proteins (Federspiel et al., 2016). Therefore, the interaction between ASK1 and Trx1 may be a lot more transient than first assumed, or, dependent upon other cellular conditions.

In Chapter 4, ASK1 and Trx1 were seen to associate and were able to form an intermolecular disulfide-linked species. Whilst the mechanism of how ASK1 and Trx1 were able to form a cross-linked species is unclear (see 4.5.6), it was seen that ASK1-Cys250 was necessary and sufficient to allow the formation of the species. One possibility that has not previously been explored is a disulfide-linked ASK1 homo-dimer (i.e. Cys250-Cys250). Such a species would have to be present at low levels, as there was no clear evidence to support a homo-dimer forming. For instance, in Figure 4.5 (page 98), where ASK1_{88-658} was incubated with Trx1 or Trx1_{C35S} and analysed by non-reducing SDS-PAGE, there were several bands—greater than 100 kDa in size—that could indicate a disulfide-linked dimer (or oligomer) of ASK1_{88-658}. However, these bands are present at the beginning of the assay, do not substantially change over time, and critically, are still present on reducing SDS-PAGE. These observations were identical when ASK1_{88-658}—
Cys250Ser was incubated with Trx1 (or Trx1C35S) in the same manner (Appendix Figure C.4c). Nonetheless, in cells, ASK1 is known to form high molecular weight, disulfide-linked species in response to H₂O₂ (Jarvis et al., 2012; Nadeau et al., 2009, 2007). Prdx1 is required for ASK1 to form these disulfide-linked species (Jarvis et al., 2012; Stöcker et al., 2018a). Nadeau et al. (2007) proposed that Trx1 acted as an oxidoreductase, reducing the ASK1 disulfide-linked species, thereby regulating ASK1 activity. The work in Chapter 4 would indicate that Trx1 can indeed serve to reduce ASK1, but Trx1 itself does not directly inhibit ASK1 activity. Taken together with the work by Federspiel et al. (2016), Trx1 may not be a specific regulator of ASK1, but rather a non-discriminant reducer of cysteine residues within ASK1.

6.3.4 Missing components of ASK1 regulation?

Whilst this Thesis points towards Trx1 not being a direct inhibitor of ASK1, there are several caveats to consider. The strength of the recombinant model system used in this work is that we are able to know the concentration and condition of proteins in an assay. However, the weakness of the system is that it will be impossible to ever recapitulate the true complexity of a cellular environment. Thus whilst Trx1 is not seen to inhibit ASK1 in this work, it cannot be extrapolated to Trx1 not being an inhibitor in vivo as other factors need to be considered.

6.3.4.1 Post-translational modification of ASK1

One aspect of native ASK1 that will not be recapitulated with bacterial expression system used in this work, is post-translational modifications of ASK1. Modifications of ASK1 could result in different behaviours, explaining the discrepancy between the work in this Thesis and in the literature. ASK1 can be phosphorylated (see Chapter 1) and methylated (Nishida et al., 2017). ASK1 can be methylated by protein arginine methyltransferases (PRMT) 1 and 5 (Chen et al., 2016; Cho et al., 2012). Methylation within the ASK1 N terminus, at Arg78 and Arg80, has been seen to increase binding of Trx1 to ASK1 (Cho et al., 2012). Arg78 and Arg80 fall outside of the regions covered by any ASK1 protein construct used in this Thesis. Therefore, it would be informa-
tive to repeat some of the experiments within this Thesis with an ASK1 construct that includes Arg78 and Arg80 (perhaps residues 2–266, providing that they do not cause instability or decreased protein solubility) and confirm whether there are differences in ASK1 activity or Trx1 binding. Recombinant rat PRMT1 has been purified from *E. coli* (Zhang and Cheng, 2003). It is, therefore, feasible to use recombinant PRMT1 to methylate ASK1 in vitro, and determine the effect of methylation on Trx1 binding and inhibition. Arg to Phe mutants could also be used to mimic the methyl group (Mostaqul Huq et al., 2006) allowing any effect on Trx1 binding to be determined. Understanding how ASK1 and Trx1 associate would still greatly benefit from a high-resolution structure. If a longer N-terminal ASK1 construct (residues 2–266) did increase ASK1’s affinity for Trx1, it would be worthwhile to attempt to co-crystallise the proteins in a complex for structure determination by X-ray crystallography.

ASK1 can also be ubiquitylated. It has been suggested that one of the mechanisms by which Trx1 can regulate ASK1 is not via directly altering ASK1 activity but rather by binding to the ASK1-TBD, promoting ubiquitylation and consequent degradation (Liu and Min, 2002). Polyubiquitylation of ASK1 within its C terminus (at six lysine residues between 946 and 957) has been shown to be required for active ASK1 signalling in response to viral infection (Yu et al., 2016). Whilst polyubiquitylation of the ASK1 C terminus may not directly modulate the interaction between ASK1 and Trx1, polyubiquitylation could affect localisation or recruitment of other factors, all of which would affect the overall signalling system.

6.3.4.2 The ASK1 signalosome

ASK1 is known to form a complex that has been termed the ‘ASK1 signalosome’ (Noguchi et al., 2005). Under resting conditions, the signalosome is at least 669 kDa in size (Federspiel et al., 2016; Noguchi et al., 2005). Using a target mass spectrometry approach, Federspiel et al. (2016) deduced that the ASK1 signalosome likely consists of two copies each of ASK1 and ASK2 and one copy of a 14-3-3 protein. ASK1 and ASK2 have repeatedly been seen to associate (Ortner and Moelling, 2007; Takeda et al., 2007; Wang et al., 1998). Perhaps then Trx1 only regulates ASK1 in context of
the full signalosome. Reconstituting the signalosome (at least ASK1 and ASK2) with recombinant protein would allow for Trx1 regulation to be tested this way.

### 6.3.5 ASK1 and activation by H₂O₂

Trx1 is widely purported as a constitutive binder of ASK1, and only when Trx1 is released, is ASK1 fully active (Fujino et al., 2007; Liu et al., 2000; Liu and Min, 2002; Saitoh et al., 1998). Trx1 is generally regarded to be released from ASK1 when Trx1 becomes oxidised by H₂O₂ (Saitoh et al., 1998). However, as covered in Chapter 5, the most likely target of H₂O₂ in vivo will be Prdxs. It is highly probable, therefore, that the interaction between ASK1 and Trx1 is much more transient, with Trx1 only associating with ASK1 to reduce oxidised-ASK1. Prdx1 would then serve to act as an activator of ASK1 by facilitating oxidation of ASK1 (Jarvis et al., 2012). In Chapter 4, ASK1 and Prdx1 were combined in an assay (4.4.7.1) with no change in ASK1 activity. However, as discussed above (6.3.4.2) perhaps ASK1 and ASK2 need to be in a complex.

Prdxs are purported to serve as mediators of disulfide relays in order to facilitate H₂O₂ signalling (Stöcker et al., 2018b). One of the best-known examples of a disulfide-relay is from yeast, between the peroxiredoxin Gpx3 and transcription factor Yap1 (Winterbourn, 2018). Gpx3 is required to transmit the oxidative signal from H₂O₂ to Yap1 but Yap1 and Gpx3 alone are not sufficient for proper transmission of the signal in vivo (Veal et al., 2003). An interacting protein—Yap1 binding protein, Ybp1—is required (Veal et al., 2003). Bersweiler et al. (2017) described Ybp1 as a sulfenic acid chaperone, explaining why Ybp1 is required. Ybp1 brings Yap1 together with the sulfenylated peroxidatic cysteine residue (i.e. C_p-SOH) of Gpx3, preventing disulfide bond formation within Gpx3 and promoting the formation of a Gpx-Yap1 inter-molecular disulfide bond (Bersweiler et al., 2017). In the Pfam database, Ybp1 belongs to the ‘Kinetochore_Ybp2 (PF08568)’ family of which the human protein glomulin is also a member (pfam.xfam.org; Finn et al., 2016). Glomulin may act as a similar sulfenic acid chaperone in a mammalian system (although not necessarily between ASK1 and Prdx1). TRAF proteins and polyubiquitlayion have well-characterised roles as protein adaptors (Rusnak and Fu, 2017; Swatek and Komander, 2016) and there-
fore could act orthogonally to Ybp1 in regulating Prdx1 and ASK1. Additionally, we may yet identify other proteins that have equivalent roles as mammalian sulfenic acid chaperones.

### 6.4 Prdx1 and H$_2$O$_2$ signalling

In this Thesis, ASK1 has been used as a model system for investigating how factors associated with oxidation (i.e., Trx1, H$_2$O$_2$ and Prdx1) can regulate protein activity in vitro. The broader question is, how do proteins get oxidised within a cellular environment? Prdxs are proposed to act as mediators of H$_2$O$_2$ signalling, becoming oxidised by H$_2$O$_2$ themselves then transmitting the signal to target proteins via inter-molecular disulfide bonds (see Figure 5.2 on page 146; Stöcker et al., 2018b).

The cytosolic mammalian Prdx, Prdx1 has been seen to be required for H$_2$O$_2$ induced activation of p38 MAPK within human myeloid leukemia U937 cells (Jarvis et al., 2012), the J774A.1 mouse macrophage cell line (Conway and Kinter, 2006) and, in this work, human myelogenous leukaemia HAP1 cells (5.5.1). In yeast (S. pombe) the Prdx, Tpx1, is required for activation of Sty1—a yeast MAPK that is orthologous to p38 and JNK—via a disulfide-relay (Day and Veal, 2010; Veal et al., 2004). In the case of Tpx1 and Sty1, the Prdx is directly activating the MAPK. In the case of Prdx1 and p38, it is thought that Prdx1 instead activates the pathway at the MAP3K level, by directly activating ASK1 (Jarvis et al., 2012). It suggests, perhaps, that Prdx-mediated activation of MAPKs is an evolutionarily conserved process, but the precise signalling partners are divergent.

Chapter 5 tested a redox proteomics approach to identify proteins that are differentially oxidised depending upon Prdx1 expression. Accordingly, it was hypothesised that additional H$_2$O$_2$ signalling substrates (including ASK1) of Prdx1 might be identified by the redox proteomics approach. Whilst the method did identify proteins, there were only $\sim$40-80 ICAT-labelled proteins that were identified per experiment. Furthermore, as the majority (more than 60%) of labelled cysteine residues originate from structural disulfide bonds (see 5.6.2.4), there is not enough to data to make any conclusions about Prdx1 as a signalling protein. The ICAT method—as deployed within this
Thesis—could be further refined, however, alternate techniques should be considered in order to investigate the role of Prdx1 in H$_2$O$_2$ signalling.

### 6.4.1 Alternative methods to investigate Prdx1 contribution to H$_2$O$_2$ signalling

Other approaches could be used to investigate the role of Prdx1 in H$_2$O$_2$ signalling. For example, van der Reest *et al.* (2018) used a redox proteomics approach to look at protein cysteine oxidation in response to metabolic perturbation. A differential alkylation strategy was used, similar to that used in Chapter 5. There were a few major differences that van der Reest *et al.* (2018) used, chiefly that they used a combination of N-ethylmaleimide (NEM) and isotopically labelled IAM. Reduced cysteines were firstly labelled with their IAM reagent followed by reduction and labelling of all other cysteines with NEM—an approach van der Reest *et al.* (2018) dubbed “stable isotope cysteine labelling with iodoacetamide (SICyLIA)”. Importantly, this approach does not feature an enrichment step. Instead, there is complete fractionation of an entire lysate—from cell lines and mouse tissue—using HPLC before subsequent LC-MS/MS analysis of individual fractions. As the SICyLIA method does not rely on enrichment it can give a much better overview of the whole proteome. Van der Reest *et al.* (2018) were able to identify on the order of 10$^5$ peptides, representing 2,000–3,500 cysteine-containing proteins—one of the most in-depth redox proteomics studies that has been published. The main drawback of the SICyLIA method is the total fractionation approach requires extensive LC-MS/MS time. Nonetheless, the SICyLIA approach would also increase the coverage of lower-abundance proteins—proteins that are more likely to represent signalling proteins—offering a marked increase in the coverage that was achieved using the ICAT method as in Chapter 5. The experimental aspect is relatively straightforward and would be easily translatable to another biological question, such as with the contribution of Prdx1 to H$_2$O$_2$ signalling.

An alternative to looking at protein oxidation is instead to monitor other aspects of cell signalling. For example, as Prdxs are linked to regulation of protein kinase signalling—either by activating kinases or inactivating phosphatases (Holmström and
Finkel, 2014)—loss of Prdx1 may alter cellular phosphorylation levels, ‘the phospho-proteome’. The use of mass spectrometry to look at the phosphoproteome (phosphoproteomics) has provided an in-depth view of phosphorylation during the cell cycle (Olsen et al., 2006, 2010). Comparing the phosphoproteomes of WT and Prdx1 KO cells in response to H$_2$O$_2$ would inform as to whether protein kinase signalling is altered by Prdx1 expression.

Moving away from a proteomics approach, determining changes in gene expression (transcriptomics) in response to H$_2$O$_2$ could be an alternative approach. Fomenko et al. (2011) compared the transcription responses of *S. cerevisiae* in response to H$_2$O$_2$ in WT strains and strains that had all Prdxs and Gpxs genetically deleted. Strikingly, Fomenko et al. (2011) saw that without Prdxs and Gpxs, there was almost no transcriptional response to H$_2$O$_2$, indicating that the peroxidases are required to regulate the yeast H$_2$O$_2$ adaptation response. A similar approach could be taken in mammalian cells, comparing the transcriptional response to H$_2$O$_2$ in WT and Prdx1 KO cells. An advantage of looking at responses in transcription—or even phosphorylation—rather than protein oxidation, is that it may no longer be necessary to protect the native oxidation status of the proteome (such as with thiol blocking using IAM). However, it would still be an important control to ensure that there is minimal effect of artefactual oxidation on transcription during sample preparation.

Stöcker et al. (2018a) used a novel approach to investigate the effect of Prdx1 and 2 expression on protein oxidation within HAP1 and HEK293T cells. They treated cells—that were either KO or knock-down for Prdx1 and 2—with H$_2$O$_2$ and analysed changes in protein oxidation by a combination of Trx1 substrate trapping, western blot and mass spectrometry. Overall their approach demonstrated that Prdx1 and 2 are required for proteins to become oxidised *in vivo*. A limitation of the work by Stöcker et al. (2018a), is that it does not directly link protein oxidation with signalling events (i.e. protein phosphorylation). Additionally, as proteins were identified by shotgun MS, there is less sensitivity than a direct MS-method. Nonetheless, there is a clear demonstration that Prdx1 and 2 are important in protein oxidation, highlighting that both Prdx1 and 2 act as more than a peroxidase.
6.4.2 Do Prdxs contribute to H$_2$O$_2$ signalling?

In Chapter 5, several models were outlined concerning the mechanisms of how Prdxs may contribute to H$_2$O$_2$ signalling—that they did not and H$_2$O$_2$ directly oxidises targets (the null hypothesis); that inactivation is required (the floodgate hypothesis); or that Prdxs actively directly transmit a signal from H$_2$O$_2$ (the disulfide-relay model). It will likely never be possible to completely rule out direct oxidation, as the small size of H$_2$O$_2$ makes it ideal to oxidise relatively inaccessible cysteine residues, such as with the EGF receptor (Paulsen et al., 2012). More broadly, there is sufficient evidence to show that Prdxs contribute to H$_2$O$_2$ signalling. As exemplified by Stöcker et al. (2018a) and Fomenko et al. (2011), loss of Prdxs results in less protein oxidation and a lack of a transcriptional response. The work by Stöcker et al. (2018a) and Fomenko et al. (2011) also strongly suggests that Prdxs are direct mediators of H$_2$O$_2$ signalling. However, Stöcker et al. (2018a) do not have a signalling read-out of the importance of Prdx-mediated protein oxidation and Fomenko et al. (2011) do not demonstrate that Prdxs are directly transmitting H$_2$O$_2$ signals. Ultimately, a combination of methods—such as outlined above (6.4.1)—are needed to link protein oxidation with Prdxs and a downstream consequence. Furthermore, using a recombinant in vitro system, such as was done in this Thesis with ASK1, would allow for a powerful validation of any effects seen from these wider studies.
6.5 Thesis Conclusions

This Thesis sought to investigate the regulatory mechanisms of the protein kinase, ASK1, and determine whether the ICAT proteomics method would be feasible for examining the role of the cytosolic peroxidase, Prdx1, in H$_2$O$_2$ signalling.

Prdx1 is required for H$_2$O$_2$ induced activation of the MAPK, p38, in HAP1 cells. A redox proteomics method was tested, using ICAT-labelling to monitor protein oxidation in HAP1 cells. Whilst ICAT-labelled proteins were detected, the deployed ICAT method was not sensitive enough to identify sufficient numbers of differentially oxidised proteins, and an alternative method is recommended for future studies.

The N terminus (residues 88–658) of ASK1 is directly inhibitory of ASK1 kinase activity. Inhibition can be regulated by the TBD within the ASK1 N terminus. The TBD can regulate access of the substrate, MKK6, to a postulated scaffolding region within the N-terminal PH domain. The PH domain may be able to promote a conformational change within MKK6, making it a better substrate for phosphorylation by the ASK1 kinase domain.

ASK1 is widely thought to be directly inhibited by the oxidoreductase, Trx1. The ASK1-TBD is able to interact with Trx1 and Trx1 and ASK1 are able to form an inter-molecular disulfide bond. ASK1-Cys250 is necessary and sufficient for the mixed-species disulfide to form between ASK1 and Trx1. However, Trx1 was not seen to inhibit ASK1 in vitro.

Overall this work has used both top-down and bottom-up approaches to build upon our knowledge of how MAP3Ks—such as ASK1—are regulated, and H$_2$O$_2$ as a signalling molecule. Higher resolution techniques—cyro-EM and mass spectrometry, for example—are required to understand the true nature of Trx1 in ASK1 regulation and the contribution of Prdx1 in H$_2$O$_2$ signalling.
References


### Appendix A

**Table A.1:** Primers used in colony PCR and sequencing

<table>
<thead>
<tr>
<th>Description</th>
<th>Sequence (5' to 3')</th>
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<td>T7</td>
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<td>T7 Term</td>
<td>GCTAGTTATGCTCAGCGG</td>
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<td>MalE</td>
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<td>DuetUp2</td>
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<tr>
<td>DuetDown</td>
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<tr>
<td>DuetUp1</td>
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<tr>
<td>Polyhedrin</td>
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<td>SP6</td>
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1—Also called pET Upstream, 2—For sequencing pcDNA3 mammalian expression vector (see A.1).

**Table A.2:** Primers used to generate LIC-PCR inserts and ASK1 cDNA

<table>
<thead>
<tr>
<th>Primer</th>
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<tr>
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<td>CAGGGAGCCCGGTAGCAGGAGCGGAGGACGAG</td>
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<td>ASK1_941_RV</td>
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All sequences are for human. FW—forward, RV—reverse. Numbers denote residue in the protein product. Underlined nucleotides denote LIC overhangs.
Table A.3: Primers used to generate restriction enzyme inserts

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<td>Trx1_2_FW_NdeI</td>
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All sequences are for human. Numbers denote residue in the protein product. Underlined nucleotides relevant restriction enzyme site.

Table A.4: Primers used for introduction of mutations in protein sequences

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<td>StrepII_Generic_FW</td>
<td>TCACGACGTGGAGCCACCCCGAATTCGAAAAAGTAACCGGCTTCTCTCG</td>
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<td>CTGCGGGTGCTCCATGTGACCCCTTCTCTCTCTGTATGTTGTTTCAC</td>
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<td>ASK1_940_StrepII_RV</td>
<td>CTGCGGGTGCTCCATGTGCAAACCTTATGTTTTCAC</td>
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Numbers denote residue in the protein product. Underlined nucleotides denote substitution sites. Italicised nucleotides denote StrepII tag.
Table A.5: Oligonucleotides used in cloning mammalian LIC expression vector

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</tr>
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<td>CTCTTGGGCTGCCTTTCAGGGACCCGGGTACCCTGGGCTTCCTCGAG</td>
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<tr>
<td>pcDNA3-StrepII-LIC-RV</td>
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<td>AGCCGCGGACTTTTCGAACTGCGGGTGGCTCCATGCTGATGCCATGGTG</td>
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FW-forward, RV-reverse. Start site (ATG) is in bold. KpnI sites are underlined. EcoRI sites are italicised. HindIII sites are italicised and underlined.

Figure A.1: SEC molecular weight standards. SEC protein standards run on either a S200I (a) or S75 (b) column. Protein standards: 1) Thyroglobulin, 669 kDa, 0.32 mg/mL. 2) Apoferritin, 400 kDa, 0.4 mg/mL. 3) Alcohol dehydrogenase (ADH) 150 kDa, 0.2 mg/mL. 4) BSA, 66 kDa, 0.4 mg/mL. 5) Carbonic anhydrase, 29 kDa, 0.12 mg/mL. 6) Ubiquitin, 8.9 kDa, 0.4 mg/mL. 250 µL of the protein standards mix, at the indicated concentration, was injected and run at a flow rate of 0.5 mL/minute in a buffer containing 300 mM NaCl, 10 mM HEPES (pH 7.6). All standards were from Sigma (catalogue number MWGF1000) with the exception of ubiquitin, which was a kind gift from Jack Curry (Mace laboratory).
Figure A.2: Example protein purifications. Continued on next page
Figure A.2: Example protein purifications. Continued on next page
Figure A.2: Example protein purifications. Continued on next page
Figure A.2: Example protein purifications. Relates to main Table 2.7. a) Schematic showing order of samples shown on SDS-PAGE gels and schematic of full-length ASK1 for reference. b-n) Purifications of indicated protein constructs listed in main Table 2.7. For an explanation of SDS-PAGE samples see a). IEX—anion exchange chromatography (ResourceQ); SEC—size exclusion chromatography on either Superdex 75 (S75) or 200 Increase (S200Inc) columns. For IEX traces, the conductivity trace is shown in yellow. Chromatogram traces for SEC molecular weight marker protein standards are shown in Appendix Figure A.1.
Table A.6: Configuration and contact files for CORAL modelling

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NTER and CTER define the number of extra N- and C-terminal residues in the protein sample not in the respective PDB file. LINK defines residues between linked proteins in a given PDB file. TBD-Robetta is the Robetta model used in this work (see Appendix Figure C.11). ASK-269-658 is from PDB ID: 5ULM (Weijman et al., 2017). Trx1 is from PDB ID: 3QFB (Fritz-Wolf et al., 2011). Lys (lysozyme) is from PDB ID: 2I2L (Vetter et al., 1996). Trx-Lys.pdb is the output from Trx1-Lys. For the contacts, ‘dist’ defines the distance (in Å) between two atoms (defined in the row below). Refer to the CORAL manual (www.embl-hamburg.de/biosaxs/manuals/coral; Petoukhov et al. 2012) for further information.

A.1 Cloning of LIC-compatible mammalian expression vector

A LIC-compatible mammalian expression vector was designed to feature an N-terminal StrepII (WSHPQFEK) tag, and 3C-protease cleavage site (SAALEVLFQGPG) within a pcDNA3 vector backbone. In this manner, any inserts generated for E. coli protein expression could also be inserted into pcDNA3. Oligonucleotides were designed and ordered from Sigma (the sequences are listed in Appendix A.5).

Linearisation of pcDNA3 vector Two and a half micrograms of pcDNA3 (Invitrogen) was linearised with 10 units of EcoRI and HindIII in SuRE/Cut Buffer B (all Roche) in a 50 µL reaction. DNA digest occurred for 4 hours at 37 °C. The restriction enzymes heat inactivated at 65 °C for 15 minutes. Linearised DNA was purified by agarose electrophoresis (2.3.5) and gel extraction (2.3.6).
Annealing of oligonucleotides to form LIC cassette  Oligonucleotides (sequences are listed in Appendix A.5) were designed and ordered from Sigma. Oligonucleotides were resuspended in UltaPure water to a final concentration of 100 µM (2.3.1). The oligonucleotides were designed so that when annealed, they would have sticky-ends that were compatible with EcoRI–HindIII linearised DNA.

Each stock oligonucleotide was diluted 10-fold in UltraPure water. Oligonucleotides were annealed in a reaction consisting of 1 µL of each diluted oligonucleotide, 5 µL of T4 DNA ligase buffer, made up to a final volume of 50 µL with UltraPure water. The reaction was mixed by pipetting before being centrifuged briefly and placed in a pre-heated 90 °C heat block (Select Bio-Products). The oligonucleotides were incubated for two minutes before the heat block was switched off and allowed to cool, slowly, to room temperature. Annealed oligonucleotides were further diluted 10-fold in UltraPure water before quantification by NanoDrop (2.3.4).

Ligation of linearised vector and LIC cassette  The LIC-cassette (insert) and linearised vector were mixed in a 1:3 vector:insert molar ratio. The ligation reaction contained 100 units of T4 DNA ligase, 2 µL of T4 DNA ligase buffer, 2.5 ng of insert and 25 ng of linearised pcDNA3 in a 20 µL reaction made up to volume with UltraPure water.

The ligation reaction was incubated at room temperature for 10 minutes. Initial ligations yielded no successful incorporation of the LIC-cassette insert. In order to circumvent this problem, the ligation reaction was repeated and subsequently digested with BamHI (as successfully EcoRI–HindIII digested and ligated vector would no longer contain a BamHI site). The ligation reaction (~8 µL) was incubated in a reaction with 2.5 units BamHI, 2 µL, SuRE/Cut Buffer B made up to 20 µL total volume with UltaPure water. BamHI digest was incubated at 37 °C for 2 hours before heat inactivation at 65 °C for 20 minutes. Four microlitres of the digested ligation reaction was transformed into chemically competent MC1061 cells (2.3.11). Colonies were screened by colony PCR (2.3.12) using SP6/T7 Term primers (Appendix A.1). A single positive colony was used to inoculate 3 mL of sterile LB with ampicillin (100 µg/mL) for mini-prep isolation (2.3.3) and subsequent sequencing.
A.2 Transient transfection of HAP1 cells

All DNA transfections were performed using Lipofectamine2000 (Invitrogen). Cells were grown as in 2.11.2. The day before transfection, cells were seeded in a six-well plate $5 \times 10^5$ cells per well in 3 mL of complete media. On the day of transfection, the media was removed and cells gently washed with PBS followed by the addition of 1 mL of pre-warmed IMDM base media (i.e. media with no FBS or antibiotic). The plate was returned to the $37 \, ^\circ C$ incubator for 30 minutes. During this time, the DNA-Lipofectamine mix was set up. For every well to be transfected, 150 µL of base IMDM were added to a sterile tube, followed by 9 µL of Lipofectamine2000. Plasmid DNA (2.5 µg per well) and 150 µL of base media were added to a separate tube. The DNA-media mix was filter-sterilised using a 0.2 µm filter-syringe. The DNA-media and Lipofectamine-media were then added together in equal volume (150:150 µL per well). The mixture was vortexted briefly before incubating at room temperature for 10 minutes. 250 µL of the complete DNA-Lipofectamine complex was added to each well (equivalent to 2 µg of DNA per well). The cells were returned to the incubator for 6 hours. After incubation, an additional 2 mL of complete pre-warmed media was added to the cells and they were left for between 24 and 48 hours until analysis by western blot (2.5).
Appendix B

Figure B.1: The N terminus of ASK1 is inhibitory to ASK1 kinase activity. Replicate assay used for quantification in addition to main Figure 3.3. The assay was performed identically to main Figure 3.3 except a lower concentration (0.01 µM) of ASK1 was also tested.

Figure B.2: Effect of phosphatase co-expression on ASK188–941 activity. ASK188–941 with and without lambda phage protein phosphatase (λPP) co-expression. The assays were carried out at room temperature with 3 µM M KK6, 2 mM DTT, the indicated amounts of ASK188–941, started with the addition of 50 µM ATP. Assays were analysed by Phos-Tag SDS-PAGE and visualised with Coomassie. High-contrast of the no co-expression gel shows ASK188–941 (which is auto-phosphorylated during expression) as multiple bands on Phos-Tag SDS-PAGE. The λPP co-expressed ASK188–941 runs as a single band. The co-expressed assay is reproduced from main Figure 3.3c.
Figure B.3: Addition N-terminal ASK1 regions (up to 1 µM) does not alter kinase activity. Blots used for quantification for main Figure 3.5. Replicate (Rep) 1 is shown in main Figure 3.5.
**Figure B.4:** *Addition of excess ASK1<sub>269-658</sub> enhances phosphorylation of MKK6.* Three replicate (Rep) experiments used for quantification in Figure 3.6d. Replicate 1 is shown in the main Figure 3.6.
**Figure B.5:** Addition of excess of ASK1<sub>88–266</sub> has no effect on ASK1 kinase activity. a) Schematic of protein constructs used in this assay. b) Kinase assay conducted with 3 µM MKK6 and 0.01 µM ASK1<sub>669–941</sub> and 2 mM DTT and was performed at room temperature. ASK1<sub>88–266</sub> was added to the assay with concentration up to 30 µM. The assay was started by the addition of 50 µM ATP. This assay was analysed by western blot against p-MKK6 with Ponceau S stained membrane shown for loading control.

**Figure B.6:** Addition of ASK1<sub>269–658</sub> does not change MKK6 phosphorylation by rMEKK1. a) ClustalOmega alignment (Li et al., 2015) of the kinase domains of human ASK1, human MEKK1 and rat MEKK1 (rMEKK1). Identity=31.8%; *—identical; :—strongly similar; .—weakly similar. b) Schematic of protein constructs in the assay. MEKK1 annotated according to UniProt (www.uniprot.org; The UniProt Consortium, 2017): S—SWIM-type zinc finger; R—RING-type zinc finger. c) rMEKK1 (residues 1160–1493) used in this assay (purified from Sf9 cells), provided by Abigail Burgess (Mace laboratory). d) In vitro kinase assay conducted with 3 µM MKK6, 2 mM DTT and rMEKK1<sub>1160–1493</sub> (at indicated concentrations), performed at room temperature. ATP (50 µM) was added to start the reaction. The assay was analysed by western blot against p-MKK6. Ponceau S stained membrane is shown for loading control.
Figure B.6: Addition of excess of ASK1269–658 does not change phosphorylation of MKK6 by rat MEKK1. Figure legend on previous page.
Figure B.7: Alignment of ASK proteins. Continued on next page.
Figure B.7: Alignment of ASK proteins. Continued on next page.
Figure B.7: Alignment of ASK proteins. a) Schematic showing overview of human ASK1.
b) Alignment of ASK1, 2 and 3. Numbering denotes human ASK1. Position of mutations in
Chapter 3 are denoted by a blue circle, cysteine residues are denoted by a red triangle.
Alignment made using ClustalOmega and displayed using ALINE (Bond and Schüttelkopf,
2009; Li et al., 2015).
**Figure B.8:** Gels used for quantification in for Figures 3.7 and 3.8. a) Purified ASK188–941 mutant proteins. b) Phos-Tag gels used for quantification in main Figures 3.7 and 3.8.
Figure B.9: Comparison of M KK6 structures. Three structures of M KK6 are available in the PDB: 3VN9 (in red); 3ENM (in orange) and 3FME (in green). 3VN9 is unphosphorylated and the only structure where the activation-loop is visible (Matsumoto et al., 2012). 3ENM is a phosphomimetic (Ser207Asp, Thr211Asp), in an ‘inhibited’ state (Min et al., 2009). 3FME is a phosphomimetic (Ser207Asp, Thr211Asp) in an ‘active’ state. An overlay (based on the C-terminal lobes) of all three structures is shown below, with all three structures in grey. The phosphorylation sites (Ser207 and Thr211) from 3VN9 are shown in red. The αC-helices of all three structures are shown (colours matching the individual structures above).
**Appendix C**

**Figure C.1:** *ASK1 and Trx1 interact* in vitro. Relates to main Figure 4.4. a) His$_6$-tagged ASK1 constructs bound on resin resolved by SDS-PAGE. b) (Top) Full Coomassie stained gel of pull-down. Western blot images for single channels—αHis$_6$ (middle), αTrx1 (bottom).
Figure C.2: *ASK1 and Trx1 can form an inter-molecular disulfide bond.* a) Schematic showing protein constructs used in this assay. Trx1 and ASK1_{88–338} were purified using only nickel-affinity. b) 5 µM ASK1_{88–338} was incubated with Trx1_{C35S} (5 µM) in PBS (pH 7.4) at 4 °C for the times indicated. At each time point, a sample was removed and added to a non-reducing SDS-PAGE buffer containing 20 mM NEM. Samples were analysed by SDS-PAGE. For reducing SDS-PAGE, the samples were incubated with 100 mM DTT for 5 minutes at room temperature. Proteins were visualised with Coomassie.
Figure C.3: ASK1-Cys250 is essential in forming an inter-molecular disulfide bond with Trx1. Relates to main Figure 4.6. a) Full blots and gels for main Figure 4.6. b) Trx1 and ASK1 were incubated identically as in main Figure 4.6, except 20 µM H₂O₂ was added at beginning of incubation.
**Figure C.4:** ASK1-Cys250 is required to form an inter-molecular disulfide bond with Trx1. Figure legend on next page.
**Figure C.4:** *ASK1-Cys250 is required to form an inter-molecular disulfide bond with Trx1.* Relates to 4.4.2 and 4.5. a) Schematic of proteins in this Figure. b) Trx1 alone gels for main Figure 4.5. c) and d) Identical to main Figure 4.5, except either Cys250Ser (c) or Cys200Ser (d) was used. Cys200Ser formed a DTT-sensitive species, but Cys250Ser did not. For further details see main Figure 4.5.

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**Figure C.5:** *ASK1<sub>88–266</sub> Cys250-only can form a disulfide-linked species with Trx1.* a) Schematic showing proteins b) ASK1<sub>88–266</sub> with only C250 (all other cysteines are mutated to Ser) was co-expressed with (untagged) human Trx1<sup>C35S</sup> in *E. coli*. Shown is purified proteins after nickel affinity purification and IEX. On non-reducing SDS-PAGE (-DTT), the proteins run predominately as a single ~30 kDa species and as two bands on reducing SDS-PAGE (+DTT). Proteins were visualised with Coomassie.
Relates to 4.4.4.1. a) Schematic showing constructs in this Figure. N-terminal-MBP fused to Trx1\textsuperscript{C35S}, described in the Figure as MBP-Trx1. An ASK1 triple cysteine mutant (ASK1\textsubscript{88–658C206S, C225,226S}), with a C-terminal StrepII-tag, was also used (referred to as ASK1 in the remainder of this legend). b) Nickel-purified ASK1 and MBP-Trx1 were reduced with 2 mM DTT for one hour, on ice, before being purified by IEX in non-reducing conditions. IEX purified ASK1 and MBP-Trx1 were combined at roughly equimolar amounts (in a buffer containing 10 mM HEPES (pH 7.4), 300 mM NaCl) before being incubated at either 10 or 30 minutes at room temperature, or for 16 hours at 4 °C. At the endpoint, samples were alkylated and denatured (in SDS sample buffer containing 50 mM IAM) for 30 minutes at room temperature, in the dark, before analysis by SDS-PAGE. Proteins were visualised with Coomassie. For reducing gels, samples were incubated with 100 mM DTT for 5 minutes at room temperature.

Figure C.6: Trx1-fusion proteins can form a mixed-species disulfide bond with ASK1.
Figure C.7: *SEC and cleavage of MBP-Trx1<sup>C35S</sup>*. Relates to 4.4.4.1. a) MBP-Trx1<sup>C35S</sup> was incubated with ASK1<sub>88-658</sub><sup>C206S, C225,226S</sup> (see Figure 4.7 for details), and subjected to SEC on an S200I column (running at 0.5 mL/min, in a buffer of 10 mM HEPES (pH 7.6), 300 mM NaCl). Arrowheads indicate molecular weight standards. The ASK1-Trx1-MBP complex eluted before the 150 kDa marker. b) Elution fractions from (a) were subjected to non-reducing and reducing SDS-PAGE. c) Elution fractions from (a) were incubated with an excess of 3C-protease—which cleaves the MBP-tag from Trx1 (see Methods (2.3.15) and Appendix C.10)—yielding the expected 1:1 (75 kDa), ASK1<sub>88-658</sub>-Trx1 complex.
Figure C.8: Preparation of ASK1-Trx1-Lys species for SAXS and comparison of ASK1_{88-658} WT and Cys250Ser. a) Reducing (+ DTT) gels for main Figure 4.8. Samples were incubated with 100 mM DTT at room temperature for 5 minutes before SDS-PAGE. b) and c) ASK1_{88-658} WT (b) or Cys250Ser (c) was incubated with Trx1_{C35S}-Lys at 37 °C for the times indicated and analysed by SDS-PAGE, see main Figure 4.7 for methods. Proteins were visualised with Coomassie.
Figure C.9: SAXS scattering and Guinier plots. Relates to 4.4.5. i) Scattering plots of Log(I) vs q, ii. Ln(I) vs q^2 for a) ASK1 (ASK1_{88–658}^{C206S, C225,226S}) alone, b) Trx-Lys (Trx1^{C35S}-Lys) alone and c) The disulfide-linked ASK1-Trx1-Lys complex. d) Non-normalised P(r) curves.
a) ASK1_{88–658}^{C206S, C225,226S},

- gpgSRRTTVAYVINEASQGQLVVAESEALQSLREACETVGATLETHLFGKL
  DFGGETTVLDRFYNAIDAVVEMSDFARQPSLFYHLGVRESFSMANNILYCDTN
  NDSLSQLKELICIQNKNTMSTGNYTFVYPMTHPHNKVYSSDSFMKGLTELM
  MQNPELGLPICLPLVDRFIQLLVAASQASSQYFRESILNDIRKARLYT
  GELAAELARIQRVDINELVTADIVINLLSRYQDYDSVIKLVETLEKL
  LPFTDLLASHHHVKFHYAFALRNRNLPGDRAKALDIMPVMQSEGQVASD
  MLYCLVGRKMMFLDFSDNFTDTSERDGHASWFKKAFSEEPTLQSGINYAV
  LLLAAHGHQFESSFELRKGVKLSSLLGKKGKNEKLKQSYEVSFGFFGASVL
  ANDHMRVQIAEKFLKJKTPAWLYKSIVETILIYKHFKLITEQPVKAE
  QVInvDFWMDFLVEATKTDVTVRFPVILEPTKIYQPFSLYSINVEEKTIS
  WHVLPDKKCHVWNSASSVQGVSISKFEERCCFLYVLHNSDFQIYFC
  TELHCKKFFVEMVTITEEGSASSHPQ

b) Trx1^{C35S,-Lys}:

- MVKQIESKTAFQEAALADAAGDKLVVDFSATWCGPSKMIKPFFHSLSEKY
  SNVIFLEVDDCQDVASECEVKSMPTFQQFKGGQKVGEFSGANKEKLE
  ATINELVleMNIFEMLRIDEGLRKYLKDTEGYTTIGHLLTKPSLNAAK
  SELDKAIRMRTANGTVDAAEIKLFQDVDAARRGILNKVPYDLSAVA
  RRALINMVQMQGETVGAVTSNSRLMQQKRWDEAAAVNLAKSRWYN
  QTNPRAKVRTITFRTGTWDAYKLNVEHHHHHH

c) MBP-Trx1^{C35S}:

- HHHHHHMKIEEGLKVIWNGDKGNYGLAEVGKKFKEDTGIKVTEHPD
  KEERFKPQAATGDGPDIIWFHARDFGGYAGSLUEEITLPDKAFQDKLY
  PFTWDARVRYNGKLIAIPAEVEALSILYNKDLPNPPEKWEEKPVGK
  AKGKSLMFLQEPYFTWPLIAADDGYYAFKYENGKDYIKDVGVNAGAK
  KAGLTFVLDDLKINHKMNADTDYSIAAFAAFNKGETAMTINGPWAWSNIDT
  SKVNVYGTVDLPTFKQGSPKTFVGVLASAGAASNPKELAKEFLENLYLTD
  EGLEAVNKDKPGLAVALSYEELAKDPRIAATMENQGKEMPINPQM
  SAFWYAARVTAAGNARQTVDEALKDAQTNSITSLYKAGENLYTFQG
  GSLELVFQgpgVKQIESKTAFQEAALADAAGDKLVVDFSATWCGPSKMIKP
  FFHSLSEKYSNVIFLEVDDCQDVASECEVKSMPTFQQFKGGQKVGEFS
  GANKEKLEATINELV

Figure C.10: Protein sequences for SAXS constructs. a) ASK1_{88–658}^{C206S, C225,226S}, construct used for SAXS. Lower case, underlined 'gpg' denotes the residues left from the His_{6}-tag after 3C cleavage, bold and underlined residues denote Cys to Ser mutations, letters in italic represent the (unused) C-terminal Strep-II tag. b) Trx1-Lys construct used for SAXS. Bold and underlined residues denote Cys to Ser mutations, the underlined 'le' represents the linker between the C terminus of Trx1 and N terminus of T4 phage lysozyme. Letters in italics denote the (unused) C-terminal His_{6}-tag. c) MBP-Trx1 fusion protein. Letters in italics denote the N-terminal His_{6}-tag, the underlined 'LEVLFQgpg' indicates the 3C cleavage site between MBP and Trx1, bold and underlined residues denote Cys to Ser mutations within Trx1.
Figure C.11: De novo ASK1\textsubscript{88–266} Robetta models. The Robetta server (Kim et al., 2004) was used to generate a de novo model for ASK1\textsubscript{88–266}. Five models were generated. a) The model used in the SAXS CORAL modelling. b) Overlay of the three most similar models (including a). All models feature a globular protein with an α-β-sandwich. Cys250 is located N-terminal to the final α-helix within the models. c) Shows the last two generated models (i. and ii.), which are, overall, more extended and disordered. Models were rendered in PyMol. N term—N terminus; C term—C terminus.
**Figure C.12:** Addition of DTT does not alter \( \text{ASK1}_{88-941} \) kinase activity. a) Schematic of proteins in this assay. b) Flow-diagram showing the order of addition of proteins. c) and d) Kinase assay, performed at room temperature, with \( \text{ASK1}_{88-941} \)—2 µM in c) and 6 µM in d)—and MKK6 (3 µM) and the addition of indicated amounts of DTT. The assay was started with the addition of 50 µM ATP and analysed via Phos-Tag SDS-PAGE. Proteins were visualised with Coomassie.
Figure C.13: Trx1 does not inhibit ASK1_{88-941} activity in vitro under reducing conditions. 

a) Schematic of proteins in this assay. b) and c) Kinase assay (performed at room temperature) with 2 mM DTT and titrating in Trx1 (b) or Trx1^{C35S} (c). The assay was started with the addition of 50 µM ATP and analysed via Phos-Tag SDS-PAGE. Proteins were visualised with Coomassie. Note—Under the conditions used, Trx1 migrates off the end of the gel and so cannot be seen.
Figure C.14: Pre-incubation of ASK1 and Trx1 does not inhibit ASK1 kinase activity.

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**Figure C.14:** Pre-incubation of ASK1 and Trx1 does not inhibit ASK1 kinase activity. a) Schematic showing protein constructs used in the assay. b) Flow diagram showing the order proteins were combined. c) and d) Kinase assay, similar main Figure 4.13 except with ASK1\textsubscript{88-941} (1 µM) and indicated concentrations of Trx1 (c), or Trx1\textsuperscript{C35S} (d), incubated together under non-reducing conditions for 16 hours at 4 °C. After the incubation, a sample was taken and alkylated with 10 mM IAM before running on non-reducing SDS-PAGE (i). The remaining sample was used for an in vitro kinase assay with MKK6 (3 µM). The assay performed at room temperature and started with the addition of ATP (50 µM). The kinase assay was analysed by Phos-Tag SDS-PAGE. Proteins were visualised with Coomassie.

**Figure C.15:** Trx1 does not inhibit full-length ASK1 in vitro. Relates to main Figure 4.14. Assay is identical to Figure 4.14 except for the inclusion of the indicated amount of DTT. a) Flow-diagram showing order proteins were combined. b) Phos-Tag SDS-PAGE and western blot (probing for p-MKK6) of the same samples.
Figure C.16: Prdx1 reduction and desalting. a) Prdx1 reduction and desalting. i.
Schematic of protocol and samples shown on SDS-PAGE in ii. Prdx1 was reduced with 10 mM DTT (in a buffer containing 10 mM HEPES (pH 7.6), 300 mM NaCl) for 16 hours at 4 °C before being desalted twice through a 0.5 mL Zeba Spin column (Thermo Scientific). The spin column was pre-equilibrated in 10 mM HEPES (pH 7.6) 300 mM NaCl (that had been dialysed against the same buffer containing 10 µg/mL bovine catalase (Sigma)). b) Treatment of 10 µM Prdx1 with the indicated concentration of H₂O₂ for the times indicated. At each time point, a sample was removed and the reaction was stopped by plunging it directly into SDS sample buffer with 10 mM IAM. Disulfide-linked dimerisation (Prdx1₂) occurs following H₂O₂ treatment.
Figure C.17: *ASK1*$_{2–1374}$ kinase activity is not changed by addition of Prdx1 and H$_2$O$_2$.

a) Schematic of proteins used in this assay. b) Flow diagram showing order proteins were combined. c) Prdx1 was reduced and desalted (as in Appendix C.16) and added (at the concentrations shown) to ASK1$_{2–1374}$ (approximately 0.1 to 0.5 µM, see main Figure 4.14). The proteins were incubated with the indicated amounts of H$_2$O$_2$ for 5 minutes at room temperature, before being added to M KK6 to perform the *in vitro* kinase assay, with 3 µM M KK6, started with the addition of 1 mM ATP. The assay was visualised by western blot, probing for p-MKK6. A Ponceau S stained membrane is shown for loading control.
**Figure C.18:** Inhibition of MKK6 phosphorylation following incubation with Trxl and \( \text{H}_2\text{O}_2 \). ASK1\textsubscript{88–941} alone for main Figure 4.17. a) Flow diagram of assay. ASK1\textsubscript{88–941} was incubated with the indicated concentrations of \( \text{H}_2\text{O}_2 \) for 15 minutes at room temperature. A sample was taken for non-reducing SDS-PAGE shown in b). The remainder used in a kinase assay with MKK6 and analysed by Phos-Tag SDS-PAGE, shown in c). For further details, see main Figure 4.17.
**Figure C.19: ASK1 secondary structure prediction.** Secondary structure prediction for human ASK1\textsubscript{88–266} numbers refer to the position in the protein sequence. e—extended; h—helix; t—turn. The stars indicate the position of the cysteine residues. Cys250 is the only cysteine predicted to be N-terminal to a helix. The prediction was generated using the ProteinCCD webtool (https://ccd.rhpc.nki.nl/; Mooij et al., 2009) with the HNN (Guermuer, 1997), DPM (Deleage and Roux, 1987) and Predator (Frishman and Argos, 1996) prediction servers. The figure was generated using Aline (Bond and Schütte-lkopf, 2009). See also Appendix Figure B.7 for a conservation plot.
Figure C.20: MKK6 cysteine residues. a) ClustalOmega (Li et al., 2015) alignment of human MKK6 and the related MAP2K, MKK3. The cysteine residues in MKK6 are indicated by a yellow dot. The activation loop (AL) is indicated by the black line. MKK6 phosphorylation sites (Ser207 and Thr211 for human) are indicated. The start of the structure in (b) is indicated. b) Crystal structure of unphosphorylated MKK6 (PDB ID: 3VN9; Matsumoto et al., 2012) rendered as a cartoon in PyMol. The cysteine residues and phosphorylation sites are shown as spheres in yellow and red, respectively.
Appendix D

Figure D.1: *Prdx1 is required for p38 response in HAP1 cells.* Relates to main Figure 5.6). Full blots for p38 response for H_2O_2 treatment of HAP1 WT and Prdx1 KO cells. Prdx1 bands are visible in the α-tubulin image as the blot was not stripped before re-probing for α-tubulin.
Figure D.2: Unspecific bands with p38α antibody and poor Prdx1 over-expression. a) WT HAP1 cells treated with 100 µM H2O2 for times indicated, lysed with a RIPA buffer, subjected to western blot and probed for p38α (used at a 1 in 2,000 dilution, Novus Biologicals, catalogue number: NB100-56665). The predicted size of p38α is indicated. The samples on this blot are the same as in as in main Figure 5.5. Other experiments gave similar results. b) StepII-tagged Prdx1, or green fluorescent protein (GFP; in a modified StrepII-LIC-pcDNA3 vector, see Appendix A.1) was transfected into HAP1 cells (see Appendix A.2 for method). Cells were lysed in RIPA buffer and resolved by western blot, probing for Prdx1, StrepII-tag and α-tubulin (loading control). GFP was used as a control transfection plasmid. The arrowhead indicates poor exogenous Prdx1-expression in Prdx1 KO cells.
**Figure D.3:** *IAM blocks reduced cysteines.* a) Flow diagram for differential protein labelling with 5-iodoacetamidofluorescein (IAF). Proteins were either not labelled with IAF, reduced with DTT and labelled with IAF or alkylated (with IAM or NEM), reduced and labelled with IAF. Alkylation occurred at room temperature for 30 minutes, in the dark. Reduction occurred at 50 °C for 15 minutes. IAF-labelling occurred at room temperature, 30 minutes in the dark. In between each step, the lysate was precipitated with 10% (v/v) (as in Methods, 2.11.3.2). b) IAF-labelled protein were visualised with in-gel fluorescence on a LAS-3000 (FujiFilm), before staining with Coomassie to visualise total protein.

M—protein marker; lanes 1 and 5—no IAF; lanes 2 and 6—total labelling; lanes 3 and 7—pre-blocking with IAM before labelling; lanes 4 and 8—pre-blocking with NEM.
Figure D.4: Technical replicates of H$_2$O$_2$ treated, ICAT-labelled WT or Prdx1 KO HAP1 cells. Relates to 5.5.3. See main Figure 5.11 for an overview of the experiment. a) Venn diagrams showing overlap between the duplicate LC-MS/MS runs on the same sample. Only proteins that had a ‘high confidence’ score in Proteome Discoverer were carried forward. Numbers in parenthesis indicate additional proteins that were found, but were only labelled in either the heavy or light channel. b) Standard deviation of the protein ICAT ratios from duplicate LC-MS/MS runs. c) Percentage of identified proteins that were ICAT-labelled. Only proteins found in both LC-MS/MS runs were carried forward.

Figure D.5: ICAT-labelled proteins from either H$_2$O$_2$ treated WT or Prdx1 KO HAP1 cells. a) and b) HAP1 cells were treated with 100 µM H$_2$O$_2$ (or an equal volume of water), for 2.5 minutes. Cells were lysed and ICAT-labelled as for the untreated WT/Prdx1 KO pilot trial (see 5.5.2). Graphical display of Tables D.2 and D.4. a) WT HAP1 cells. The ICAT ratio is heavy (H) (H$_2$O$_2$ treated) over light (L) (untreated). b) Prdx1 KO HAP1 cells. The ICAT ratio is light (L) (H$_2$O$_2$ treated) over heavy (H) (untreated). Note—these experiments were only carried out once.
**Figure D.5:** ICAT-labelled proteins from either H₂O₂ treated WT or Prdx1 KO HAP1 cells.

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**Figure D.6:** ICAT-labelled protein and cysteine origins. Relates to main Figure 5.14. a) Percentage of total ICAT-labelled protein that originate from disulfide bonds. b) Breakdown of sub-cellular origins of identified ICAT-labelled proteins. Annotated manually according to UniProt (www.uniprot.org; The UniProt Consortium, 2017).
### Table D.1: Single-channel ICAT-labelled proteins for untreated HAP1 (WT vs Prdx1 KO HAP1) cells

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Description</th>
<th>Accession code</th>
<th>ICAT Ratio (WT/Prdx1 KO)</th>
<th>Cysteine labelled</th>
<th>Known to form disulfide? (partner)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene Name</td>
<td>Description</td>
<td>Accession code</td>
<td>Rep 1 Run 1</td>
<td>Rep 2 Run 1</td>
<td>Rep 3 Run 1</td>
</tr>
<tr>
<td>GPC3</td>
<td>Glypican-3</td>
<td>P51654</td>
<td>1.65 Run 1</td>
<td>1.35 Run 1</td>
<td>0.01 Run 1</td>
</tr>
<tr>
<td>CANX</td>
<td>Calnexin</td>
<td>P27824</td>
<td>1.16 Run 1</td>
<td>0.93 Run 1</td>
<td>100.00 Run 1</td>
</tr>
<tr>
<td>NDUFB10</td>
<td>NADH dehydrogenase 1 beta subcomplex subunit 10</td>
<td>O96000</td>
<td>0.01 Run 1</td>
<td>1.30 Run 1</td>
<td>1.24 Run 1</td>
</tr>
<tr>
<td>TF</td>
<td>Serotransferrin</td>
<td>P02787</td>
<td>1.52 Run 1</td>
<td>0.79 Run 1</td>
<td>0.79 Run 1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>P04406</td>
<td>1.17 Run 1</td>
<td>0.43 Run 1</td>
<td>0.70 Run 1</td>
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<tr>
<td>RPS12</td>
<td>40S ribosomal protein S12</td>
<td>P25398</td>
<td>1.25 Run 1</td>
<td>0.01 Run 1</td>
<td>0.01 Run 1</td>
</tr>
<tr>
<td>HCCS</td>
<td>Cytochrome c-type heme lyase</td>
<td>P53701</td>
<td>1.18 Run 1</td>
<td>1.07 Run 1</td>
<td>100.00 Run 1</td>
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<tr>
<td>TUBA1B</td>
<td>Tubulin alpha-1B chain</td>
<td>P68363</td>
<td>1.74 Run 1</td>
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<tr>
<td>GALNT2</td>
<td>Polypeptide N-acetylgalactosaminyltransferase 2</td>
<td>Q10471</td>
<td>1.18 Run 1</td>
<td>0.20 Run 1</td>
<td>0.01 Run 1</td>
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<tr>
<td>ALB</td>
<td>Serum albumin</td>
<td>P02768</td>
<td>0.01 Run 1</td>
<td>0.90 Run 1</td>
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</table>

Manually annotated using UniProt (www.uniprot.org; The UniProt Consortium, 2017). ICAT ratio = heavy (Prdx1 KO) / light (WT). If a peptide was only found in one channel (heavy or light) Proteome Discoverer gives the peptide a value of 100 for the channel identified. Thereby a value of 100 means a protein was only found in the heavy channel, if it is only found in the light channel the ratio will equal 1/100 (i.e. 0.01). Underlined numbers were used to calculate the ‘mean’. 256
### Table D.2: ICAT-labelled proteins from H₂O₂ treated WT HAP1 cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Accession code</th>
<th>ICAT ratio (+H₂O₂/untreated)</th>
<th>Cysteine labelled</th>
<th>Known to form disulfide? (partner)</th>
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</thead>
<tbody>
<tr>
<td>CNPY2</td>
<td>Protein canopy homolog 2</td>
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<td>0.73</td>
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<tr>
<td>TUBA1B</td>
<td>Tubulin alpha-1B chain</td>
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<td>0.73</td>
<td>115</td>
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<tr>
<td>TXNDC5</td>
<td>Thioredoxin domain-containing protein 5</td>
<td>Q8NBS9</td>
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<tr>
<td>POFUT1</td>
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<td>SOD1</td>
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<td>AK2</td>
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<td>GLG1</td>
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<td>Cathepsin L1</td>
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<td>PPT1</td>
<td>Palmitoyl-protein thioesterase 1</td>
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<td>PPIA</td>
<td>Peptidyl-prolyl cis-trans isomerase A</td>
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<td>LDHB</td>
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<td>P07195</td>
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Table D.2: ICAT-labelled proteins from H$_2$O$_2$ treated WT HAP1 cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Accession code</th>
<th>ICAT ratio (+H$_2$O$_2$/untreated)</th>
<th>Cysteine labelled</th>
<th>Known to form disulfide? (partner)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERGIC1</td>
<td>Endoplasmic reticulum-Golgi intermediate compartment protein 1</td>
<td>Q969X5</td>
<td>1.05</td>
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<tr>
<td>PRKCSH</td>
<td>Glucosidase 2 subunit beta</td>
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<td>AHSG</td>
<td>Alpha-2-HS-glycoprotein</td>
<td>P02765</td>
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<td>Yes (62)</td>
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<tr>
<td>HCCS</td>
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<tr>
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<tr>
<td>NDUFB10</td>
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<td>CD276 antigen</td>
<td>Q5ZPR3</td>
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<td>Lysosomal Pro-X carboxypeptidase</td>
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<td>TMED2</td>
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<td>CD59 glycoprotein</td>
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<td>Q96000</td>
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<td>Yes (90)</td>
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Table D.2: ICAT-labelled proteins from H$_2$O$_2$ treated WT HAP1 cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Accession code</th>
<th>ICAT ratio (+H$_2$O$_2$/untreated)</th>
<th>Cysteine labelled</th>
<th>Known to form disulfide? (partner)</th>
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<tbody>
<tr>
<td>CMC2</td>
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<td>Q9NRG2</td>
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<td>37</td>
<td>Yes (24)</td>
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<td>CCDC58</td>
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<td>No</td>
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<td>FAM136A</td>
<td>Protein FAM136A</td>
<td>Q96C01</td>
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<td>UQCRHL</td>
<td>Cytochrome b-c1 complex subunit 6-like</td>
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<td>81</td>
<td>Yes (37)</td>
</tr>
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<td>P4HA1</td>
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Annotated manually using UniProt (www.uniprot.org; The UniProt Consortium, 2017). This data is displayed graphically in Appendix Figure D.5a.
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<th>Cysteine labelled</th>
<th>Known to form disulfide? (partner)</th>
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<td></td>
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<td></td>
<td>Run 1</td>
<td>Run 2</td>
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<td>TF</td>
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Annotated manually using UniProt (www.uniprot.org; The UniProt Consortium, 2017). ICAT ratio = heavy (treated) / light (untreated). If a peptide was only found in one channel (heavy or light) Proteome discoverer gives the peptide a value or 100 for the channel identified. Thereby a value of 100 means a protein was only found in the heavy channel, if it is only found in the light channel the ratio will equal 1/100 (i.e. 0.01).
Table D.4: ICAT-labelled proteins from H$_2$O$_2$ treated Prdx1 KO HAP1 cells

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<tr>
<th>Gene name</th>
<th>Description</th>
<th>Accession code</th>
<th>ICAT ratio ($+H_2O_2$/untreated)</th>
<th>Cysteine identified</th>
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<tr>
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<td>Q9NRP2</td>
<td>0.52</td>
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<td>NDUFB10</td>
<td>NADH dehydrogenase 1 beta subcomplex subunit 10</td>
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<td>TIMM10</td>
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<td>CALR</td>
<td>Calreticulin</td>
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<td>UBE2D3</td>
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<td>CANX</td>
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Table D.4: ICAT-labelled proteins from H$_2$O$_2$ treated Prdx1 KO HAP1 cells

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<th>Gene name</th>
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<th>Known to form disulfide? (partner)</th>
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<td>CHCHD3</td>
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<td>CD59</td>
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<td>LMAN2</td>
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<td>SOD1</td>
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<td>F11R</td>
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<td>CNPY2</td>
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<td>GALNT2</td>
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<td>LAMP2</td>
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<td>HCCS</td>
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<td>HLA-A</td>
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<td>P01891</td>
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Continued on next page
Table D.4: ICAT-labelled proteins from H₂O₂ treated Prdx1 KO HAP1 cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Accession code</th>
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<th>Cysteine identified</th>
<th>Known to form disulfide? (partner)</th>
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<tbody>
<tr>
<td>GLG1</td>
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<td>1079</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>PRDX4</td>
<td>Peroxiredoxin-4</td>
<td>Q13162</td>
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<td>51</td>
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<tr>
<td>BSG</td>
<td>Basigin</td>
<td>P35613</td>
<td>1.25</td>
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<tr>
<td>LTF</td>
<td>Lactotransferrin</td>
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<td>LTF (partner)</td>
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<td>FAM136A</td>
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<td>ENO1</td>
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<td>NPTN</td>
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<td>Actin, cytoplasmic 1</td>
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<td>TXNDC5</td>
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<td>AHSG</td>
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Table D.4: ICAT-labelled proteins from H$_2$O$_2$ treated Prdx1 KO HAP1 cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Accession code</th>
<th>ICAT ratio (+H$_2$O$_2$/untreated)</th>
<th>Cysteine identified</th>
<th>Known to form disulfide? (partner)</th>
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<tr>
<td>GPC4</td>
<td>Glypican-4</td>
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Annotated manually using UniProt (www.uniprot.org; The UniProt Consortium, 2017). a—Prdx4 Cys51 is in the N-terminal signal peptide. A graph of this data is in Appendix D.5b

---

Table D.5: Single-channel ICAT-labelled proteins from H$_2$O$_2$ treated Prdx1 KO HAP1 cells

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<th>Gene name</th>
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<th>Cysteine labelled</th>
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<td>GPC3</td>
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Annotated manually using UniProt (www.uniprot.org; The UniProt Consortium, 2017). ICAT ratio=light (treated)/heavy (untreated). If a peptide was only found in one channel (heavy or light) Proteome discoverer gives the peptide a value or 100 for the channel identified. Thereby a ratio value of 100 means a protein was only found in the light channel, if it is only found in the heavy channel the ratio will equal 1/100 (i.e. 0.01)
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<td>HAP1 (untreated)</td>
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<td>Mean</td>
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<td>SD</td>
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<td><strong>Standard Error</strong></td>
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<td>Amplitude</td>
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<td><strong>95% CI (profile likelihood)</strong></td>
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<td>Y values</td>
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Relates to main Figures 5.9 and 5.12.
Appendix E

Publications arising from this Thesis
Structural basis of autoregulatory scaffolding by apoptosis signal-regulating kinase 1

Johannes F. Weijman, Abhishek Kumar, Sam A. Jamieson, Chontelle M. King, Tom T. Caradoc-Davies, Elizabeth C. Ledgerwood, James M. Murphy, and Peter D. Mace

Biochemistry Department, School of Biomedical Sciences, University of Otago, Dunedin 9054, New Zealand; Australian Synchrotron, Clayton, VIC 3168, Australia; The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC 3052, Australia; and Department of Medical Biology, University of Melbourne, Parkville, VIC 3052, Australia

Edited by Melanie H. Cobb, University of Texas Southwestern Medical Center, Dallas, TX, and approved February 1, 2017 (received for review December 19, 2016)

Apoptosis signal-regulating kinases (ASK1–3) are apical kinases of the p38 and JNK MAP kinase pathways. They are activated by diverse stress stimuli, including reactive oxygen species, cytokines, and osmotic stress; however, a molecular understanding of how ASK proteins are controlled remains obscure. Here, we report a biochemical analysis of the ASK1 kinase domain in conjunction with its N-terminal thioredoxin-binding domain, along with a central regulatory region that links the two. We show that in solution the central regulatory region mediates a compact arrangement of the kinase and thioredoxin-binding domains and the central regulatory region actively primes MKK6, a key ASK1 substrate, for phosphorylation. The crystal structure of the central regulatory region reveals an unusually compact tetrameric repeat (TPR) region capped by a cryptic pleckstrin homology domain. Biochemical assays show that both a conserved surface on the pleckstrin homology domain and an intact TPR region are required for ASK1 activity. We propose a model in which the central regulatory region promotes ASK1 activity via its pleckstrin homology domain but also facilitates ASK1 autoinhibition by bringing the thioredoxin-binding and kinase domains into close proximity. Such an architecture provides a mechanism for control of ASK-type kinases by diverse activators and inhibitors and demonstrates an unexpected level of autoregulatory scaffolding in mammalian stress-activated MAP kinase signaling.

Significance

Phosphorylation catalyzed by protein kinases governs many aspects of cellular behavior. Apoptosis signal-regulating kinases (ASK1–3) trigger responses to stress, but the structural basis of their regulation remains unclear. Here, we show that a domain directly adjacent to the ASK1 kinase domain promotes activity of ASK1 on a key substrate and also orient an additional ASK1 domain nearby to suppress kinase activity. The structure of this regulatory domain appears to be shared by all ASK kinases and provides a versatile mechanism to control ASK activity in response to various stress stimuli.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition. The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 5ULM).

1J.F.W. and A.K. contributed equally to this work.

2To whom correspondence should be addressed. Email: peter.mace@otago.ac.nz.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1620813114/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1620813114
to negatively regulate activity. Under conditions of redox stress, thioredoxin dissociates with and TRAF proteins associate with the thioredoxin-binding and kinase domains of ASK1. This so-called "domain of unknown function" (PFAM domain DUF4071) corresponds to the region proposed to associate with TRAF proteins during ASK1 activation. The crystal structure reveals a surprisingly compact fold, with core features that are highly conserved in all human ASK proteins and in ASK orthologs throughout metazoa. Our biochemical and biophysical analyses reveal conserved residues that regulate ASK1 activity in both positive and negative manners, and show that the compact fold of the central regulatory region is crucial for bringing the thioredoxin-binding and kinase domains into close proximity and priming MAP2K substrates for phosphorylation.

Relative little is known about the structural basis of ASK1 regulation, and thus previous studies necessarily have relied on prediction and deletion-based analysis. Although such approaches have identified various important regions for regulation of ASK proteins, the lack of atomic resolution data still confounds our understanding of how ASK proteins respond to diverse stimuli. For instance, very little is known about how thioredoxin or TRAF proteins actually might influence the recruitment of substrates to the ASK signalosome, or control the kinase activity of ASK1 on its substrate MAP2Ks. Here we present the first structure of a regulatory domain of ASK1, that of the central region that links the thioredoxin-binding and kinase domains of ASK1. This so-called "domain of unknown function" (PFAM domain DUF4071) corresponds to the region proposed to associate with TRAF proteins during ASK1 activation. The crystal structure reveals a surprisingly compact fold, with core features that are highly conserved in all human ASK proteins and in ASK orthologs throughout metazoa. Our biochemical and biophysical analyses reveal conserved residues that regulate ASK1 activity in both positive and negative manners, and show that the compact fold of the central regulatory region is crucial for bringing the thioredoxin-binding and kinase domains into close proximity and priming MAP2K substrates for phosphorylation.
phosphorylation. This model provides a structural template on which to interpret various proposed mechanisms of ASK kinase regulation by different binding partners.

Results

The ASK1 Central Regulatory Region Promotes MKK6 Phosphorylation. The N-terminal region of ASK1 has been proposed to interact with various partners to regulate ASK kinase activity (Fig. 1A). To develop a quantitative system to analyze ASK1 activity, we used in vitro kinase assays and Phos-tag SDS/PAGE to compare the phosphorylation of kinase dead MKK6 by either the isolated ASK1 kinase domain (Fig. 2A) or the isolated ASK1 kinase domain and ASK1(88–941) (Fig. 2B). This observation supports previous reports that the N-terminal region of ASK1 suppresses kinase activity (19), and shows that a reconstituted in vitro system is a useful tool for detailed analysis of ASK1 regulation.

To gain greater insight into the role of the N-terminal portions of ASK1, we designed further constructs based on secondary structure prediction. These constructs encompassed residues 88–206 (the thioredoxin-binding domain) and residues 269–658 (the central regulatory region) (Fig. 1A). Following expression in Escherichia coli and purification to homogeneity (Fig. S1A), we tested how these additional domains affected ASK1 kinase activity in vitro. In these experiments, MKK6 phosphorylation was monitored using Western blot analysis, because phospho-MKK6 comigrated with the central regulatory region on Phos-tag SDS/PAGE. Surprisingly, activity of the wild-type (WT) ASK1 kinase domain was greatly enhanced by the addition of 30 μM ASK1(269–658) (Fig. 1C and D). We did not observe any equivalent enhancement or inhibition of MKK6 phosphorylation by the thioredoxin-binding domain of ASK1 when present in similar concentrations (Fig. 1E).

Although these results are consistent with published experiments showing that ASK1 lacking the thioredoxin-binding domain is more active than full-length ASK1 in cells (19), it has not previously been suggested that the central regulatory region is capable of stimulating ASK1 kinase activity in vitro. In our simplified assay system, there are two potential mechanisms by which the central regulatory region could enhance MKK6 phosphorylation: allosterically activating the ASK1 kinase domain or priming the MKK6 substrate for phosphorylation. At concentrations of the central regulatory region up to 100-fold greater than ASK1 kinase (0.01 μM) but well below substrate MKK6 levels (3 μM), there was no significant rate enhancement (Fig. S2). Such a dose response where excess levels of the ASK1 central regulatory region relative to the substrate, rather than active kinase, are required is most consistent with the idea that the central regulatory region acts by binding to the substrate, MKK6. Attempts to investigate activity of the central regulatory region and kinase domain in one polypeptide, without the thioredoxin-binding domain, were hampered by the fact that a construct comprising residues 269–941 was completely insoluble when expressed in E. coli.

The following experiments demonstrate two interesting concepts. First, a region outside of the ASK1 kinase domain can associate with downstream kinase kinases, thereby acting as a scaffold for substrate recruitment. Second, because rate enhancement occurs when the kinase and central regulatory region are on separate polypeptides, the central regulatory region actively promotes a state of MKK6 that is primed for phosphorylation. In offering an explanation of how this might occur, we surveyed the available structures of MKK6. The three available crystal structures exhibit three different conformations of the activation loop, and movement of up to 18 Å in the N-terminal end of the α-helix, indicating that it is relatively flexible (Fig. S1B). Two of these structures contain phosphomimetic mutations (PDB ID codes 3FME and 3ENM), but the structure of unphosphorylated MKK6 (PDB ID code 3VN9) shows that the phosphorylation target residues in the activation loop (Ser207/Thr211) are buried, and that the activation loop is stabilized by the α-helix. Thus, we propose that by manuevering the α-helix, it is plausible that ASK1(269–658) could manipulate access to the phosphorylation target residues and thereby “prime” MKK6 for phosphorylation (Fig. 1F). This concept provides another layer of complexity to the thoroughly investigated biology of MKK6 dual phosphorylation by ASK1 (35, 36), and raises the question of how the central regulatory region plays such an active scaffolding role.

Structure of the Central Regulatory Region of ASK1. To gain insight into how the central regulatory region might prime MKK6 for phosphorylation, we expressed and purified human ASK1 residues 269–658 from E. coli, crystallized it, and solved its crystal structure to a resolution of 2.1 Å (Fig. 2A and Table 1). The final structure contains two molecules in the asymmetric unit, which are essentially identical and share an rmsd of 0.04 Å. Given the various reports of multimerization in ASK1 regulation, we also tested whether any of the crystal contacts that we observed could play
a role in the formation of ASK(269–658) dimers. Neither size exclusion chromatography (SEC) coupled to both SEC-MALLS nor small-angle X-ray scattering (SEC-SAXS) indicated a tendency of ASK1(269–658) to progressively fold back on itself and form a compact arrangement. Crucially, the crystallized ASK1 structure contains the predicted NCC region (residues 297–324) (19). The structure shows that residues 297–324 reside stably within TPRs 1 and 2, forming numerous interactions with surrounding TPR helices. Thus, although it is clearly important for ASK1 structure, the NCC is more accurately described as an integral part of the TPR domain and seems unlikely to directly mediate conventional coiled-coil type oligomerization.

The pleckstrin homology domain of ASK1 adopts the typical form of two antiparallel α-helical pleckstrin fold, but lacks the tropomyosin fold found within the terminal helix of most conventional pleckstrin homology domains (38). It is not uncommon for widely disparate sequences to produce the pleckstrin homology fold, and in ASK1 the lack of a locking tropomyosin residue (which is Phe646 in ASK1) may explain why it had not been recognized previously. The interface between the base of the pleckstrin homology domain and the TPR region is highly hydrophobic and forms an extensive network of interactions with helices 12 and 14 from TPRs 6 and 7 (Fig. S3). The complementarity of these interactions, in conjunction with scattering data described above, suggests that the intimate association between the pleckstrin and pleckstrin homology domain of ASK1 is the stable form of ASK1 in solution. In line with this idea, attempts to express the isolated ASK1 pleckstrin homology domain in the absence of the TPR region yielded completely insoluble protein, in contrast to the solubility and stability of ASK1(269–658).

**Table 1. Crystallographic data**

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and analyzed their ability to phosphorylate MKK6 using Phos-tag kinase assays. We hypothesized that if the conserved surface is important for autoinhibition (as observed in Fig. 1F), then the activity of mutants should be increased, whereas if the surface is important for priming MKK6 (Fig. 1C), then the Phe623Glu and Asp632Arg mutants should show decreased activity. Both mutants had markedly lower initial rates of MKK6 phosphorylation (Fig. 3C and D), clearly supporting the hypothesis that the conserved pleckstrin homology surface plays an important positive role in facilitating MAP2K phosphorylation by ASK1. The position of the ASK1 pleckstrin homology domain directly adjacent to the kinase domain makes it an ideal location for transient docking of MAP2Ks for both localization and priming of the activation loop for phosphorylation.

Having established that the conserved pleckstrin homology surface is important for ASK1 activity on downstream substrates, we next sought to understand how the remainder of the conserved closed TPR facilitates signal control and reduced activity of ASK1(88–941) relative to the isolated kinase domain.

**Closed TPR Interactions Facilitate ASK Kinase Regulation.** In the compact arrangement of the central regulatory region, its N and C termini are separated by only ∼50 Å (Fig. 2A). Based on previous reports showing that deleting the thioredoxin-binding domain leads to more active full-length ASK1, we hypothesized that a major role of the central regulatory region is to bring the thioredoxin-binding domain into close proximity to the kinase domain to inhibit its activity. Other possible effects of such an interaction may be to protect the β5–β7 pleckstrin homology surface and impede MAP2K recruitment and priming (Fig. 1). The most long-range of these contacts involve cation stacking between Trp509 from helix 13 and Arg322 in TPR 2 (Fig. 4A). Trp509 is also one of the most conserved residues across the sequence logo of diverse occurrences of DUF4071 in PFAM. To investigate the importance of the closed TPR interactions, we designed two mutants, one substituting Trp509 with glutamate and the other replacing Arg395 with glutamate. These mutants serve two different purposes. Trp509Glu could reasonably be expected to form a salt bridge with Arg322 and maintain the overall TPR architecture, but to disrupt ASK1 function if the closed TPR interaction is mobile and Trp509 takes on a different conformation in an active signaling form. Arg395Glu was designed as a disruptive mutant to destabilize the compact closed TPR structure. Analyzing both the time course and initial rates of MKK6 phosphorylation by these ASK1 variants showed that disruption of the closed TPR by Arg395Glu markedly reduced ASK1 activity, whereas Trp509Glu was indistinguishable from WT protein (Fig. 4B and C). Based on these findings, we conclude that the closed TPR must remain intact to allow the full activity of ASK1(88–941).

Trp476 is another notable residue conserved within the ASK1 central domain. Trp476 is one of three invariant residues over the consensus definition of DUF4071, along with Trp509, and the pleckstrin homology domain. In contrast to the latter two residues, which play clear roles in stabilizing the structure, Trp476 is unusually surface-exposed. It is located at the N-terminal end of TPR helix 11 (residues 475–488) and faces toward the center of the closed TPR domain. To ascertain a function of Trp476, we mutated the residue to serine in the context of ASK1(88–941). Surprisingly, ASK1 W476S phosphorylated MKK6 more effectively than WT protein (Fig. 4A). This finding is consistent with Trp476 playing a role in stabilizing autoinhibitory interactions that suppress the activity of the kinase domain.

Interacting closed TPR residues (displayed in Fig. 4A) are remarkably well conserved among ASK-type kinases (Fig. S4). Namely, kinases including human ASK1, ASK2, and ASK3; Drosophila ASK1 and NSY1; and the C. elegans ASK homolog all maintain residues that mediate long-range TPR interactions.
suggesting that this compact fold and function are highly conserved. Overall, these results, along with previous studies showing that deletion of residues 297–324 (the NCC) disrupts ASK1 regulation, show that the integrity of the TPR region is important for both function and regulation of ASK1 signaling. Whereas conformational changes cannot be discounted, it appears that a major role of the central closed TPR of ASK1 is to bring the kinase domain into relative proximity of the N-terminal (thioredoxin-binding) regulatory domain to mediate the regulation of kinase activity.

Architecture of the ASK Autoregulatory Scaffold in Solution. To investigate how the architecture of the thioredoxin-binding and central regions of ASK1 may facilitate signal regulation, we turned to SAXS analysis, first analyzing the ASK1 N-terminal regulatory region ASK1(88–658) alone (Table 2). Guinier analysis showed that the sample was monodispersed, and under the reducing SEC-SAXS conditions when optimal data were collected, we found no evidence of the dimerization previously shown to occur through the NCC region of ASK1. Because the structure of the N-terminal thioredoxin-binding domain has not been solved, we used the Robetta server to generate a homology model of ASK1(89–266), which predicted a globular fold based around an α-β sandwich (Fig. S5)(42).

Because our earlier SAXS analysis of the central regulatory region alone showed a stable fold and limited flexibility, when analyzing scattering data, we treated residues 89–266 and 272–654 as two separate rigid bodies. Using BUNCH (43), we generated a model for ASK1(88–941) (1 μM) WT or indicated pleckstrin homology domain mutants. Quantitation of independent triplicate experiments is shown alongside as mean values, with error bars representing SEM. (D) Rates of MKK6 phosphorylation calculated over the first 5 min from C. Error bars represent the SD of the linear rate fit.

Fig. 3. The ASK1 pleckstrin homology domain mediates protein-protein interactions and activity. (A, Left) Superposition of the ASK1 pleckstrin homology domain (purple) with the canonical pleckstrin homology domain of phospholipase C (PLC, in yellow; PDB ID code 1MAI) (77). (A, Right) Three electrostatic surface representations calculated in APBS (78) for the pleckstrin homology domains of PLC bound to Ins(1,4,5)P3, ASK1, and a model of the same region of ASK2 generated using MODELER. (Conservation between ASK1 and ASK2 can be viewed in the alignment in Fig. S4) (B) Surface representation of ASK1(1269–658), color-coded according to the degree of conservation in the alignment in Fig. S4. The least conserved residues are in cyan; the most conserved, in maroon. Areas of high conservation are also indicated with circles. (Inset) Close-up view of the conserved pleckstrin homology surface. (C) Phos-tag SDS-PAGE monitoring MKK6 (3 μM) phosphorylation by ASK1(88–941) (1 μM) WT or indicated pleckstrin homology domain mutants. Quantitation of independent triplicate experiments is shown alongside as mean values, with error bars representing SEM. (D) Rates of MKK6 phosphorylation calculated over the first 5 min from C. Error bars represent the SD of the linear rate fit.

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and kinase domains (Fig. 5D). We also collected scattering data from the mutant ASK1(88–941) W476S construct, which displayed elevated activity (Table 2 and Fig. 6 C–E). Apart from a small reorientation of the kinase domain, the W476S model did not differ markedly from WT protein in its overall arrangement. Attempts to collect data from inhibitory mutants within the pleckstrin homology domain were hampered by protein instability at high concentrations.

The main conclusion that we draw from these solution studies is that ASK1(88–941) likely exists in dynamic continuum between an active open form and a closed conformation in which the thioredoxin-binding domain is in close proximity to the ASK kinase domain. Subtle changes (such as the W476S mutation) can alter the structural ensemble present in solution, but we are reticent to propose specific interdomain contacts, given that our modeling relies on a de novo model of the thioredoxin-binding domain. In the proposed model, the thioredoxin-binding domain is ideally placed to inhibit activity of the ASK kinase domain and impede access to the MKK6 activating surface of the ASK1 pleckstrin homology domain—in effect acting as a stimulus-responsive toggle to control ASK1 kinase activity and substrate recruitment and priming.

Discussion
Based on the results of our biochemical, biophysical, and structural experiments, we are now able to put forward a model to interpret the regulation of ASK proteins (Fig. 6). The central regulatory region spans ~400 residues between the thioredoxin-binding domain and the kinase domain. More importantly, it provides a platform for the recruitment and priming of MAP2K substrates, as well as a link that brings the N-terminal thioredoxin-binding domain and C-terminal kinase domains of ASK1 into proximity for autoinhibition. Although our experiments have focused on ASK1, functional residues also are highly conserved in ASK2 and ASK3, and from mammals to nematodes, and so this architecture is likely to be functionally conserved throughout ASK-type kinases.

The presence of adjacent pleckstrin homology and kinase domains is reminiscent of the domain architecture of AKT proteins. This similarity is only superficial, however, given that AKT pleckstrin homology domains are bona fide binders of phosphoinositides. Structures of near full-length AKT1 have revealed that
its pleckstrin homology domain and kinase antagonize each other in a reciprocal manner—the pleckstrin homology domain forces the kinase into an inactive confirmation, and the kinase domain blocks the phospholipid binding site of the pleckstrin homology domain (45, 46). Instead, it appears that the pleckstrin homology domain plays a positive role in ASK1 activity, closer to that of the pleckstrin homology domain within the yeast MAP kinase scaffold Ste5 (40). Ste5 contains a predicted pleckstrin homology domain that has been shown to bind the MAP3K Ste11 and promote activation of the mating pathway (39). In contrast to Ste5, ASK proteins already contain a MAP3K domain, and use their pleckstrin homology domain as a recruitment site for their primary substrate, MAP2Ks, thereby forming their own scaffold.

Whereas some scaffold proteins act passively by colocalizing participating active signaling proteins, other scaffolds play more active roles by activating or deactivating participating proteins to promote signal fidelity (47). For instance, in mammals, KSR1/2 act as scaffolds in the RAF-MEK-ERK MAPK pathway and promote signaling by forming RAF-KSR pseudokinase heterodimers that activate RAF kinase activity (48, 49). Directly relevant to our MKK6-ASK1 data, the yeast Ste5 scaffold contains a von Willebrand type A (VWA) domain that primes the Fus3 MAPK for phosphorylation by the MAP2K Ste7 (50). We propose that the ASK pleckstrin homology domain plays a role analogous to that of the VWA domain of Ste5, promoting a conformation of MKK6 that is primed for phosphorylation. Beyond the aforementioned Ste5 from yeast, there have been few examples of substrate priming of MAP2Ks described in metazoan MAPK pathways. The diversity of the activation loop and αC helix conformations observed for various MAP2K proteins suggests that they may be particularly sensitive to such regulation (33, 34, 51–53).

Previous work has shown the isolated ASK1 kinase domain is intrinsically active (35, 44). Cell-based studies also have shown that ASK1 lacking the N-terminal thioredoxin-binding domain is more active than full-length protein (19), suggesting that it plays an important role in suppressing ASK1 activity. Our experiments, which used recombinant proteins in the absence of other possible interactors, show the seemingly contradictory results that ASK1(88–941) is autoinhibited relative to the kinase domain alone, but that the central regulatory region promotes MKK6 phosphorylation in trans. Our point mutants surrounding the closed TPR also show an interesting dichotomy; disruption at the core of the closed TPR (R395) abrogates ASK1 activity, whereas mutation of the surface-exposed W476, which presumably retains the overall structure of the activation loop priming, and phosphorylation. Beyond the aforementioned Ste5 from yeast, there have been few examples of substrate priming of MAP2Ks described in metazoan MAPK pathways. The diversity of the activation loop and αC helix conformations observed for various MAP2K proteins suggests that they may be particularly sensitive to such regulation (33, 34, 51–53).

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The ASK1 kinase domain has been shown to form a relatively tight dimer (with a dissociation constant of ~0.2 μM) (44), and it is possible that formation of a kinase domain dimer could represent the activated state of ASK proteins. ASK1(88–941) was monomeric in solution in our SAXS experiments, in contrast with previous reports regarding the isolated kinase domain (28, 44), which may provide some insight into a potential activation mechanism. Our model do not preclude ASK kinase dimer formation, but the scattering data do suggest that it occurs less readily with longer proteins than the kinase domain. However, the presence of the C-terminal region of ASK1, which likely predisposes the protein to form oligomers, could enable kinase dimerization to predominate in the context of the full-length protein. In addition, 14-3-3 proteins bind adjacent to the kinase domain and can themselves form dimers (28). Active kinase dimers also would be analogous to RAF MAP3Ks, which have been the topic of intense study in the ERK pathways (54–57). Our functional experiments are consistent with ASK1 regulation in either a monomer form or a dimer form. In this regard, one possibility is that MKK6 primed by one pleckstrin homology domain could be phosphorylated by ASK1 kinase across the kinase dimer interface. There is a wealth of data suggesting that ASK1 functions as part of an oligomeric multi-protein complex, and our observation of autoregulatory scaffolding could be amplified or regulated in the presence of ASK1–3 oligomers. No doubt many intriguing questions remain to be addressed by future biochemical and structural studies investigating how ASK1–3 oligomerization affects kinase regulation.

The precise molecular basis for manipulation of the autoregulatory scaffold from ASK-type proteins by various partners is a clear avenue for future study. For instance, thioredoxin forms both covalent and noncovalent complexes with the thioredoxin-binding domain (6, 30), either of which may be capable of interfering with the regulation of kinase activity in the context of the higher-order assembly known as the ASK signalsome. Cysteine residues that are essential for activation of ASK1 by reactive oxygen species are located very close to the linker between the central and thioredoxin-binding domains of ASK1 (31, 58). It is easy to envisage that disulfide bond formation by these cysteines could restrict ASK1 dynamics in a conformation that favors MAP2K recruitment and activity. Furthermore, the central regulatory region is also essential for binding of TRAFs to ASK proteins (7, 10, 59), which could disrupt the autoregulatory arrangement between the ASK1 kinase and thioredoxin-binding domains.

In conclusion, the model provided here for autoregulatory scaffolding by ASK1 N-terminal regulatory domains is an enticing framework on which to interpret the various reported stimuli that control ASK1 activity. In addition, the role of substrate kinase priming in MAP kinase signaling has been underappreciated, and it will be intriguing to observe the prevalence of substrate priming for ASK-type kinases on different substrates, for other MAP3K-MAP2K phosphorylation events, or for MAP2K-MAP3K activity. Further insight into each of these phenomena will allow a greater understanding of how ASK-type proteins become dysregulated in disease, as well as the fundamental regulation of kinase signaling networks.

Materials and Methods

Protein Expression and Purification. For biochemical studies and native crystallization, all proteins were expressed in E. coli BL21(DE3). Fragments of the gene encoding ASK1 were amplified from the MegaMan Human Transcriptome Library (Agilent) and cloned into modified pET-LIC vectors (a kind gift from the Netherlands Cancer Institute Protein Facility, with funding from Grant 175.010.2007.001). ASK1(269–658) and ASK1(388–658) were expressed
incorporating an N-terminal 6xHis tag and 3C protease cleavage site. ASK1(88–941) was cloned with the same N-terminal 6xHis tag and 3C protease cleavage site, but also with an additional Strept tag at its C terminus, and coexpressed with human thioredoxin-1 and lambda protein phosphatase. All mutants were generated using the QuickChange Mutagenesis Kit (Agilent).

ASK1(269-658) and ASK1(88-658) were initially purified by Ni²⁺ affinity chromatography and then purified to homogeneity after cleavage with 3C protease using anion-exchange chromatography (Resource Q) and size exclusion chromatography (Superdex 200 Increase 10/300). Purification proceeded as for native proteins.

SEC-MALLS. SEC-MALLS was conducted using a Wyatt Dawn B+ detector (Wyatt Technology) coupled to a Superdex 200 10/300 column (GE Healthcare) and a refractive index detector. Samples were run in 10 mM Hepes (pH 7.6), 500 mM NaCl, 5% (v/v) glycerol, and 0.2 mM TCEP and loaded at 2.2 mg/mL. All data were analyzed using ASTRA V software.

Purification proceeded as for native proteins. ASK1(88–941) was purified by Ni²⁺ affinity chromatography and cleaved overnight using 3C protease while dialyzing against a buffer consisting of 50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, and 2 mM DTT. Dialyzed protein was then subsequently dialyzed against 50 mM Tris pH 8.0, 150 mM NaCl. Samples for enzymatic assays were used directly, for SAXS analysis, eluted protein was further purified using size exclusion chromatography (Superdex 200 Increase 10/300).

Kinase dead MMK6 (MKK6 K82A) was purified as an N-terminal 6xHis tag and 3C protease cleavage and further purified by size-exclusion chromatography (Superdex 200 Increase 10/300).

Crystalization and Structure Solution. ASK1(269-658) was initially crystallized in 0.2 M sodium fluoride and 20% (v/v) PEG 3350. Poor initial diffraction was improved through seeding and additive screening, with final data collected from crystals grown in 0.1 M sodium fluoride and 10% (v/v) PEG 3350 and frozen with the addition of 20% (v/v) glycerol. The crystal was solved by single-wavelength anomalous dispersion, using a 2.9-Å peak dataset created by merging data from two separate selenomethionine-labeled ASK1 crystals collected at 0.9793 Å. Thirteen of 14 possible selenium sites from two ASK1 monomers in the asymmetric unit were located using Phenix.Analysis, eluted protein was further purified using size exclusion chromatography (Superdex 200 Increase 10/300).

Cryoprotection. Crystals were flash-frozen for storage in 10 mM Hepes pH 7.6, and 2 mM DTT. Selenomethionine-labeled ASK1(269-658) was passivated by incubating with 2.5 M ethanedithiol (EDT) overnight using medium (Molecular Dimensions) according to the manufacturer’s instructions.

The final model has excellent geometry and, aside from several disordered loops connecting TPR helices, is clearly defined in both molecules of the asymmetric unit. Structural figures were generated using UCSF Chimera (66).

SAXS. SAXS data collection was performed at the Australian Synchrotron SAXS/WAXS beamline using an inline gel filtration chromatography setup (67), essentially as described previously (68–71). Summary statistics for data collection and analysis are reported in Table 2. Here 50 μL of purified recombinant ASK1(269-658) at 7.7 mg/mL, ASK1(88-658) at 9.7 mg/mL, or ASK1(88-941) at 9.3 mg/mL (WT) or 15.5 mg/mL (M5) was injected onto an inline Superdex 200 15/10 column (GE Healthcare) and eluted at a flow rate of 0.2 mL/min via a 150 mM NaCl, 10 mM Hepes (pH 7.5), 5% (v/v) glycerol, and 0.2 mM TCEP at 12 °C. Coflow SAXS was used to minimize sample dilution and maximize signal to noise (72).

Scattering data were collected in 2-° exposures over the course of the elution and 20 intensity plots with consistent scatter intensities from the peak of the sizing chromatography run were radially averaged, normalized to sample transmission, and background subtraction performed using Scatterbrain software (Stephen Mudie, Australian Synchrotron). Background scatter was assessed by averaging scattering profiles from earlier in the size exclusion chromatography run, before protein elution. Guinier analysis of each scatter pattern across the entire elution peak showed consistent radius of gyration (Rg) values, and superimposable averages were generated. Four profiles for ASK1(269-658), eight profiles for ASK1(88-658), 21 profiles for ASK1(88-941), and four profiles for ASK1(88–941) W476E were averaged and background-subtracted using Scatterbrain to generate the averaged scatter patterns presented in the manuscript. Guinier data analyses were performed using PRIMUS (73). Indirect Fourier transform with GNOM (74) was used to obtain the distance distribution function, P(R), and the maximum dimension, Dmax, of the scattering particle. CRYSON (75) was used to calculate theoretical scattering curves from crystal structure atomic coordinates and compare them with experimental scattering curves.

Kinase Assays. Each kinase assay was carried out at room temperature with final concentrations of 25 mM Hepes pH 7.6, 20 mM MgCl₂, 2 mM DTT, 100 mM NaCl, and 3 μM kinase dead MKK6, along with 0.01–1 μM kinase and 0.01–30 μM ASK1 regulatory domains and 50 μM ATP. Assays were set up as master mixes containing all components except kinase and ATP, to ensure equal substrate addition to all reactions. For kinase assays, including separate ASK1 regulatory domains, ASK1’ kinases was added to the master mix. The master mix was divided into two, one for an empty reaction, and the other for the addition of the kinase. Samples were mixed and incubated at room temperature for 30 min. Kinase assays were stopped by the addition of ATP. At each time point, an aliquot from each tube was removed in parallel, and the reaction was terminated by immediate mixing into 4× Laemmli sample buffer [240 mM Tris pH 6.8, 32% (v/v) glycerol, 8% (v/v) SDS, and 0.02% (v/v) bromophenol blue]. Samples were briefly spun down and stored at −80 °C for further analysis.

For analysis by Phos-tag gels, 15-μL thick Phos-tag analysis gels were hand- poured to contain final concentration of 20 μM Phos-tag, 100 μM MnCl₂, and 10% (v/v) polyacrylamide. Gels were run as conventional SDS-PAGE gels. Total protein was visualized with Coomassie brilliant blue and imaged with an Odyssey Fc imaging system (LI-COR) in the 700 channel. Quantitation was performed by measuring the intensity of both phosphor- ylated and unphosphorylated MKK6 bands. The intensity of phosphorylated bands was expressed as a fraction of total intensity, and corrected to an absolute concentration by multiplying the fraction of phosphorylated spe- cies by the 3 μM total concentration of MKK6 in all assays. Using mass spectrometry, we confirmed that phosphorylation of MKK6 followed the precisely ordered phosphorylation events established by Humphreys et al. (35), with the more rapidly appearing band on Phos-tag SDS-PAGE corre- sponding to Thr211 of MKK6 (Figs. 3 and 4).

For analysis by Western blot, samples were run in a conventional manner and transferred by a semidy method to 0.45 μM nitrocellulose (GE Healthcare). Total protein transferred to membrane was visualized by staining membrane in 0.5% (v/v) Ponceau S solution for 5 min at room temperature. Excess Ponceau S was removed by rinsing in distilled water. Ponceau S-stained blots were imaged using the Odyssey Fc imaging system in the 800 channel. After imaging, blots were rinsed further before blocking in 5% (v/v) BSA in Tris-buffered saline (TBS) for 1 h at room temperature. Blots were then incubated with rabbit polyclonal [p-MKK3/6 (Ser187), sc-7994-R; Santa Cruz Biotechnology] and rabbit [p-MKK6 (Thr211), sc-834(22); Santa Cruz Biotechnology] antibodies, diluted in 5% (v/v) milk, and incubated for 1 h. Membranes were then washed three times for 5 min each in TBST before incubation with secondary antibody (goat anti-rabbit IRdye 680LT; LI-COR) diluted 1:2,500 in TBSB with 1% (v/v) BSA and allowed to bind overnight at 4 °C. Blots were then washed three times for 5 min each in TBS before incubation with secondary antibody (goat anti-rabbit IRdye 680LT; LI-COR) diluted 1:2,000 in TBSB with 1% (v/v) BSA. The secondary antibody was allowed to bind for 1 h at room temperature before being washed another three times in TBSB. Blots were developed in the Odyssey Fc imaging system. Quantification of blots and Phos-tag gels was performed using ImageStudio (LI-COR).

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Fig. S1. (A) Example purified sample proteins from each of the constructs used in this work. (B) Illustration of MKK6 conformational flexibility. (Left) An overlay of the three crystal structures of MKK6 currently available in the protein databank. All proteins are colored gray, with respective αC helices in yellow (2VN9), pink (3ENM), and green (3FME). The distance between the N terminus of the αC helix in 3ENM and 3FME is indicated. An illustrative model was created using Modeller to model in the disordered activation loop of structure 3FME. (Right) A comparison of 3FME with the Modeller output, with the activation loop indicated. The Modeller model is shown in Fig. 1F. (C) Purified point mutants of ASK1(88–941) used for the assays shown in Figs. 3C and 4B. A schematic summarizing the constructs is shown above, indicating that the construct containing only the central regulatory region and kinase was insoluble and so could not be tested in activity assays. (D) Preparative size-exclusion chromatography of ASK1(88–941) with molecular weight standards indicated. The majority of ASK1 protein elutes later than the 160-kDa marker, consistent with it behaving as a (~100-kDa) monomeric species. Peak fractions elute at a concentration of ~40 μM based on A280.
**Fig. S2.** Relates to Fig. 1C. MKK6 phosphorylation by 0.01 μM ASK1 kinase domain with concentrations of ASK1(269–658) substoichiometric to the substrate (MKK6), but up to 100-fold excess to the ASK1 kinase domain. MKK6 is held constant (3 μM), and phosphorylation is monitored by Western blot analysis. Quantitation of independent triplicate experiments is shown. Each band was normalized to the band intensity of the kinase alone at the 10-min time point for that experiment. Mean values are plotted, with error bars representing the SEM.

**Fig. S3.** Detailed view of the interface between TPR repeats 6 and 7 and the pleckstrin homology domain. Hydrophobic residues at the core of the interface are indicated.
Fig. S4. Sequence alignment of ASK1–3 and orthologs from various species over the central regulatory domain, with the secondary structure indicated. Point mutants generated in this work are indicated above with a blue circle. The figure was generated using ALINE (79).

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Fig. S5. (A) BUNCH model of ASK1(88–941) with three closely related Robetta models of the thioredoxin-binding domain overlaid. (B) As for A, but with all five models output by Robetta shown. The two additional models in B have a similar beta sheet core but differ in their decorating helices.
Fig. S6. (A and B) Interatomic distance distributions of ASK1(88–658) (A) and ASK1(88–941) (B). (C) Overlay of experimental scattering data (black circles) and scattering profile calculated using BUNCH for the model of ASK1(88–941) W476S. A Guinier plot for the dataset is shown below, indicating that aggregates do not measurably contribute to the scattering profile. (D) Interatomic distance distributions of ASK1(88–941) W476S. (E) Surface representation of the BUNCH model of ASK1(88–941) W476S, with the thioredoxin-binding domain in gray, the TPR region in yellow, and the pleckstrin homology domain in purple. Residues that affect activity when mutated are indicated in red (F623 and D632) and blue (W476). Agreement between the experimental data and the calculated scatter pattern is indicated by $\chi = 0.55$. Weijman et al. www.pnas.org/cgi/content/short/1620813114 5 of 5
The role of peroxiredoxin 1 in redox sensing and transducing
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Abstract
Peroxiredoxin 1 is a member of the ubiquitous peroxiredoxin family of thiol peroxidases that catalyse the reduction of peroxides. In recent years eukaryotic peroxiredoxins have emerged as a critical component of cellular redox signalling, particularly in response to alterations in production of hydrogen peroxide. Peroxiredoxins are exquisitely sensitive to oxidation by hydrogen peroxide making them key peroxide sensing enzymes within cells. Evidence gathered over the last decade suggests that in addition to sensing the redox signal, peroxiredoxins have a major role in transducing this signal to downstream signalling proteins, ultimately contributing to regulation of diverse cellular processes including proliferation, differentiation and apoptosis. In this review we present the three current models for the sensing and signal transducing roles of peroxiredoxins, with a specific focus on mammalian peroxiredoxin 1. The evidence for each mechanism is discussed and areas for future work are identified.

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Changes in the redox state of cells are intimately involved in regulating a multitude of cell processes. Key questions revolve around how changes in the redox state are sensed, and then how this information is transduced into a specific change in cell function. A critical molecule in redox signalling is the mild oxidant hydrogen peroxide (H₂O₂). H₂O₂ is a strong candidate for a second messenger in redox signalling because its production in cells can be regulated, it is relatively stable compared to many cellular oxidants, and it has a preference of oxidation of cysteine thiols in proteins [1–3]. Reversible thiol oxidation has emerged as an important and widespread mechanism of controlling protein function [4,5]. The use of thiol labelling combined with quantitative proteomics by mass spectrometry is leading the way in identifying signalling enzymes within cells. Evidence gathered over the last decade suggests that in addition to sensing the redox signal, peroxiredoxins have a major role in transducing this signal to downstream signalling proteins, ultimately contributing to regulation of diverse cellular processes including proliferation, differentiation and apoptosis. In this review we present the three current models for the sensing and signal transducing roles of peroxiredoxins, with a specific focus on mammalian peroxiredoxin 1. The evidence for each mechanism is discussed and areas for future work are identified.

Keywords: Peroxiredoxin, Redox signalling, Disulfide exchange, Hydrogen peroxide

1. Enzymology of peroxiredoxin 1
All peroxiredoxins use thiol residues to reduce peroxides. The
PeroxiRedoxin classification indEx (PREX) database categorises peroxiredoxins into six subfamilies [13,16]. In mammalian species there are six peroxiredoxins, Prdx1-4 (Ahp/Prdx1 subfamily), Prdx5 (Prdx5 subfamily) and Prdx6 (Prdx6 subfamily). Prdx1-4 utilise a typical 2-Cys mechanism, whereas Prdx5 uses an atypical 2-Cys mechanism and Prdx6 has a 1-Cys mechanism [17]. These Prdx1 family members differ in subcellular location with Prdx1 and 2 primarily cytosolic, Prdx3 mitochondrial and Prdx4 present in the endoplasmic reticulum lumen. Expression levels of each vary across different cell types [17,18].

Mammalian Prdx1 is a typical 2-Cys peroxiredoxin and is estimated to be present at 15–60 μM in the cytosol of mammalian cells [19,20]. In a mechanism common to all 2-Cys peroxiredoxins, Prdx1 functions as a head-to-tail homodimer, with each monomer containing a peroxidatic Cys (CP) and a resolving Cys (CR) (residues 52 and 173 respectively in Fig. 1A&B). The CP thiol has a low pKa and thus is deprotonated at physiological pH. The thiolate (CpS−) reacts with peroxide to form a sulfenic acid (CpSOH), which condenses with CRSH on the other monomer to form a disulfide bond (Fig. 1B). Thus each active homodimer has the capacity to form two disulfide bonds and reduce 4 molecules of peroxide. The disulfide is then reduced in a disulfide exchange reaction originally postulated to be catalysed by thioredoxins [21]. The second order rate constant for reduction of peroxide has not been reported for a mammalian Prdx1 but, based on the homology of the active site with other mammalian 2-Cys peroxiredoxins (Fig. 2), is expected to be in the range of 107-108 M−1 s−1 [22,23]. As an alternative to disulfide formation, the CpSOH can further react with peroxide to form sulfinic (CpSO2H) and sulfonic (CpSO3H) acids, commonly referred to as over- or hyper-oxidation [13,24,25]. These forms are catalytically inactive, with CpSO2H slowly reduced by sulfiredoxin in an ATP-dependent reaction [26,27] and CpSO3H regarded as irreversible.

An ongoing question in the field has been given an antioxidant role of peroxiredoxins, why should they be so susceptible to inactivation by their own substrate?

In addition to inactivation by hyperoxidation, the activity of Prdx1 is regulated by other post-translational modifications. The best understood of these is phosphorylation, which can both inhibit [28–32] and activate [33] the peroxidase activity of Prdx1. Prdx1 activity is also inhibited by S-nitrosylation [34]. Prdx1 can be glutathionylated at three of its cysteine residues: C52, C83, and C173 in vitro and in response to H2O2 treatment in cultured cells [35]. The significance of glutathionylation of Prdx1 in redox signalling is as yet unknown, but may represent a possible regulatory mechanism or an alternative recycling mechanism as proposed for Prdx2 [36].

High throughput mass spectrometry approaches as indexed by the PhosphoSitePlus resource [37] indicate modification of Prdx1 by ubiquitylation, sumoylation, acetylation and succinylation, although the physiological roles of these modifications await further investigation.

2. Tertiary and quaternary structure of peroxiredoxin 1

Structures of five mammalian Prdx1 have been reported: oxidised rat C83S Prdx1 (PDB accession code 1QQ2) [38], rat C52S Prdx1 (PDB accession code 2Z9S) [39], oxidised human C83S Prdx1 (PDB accession code 4XCS), human C71S, C83E, C173S Prdx1 in complex with suliredoxin (PDB accession code 2RII) [40] and human C52D, C71S, C83E, A86E, C173S, K185C Prdx1 in complex with sulfiredoxin, ATP and Mg2+ (PDB accession code 3HY2) [41]. In common with other members of the Ahp/Prdx1 subfamily, reduced mammalian Prdx1 exists in equilibrium between fully folded (FF) and locally unfolded (LU) states (Fig. 1A&B). The structure of the active site pocket in the FF state of the protein optimises H2O2

Fig. 1. Structural features of Prdx1. A Dimeric rat C52S Prdx1 (PDB accession code 2Z9S). The Prdx1 homodimer contains two centrally located active sites with C83 at the periphery. The C173 C83 (inset) is 13.9 Å from the catalytic mutant C52, S52 that exists in a fully folded (FF) helix. B Dimeric rat C83S Prdx1 (PDB accession code 1QQ2). The homodimer exists with active site cysteines oxidised as a disulfide bond (inset); with the C52 (C52) now occupying a locally unfolded (LU) loop at the conclusion of the catalytic cycle. C Decameric rat C52S Prdx1 (PDB accession code 2Z9S). The decamer exists in a toroidal (422) configuration, with each dimer shown as a blue and green monomer pair. D C83-C83 disulfide bond at dimer-dimer (DD) interface of decameric rat C52S Prdx1 (PDB accession code 2Z9S). Colouring as in Fig. 1C and disulfide bonding C83 sulfurs shown in yellow.
binding and oxidation of the C$_n$ thiolate to sulfinic acid. However in the FF state, C$_n$ of one monomer is ~14 Å away from C$_n$ of the second monomer (Fig. 1A), sufficiently slowing condensation of C$_n$SOH with C$_n$SH so as to make further oxidation to sulfinic acid kinetically competitive with disulfide bonding between C$_p$ and C$_R$ [13,42]. Rearrangement of the active site to the locally unfolded (LU) state repositions C$_n$ to enable disulfide bond formation with C$_p$ (Fig. 1B). The rate of transition from the FF to the LU state is thought to explain, at least in part, the varying reactivity’s of peroxiredoxins to H$_2$O$_2$. This sensor and signalling role is consistent with gene expression analysis suggesting the main role of peroxiredoxins in expression of key active site residues and H-bonds conserved (Fig. 2). An intriguing feature of peroxiredoxins is the assembly of the dimers into higher order toroidal (2$_2$$\times$5) decamers or (2$_2$$\times$6) duckedecamers. Human Prdx1 forms decamers (Fig. 1C) in vitro and in vivo with the equilibrium between decamer and dimer reported to be influenced by protein concentration, ionic strength, redox state, glutathionylation and S-nitrosylation [19,34,39,44]. The critical transition concentration for reduced human Prdx1 in vitro has been reported as 1.3 µM [45] suggesting it will exist mainly as the decamer in vivo. In general the equilibrium is pushed towards the decameric state under reduced and hyperoxidised conditions, whereas oxidation favours the dimeric form although this has not yet been formally demonstrated for a mammalian Prdx1. Prdx1 is unique among the 2-Cys peroxiredoxins in containing a third cysteine residue (C83 in hPrdx1) that is conserved in all but four vertebrate Prdx1 and sits at the dimer-dimer interface of the decamer (Fig. 1D). While disulfide bond formation between adjacent C83 residues of Prdx1 at the dimer-dimer interface was initially reported to be necessary for formation of the decamer [44], it is apparent from mutagenesis studies and other crystallographic data that this feature is not essential for decamernisation and is likely dependent on the crystallisation conditions used [39]. Instead decamer formation involves hydrophobic interactions and van der Waals contacts between adjacent dimers [39]. Overall the dimer-dimer interface is a region of lower homology (Fig. 2B) and it is not known if Prdx1 dimers from different species could form heterodecamers.

Peroxiredoxins also form higher order complexes that are proposed to lose peroxidase activity and gain chaperone activity [46], and this has been observed for Prdx1 in vitro [44]. Details such as what functional role if any oligomers play in vivo, as well as how this process influences signalling functions of peroxiredoxins remain unclear.

3. Current models of the role of Prdx1 in signal transduction

The role of Prdx1 in redox sensing and transducing is complex. Here we discuss the three current models for the role of Prdx1 as a redox sensor and transducer, giving examples that support each model. These models are not necessarily mutually exclusive, and it is highly likely that the role of Prdx1 varies with cell type, stimulus and other factors.

3.1. Prdx1 as the redox sensor and transducer: the signal peroxidase model

Peroxiredoxins have been proposed to play an active role in redox signalling whereby they sense the H$_2$O$_2$ signal and transduce this signal by catalysing disulfide formation in target proteins, sometimes referred to as a disulde relay. Early work in yeast demonstrated the functional importance of formation of an inter-molecular disulfide between the Schizosaccharomyces pombe peroxiredoxin Tpx1 and the p38/JNK homologue Sty1 [47], and between the Saccharomyces cerevisiae peroxiredoxin Gpx3 and the AP-1 homologue Yap-1 [48] in response to increasing concentrations of H$_2$O$_2$. This sensor and signalling role is consistent with gene expression analysis suggesting the main role of peroxiredoxins in yeast was not as antioxidants but as regulators of gene expression [49].

The first evidence for the ability of mammalian Prdx1 to catalyse disulfide formation in a target came from a study of the role of hPrdx1 in the regulation of apoptosis signalling kinase 1 (ASK1). ASK1 is a key redox-sensitive protein kinase that activates p38 and JNK MAP kinase pathways [50]. ASK1 is oxidised upon treatment of cells with H$_2$O$_2$, with oxidation being necessary for full activation in response to a redox stress [51,52]. Co-immunoprecipitation experiments demonstrated that Prdx1 forms a transient DTT sensitive and covalently-linked intermediate with ASK1 in cultured cells [53]. This result suggested that Prdx1 is able to catalyse oxidation of ASK1 to a disulfide bond-linked oligomer. In the absence of Prdx1,
H$_2$O$_2$-induced ASK1 oxidation and subsequent p38 phosphorylation was inhibited, demonstrating the requirement for the presence of active Prdx1 for signalling.

These observations led to the proposal of a signal peroxidase model for control of H$_2$O$_2$ signalling by peroxiredoxins in mammalian cells (Fig. 4A) [53]. In this model, peroxiredoxins “sense” the H$_2$O$_2$ by oxidation of C$_5$S$^-$ to C$_5$OH. The H$_2$O$_2$ signal is subsequently transduced to target proteins (in yellow in Fig. 4) via formation of a transient intermolecular disulphide bond between the peroxiredoxin and the target protein. Supporting data for the signal peroxidase model of Prdx1 function has come from recent finding of putative disulphide relays between Prdx2 and both signal transducer and activation of transcription 3 (STAT3) [54], and Dj-1 [55]. STAT3 undergoes oxidative regulation in response to H$_2$O$_2$ with its transcriptional activity attenuated upon oxidation [56]. STAT3 and Prdx2 were found to associate via a mixed disulphide intermediate in response to both H$_2$O$_2$ and cytokine treatment of cells, and loss of Prdx2 inhibited STAT3 oxidation. Dj-1 (also known as PARK7) is a 20 kDa, redox sensitive protein with many attributed functions including peroxidase and cytoprotective properties [57,58]. Prdx2 has been proposed to act as a sensor of H$_2$O$_2$ to facilitate the dimerization of Dj-1 whereby a disulphide linked species between Prdx2 and Dj-1 subsequently condenses to produce a Dj-1 disulphide linked homodimer [55].

Currently it is unclear whether disulphide bond formation in Prdx1 targets such as ASK1, (or indeed Prdx2 targets STAT3 and Dj-1) occurs via disulphide exchange (reaction 2 in Fig. 4A), or whether the peroxiredoxin C$_5$SOH resolves with a cysteine in the target protein rather than with C$_5$SH of the other peroxiredoxin monomer (reaction 1 in Fig. 4A). Disulphide exchange is characteristically catalysed by thiol oxidoreductases containing a conserved CXXC motif within a thioredoxin fold [59–61]. This is the mechanism by which
thioredoxins reduce cytosolic and mitochondrial peroxiredoxins, and endoplasmic reticulum (ER) oxidoreductases such as protein disulfide isomerase reduce the ER localised Prdx4 [62,63]. While it is well established that intramolecular disulfide exchange can occur in non-thiol oxidoreductases [33,64], there is little direct evidence for intermolecular disulfide exchange with a non-thiol oxidoreductase. There is however direct support in the literature for the reaction of CPSOH of a 2-Cys peroxiredoxin with a target thiol group [65]. This currently seems the most likely mechanism for Prdx1-catalysed disulfide formation in target proteins. Indeed, the structural features of 2-Cys peroxiredoxins that delay the reaction of CrSOH with CPSH may be an adaptation that enables CrSOH to condense with a thiol in a target protein to transduce the peroxide signal.

3.2. Prdx1 as the redox sensor: the protein interaction model

A second model for Prdx1 function in H$_2$O$_2$ sensing and signalling involves non-covalent interaction of Prdx1 with target proteins (Fig. 4B). Prdx1 has been reported to interact with a wide range of proteins [66], and the Biogrid interaction database [67] currently lists 130 unique Prdx1 interactors (http://thebiogrid.org/111089/table/homo-sapiens/prdx1.html, accessed 22/6/16). A difference between the binding affinities of reduced, oxidised or potentially hyperoxidised Prdx1 for the target protein would enable transduction of the redox signal to target proteins that do not contain redox active cysteine residues. To date the relationship between interaction with the target protein and the oxidation state of Prdx1 has only been considered in a few cases. The dual-specificity lipid phosphatase and tensin homologue (PTEN) is one such protein. PTEN is inactivated upon formation of an intramolecular disulfide bond following treatment with H$_2$O$_2$ [68] or growth factors such as epidermal growth factor [68,69], with reduction catalysed by Trx1 [70]. Prdx1 has been reported to interact with PTEN, although in one case this interaction was shown to protect PTEN from oxidation, with Prdx1 dissociating following...
H$_2$O$_2$ treatment [71], whereas in another case Prdx1 interacted more strongly with oxidised than reduced PTEN [72]. This disparity suggests we still have much to learn regarding the role of Prdx1 in regulation of PTEN activity. Prdx1 has been also been reported to interact with non-peroxidase proteins and regulate their activity without changing their redox state. Prdx1 interacts with mammalian Ste20-like kinase-1 (Mst-1) in an oxidation state- and oligomerization-dependent manner, stimulating Mst-1 autophosphorylation and activation by triggering dissociation of the inhibitory C-terminal domain of Mst-1 [73]. The transcription factor c-Myc has also been identified as a Prdx1 interacting protein, with Prdx1 inhibiting c-Myc mediated transcription and potentially acting as a tumour suppressor [74].

Further analysis of Prdx1 interacting partners will be an important approach for ascertaining the roles of Prdx1 as a signal transducing molecule in global cellular function. A large number of these potential interactors have only been identified in high-throughput mass spectrometric-based methods. The high cellular expression of Prdx1 makes artefactual identification of interactions a strong possibility, and thus further validation of much of this data is required. In addition many studies investigating Prdx1 interactions have used overexpression of tagged Prdx1, however as the presence of a tag alters peroxiredoxin activity [75] it is unclear if these results are relevant for endogenous Prdx1.

### 3.3. Peroxiredoxin inactivation as the redox sensor: the floodgate model

The quandary between the abundance and high reactivity of peroxiredoxins with H$_2$O$_2$ on the one hand, and the clear demonstration that regulated oxidation of non-peroxidase proteins occurs in cells on the other, was first addressed in terms of the susceptibility of eukaryotic peroxiredoxins to hyperoxidation [42]. In its original version the floodgate model proposed that at low concentrations, H$_2$O$_2$ is reduced by peroxiredoxins and is not available as a second messenger for redox signalling (Fig. 4C). As the concentration of H$_2$O$_2$ rises, for example as a result of stimulation of NADPH oxidase activity, the peroxidase function of peroxiredoxins is inactivated by hyperoxidation, thereby allowing H$_2$O$_2$ to accumulate and oxidise target proteins directly. However there is little evidence for widespread peroxiredoxin hyperoxidation in response to H$_2$O$_2$-generating stimuli and it now seems this model as originally proposed is unlikely to be a major contributor to transduction of the H$_2$O$_2$ signal. Instead, the concept of localised inactivation of peroxidase activity by other post-translational modifications is gaining increasing support (Fig. 4C). In this scenario, localised and regulated inactivation of peroxiredoxins, for example by phosphorylation [28,32], enables localised build-up of H$_2$O$_2$ which can directly oxidise a target protein in the immediate vicinity. This mechanism has been suggested to explain the role of Prdx1 in control of cell cycle progression, whereby inhibitory phosphorylation of Prdx1 at Thr90 by Cdk1 at the centrosome allows local accumulation of H$_2$O$_2$ so a centrosome-bound phosphatase can be directly oxidised and thus inhibited, preventing early degradation of mitotic activators [28]. However it still remains unclear how H$_2$O$_2$ can accumulate at the centrosome to enable oxidation of the phosphatase. Lim et al. [28] suggested that H$_2$O$_2$ is channelled to the centrosome from its site of production, but there is currently no mechanistic explanation for this. In addition, even if Prdx1 is inactive, cytosolic Prdx2, GpX enzymes and even GSH are likely to outcompete signalling proteins for reaction with H$_2$O$_2$ [11].

An alternative role of peroxiredoxin hyperoxidation in a yeast cell’s response to oxidative stress has recently proposed, whereby hyperoxidation is a means of removing peroxiredoxin as a substrate for thioredoxin, thus enabling thioredoxin to reduce other oxidised proteins [76]. It remains to be determined if this mechanism is relevant for mammalian peroxiredoxins.

### 4. Reversing the redox signal — the role of thioredoxins, thioredoxin reductase and NADP$^+$/NADPH

The activity of Prdx1 in redox signalling is controlled not only by variations in H$_2$O$_2$ levels, but also by changes in thioredoxin activity, TrxR activity and the NADP$^+$/NADPH ratio. Thioredoxins have two potential functions in Prdx1-mediated redox signalling. They may directly reduce Prdx1, or they may reduce the targets oxidised by Prdx1, thus providing a reversal of the redox signal. Completion of the Prdx1 catalytic cycle ultimately requires reducing equivalents derived from NADPH. This links Prdx1 activity to metabolic events, and implies that Prdx1 activity and thus its role in redox sensing and transducing will vary even in the absence of endogenous production of H$_2$O$_2$. Indeed the levels of oxidised mitochondrial Prdx3 have been found to increase when NADPH levels decrease in the absence of nicotinamide nucleotide transhydrogenase [77]. The circadian variation in peroxiredoxin oxidation has been linked to an altering NADP$^+$/NADPH ratio [78]. Similarly, inhibition of thioredoxin reductase causes basal oxidation of cytosolic and mitochondrial peroxiredoxins [79].

### 5. Future perspectives

In the relatively short time since their initial identification substantial progress has been made in understanding the basic enzymology of peroxiredoxins. The next step is to gain a full understanding of how this enzymology relates to the function of peroxiredoxins in vivo. Teasing out the specific functions of the different peroxiredoxins in signal transduction will be experimentally challenging as potential intermediates are formed transiently and are at low levels. Progress in this area will require development and use of methodologies that allow trapping of intermediates. In the short term it is critical to prove the formation of direct peroxiredoxin-S-S-target linkages in mammalian cells via a technique such as LC-MS/MS, and to ascertain which of the multitude of peroxiredoxin interactions are specific and modulated by the redox state of the peroxiredoxin. More information is also required addressing the role of alterations in the Trx/TrxR/NADPH system in controlling peroxiredoxin activity. The recent development of kinetic models [80–82] attempting to explain H$_2$O$_2$ signalling is a promising start, and obtaining more information on the in vivo roles of peroxiredoxin will improve these models so that they better explain redox signalling on a whole cell basis.

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.abb.2016.10.009.

### References


