Cytoskeletal and mitotic partners for synthetic lethal targeting of E-cadherin-deficient carcinoma

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Abstract

Hereditary diffuse gastric cancer (HDGC) is the familial predisposition to poorly-differentiated, diffuse gastric adenocarcinoma. It is inherited in an autosomal dominant manner and shows high penetrance for diffuse gastric cancer as well as lobular breast cancer. Due to the unreliability of endoscopic detection, the primary management strategy for HDGC is prophylactic total gastrectomy. There is an urgent need for effective drugs for use in conjunction with screening in order to mitigate reliance on surgery.

Germline inactivating mutation of the tumour suppressor gene E-cadherin (CDH1) and its functional relatives accounts for the majority of HDGC diagnoses. E-cadherin is a transmembrane protein that plays key roles in establishing and maintaining cell-cell adhesions, apicobasal polarity and mitosis. Although not amenable to conventional drug targeting, the loss of E-cadherin is predicted to create vulnerabilities that can be targeted.

Synthetic lethal interactions occur between two genes where only inactivation of both genes is lethal, while inactivation of either gene alone is tolerated. Candidate synthetic lethal targets were silenced with shRNA and drugs in an isogenic (CDH1+/− and CDH1+/+) MCF10A background. Quantitation of gene expression and analysis of cell viability following treatment was used to assess synthetic lethal effect.

In this investigation, microtubule-associated targets MAST2 and MAP1B, and mitotic regulators Aurora-A and -B kinase, were identified as vulnerabilities in an E-cadherin-deficient context. Microtubules have been the targets of many drugs since the advent of chemotherapy, and represent a central set of functions that become perturbed in E-cadherin-deficient cells. Given the role of E-cadherin, it is rational to target microtubule and mitotic processes and further investigation of agents that target these functions is warranted.
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Cytoskeletal and GPCR partners for synthetic lethal targeting of E-cadherin-deficient tumours

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Premise

- Germline mutation of E-cadherin (CDH1) predisposes individuals to hereditary diffuse gastric cancer (HDGC), a syndrome characterised by susceptibility to poorly differentiated diffuse gastric cancer (DDC) and lobular breast cancer (LBC).

Fig 1. Local HDGC family
Pedigree of an HDGC family with a genuine CDH1 mutation. Individuals carry a 70% lifetime risk of developing DGC and women an additional 40% risk of LBC.

- Loss of CDH1 expression disrupts cell-cell adhesion, cytoskeletal organization, polarity & survival signalling. These selective advantages come with new vulnerabilities that may be exploited in cancer therapy.

Fig 2. CDH1 maintains epithelial function
E-cadherin is a membrane-spanning protein with key roles in cellular signaling, cell-cell adhesion and maintenance of cytoskeletal morphology.

- The primary management strategy for HDGC is prophylactic gastrectomy due to the unreliability of endoscopic detection, and there is a need for targeted drugs to mitigate reliance on surgery and non-targeted chemotherapies.

Prevention

- Early HDGC presents as indolent, multifocal stage T1a adenocarcinoma, which argues against additional genetic defects, suggesting the early disease is attributable to CDH1 silencing alone.

- Homogenous disease will respond more uniformly to a specific drug than advanced, genetically heterogeneous disease.

Fig 3. Early HDGC pathology
Stomach map from total gastrectomy of early HDGC patient with multifocal adenocarcinoma (left); HDGC biopsy showing poorly-differentiated, diffuse phenotype in the submucosa (below).

- Chemopreventative drugs could be administered every 2-5 years to at-risk individuals, allowing the early disease focus to be eliminated before more genetic defects fuel progression.

Promise

- Two genes are “synthetic lethal” when only their simultaneous inactivation leads to death.

Fig 4. Synthetic lethality
An inactivating mutation in cancer cells but not in normal cells can create opportunities to selectively kill cancer cells.

- A synthetic lethal target will reduce cell viability only in cells carrying a specific loss of function mutation - namely in E-cadherin gene CDH1.

Process

- To interrogate any synthetic lethal effect, a genome-wide shRNA screen was performed in isogenic (CDH1+/+ and CDH1−/−) epithelial cell lines.

- MCF10A mammary epithelial cells were used due to their normal genetic background - important in an early disease model - and relevance to HDGC.

- 2500 potential synthetic lethal genes were elucidated in the screen, enriched for cytoskeletal functions and survival signalling pathways.

Table 1. Candidate ontology
Examples of gene classes elucidated in shRNA screening.

- Selected candidate genes were validated with lentiviral shRNA-knockdown, supported by treatment with biologically pertinent drugs.

Proof

- Total nucleic counts following treatment validate the presence of synthetic lethal interactions between CDH1 and selected candidate genes.

Fig 5A. shRNA silencing
Total nucleic counts 72h after shRNA-vector silencing of candidate genes, validating the presence of a synthetic lethal effect.

- Normalising controls are non-silencing shRNA for shRNA-knockdown and mock DMSO for drug treatment.

Fig 5B. Drug treatment
Total nucleic counts following treatment with drugs targeting candidate gene products - further evidence of a synthetic lethal effect.

Product

- This supports the development of a novel class of synthetic lethal drugs for the treatment of CDH1-deficient cancers.

- As CDH1 loss is a common feature of sporadic adenocarcinoma, associated with epithelial-mesenchymal transition and poor prognosis, this work may also improve the management of sporadic DGC and LBC.

- Validation of candidates from screening shows the screen is robust, and leaves a plethora of genes for further investigation.

Acknowledgements

New Zealand Society for Oncology 2015 conference poster prize winner
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List of abbreviations

˚C – degrees Celsius
µ – micro
Ab – antibody
AK – aurora kinase
AKi – AK inhibitor
APS – ammonium persulfate
ART – assisted-reproductive therapy
ATP – adenosine triphosphate
AURKA – Aurora-A kinase
AURAB – Aurora-B kinase
BCA – bicinechninic acid
CDH1 – E-cadherin
cDNA – complementary DNA
Ct – cycle threshold
DAPI – 4’’,6’’-diamidino-2-phenylindole
ddH₂O – double-distilled H₂O
DGC – diffuse gastric cancer
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
DNase – deoxyribonuclease
EGF – epidermal growth factor
FDA – Food and Drug Administration
FBS – fetal bovine serum
g – grams
G – gauge
GC – gastric cancer
gDNA – genomic DNA
hCMV – human cytomegalovirus
HDAC – histone deacetylase
HDACi – histone deacetylase inhibitor
hr – hour
HDGC – hereditary diffuse gastric cancer
HRP – horse radish peroxidase
hrs – hours
ie – id est
LB – lysogeny broth
LBC – lobular breast cancer
m – milli
M – molar
MAP1B – microtubule-associated protein 1B
MAST2 – microtubule-associated serine/threonine kinase 2
Mc – monoclonal
min – minute
mins – minutes
MOI – multiplicity of infection
mQH₂O – Milli-Q H₂O
mRNA – messenger RNA
n – nano
PAGE – polyacrylamide gel electrophoresis
PBS – phosphate buffered saline
Pc – polyclonal
PCR – polymerase chain reaction
pDNA – plasmid DNA
PGD – pre-implantation genetic diagnosis
PI – propidium iodide
PVDF – polyvinylidene difluoride
qPCR – quantitative PCR
RNA – ribonucleic acid
rpm – revolutions per minute
RT – real-time
RTase – reverse transcriptase
s – second/seconds
SDS – sodium dodecyl sulfate
shRNA – short hairpin RNA
siRNA – short interfering RNA
SOC – super optimal broth with catabolite repression
t – time
T - temperature
TBS – Tris-buffered saline
TBST – Tris-buffered saline with tween-20
TEMED – tetramethylethylenediamine
TU – transducing units
VSVG – vesicular stomatitis Indiana virus envelope glycoprotein
WT – wild-type
1. Introduction

In the post-genomic era, there is an ever growing understanding of the molecular mechanisms that drive cancer. This understanding facilitates the development of specific, targeted treatments that are both increasingly effective and carry reduced side effects. While this has contributed to a decrease in cancer mortality, incidence has not fallen as sharply, a trend that reflects focus upon the treatment rather than the prevention of the disease.

Germline mutation of the tumour suppressor gene E-cadherin (CDH1), predisposes individuals to hereditary diffuse gastric cancer (HDGC). HDGC is highly penetrant, with a 70% lifetime risk of diffuse gastric cancer and an additional 40% risk of lobular breast cancer in women (Vogelaar et al., 2015). The primary management strategy for HDGC is prophylactic gastrectomy due to the unreliability of endoscopic detection (Charlton et al., 2004). Consequently, there is a need for chemoprevention drugs which can be used in conjunction with endoscopy to mitigate the reliance on surgery.
In hereditary cancer syndromes, the molecular driving mechanisms are more predictable than in sporadic cancers and thus more amenable to a chemoprevention approach. A multifaceted approach, combining bioinformatics and high-throughput screening, provides one way to build an unbiased view of the molecular vulnerabilities that could be exploited for the chemoprevention of familial cancers. A chemoprevention approach will both decrease the long-term burden on the health system and provide improved outcomes for at-risk individuals.

Inactivating mutations in tumour suppressor genes are less accessible drug targets due to the absence of a perturbed protein. However, inactivation of tumour suppressor genes is predicted to create vulnerabilities that may be targeted with a synthetic lethal approach. The development of a novel class of 'synthetic lethal' drugs could be used for the chemoprevention of HDGC and also the improved management of sporadic diffuse gastric cancer and lobular breast cancer.
1.1. Cancer

1.1.1. Statistics

Cancer mortality rates (in the US) have steadily decreased at about 1% per year over the last decade (AACR, 2012), owing primarily to development of powerful new targeted therapies and improved screening. While rates are down, the total numbers diagnosed and dying are increasing (AACR, 2012). While the primary factor for this is an aging population, the increased exposure to hazardous environmental factors (processed food, atmospheric pollution, smoking, etc), and increasingly sedentary and high-stress lifestyles also contribute to increasing incidence (Crew & Neugut, 2006)

Incidence and mortality rates of gastric cancer has fallen significantly over the last 50+ years, though this trend is now flattening as the Western lifestyle prevails (Crew & Neugut, 2006). Although the mortality rate is decreasing, gastric cancer affects almost one million people annually, and was responsible for 720,000 deaths in 2012 – the second biggest killer only to lung cancer. Nearly two-thirds of cases occur in developed countries and incidence rates vary up to ten-fold (Crew & Neugut, 2006; Torre et al., 2012). Countries with high rates of Helicobacter pylori (H. pylori) infection and consumption of processed and fermented food show above-average rates of gastric cancer. For example, South Korea has a rate of 50 per 100,000, while in New Zealand 8 per 100,000 are affected by the disease (the global average is ~14 per 100,000; Torre et al., 2012). In New Zealand, males aged 25-44 who died of cancer most commonly died of gastric cancer (NZMOH, 2010).

1.1.2. Gastric cancer

According to Lauren's classification, gastric cancer can be separated into two primary types: intestinal and diffuse (Lauren, 1965), each with distinct genetic profiles,
pathogenesis, epidemiology and morphology. In the intestinal type, cohesive cells form a mass of gland-like tubular structures that form visible lumps and are more amenable to detection. In the diffuse type, signet ring cell (SRC)-esque cells and associated fibroblasts thicken the stomach wall without forming a solid mass, making detection more difficult. Even advanced cancers may go undetected if there is no visible perforation of the lumen (Hanaoka et al., 2010). Diffuse gastric cancer (DGC) can be discriminated from intestinal gastric cancer based the absence of CDH1 expression (Becker et al., 1994).

While the intestinal type carries a stronger environmental etiology, the diffuse type shows a stronger genetic contribution. Only the diffuse type is associated with HDGC, and because of the difficulty in detecting DGC by endoscopy, more cases are diagnosed at late stages and carry poorer prognoses (Torre et al., 2012). Recently, gastric adenocarcinoma has been classified further into four molecular subgroups: tumours positive for Epstein-Bar virus (EBV); microsatellite unstable tumours; genomically stable tumours; and genomically unstable tumours (TCGA Research Network, 2014). Genomically stable tumours show frequent RHOA and CDH1 mutations while genomically unstable tumours show amplifications in receptor proteins and receptor tyrosine kinases (RTKs) linked to abnormal growth signalling.

1.2. Hereditary diffuse gastric cancer

1.2.1. History

The existence of familial gastric cancer has been known about since the 1800s when multiple cases of gastric cancer were observed in the Bonaparte family. By some accounts, while on his deathbed, Napoleon proclaimed that he “has [his] father's stomach” - although others believe he succumbed to arsenic poisoning (Sokoloff, 1938). The first link between germline CDH1 mutation and familial DGC was made in 1998 (Guilford,
1998). It is estimated that inherited cancer predisposition syndromes accounting for 1-3% of gastric cancer incidence, while the rest is sporadic. Other syndromes associated with occasional cases of gastric cancer include Li-Fraumeni syndrome, Lynch syndrome and familial adenomatous polyposis, among others (Vogelaar et al., 2015).

1.2.2. HDGC diagnosis

Hereditary diffuse gastric cancer (HDGC) is inherited in an autosomal dominant manner (figure 1.1). It is characterised by germline mutation of \textit{CDH1}, the gene encoding the cell-cell adhesion protein E-cadherin. HDGC is highly penetrant, with at-risk individuals harbouring a 70% lifetime risk of developing DGC (figure 1.1). Women carry an additional 42% lifetime risk of developing lobular breast cancer (LBC, Vogelaar et al., 2015).

The clinical criteria for HDGC-like families (including second-degree relatives) to be tested for germline \textit{CDH1} mutation is: (1) two or more gastric cancers at any age with one confirmed DGC; (2) both DGC and LBC with one before age 50; and (3) one confirmed DGC before age 40 (van der Post et al., 2015). Among families that fit this criteria the average age of onset is 38 years old, and it is estimated \textit{CDH1} mutation will account for 25-40% of cases (Hansford, 2015; Vogelaar et al., 2012). A small number of families carrying \textit{CTNNA1} mutations have also met this criteria, and share many clinical similarities

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{HDGC family pedigree. Partial pedigree of original HDGC family harbouring germline \textit{CDH1} mutation. Black fill represents those affected by diffuse gastric cancer, slash indicates individual is deceased (Guilford et al., 1998).}
\end{figure}
with CDH1 mutation-positive families (Majewski et al., 2013). HDGC is best defined by mutations in CDH1 or related genes, both in function (ie, adhesion molecules) or regulation (ie, transcriptional activator of CDH1; Hansford, 2015). It is likely more genes associated with HDGC will be identified through whole genome or exome next-generation sequencing approaches, although to date no additional compelling genes have been found (P Guilford, pers. comm., 2016).

1.2.3. Pathogenesis

Most HDGC patients with CDH1 mutation develop asymptomatic/preclinical, indolent, multifocal signet ring cell carcinomas (SRCC), enriched in the body-antral transitional zone of the stomach (figure 1.2). These early foci sometimes proliferate slowly, evident by the lack of mitotic cells and low Ki-67 expression (Humar et al., 2009), suggesting E-cadherin-deficient cells lack a major selective growth advantage. However, further genetic and epigenetic changes may drive neoplastic growth and fuel the progression of the disease. Risk factors for DGC are discussed below (chapter 1.4.4).

![Figure 1.2. Multifocal tumours in early HDGC. Stomach of HDGC individual, showing the density and localisation of indolent, multifocal stage T1a SRCC. This patient had >125 identified lesions. Adapted from Charlton et al., (2004).](image)
A proposed model for HDGC development starts with carcinoma in situ, corresponding to signet ring cell (SRC) presence in the superficial lamina propria that follows a pagetoid (upwards) spread around the edge of the gland (Humar et al., 2009). The disease may then spread throughout the lamina propria, followed by thickening of the gastric wall by sustained tumour cell infiltration in a disease process called linitis plastica. Like sporadic DGC, advanced HDGC mainly presents as linitis plastica with diffuse infiltration of the gastric wall.

The stomach is comprised of distinct role-specific tissue layers (figure 1.3): the upper mucosa, submucosa, muscularis and serosa. The upper gastric mucosa is the mucous membrane layer that contains the gastric pits and glands and is comprised of epithelium, lamina propria and the muscularis mucosae. Epithelium forms the upper layer of structures such as glands and sits atop a basement membrane, while the rest of the mucosa is lamina propria. Together, the epithelium and lamina propria sit atop the muscularis mucosae.

Figure 1.3. Anatomy of the stomach. DGC is thought to originate from hyperproliferative progenitor cells in the upper neck of the gastric glands in the gastric mucosa. Adapted from Britannica (2003).
There are three types of glands: cardiac (proximal part of stomach), oxyntic glands (most common), and pyloric glands, each with unique secretory roles. DGC is thought to originate from the hyperproliferative mucous neck progenitor cells in the upper isthmus of a gastric (most likely oxyntic) gland. The leading hypothesis for the mechanism of cancer initiation following E-cadherin \((CDH1)\) loss posits that disruption of polarity in gastric progenitor cells leads to abnormal mitotic spindle orientation and subsequent division out of the epithelial plane (Humar & Guilford, 2009).

### 1.3. E-cadherin

Epithelial tissues line the surfaces and cavities of structures throughout the body. The primary functions of mammary and gastric epithelium is physical protection, controlling transport and secretion. E-cadherin is highly expressed in gastric epithelium, playing roles in maintaining tissue integrity, cell polarity, intercellular signalling and the regulation of mitosis (Nose et al., 1990). E-cadherin is encoded by \(CDH1\) (16q22.1) and consists of a single transmembrane domain, a 150-amino acid cytoplasmic domain, and a 550-amino acid ectodomain comprising five tandem repeats (figure 1.4).

![Figure 1.4. Interactions of E-cadherin.](image)

The extracellular domain of E-cadherin forms adherens junctions with adjacent cells, while the cytoplasmic domain directly links to the cytoskeleton and facilitates signal transduction. Adapted from Perry et al., (2012).
The ectodomain of E-cadherin forms Ca\(^{2+}\)-dependent adherens junctions with the E-cadherin of an adjacent cell to facilitate the organisation of polarised tissue monolayers. Radial actin filaments interact with adherens junctions via a cadherin/catenin complex to create a dynamic tension-based cytoskeletal plane across the apical surface of epithelial layers (Vaezi et al., 2002). Adherens junctions bear the force between epithelial cells and are important for maintaining cell shape and transport physiology while providing a barrier against microbial infection and gastric secretions (Turner et al., 2009).

The cytoplasmic tail forms a complex with catenins that are in turn directly linked to the cytoskeleton. This interaction helps maintain polarity, cellular architecture and aids in receptor recycling. The tail also stabilises signaling complexes inside the membrane, and is directly involved in the transduction of pathways including Wnt/β-catenin, PI3K/AKT and Rho GTPase signalling (van den Bossche et al., 2012).

E-cadherin mediated cell-cell adhesion provides spatial cues that promote and establish apical-basal polarity. Cytoskeletal filament interaction with adherens junctions at the apicolateral corners is central to establishing polarity, though the mechanism is not clearly understood. Mitotic spindle orientation must be tightly controlled in the Z-axis in order to preserve the architecture of the tissue (Fleming et al., 2007). E-cadherin acts as a spatial cue within the cell to direct mitotic spindle orientation from the centrosome (den Elzen et al., 2009). Ordinarily, cleavage furrow formation is immediately followed by adherens junction formation, which means apicobasal polarity is maintained during all stages of mitosis.

E-cadherin is directly involved in signal transduction of pathways such as Wnt/β-catenin and PI3K/AKT (Lau et al., 2011). Wnt signalling controls stem cell pluripotency and cell
fate decisions through transcriptional activation by β-catenin (Jamora & Fuchs, 2002), while PI3K/AKT signalling feeds in to important proliferation and survival pathways (Lau & Leung, 2012). Once activated, AKT translocates to the nucleus where it regulates transcription of cell survival, cell cycle and migration genes. AKT signalling is disrupted following the loss of E-cadherin mediated cell-cell adhesions, leading to retarded cell proliferation and an increased propensity for transformation (Reddy et al., 2009; Huber et al., 2005).

β-catenin contains a default nuclear localisation signal, though it is regularly bound at the cell membrane to the cytoplasmic tail of E-cadherin. In the absence of E-cadherin, β-catenin can freely migrate towards the nucleus and activates the transcription of Wnt-controlled genes. Wnt target genes, such as c-MYC or cyclin D1, are associated with increased growth, invasion and cellular transformation. (Lin et al., 2000, He et al., 1998). Further, β-catenin is degraded by GSK3B, which is inactivated by active AKT. Constitutive activation of PI3K/AKT therefore reinforces nuclear β-catenin signalling (Lau et al., 2011).

1.3.1. E-cadherin-deficient tumourigenesis

Early DGCs are frequently signet ring cell carcinoma (SRCC). Lineage markers of early SRCC foci point towards mucous neck cells that have invaded the basement membrane as the primary origins (Humar et al., 2007). Early SRCC cells can express markers for a variety of cell types including pit, neck and chief cells, accordant with defective distribution of factors that determine cell fate (Humar et al., 2009). E-cadherin loss leads to cell division outside of the epithelial plane and the formation of early SRCC foci.

In cultured adherent cells, Ca²⁺-depletion abolishes correct spindle orientation by disrupting adherens junction-mediated cell adhesion (Yap et al., 2009). Similarly,
expressing only the endodomain of E-cadherin without the extracellular linker ectodomain, or inhibiting ectodomain interactions, leads to incorrect spindle orientation (Yap et al., 2009). As the cytoplasmic anchorage points for the cytoskeleton were not ablated in these studies, this indicates the loss of adherens junction-mediated cell adhesion alone is sufficient to affect the mitotic spindle angle. Furthermore, inducible double negative E-cadherin knockout mice showed increased frequency of epithelial anaphase angles above 10 degrees (den Elzen et al., 2009). Loss of the extracellular linker domain is likely to account for the majority of E-cadherin-deficient pathogenesis. It is postulated that disrupted spindle orientation leads to hypertrophy of the gastric crypt epithelium, as orientation of the preceding cell division tends to be maintained in subsequent divisions (Humar & Guilford, 2009).

In gastric cancer, the basement membrane must be degraded or otherwise perforated for a misaligned daughter cell to be displaced into the lamina propria. A potential mechanism for this involves gastric proteases penetrating the basement membrane due to the loss of epithelial barrier function. Proteases that are likely responsible for this include pepsinogen, matrix metalloproteases and collagenases – some of which have been shown to be upregulated in response to E-cadherin downregulation (Radisky & Radisky, 2010; Morohara et al., 2006). Cellular invasion through the basement membrane into the lamina propria is considered evidence for diagnosis of invasive cancer (Okabayashi & Shima, 2013).
Figure 1.5. Model of early events in HDGC. (Left to right): (i) E-cadherin loss leads to misaligned division; (i) division out of epithelial plane is maintained; (iii) accumulation of dividing cells without a mass or perforation; (iv) invasion of submucosa, associated with EMT and metastasis. Adapted from Humar & Guilford (2009).

E-cadherin downregulation is a hallmark of the epithelial-mesenchymal transition (EMT), although is insufficient to trigger the event alone (Chen et al., 2014). During an EMT, cells gain invasive and migratory capabilities that are associated with mesenchymal phenotype (Huber et al., 2005). Lymphatic and vascular structures reside in the submucosa below the lamina propria, and growth of the tumour into these circulatory systems can lead to cells being further displaced into distal tissues, initiating metastasis. Though E-cadherin loss alone is insufficient to confer a selective growth advantage, the loss of adhesion and spatial cues for polarity, combined with increased pro-survival signalling, gives rise to an invasive, poorly-differentiated phenotype.
1.3.2. Genetic abnormalities of CDH1

The majority of pathogenic CDH1 abnormalities in HDGC are inactivating mutations (frameshift, splice site, nonsense) although many missense mutations have also been identified. Germline mutations of CDH1 are found evenly distributed throughout the 16 exons of the gene without regional clustering (Hansford et al., 2014). This is in contrast to sporadic diffuse gastric cancer, where mutations cluster in exons 7-9 (Fitzgerald, 2004).

![Figure 1.6. Genetic abnormalities of CDH1. Mapping of germline CDH1 mutations described to date by mutation type and location on CDH1 transcript. Adapted from Hansford et al., (2014).](image)

Although very abundant SRCC foci have been described in particular families, no correlations between phenotype and location or type of mutation has yet been made (Guilford et al., 2010). The somatic inactivation of the second CDH1 allele triggers the initiation of HDGC pathology/pathogenesis and is most commonly gene promoter hypermethylation (over 50%; Humar et al., 2009). Interestingly, epigenetic downregulation of CDH1 first occurs by histone deactylation and subsequent DNA methylation (Dumont et al., 2008).
1.3.3. Risk factors

It remains unclear why DGC and LBC are the only frequent tumours associated with germline CDH1 mutation. However, risk factors for further genetic and epigenetic damage in the gastric epithelium include increased carcinogen exposure or activity (particularly salted, preserved, cured foods; smoking, alcohol), hypoxia, chronic inflammation, obesity, *Helicobacter pylori* (*H. pylori*) and, more recently, Epstein-Barr virus infection (TCGA, 2014; Torre et al., 2012).

Chronic inflammatory responses such as gastritis lead to an increase of cyclooxygenase-2 (COX-2) and prostaglandin E2 which induce transcriptional repressors of CDH1 (Wild & Hardie, 2003; Dohadwala et al., 2006). The transcriptional repression of CDH1 may lead to stable promoter methylation and a long-term reduction in CDH1 expression (Niwa et al., 2010).

*H. pylori* infection is the primary risk factor for sporadic gastric cancer, with geographic variation of cancer incidence paralleling infection rates, although this factor is more pertinent to the intestinal subtype than the diffuse (Okabayashi & Shima, 2013). *H. pylori* infection leads to cell junction protein translocation away from the membrane – a possible mechanism for displacement of a daughter cell outside the epithelial plane which is then maintained in future divisions. This may be a unique mechanism that does not fit the traditional paradigm of a “second hit” mutation, although sufficient for initiation of the cancer because out-of-plane division angles are maintained. As gastric progenitor cells may already have decreased CDH1 expression compared to their differentiated counterparts, these cells may be more vulnerable to environmentally-induced CDH1 downregulation.
1.4. Clinical management

1.4.1. Primary prevention

Because gastric cancer is difficult to treat effectively, the best approach for improving clinical outcomes is primary prevention. The most modifiable risk factors are salt/nitrite consumption, fruit/vegetable consumption, alcohol consumption, tobacco smoking, stress and *H. pylori* infection (Uemura *et al.*, 1997). Global improvement of sanitary and housing conditions, as well as use of refrigeration, has led to decreased *H. pylori* infection (La Vecchia, 1990). Antioxidant intake such as vitamins C and E and β-carotene may show a preventive role through protection from DNA damage, though the effect is most pronounced in populations with low micronutrient intake (Yuan *et al.*, 2004). In white Americans, it is estimated that between 40-70% of carcinomas may be prevented by controlling modifiable risk factors, emphasizing the need to prioritise primary prevention (Song & Giovannucci, 2016).

COX-2 plays roles in the inflammatory response and may be carcinogenic in gastric cancer (GC) via induction of *CDH1* repressors. Expression of COX-2 is induced by tobacco, acidic conditions and *H. pylori* infection (McCarthy *et al.*, 1999), therefore COX-2 inhibiting drugs (ie, aspirin, non-steroidal anti-inflammatory drugs) or foods (curcumin from turmeric) may be chemopreventive in GC (Uefuji *et al.*, 2000). Non-psychotropic cannabinoids such as cannabigerol (CBG) have potent effects in counteracting intestinal inflammation (Izzo & Camilleri, 2009), and have also been identified as potential chemopreventive agents for GC (Borrelli *et al.*, 2014).

1.4.2. Genetic testing

Genetic counseling is an essential aspect of the clinical management of HDGC families. Clinical guidelines recommend a full multidisciplinary team comprising expertise in gastric
surgery, gastroenterology, breast oncology, pathology, psychosocial support and nutrition (Vogelaar et al., 2015; van der Post et al., 2015). In New Zealand, individuals from identified or suspected HDGC families undergo genetic testing with informed consent from age 16 to determine \textit{CDH1} mutation status. This analysis is carried out with a combination of Sanger sequencing and Multiplex Ligation-dependent Probe Amplification to assess the entire open-reading frame, including exon-intron boundaries, and copy number analysis of individual exons (van der Post, 2015). Testing of younger family members prior to age of informed consent is also considered on a case-by-case basis.

However, this analysis is imperfect because \textit{CDH1} is large (~5kb) with broad mutation distribution. In some instances, it may also be difficult to determine whether a mutation is pathogenic or a benign polymorphism. While over 155 pathogenic germline variants of \textit{CDH1} have been published worldwide (figure 1.6), and many others still unpublished, there are likely to be further polymorphisms of reduced penetrance or unknown significance yet to be identified. Further, \textit{CDH1}-related genes such as catenins (ie, \textit{CTNNA1}) or transcriptional activators of \textit{CDH1} can contribute to HDGC in clinically similar ways.

\subsection{1.4.3. Prophylactic gastrectomy}
DGC is difficult to detect in its early stages due to its diffuse phenotype and often absence of a visible mass. Multiple small SRCC foci have been found in almost all germline \textit{CDH1} mutation carriers, even as young as 15 (Charlton et al., 2004). Thus, the only existing option to eliminate risk of DGC in HDGC families is prophylactic total gastrectomy. This procedure involves the total removal and resection of the stomach (figure 1.7) and is curative if there has been no metastatic spread of the disease. The procedure itself reports a 0-6% mortality risk and is lowest in young, healthy individuals (Haverkamp, 2015).
Despite laparoscopic techniques, total gastrectomy is a major surgery and leaves patients with 100% lifetime morbidity (Huscher et al., 2005). Morbid effects commonly include weight loss, diarrhoea and altered eating habits because of reduced gastric secretions and transit time. Fat, iron and vitamin A/D/E/K/B12 malabsorption and deficiency also occur with the resultant nutritional and metabolic problems requiring careful monitoring.

1.4.4. Endoscopic screening

In Japan, workforce endoscopic screening has led to detection of half of gastric cancers at an asymptomatic stage, effectively halving the mortality rate for sporadic gastric cancer since 1970 (Yamaguchi, 2002). Based their level of effectiveness, the current screening recommendations for individuals with HDGC who decline gastrectomy are upper endoscopy with multiple biopsies on an annual basis. The endoscopic process can involve white light gastroscopy or chromoendoscopy (figure 1.8; Shaw et al., 2005; Okabayashi & Shima, 2013).
The drawbacks of these methods include difficulty in identifying submucosal lesions and sampling bias in macroscopically normal appearing mucosa. Expert histopathological identification of early SRCC is recommended and can be achieved with biopsies.

In one clinical HDGC report, SRCC foci were found in 2/23 patients using preoperative white light gastroscopy. However, pathological examination of the resected stomach identified foci in 22/23 patients, highlighting the difficulty of detecting early lesions with routine endoscopy (Charlton et al., 2004).

White light endoscopy alone is an imperfect method because DGC infiltrates down without ulceration or distortion of the epithelium. Consequently, advanced cancer in one individual of a routinely screened New Zealand cohort of 30 CDH1 mutation carriers escaped detection and the patient subsequently died (P Guilford, pers. comms., 2015). Chromoendoscopy relies on a pH-sensitive dye, such as Congo red or indigo carmine, that allows the visualisation of acid secreting mucosa. Chromoendoscopy with Congo red
following gastric secretory stimulation by pentagastrin may help highlight areas of interest (Shaw et al., 2005). Though the method is more sensitive, it has been criticised for being toxic and is still not able to detect foci <4mm (Marti et al., 2005). Toxicity is even less desirable in circumstances requiring constant surveillance, such as in HDGC families, and some have recommended against use of these agents (Marti et al., 2005). Current surveillance methods may be succeeded by emerging technologies such as narrow band imaging, blue laser imaging and confocal laser endomicroscopy (Dunbar & Canto, 2008), to allow detection of sub-luminal foci in vivo.

Mapping of early SRCC foci in some studies has identified densities up to five times higher in the body-antral transitional zone than in the body or antral zones alone (Charlton et al., 2004; Fujita et al., 2012). Such a high density of cancer foci, in a zone comprising under 10% of the gastric mucosal surface, supports the use of targeted transitional zone biopsies in HDGC surveillance. Biopsies targeted to pale areas are also performed, although this is affected by pale scarification from previous biopsies and may increase the chances of bleeding and biased sampling (Vogelaar et al., 2015). The current clinical guideline is for random biopsies to be taken from all areas of the stomach (van der Post, 2015).

Faced with an additional 42% risk of developing breast cancer, it is recommended that women with a CDH1 mutation undergo annual breast MRI screening and may also consider prophylactic mastectomy. This option is reviewed on case-by-case basis because the cancer has a lower penetrance. Some guidelines recommend using selective oestrogen receptor modulators or aromatase inhibitors for chemoprevention (van der Post, 2015). In Australia, which has a similar cancer picture to New Zealand, it is estimated that the lives of up to 300 GC patients could be saved per year through widespread uptake of a simple stool test that tests for the presence of blood, with positive tests warranting further
investigation (NZSO Physician, 2015). However, it is unclear whether these tests would be useful for DGC and HDGC in particular.

1.4.5. Psychosocial effects
The psychological toll of concern over a cancer diagnosis is also a factor faced by at-risk individuals who opt for surveillance over surgical prophylaxis. The main psychosocial problems include fear and anxiety in coping with cancer, practical (i.e., life insurance) and family issues (Vogelaar et al., 2015). Although the effects are difficult to qualify or quantify, prophylactic surgery on physically and psychologically fit individuals may mitigate some concerns. However, surgery-related morbidity can carry its own psychological burden.

1.4.6. Prognosis
Currently, the 5-year survival rate for DGC does not exceed 30% (Correa, 2013; Stiekama et al., 2013), reflecting the hazards posed by delayed detection of clinically-silent early disease. This exemplifies the benefit that young, healthy individuals may reap from a prophylactic total gastrectomy, regardless of the long list of morbidities. As few as 10% of patients who develop invasive, symptomatic DGC have curable disease (Koea et al., 2000). DGC is more inclined to spread via peritoneal dissemination, and is considered invasive disease following growth into the lamina propria. Locally advanced and metastatic gastric cancer has 5-year survival rates as low as 0% (Ridwelski et al., 2001). As well as the need to improve detection methods, this highlights the need for better chemotherapeutic and/or chemopreventive drugs.

1.4.7. Early tumour homogeneity
Tumour heterogeneity – where different cells from the same tumour show distinct phenotypes and genotypes – presents a significant obstacle in ridding a patient of cancer
This is because additional genetic and epigenetic changes may confer protection against one or more forms of treatment. In early HDGC, the presence of synchronous, indolent foci argues against the presence of additional mutation events, suggesting the earliest disease is attributable to \textit{CDH1} silencing alone.

\textbf{Figure 1.9. Tumour heterogeneity and post-therapy relapse.} Heterogeneous tumours are difficult to fully treat due to the distinct phenotypes produced through additional genetic events during tumour progression and evolution. This feature is absent from early HDGC tumours, suggesting that drug regimentation may elicit a strong, uniform response. Adapted from Meacham (2013).

Multiple cancer foci in early HDGC should be equally amenable to a given drug treatment as they are homogeneous and have not yet acquired further genetic changes. With a chemopreventive approach, early foci could, in principle, be eliminated before further genetic changes fuel progression. Thus, drugs that are selectively lethal to E-cadherin-deficient epithelial cells could be administered to at-risk individuals in HDGC families to prevent or delay the development of advanced disease.

\textbf{1.5. Chemotherapy}

\textbf{1.5.1. History}

The era of chemotherapy began during World War II. Research into agents of chemical warfare lead to the discovery of a genotoxic (DNA damaging) compound called nitrogen mustard, which was found to be effective against lymphoma by rapidly killing growing cancer cells via DNA damage. Since then, a plethora of drugs have been discovered that target a variety of functions in cancer cells.
Traditional cytotoxic chemotherapies act by damaging DNA to kill rapidly dividing cells, exploiting one of the main properties of most cancer cells. However, this also leads to harm in cells that divide rapidly in normal condition such as cells in the digestive tract and hair follicles, leading to gastrointestinal toxicity, hair loss and other side effects. In the 20th century the focus was cytotoxic drugs, while that has now shifted to targeted therapies (Baguley 2002). Targeted therapies block the growth of cancer cells by interfering with specific molecules using specific, targeted drugs. Targeted therapies are chosen or designed to interact with their target, while traditional chemotherapies were identified because they kill cells, with little thought for their mechanism of action.

1.5.2. Chemotherapy in diffuse gastric cancer

The most effective treatment for patients with sporadic gastric cancer is perioperative (before and after surgery) chemotherapy combined with total gastrectomy (Cunningham et al., 2006). Drug regimentation includes infused cycles of epirubicin, cisplatin and daily infused 5-fluorouracil (5-FU). This approach was more effective than surgery alone and reports a 5-year survival rate of 36% for advanced, resectable cancers. This highlights the utility of effective drugs for further improving patient outcomes, although these first-line treatment modalities are non-targeted chemotherapies that rely on rapid cell proliferation. Furthermore, there is little available data on the value of perioperative chemotherapy specifically in the diffuse subtype (P Guilford, pers. comms., 2016). Drug regimens vary across countries and providers, and may also include gemcitabine, capecitabine, carboplatin, oxaliplatin and docetaxel (Cunningham et al., 2006; Barone et al., 2007).
1.5.3. Side effects
Chemotherapy often involves a raft of side effects including nausea, ulceration, pain, diarrhoea, appetite/weight loss, fatigue, light sensitivity and hair loss. The common side effects of GC chemotherapy include severe nausea, mouth sores and appetite suppression (Cunningham et al., 2006; Chemocare, 2016). Adjuvant anti-emetic, anti-nausea and chemoprotective drugs such as dexrazoxane (protects from cardiotoxicity) and amifostine (reduces incidence of infection and fever) are used to reduce side effects of cancer chemotherapy. Anti-nausea drug ondansetron (Zofran) was voted one of the top 5 advances in modern oncology” (Chustecka, 2014), underpinning the need for chemotherapies with reduced side effects. Though some effects will pass, late and potentially permanent side effects of chemotherapy include cataracts, early menopause, heart problems, liver problems, nerve damage, reduced lung capacity and damaged vasculature (Janunger et al., 2002).

1.5.4. Standard targeted drug paradigm
Drug targeting involves inhibiting an aberrant protein that is critical to the biology of the cancer. For example, in HER2+ breast cancer, where overexpression of the HER2 receptor leads to an increased growth factor signalling, trastuzumab (Herceptin) works by binding and blocking the receptor on the surface of the breast cancer cell (Verma et al., 2012). Similarly, vemurafinib (Zelboraf) targets gain of function oncogenic driver BRAFV600E in melanoma (Sosman, 2012). However, as E-cadherin is a tumour suppressor protein whose function is lost, its absence means it cannot be a conventional drug target (figure 1.10). Nevertheless, an inactivating mutation in cancer cells but not in normal cells can create opportunities to selectively kill cancer cells via synthetic lethal targeting.
Figure 1.10. Loss of function and gain of function mutations drug paradigm. While gain of function mutations in oncogenes present as clear targets for therapy, loss of function mutations in tumour suppressor genes leaves no clear targets. Adapted from Brough et al., (2011).

1.5.5. HDGC chemoprevention

While there is a need for improved detection methods and more effective treatments, there has been little focus on the chemoprevention of DGC or LBC in HDGC families. Ideally, the indolent stage T1a foci could be eliminated before their growth is advanced by further genetic events. Such chemopreventive therapies could be applied every periodically (perhaps every 2-5 years) in at-risk individuals, supported by annual endoscopic surveillance. This precautionary approach to chemoprevention may be more cost effective than surgery and would significantly reduce patient morbidity.

1.6. Synthetic lethality

1.6.1. Synthetic lethal definition

Synthetic lethal interactions were first described almost 100 years ago in *Drosophila melanogaster* (*D. melanogaster*), where the inheritance of two specific mutations led to a lethal phenotype (Metz & Bridges, 1917). Two genes are 'synthetic lethal' when only their
simultaneous inactivation leads to death, while individual inactivation leaves cells viable (figure 1.11).

Figure 1.11. Synthetic lethal concept. Two genes are synthetic lethal when only their simultaneous inactivation leads to death. In cancer therapy, the effect implies inhibiting one gene (B) in a context where the other gene (A) is defective should be selectively lethal to cancer cells.

Synthetic lethal interactions are attractive targets in loss-of-function cancers, where the absence of a gene product is the primary driver of tumourigenesis. Investigating synthetic lethal interactions in E-cadherin-deficient cells may present clinically relevant vulnerabilities that are amenable to targeted therapy. In this context, ‘synthetic’ is borrowed from the Greek synthetikós which means to put together. Indeed, put together, inactivation of a synthetic lethal partner gene in an E-cadherin-deficient context will lead to selective killing of the cancer cell while leaving other cells unaffected.

1.6.2. Synthetic lethal screening

Early screening efforts utilised model organisms such as D. melanogaster, and then Caenorhabditis elegans (C. elegans) and Saccharomyces cerevisiae (S. cerevisiae), allowing for large mutant crossing matrices and the generation of complex interaction
networks (Brough et al., 2011). For example, a synthetic genetic array assay in *S. cerevisiae* allows strains with a single query gene disruption to be crossed with an array of yeast strains with over 4700 deletions and any synthetic lethal interactions are subsequently detected by the absence or poor growth of particular mutant crosses (Tong et al., 2001).

Model organism drawbacks and new technology means synthetic lethality screens can now be performed in human cell lines with RNA-interference (Farmer et al., 2005; Telford et al., 2015). An unbiased, genome-wide approach to elucidating targets would support the development of novel classes of synthetic lethal drugs for E-cadherin-deficient tumours. Uncovered vulnerabilities may also be amenable to existing drugs, and thus merit the repurposing of existing drugs in new therapeutic applications as well as in combination for synergistic effect. Furthermore, chemical screens in human cell lines are also important for identifying pharmacological targets and vulnerabilities. Compound screening in human cell lines with both known and unknown drug libraries is a potentially rapid method of elucidating susceptibilities. In this case, lead compounds can be identified, even if their targets or mechanisms are still unknown.

### 1.6.3. Synthetic lethality in action

Targeting of DNA-repair enzyme poly-ADP ribose polymerase (PARP) illustrates the clinical utility of a synthetic lethal approach to chemotherapy (Ashworth, 2008). Cells with inactivating mutations in DNA-repair enzymes BRCA1/2 become reliant on PARP for DNA repair. Inhibition of PARP ablates essential dsDNA repair and promotes apoptosis in BRCA1/2-deficient cells (Farmer et al., 2005). Olaparib (Lynparza) is a targeted therapy targeting PARP and leads to strong clinical responses in breast and ovarian cancer patients with BRCA1/2 mutations (Tutt et al., 2010). Aligned with the synthetic lethal
principle, olaparib does not produce as many adverse side effects associated with conventional chemotherapy (Tutt et al., 2010).

Synthetic lethal interactions can occur between genes acting in the same pathway or in distinct, compensatory pathways (Hartman et al., 2001). As expected, components of the same pathway often share the same synthetic lethal partners (Tong et al., 2001). Reciprocally, there are less likely to be many partners of genetically redundant genes as systems of functional buffering can mitigate loss of function events. However, it is likely that some synthetic lethal partners are elements of function buffering networks, as is the case with PARP and BRCA1/2. There is evidence that PARP is hyperactivated when BRCA1/2 is lost, most probably to mitigate the loss of a mechanism of functional compensation for DNA repair (Brough et al., 2011). Thus, the increased expression of genes following a loss of function event may be used as evidence of a redundant mechanism to support screening methods.

1.6.4. Rationality of synthetic lethal interaction

E-cadherin-deficient cancers gain a selective advantage from altered cell-cell adhesion, cytoskeletal organisation and survival signalling. However, it is probable that these advantages come at the cost of acquired vulnerabilities that may be exploited in a synthetic lethal manner. For example, the disruption to cytoskeletal interactions promotes a diffuse phenotype through loss of cell adhesion and altered cytoskeletal organisation. However, this leaves the disorganised cytoskeleton as a clear vulnerability (figure 1.12).
Figure 1.12. Loss of E-cadherin affects cytoskeletal organisation. Apical surface of isogenic MCF10A (CDH1+/+ and CDH1-/-) cells demonstrating the effect of E-cadherin deletion on cytoskeletal organisation (Chen et al., 2014). Top row: microtubules; bottom row: actin; left column: MCF10A CDH1+/+; right column MCF10A CDH1-/- Adherens junction protein E-cadherin (red), tight junction protein ZO-1 (magenta), microtubule and actin cytoskeleton proteins α-tubulin and F-actin, respectively (green).

E-cadherin loss alters certain signalling pathways and activation of genes that may be targeted as cells become increasingly reliant on their function for survival. For example, pro-survival signalling by PI3K/AKT and Wnt pathways, or the components of cytoskeletal function may be robust synthetic lethal targets. Cells without CDH1 expression are predicted to require compensatory action to mitigate the disorganisation observed in the cytoskeleton (figure 1.12).
1.6.5. Microtubule-associated vulnerabilities

Preliminary genome-wide siRNA screening has identified a plethora of genes with evidence of synthetic lethal relationships with CDH1. Gene ontology analysis shows enrichment in GPCR proteins, PI3K/AKT pathway components and proteins involved with the cytoskeleton (Telford et al., 2015), such as microtubule-associated proteins. Microtubule-associated proteins are involved in a wide range of functions, from organisation and dynamics to transport and mitosis, with both structural and signalling roles.

Microtubule-associated serine/threonine kinase 2 (MAST2) plays a part in modulating microtubule interactions and dynamics, and was identified in the preliminary screen as potentially synthetic lethal. MAST2 interacts with dystrophin/utrophin networks to facilitate dynamics between microtubules and the cell membrane (Garland et al., 2008). In epithelial cells, utrophin particularly interacts with adherens junctions proteins, leading to a stable cytoskeleton and cell structure (Belkin & Burridge, 1995). RNA sequencing data shows an upregulation of MAST2 in E-cadherin-deficient cells, implying it may be a mechanism of compensation for cytoskeletal disorganisation.

Additional biologically relevant genes identified in the preliminary screen included microtubule-associated protein 1B (MAP1B) and the mitosis regulating family of Aurora kinases (AURKA and AURKB). In epithelial biogenesis, MAP1B plays a role in polarised growth and maintenance of plasma membrane domains, and silencing show been shown to affect polar morphogenesis (Spiliotis et al., 2008). While MAP1B is not upregulated, it is possible that further dysregulation of polarised epithelial morphogenesis is intolerable in an E-cadherin-deficient context. The Aurora kinase family regulate centrosome function and control chromatid segregation and microtubule polymerisation. Similarly, it is expected that
further defects to bipolar assembly and chromosome alignment may be lethal to E-cadherin-deficient cells.

There is a well-described arsenal of drugs targeting signalling pathways and the cytoskeleton such as the taxanes paclitaxel and docetaxel. Plant-derived compounds targeting microtubule dynamics represents some of the oldest targeted functions in cancer therapy (Wani et al., 1971). Terpene alkaloids the taxanes from the Pacific Yew tree (Taxus brevifolia) and vinca alkaloids such as vinchristine and vinblastine from the Madagascan periwinkle (Catharanthus roseus, formerly Vinca roseus) target microtubule disassembly and assembly, respectively. Historically, the advent of these drugs has been responsible for some of the most significant increases in cancer survival rates (Rowinsky et al., 1992).

1.6.6. Translational synthetic lethality

Synthetic lethal drugs may be used in the chemoprevention and/or treatment of early CDH1-deficient tumours in HDGC families. This would reduce reliance on morbid surgical prophylaxis, instead allowing at-risk individuals to manage the cancer syndrome with the administration of drugs supported by routine screening. As well as improved physical outcomes, chemopreventive methods would heavily mitigate the psychological burden of a cancer predisposition throughout a lifetime. As CDH1 loss is also common feature of sporadic adenocarcinoma, associated with epithelial-mesenchymal transition and poor prognosis, these synthetic lethal drugs may also improve the management of sporadic DGC and LBC, and possibly other advanced adenocarcinoma.
2. Materials & methods

In cancer therapy, a synthetic lethal effect implies that inhibiting one gene in a context where another (e.g., $CDH1^{+/+}$) is defective should be selectively lethal to the cancer cells. This work aims to investigate the presence of synthetic lethal interactions between $CDH1$ and putative partner genes. To do this, query partner genes are silenced in two isogenic epithelial cell lines with and without $CDH1$ expression ($CDH1^{+/+}$ and $CDH1^{-/-}$). In principle, a synthetic lethal interaction will manifest as a selective reduction in viability in cells lacking $CDH1$ expression following partner gene silencing. To interrogate synthetic lethal effect, selected candidate genes are silenced by shRNA knockdown, followed by time-course analysis of gene expression and cell viability.
2.1. Materials

2.1.1. Reagents

0.22 µm hydrophillic syringe filter – Sartorius, Madrid, Spain
0.45 µm PVDF syringe filter – Sigma-Aldrich, St. Louis, USA
0.5% EDTA trypsin – Invitrogen, Waltham, USA
2-Mercaptoethanol – Sigma-Aldrich, St. Louis, USA
30% 37.5:1 acrylamide:bis solution – Bio-Rad, Hercules, USA
5X Cell Culture Lysis Reagent – Promega, Fitchburg, USA
Actrapid Penfil Neutral insulin – Novo Nedvark, Denmark
Amersham ECL Plus Western Blotting Substrate – GE Healthcare Life Sciences, Little Chalfont, USA
Ammonium persulfate (APS) – Sigma-Aldrich, St. Louis, USA
Anti-α-tubulin monoclonal antibody T6199 (mouse) – Sigma-Aldrich, St. Louis, USA
Anti-actin polyclonal antibody A2066 (rabbit) – Sigma-Aldrich, St. Louis, USA
Anti-E-cadherin polyclonal antibody sc7870 (rabbit) – Santa Cruz, Santa Cruz, USA
Anti-mouse Alexa Fluor 555 – Thermo Fisher Scientific, Waltham, USA
Anti-mouse Alexa Fluor 647 – Thermo Fisher Scientific, Waltham, USA
Anti-mouse HRP-conjugated antibody NA934 (rabbit) – Amersham Biosciences, Buckinghamshire, UK
Anti-pericentrin polyclonal antibody ab4448 (rabbit) – Abcam, USA
Anti-rabbit Alexa Fluor 488 – Thermo Fisher Scientific, Waltham, USA
Anti-rabbit HRP-conjugated antibody NA931 (mouse) – Amersham Biosciences, Buckinghamshire, UK
Antibody diluting buffer – prepared in lab (appendix 5.1.8)
Agar bacteriology grade – Applichem, St. Louis, USA
Agar plates – prepared in lab (appendix 5.1.3)
Alisertib – Applichem, Houston, USA
Ampicillin sodium salt – Sigma-Aldrich, St. Louis, USA
BCA Protein Assay Kit – Thermo Fisher Scientific, Waltham, USA
Blocking buffer – prepared in lab (appendix 5.1.8)
Calcium chloride – Scharlau, Barcelona, Spain
CellTiter-Glo – Promega, Fitchburg, USA
Custom oligonucleotide primers – Integrated DNA Technologies, Coralville, USA
Cholera toxin – Sigma-Aldrich, St. Louis, USA
Danusertib – Applichem, Houston, USA
Dimethyl sulfoxide (DMSO) – Sigma-Aldrich, St. Louis, USA
Dulbecco’s Modified Eagle Medium (DMEM) – Invitrogen, Waltham, USA
Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) – Invitrogen, Waltham, USA
ECL Plus Western Blotting Substrate – Amersham Biosciences, Buckinghamshire, UK
Electrophoresis protein gel – prepared in lab (appendix 5.1.7)
Epidermal growth factor (EGF) – PeproTech, Rocky Hill, USA
Fast EvaGreen qPCR Master Mix – Bio-Rad, Hercules, USA
Fetal bovine serum (FBS) – Invitrogen, Waltham, USA
Fibronectin – Thermo Fisher Scientific, Waltham, USA
Fixing buffer – prepared in lab (appendix 5.1.8)
Freezing media – prepared in lab (chapter 2.2.6)
Gel running buffer – prepared in lab (appendix 5.1.6)
GIPZ lentiviral shRNA vector – Thermo Fisher Scientific, Waltham, USA
Glycerol – Scharlau, Barcelona, Spain
Glycine – Sigma-Aldrich, St. Louis, USA
Goat serum – Invitrogen, Waltham, USA
Hoechst 33342 – Thermo Fisher Scientific, Waltham, USA
Horse serum – Invitrogen, Waltham, USA
Hydrocortisone – Sigma-Aldrich, St. Louis, USA
L-glutamine – Invitrogen, Waltham, USA
Laemmli sample buffer – prepared in lab (appendix 5.1.6)
Lipofectamine 2000 – Thermo Fisher Scientific, Waltham, USA
Lower buffer – prepared in lab (appendix 5.1.6)
Lysogeny broth (LB) – prepared in lab (appendix 5.1.3)
MEM non-essential amino acids – Invitrogen, Waltham, USA
NucleoBond Xtra Midi Plus – Machery-Nagel, Düren, Germany
NucleoSpin EasyPure – Machery-Nagel, Düren, Germany
PageRule prestained protein ladder – Thermo Fisher Scientific, Waltham, USA
Paraformaldehyde (PFA) – BDH, UK
Penicillin-streptomycin – Invitrogen, Waltham, USA
Permeabilisation buffer – prepared in lab (appendix 5.1.8)
Phosphate buffered saline (PBS) solution – prepared in lab (appendix 5.1.1)
Phosphate buffered saline (Dulbecco A) tablets – Oxoid Limited, Surrey, UK
PrimeScript RT Reagent Kit – Takara Bio, Otsu, Japan
Propan-2-ol – Scharlau, Barcelona, Spain
Propidium iodide (PI) – Thermo Fisher Scientific, Waltham, USA
psPAX2 lentiviral packaging plasmid – Addgene, Cambridge, UK
Puromycin – InvivoGen, San Diego, USA
Quick-RNA MiniPrep – Zymogen, Tustin, USA
Reduced serum media (optiMEM) – Thermo Fisher Scientific, Waltham, USA
Resazurin dye – prepared in lab (appendix 5.1.5)
RNAGEM Tissue Plus – Zygem, Auckland, New Zealand
Running buffer – prepared in lab (appendix 5.1.6)
Skim milk powder – Pams, Auckland, New Zealand
Super optimal broth with catabolite repression (SOC) – prepared in lab (appendix 5.1.4)
Sodium chloride – Scharlau, Spain
Sodium dodecyl sulfate (SDS) – Sigma-Aldrich, St. Louis, USA
Sodium pyruvate – Invitrogen, Waltham, USA
Stripping buffer – prepared in lab (appendix 5.1.6)
Tetramethylethylenediamine (TEMED) – Thermo Fisher Scientific, Waltham, USA
Top10 Dh5α *Escherichia coli* (*E. coli*) – Invitrogen, Waltham, USA
Transfer buffer – prepared in lab (appendix 5.1.6)
Tris-buffered saline and tween-20 (TBST) – prepared in lab (appendix 5.1.6)
Tris ultrapure – Applichem, St. Louis, USA
Triton X-100 – Sigma-Aldrich, St. Louis, USA
Tryptone – Scharlau, Barcelona, Spain
Tween-20 – Sigma-Aldrich, St. Louis, USA
Upper buffer – prepared in lab (appendix 5.1.6)
Vectashield Mounting Medium with DAPI – Vector Labs, USA
VSVG lentiviral envelope plasmid – Addgene, Cambridge, UK
Yeast extract – Merck, Darnstadt, Germany

### 2.1.2. Equipment

0.22 µm polyethersulfone vacuum filter system – Corning, Corning, Corning, USA
0.6 mL microtube – Axygen, Union City, USA
1 mL cryovial – Nunc, Rochester, USA
1 mL syringe – BD, Singapore
1.5 mL microtube – Axygen, Union City, USA
20 G needle – Terumo, Tokyo, Japan
6-well, clear, flat-bottom tissue culture plate – Greiner Bio-One, Frickenhausen, Germany
10 mL serological pipette – Greiner Bio-One, Frickenhausen, Germany
10 mL syringe – BD, Singapore
100x20 mm cell culture dish with vent – Greiner Bio-One, Frickenhausen, Germany
15 mL Falcon tube – BD Biosciences, San Jose, USA
22x22 mm glass cover slip – Menzel-Glaser, Germany
25 mL cell culture flask – Greiner Bio-One, Frickenhausen, Germany
25 mL serological pipette – Greiner Bio-One, Frickenhausen, Germany
50 mL falcon tube – BD Biosciences, San Jose, USA
5390 Centrifuge – Eppendorf, Hamburg, Germany
5810 R Centrifuge – Eppendorf, Hamburg, Germany
60 mL syringe – BD, Singapore
75 mL cell culture flask – Greiner Bio-One, Frickenhausen, Germany
75x25 mm Gold Seal glass slide – Thermo Fisher Scientific, Waltham, USA
96-well tissue culture plate 3603 – Corning, Corning, USA
7900HT Fast Real-Time PCR system – Applied Biosystems, Carlsbad, USA
Avanti J-25 Centrifuge – Beckman, Brea, USA
Centra 3C centrifuge – International Equipment Company, Oakbrook, USA
Cell culture incubator with carbon dioxide – Binder, Tuttlingen, Germany
Cell scraper – Corning, Corning, USA
CFX-Connect Real-Time System – Bio-Rad, Hercules, USA
Cytell Cell Imaging System – GE LifeSciences, Little Chalfont, USA
Double-chambered cell-counting slide – Bio-Rad, Hercules, USA
Eclipse Ti Inverted Microscope System – Nikon, Tokyo, Japan
Fuji LAS-3000 ECL Imaging System – Thermo Fisher Scientific, Waltham, USA
Immobilon-P 0.45 µm PVDF membrane – Merck Millipore, Darmstadt, Germany
Inoculating loop – Nunc, Rochester, USA
IncuCyte FLR – Essen BioScience, Ann Arbor, USA
MicroAmp Optical 384-well plate – Thermo Fisher Scientific, Waltham, USA
MicroAmp Optical 96-well plate – Thermo Fisher Scientific, Waltham, USA
Milli-Q Ultra Pure Water Purification System – Millipore, Darmstadt, USA
Mini-PROTEAN Cell – Bio-Rad, Hercules, USA
Mr Frosty 5100 Cryo 1°C Freezing Container – Thermo Fisher Scientific, Waltham, USA
Nanodrop ND-1000 Spectrophotometer – Nanodrop Technologies, Waltham, USA
Pasteur pipette – Hirschmann, Neckartenzlingen, Germany
Pipette aid – Thermo Fisher Scientific, Waltham, USA
POLARstar Microplate Reader – BMG, Berlin, Germany
PowerPac Basic 300 V Power – Bio-Rad, Hercules, USA
TC10 Automated Cell Counter – Bio-Rad, Hercules, USA
Trans-Blot Cell – Bio-Rad, Hercules, USA
Tissue culture hood – EMAIL, Melbourne, Australia
Water bath – Semco, Vista, USA

2.1.3. Software

4peaks – Mekentosj, USA
CellProfiler – Broad Institute, Cambridge, USA
Cytell BioApps – GE Healthcare Life Sciences, Little Chalfont, USA
ImageJ + CellCounter plugin – National Institute of Health, Bethesda, USA
IncuCyte software – Essen BioScience, Ann Arbor, USA
NIS Elements 4.10 – Nikon, Tokyo, Japan
Prism 6 – Prism Software, San Diego, USA
2.1.4. Cell lines

Isogenic MCF10A (CDH1+/+ & CDH1−/−) cell line – Sigma-Aldrich, St. Louis, USA

HEK293FT cell line – donated by Dr Stephanie Hughes, Otago University, New Zealand

2.2. Cell culture

2.2.1. Cell lines

Isogenic (WT & CDH1−/) MCF10A breast cell lines were obtained from Sigma-Aldrich. CDH1 knockout was created by a 4 base-pair homozygous deletion in exon 11 (extracellular adhesion domain) by zinc finger nuclease editing, confirmed by RNAseq analysis (Beetham, 2014). HEK293FT (Human embryonic kidney cell, transformed with SV40 large T antigen) cell line was used for lentiviral particle production (chapter 2.5.3).

Upon receipt, lines were thawed and cells from early propagation were aliquoted and frozen in liquid nitrogen (chapter 2.2.6). Experiments with MCF10A cells were conducted with cells between passage 6 and 15. HEK293FT cells were used between passage 8 and 20.

2.2.2. Recovery

An aliquot of cells (chapter 2.2.6) was thawed in a 37°C water bath and resuspended in 9 mL of pre-warmed complete growth media. Cells were pelleted by centrifugation at 1000 rpm for 5 mins and the supernatant was discarded. Pellet was resuspended in 4.5 mL of complete media (chapter 2.2.3). Cells were transferred to a T25 cell culture flask and transferred to the incubator at 37°C with 5% CO₂. Media was replaced with warm, complete media at 24 hrs to remove any trace DMSO and any non-adhered cells.
2.2.3. Maintenance

MCF10A cells were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) supplemented with 5% horse serum, 20 ng/mL EGF, 0.5 µg/mL hydrocortisone, 100 ng/mL cholera toxin and 10 µg/mL insulin. Pre-mixed media was filter-sterilised using a 0.22 µm polyethersulfone filter and dispensed into 50 mL aliquots. HEK293FT cells were maintained in DMEM media supplemented with 10% FBS, 6 mM L-glutamate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 100 µg/mL penicillin-streptomycin. Pre-mixed media was filter-sterilised and aliquoted as above. Cell culture manipulations were performed under sterile conditions using aseptic technique in a class II hood. Flasks were maintained in a 37°C humidified chamber with 5% CO2.

2.2.4. Passage

Cells were passaged at 72-96 hr intervals while sub-confluent. Solutions of phosphate buffered saline (PBS), trypsin (0.05% for MCF10A; 0.025% for HEK293FT), and complete growth media were pre-warmed in a 37°C waterbath prior to passage. Media was aspirated and discarded, cells were gently washed with PBS, PBS was aspirated and discarded. Trypsin was gently added to the flask (1.5 mL for T25; 4.5 mL for T75) and returned to the incubator for 20 mins for MCF10A; 10 mins for HEK293FT. Following detachment, trypsin was neutralised with 5 mL complete media and the flask contents transferred to a 15 mL tube. Cells were pelleted by centrifugation at 1000 rpm for 5 mins. The supernatant was removed and the cells were resuspended in 1-5 mL complete media (depending on application). MCF10A cell density was determined (chapter 2.2.5) and cells were reseeded in new flasks at a density of 1.0 x 10^5 for WT and 1.5 x 10^5 for CDH1^-/- (3.0 x 10^5 and 4.5 x 10^5, respectively, for T75) and returned to the incubator. HEK293FT cells were split 1 in 6 for serial passage. Multiple T75 flasks were seeded for experiments requiring many cells.
2.2.5. Cell counting

During passage (chapter 2.2.4), pelleted cells were thoroughly resuspended in 1-5 mL of complete media. 10 µL of the cell suspension was pipetted onto a dual-chambered counting slide for cell counter and the cell density determined with a TC10 Automated Cell Counter.

2.2.6. Freezing

Cells were preserved in liquid nitrogen in freezing media. MCF10A freezing media comprised of 70% complete media, 20% horse serum and 10% DMSO; and HEK293FT freezing media comprised of 90% complete media and 10% DMSO. Following passage (chapter 2.2.4), cells were pelleted and resuspended at 1 x 10^6 cells/mL in freezing media. Aliquots of 1 x 10^6 cells were transferred into cryovials and placed in a Mr Frosty or 5100 Cryo 1°C Freezing Container in a -80°C freezer. Cells were refrigerated overnight before being transferred into liquid nitrogen for long-term storage.

2.3. Characterisation of isogenic MCF10A cell lines

2.3.1. Western blot

2.3.1.1. Protein lysate preparation

Cells were seeded into a 6-well cell culture dish in complete media at 1x10^5 per well and harvested prior to reaching confluence. Media was aspirated and the cells washed once with PBS. Cells were lysed with 1.5 mL 1X passive lysis buffer (5X Cell Culture Lysis Buffer in PBS). To maximise lysis, cells were scraped with a sterile cell scraper before being transferred to a microcentrifuge tube. Debris was pelleted by centrifugation at 2000 rpm for 5 mins, the lysate was transferred to a fresh tube and stored at -20°C until use within one month of preparation. On the day of blotting, sample was thawed on ice before a
bicinchoninic acid (BCA) assay was performed to determine protein concentration.

2.3.1.2. SDS-PAGE gel preparation

Electrophoresis gels were prepared with two components: resolving gel and stacking gel. For the resolving gel, 2.5 mL 4X lower buffer (appendix 5.1.6) and 3.3 mL 30% pre-mixed 37.5:1 bis:acrylamide was added to 4.2 mL mQH₂O. 100 µL ammonium persulfate (APS) and 10 µL tetramethylethylenediamine (TEMED) was added. The mixture was carefully poured into a gel mould and covered with 1 mL of isopropanol to prevent desiccation. Prior to adding the stacking gel, isopropanol was carefully poured off and the gel rinsed with mQH₂O. For the stacking gel, 1 mL 4X upper buffer (appendix 5.1.6) and 0.65 mL 30% pre-mixed 37.5:1 bis:acrylamide was added to 2.35 mL mQH₂O. 30 µL APS and 10 µL TEMED was added and the mixture carefully poured on top of the resolving gel. A 12 lane gel comb was used, and complete gels were used immediately or otherwise stored airtight in moist tissue paper at 4°C.

2.3.1.3. Gel electrophoresis

Protein samples were mixed 4:1 with 5X DNA loading buffer (appendix 5.1.6) and boiled at 95°C for 5 mins. Samples were centrifuged at 16k rpm for 1 min to remove bubbles and pellet residual debris. 25 µg of protein, as determined by BCA assay, was loaded into each lane of an SDS-PAGE gel. 10 µL of PageRuler Prestained Protein Ladder was also loaded onto at least one outer lane. Loaded gel was transferred to gel apparatus and running buffer (appendix 5.1.6) was added as per instructions of the apparatus. Size separation electrophoresis was carried out at a constant 200 v for 1 hr.

2.3.1.4. Protein transfer

Polyvinylidene difluoride (PVDF) membrane was cut to shape, briefly activated in 100%
methanol, and washed once with mQH$_2$O. Protein transfer sandwich components (membrane, fibre pads and filter paper) were soaked in 1X transfer buffer (appendix 5.1.6) for 15 mins prior to assembly. The transfer sandwich was assembled as per the manufacturers instructions and put in to the transfer tank. The assembly was submerged in transfer buffer and the protein transferred at 100 v for 1 hr. Membrane was washed for 5 mins with mQH$_2$O following transfer.

2.3.1.5. Antibody incubation and imaging

The membrane was washed for 10 mins three times in Tris-buffered saline and tween-20 (TBST; appendix 5.1.6). To block non-specific binding, the membrane was blocked with 5% milk powder in TBST at RT for 1 hr. The membrane was incubated on a shaker at 4°C overnight with primary antibody diluted in TBST (see appendix 5.2 for antibody dilutions). Membrane was washed for 10 mins three times after primary incubation to remove any unbound antibody. The membrane was incubated on a shaker at RT for 1 hr with secondary antibody diluted in TBST. The membrane was incubated with enhanced chemiluminescence (ECL) reagent as per the manufacturers instructions and imaged using the Fuji ECL Imaging System.

2.3.1.6. Strip and reprobe

Following imaging of the primary probe, membrane was stripped of bound antibodies using stripping buffer (appendix 5.1.6) at 50°C for 1 hr. The membrane was then washed with mQH$_2$O at RT for 1 hr. Antibody incubation and imaging was repeated with secondary probe for the loading control. Two negative controls were included without primary and secondary antibody, respectively.

2.3.2. Immunofluorescence
2.3.2.1. Cell preparation

To aid adhesion, sterile 22x22 mm cover slips were placed into a 6-well cell culture dish and incubated in 200 µg/mL fibronectin (in PBS) at RT for 60 mins. Fibronectin was aspirated and 1 x 10⁶ cells were seeded per well and incubated in 37°C with 5% CO₂ for at least 16 hours to allow for complete cell adherence.

2.3.2.2. Fixing/treatment

Blocking and antibody diluting buffers were made up fresh and all steps were performed at RT. Cells were washed three times with ~5 mL PBS prior to fixing. Cells were fixed in 2 mL of 4% paraformaldehyde (PFA; appendix 5.1.8) in the dark for 20 mins. Cells were washed again two times with PBS after fixing and then permeabilised with 2 mL of permeabilisation buffer (0.5% Triton in PBS) for 10 mins. To block non-specific binding, the cells were incubated in 2 mL of blocking buffer (5% secondary matched serum, 1% FBS in PBS) for 60 mins.

2.3.2.3. Antibody incubation

Antibodies were diluted in antibody diluting buffer (appendix 5.2 for dilutions) and 40 µL of the mixture was pipetted onto coverslips. Coverslips were then inverted in a 6-well plate and kept moist in the dark for 1 hr at RT or overnight at 4°C. To remove unbound antibody, the coverslips were washed three times in PBS prior to secondary incubation. Secondary antibody incubation was performed as above except the incubation was strictly in the dark for 1 hr at RT.

2.3.2.3. Mounting

To preserve signal strength and to stain nuclei, coverslips were mounted on glass microscope slides with one drop of Vectashield mounting medium (with DAPI). Slides were
air-dried at RT for ~20 mins, excess stain wiped away, and stored in the dark at 4°C. Two negative controls were included without primary and secondary antibody, respectively.

2.3.2.4. Imaging

Images were captured on the Eclipse Ti Inverted microscope using the fluorophore-appropriate filter. Images were edited and adjusted using ImageJ.

2.3.3. qPCR

2.3.3.1. RNA extraction

RNA was extracted using RNAGEM Tissue Plus RNA extraction kit according to the manufacturers instructions for adherent cells, including DNase treatment. 6-, 24-, and 96-well formats were used, requiring 200, 50, and 20 µL of extraction mixture. RNA was immediately synthesised into cDNA (chapter 2.3.3.2) while surplus RNA was stored at -80°C.

2.3.3.2. cDNA synthesis

cDNA was synthesised using Primescript RT Reagent kit immediately following RNA extraction (chapter 2.3.3.1), according to the manufacturers instructions for SYBR Green assay (reaction mixture in appendix 5.4). cDNA synthesis was performed using a PCR block for 15 mins at 37°C, followed by a 5 sec deactivation step at 85°C. No RNA and no reverse transcriptase (RTase) controls were included. cDNA was immediately analysed with qPCR (chapter 2.3.3.3) or stored at -20°C if used the next day. Surplus cDNA was stored at -80°C.

2.3.3.3. qPCR

qPCR was performed immediately following cDNA synthesis using Evagreen DNA Binding
Dye as per the manufacturers instructions. 6.5 and 13 µL reactions were performed in 384- and 96-well optically clear reaction plates, respectively (table 5.4), using 7900HT Sequence Detection System and CFX-Connect Real-Time System, respectively. cDNA samples were diluted 1/5 in mQH$_2$O. A standard curve was generated using existing and constant cDNA synthesised from pooled RNA from multiple cell lines.

Using existing RNAseq data (Beetham et al., 2014), reference genes were selected upon the following criteria being met: (1) high expression (>10,000 normalised reads); and (2) consistent expression (0.93 > ∆fold-change > 1.10) across isogenic MCF10A cell lines. qPCR was performed with GAPDH, PPIA, and RPL13a as reference genes to normalise expression. MAST2 and MAP1B primers (appendix 5.3) were designed to span introns to mitigate gDNA contamination. qPCR amplification steps included an initial melting and Taq polymerase activation step for 3 mins at 95˚C, followed by 40 cycles of 5 sec at 95˚C, 10 sec at 55˚C, and 30 sec at 72˚C. No cDNA control and both controls from cDNA synthesis (chapter 2.3.3.2) were used and each reaction was performed in triplicate.

2.3.3.4. Analysis

GAPDH, RPL13a, and PPIA cycle threshold (Ct) were used to normalise mRNA expression. Efficiency was determined using a standard curve as described by (Larionov, 2005). Fold change was calculated using the method described by Pfaffl (2001). With high fidelity primers, DNA content within a sample is exactly duplicated, hence log$_2$ fold-changes can be used to back-calculate relative expression. Relative expression ratio is calculated from qPCR efficiencies and the crossing point of a sample versus a control.

Efficiencies calculated between 1.95 and 2.05 were acceptable, and Cts later than 38 were ignored. All experiments included a no cDNA control and a no-template cDNA control.
2.4. Transfection

pGIPZ shRNA library was obtained in *E. coli* Dh5α bacterial glycerol stocks from Dhharmacon. For each candidate SL gene, between 3 and 10 clones containing unique shRNA sequences were obtained. See appendix 5.5 for shRNA sequence list.

2.4.1. pGIPZ shRNA vector maps

![pGIPZ vector map](image)

<table>
<thead>
<tr>
<th>Feature</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCMV</td>
<td>Human cytomegalovirus promoter drives strong transgene expression</td>
</tr>
<tr>
<td>tGFP</td>
<td>TurboGFP reporter for visual tracking of transduction and expression</td>
</tr>
<tr>
<td>Puro^®</td>
<td>Puromycin resistance permits antibiotic-selective pressure and propagation of stable integrants</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry site allows expression of TurboGFP and puromycin resistance genes in a single transcript</td>
</tr>
<tr>
<td>shRNA</td>
<td>microRNA-adapted shRNA (based on miR-30) for gene knockdown</td>
</tr>
<tr>
<td>5’ LTR</td>
<td>5’ long terminal repeat</td>
</tr>
<tr>
<td>3’ SIN LTR</td>
<td>3’ self-inactivating long terminal repeat for increased lentivirus safety</td>
</tr>
<tr>
<td>Ψ</td>
<td>Psi packaging sequence allows viral genome packaging using lentiviral packaging systems</td>
</tr>
<tr>
<td>RRE</td>
<td>Rev response element: enhances titer by increasing packaging efficiency of full-length viral genomes</td>
</tr>
<tr>
<td>WPRE</td>
<td>Woodchuck hepatitis posttranscriptional regulatory element enhances transgene expression in the target cells</td>
</tr>
</tbody>
</table>

**Figure 2.1. pGIPZ lentiviral shRNA vector map with features.** Image supplied in manufacturers catalogue.
2.4.2. Competent cells

Chemically-competent Dh5α *E. coli* cells were produced by the ice-cold calcium chloride method described by Inoue (1990) in house. Dh5α cells were streaked onto LB agar and incubated at 37°C overnight. A single colony was isolated and transferred to 100 mL LB and incubated at 37°C with agitation at 300 rpm for 3 hrs. The culture was crash cooled on ice for 10 mins before pelleting by 4k rpm centrifugation for 10 mins at 4°C. The supernatant was discarded and cells were resuspended in 10 mL ice cold 0.1 M CaCl₂ while maintained on ice throughout the protocol. Cells were pelleted as above and then resuspended in 2 mL ice cold 0.1 M CaCl₂. 70 µL glycerol was added, mixed and stored for 15 mins before a further 70 µL glycerol was added. Chemically competent cells were aliquoted, snap frozen in liquid nitrogen and stored at -70°C.
2.4.3. Heat-shock transformation

Frozen chemically competent cells (chapter 2.4.1) were thawed on ice. 10 µL of cells, together with no more than 5 µL containing no more than 100 ng of plasmid, were mixed by flicking in a 1.5 mL microcentrifuge tube and were incubated on ice for 20 mins. Tubes were heat shocked at 42°C for 1 min followed by 37°C for 3 mins before being chilled on ice for 2 mins. 250 µL of SOC (appendix 5.1.4) was added to the tube and incubated at 37°C for 45 mins with agitation. Due to varying competence, transformed cells were spread on LB agar plates at volumes of 1, 50, and 200 µL.

2.4.4. Propagation of bacterial culture

Glycerol stocks of Dh5α E. coli harbouring pGIPZ were streaked onto LB agar plates (appendix 5.1.3) containing 100 µg/mL ampicillin using aseptic technique and incubated overnight at 37°C. To ensure a clonal population, single colonies were isolated using a sterile loop and inoculated into LB (appendix 5.1.3) with 100 µg/mL ampicillin. LB did not exceed one third of the maximum volume of the vessel. For cultures over 100 mL, a 1 mL starter culture was incubated for 4 hrs without antibiotic before being transferred to larger vessel with antibiotic. Cultures were incubated at 37°C overnight or until an appropriate optical density (~1.0 Abs at 600 nm) was reached.

2.4.5. Plasmid extraction

To test which plasmid extraction kit would suit the project, a variety of kits were tested. The NucleoBond Xtra MidiPrep (Machery-Nagel) was selected from a number of potential kits (appendix 5.1.9) for moderate plasmid requirements (individual shRNA clones), while the PureLink MaxiPrep (Invitrogen) was selected for larger requirements (control shRNA clones). Kits were used as per the manufacturers instructions, however, elution buffer was slightly warmed prior to the elution step.
2.4.6. Plasmid quantitation

Plasmid was quantified using NanoDrop and Epoch as per the manufacturers instructions. Zero-calibration was performed using the respective elution buffer (chapter 2.4.5).

2.4.7. Sanger sequencing

To assess the validity of the plasmid, 200 ng of pGIPZ template and 3.2 pmol of pGIPZ insert sequence primer was made up to 5 µL with mQH₂O and submitted to Genetic Analysis Services for sequencing. Analysis was carried out using 4Peaks software and publicly available BLAST database (NCBI).

2.4.8. Neon transfection

The Neon Transfection System (Invitrogen, Waltham, USA; hereon Neon) is an electroporation method adapted from the novel capillary and electrode-based approach from Kim et al. (2008) that claims high efficiency. The basis of electroporation is the application of an electric field to cells to promote physical displacement of lipid molecules, opening a pore through the bilayer to increase the permeability of the cell membrane. As the pore fills with water to create a pathway, charged molecules like DNA can passively diffuse into the cell.

At sub-confluence, cells were passaged (chapter 2.2.4) and transfection was carried out as per the manufacturers instructions for adherent and suspension cell lines (Resuspension Buffer R; supplied) with 100 µL Neon Tips (Electrolytic Buffer E2; supplied). Neon transfection conditions were initially optimised with 5 µg plasmid and 2 x 10⁵ cells, pulsed 3 times at 1600 v for 10 ms (chapter 3.2). For a complete list of conditions tested, see appendix. Optimal conditions were 30 µg pGIPZ plasmid and 4 x 10⁵ cells. Neon Tips were changed after each transfection.
2.5. Lentiviral packaging and transduction

2.5.1. Lentiviral packaging plasmid maps & resource

Figure 2.3. VSV-G lentiviral glycoprotein envelope plasmid detailed vector map. From biovisualtech.

Figure 2.4. psPAX2 lentiviral packaging plasmid detailed vector map. From biovisualtech.
Figure 2.5. Lentiviral packaging overview. (made by the author of this thesis)

ReTrovirus = RTase = RNA genome
γ-retrovirus targets dividing cells; lentivirus targets both dividing/non-dividing cells
replication-incompetent recombinant LV = no life cycle, just payload delivery for controlled 'dosing'

Overview of WT lentiviral genome and plasmid components for producing recombinant particles

Envelopε plasmid (pVSVG)
vesicular stomatitis virus G protein (envelope glycoprotein, env)
no Ψ signal = no packaging of envelope/packaging plasmid RNA into lentivirus particle
minimal genome lacking homology to reduce recombination (true for all plasmids)
produces VSV glycoprotein that becomes the outer envelope of the lentivirus

Packaging plasmid (psPAX2)
contains gag (encodes viral capsid, matrix, nucleocapsid)
pol (RTase, Pol III, protease)
rev (binding of Rev to viral RNA containing Rev response element (RRE) allows
mRNA export out of the nucleus by a mechanism different to that of cellular mRNA)
tat (unstalls env transcription)
produces viral core, enzymes (RTase, Pol, protease, integrase), and initiates signal to package

Transfer plasmid (pGIPZ)
shRNA-containing plasmid – the payload
produces one big polycistronic transcript using WT
5' LTR as promoter
Ψ signal (RNA 2’. structure) is recognised for
transcript to be packaged into particles
contains long 3’ and 5’ LTRs to facilitate
integration
tGFP, puromycin resistance markers

pGIPZ → long RNA transcript |5’ LTR| Ψ| GFP| puroR| shRNA| 3’ LTR| packaged into virus
delivery by transduction, RNA → dsDNA by RTase, integration by LTRs/integrase, switches
to hCMV promoter, drives stable tGFP, puroR & shRNA expression
→ stable RNAi with selectable GFP and puromycin markers
2.5.2. Plasmid extraction

VSVG, PAX2, and GIPZ plasmids were prepared from LB cultures as described above (chapter 2.4.4-5). NucleoSpin MiniPrep kit was used for individual shRNA plasmids while the NucleoBond Xtra MidiPrep kit was used for plasmids required in higher amount (VSVG, PAX2, control shRNA). Overall, lentiviral packaging requires significantly less plasmid than Neon transfection – the plasmid titre from one technical replicate of Neon can produce enough virus to perform at least 10 viral replicates, up to 20 if aliquoted appropriately.

2.5.3. Lentiviral packaging

The key elements of pGIPZ, including shRNA and cell markers, are expressed together and flanked by a Psi packaging sequence and LTRs. The RNA molecule/'genome' is packed into lentiviral particles in the presence of packaging plasmids that produce coat proteins and elements for RNA integration (ie, RTase, integrase, etc). Once delivered, the pGIPZ RNA is reverse transcribed and integrated into the target cell genome, commencing expression of shRNA and markers.

HEK293FT cells were seeded at a density of $5.4 \times 10^6$ cells in 10 mL complete growth media in a T75 flask. Allowing 8 mL per flask, optiMEM supplemented with 5% FBS was filtered through a 0.22 µm filter and warmed to 37°C. Prior to making a transfection mixture, all plasmids were quantitated using the NanoDrop (chapter 2.4.6). 24 hrs post-seeding, a transfection mixture of 18.6 µg of shRNA plasmid (or control), 9.6 µg PAX2, and 4.8 µg of VSVG was filtered through a 0.22 µm hydrophillic filter directly into a 1 mL mixture of filtered optiMEM containing 55.8 µL Lipofectamine 2000 and incubated at RT for 20 mins. HEK293FT media was aspirated and the cells washed with PBS, taking care not to dislodge cells. 8 mL of 5% FBS/optiMEM was added slowly and the transfection mixture
added dropwise.

24 hrs post-transfection, growth media was aspirated, cells washed with PBS, and 6 mL of pre-warmed, complete growth media was added. 48 hrs post-transfection, HEK293FT growth media containing lentiviral particles was transferred to 15 mL falcon tubes. Media was centrifuged at 3000 rpm for 15 mins and filtered through a 0.45 µm PVDF filter. Lentiviral particles were dispensed into 500 µL aliquots and immediately frozen at -80°C. For each subsequent lentiviral experiment, an aliquot was only used once and then discarded to mitigate repeated freeze-thaw cycles.

2.5.4. Lentiviral titration

Functional viral titre was determined by performing a 2-fold dilution series using unconcentrated viral particles that were put aside during viral packaging. MCF10A WT cells were seeded at 4 x 10^3 cells per well in a black-walled, clear-bottom 96-well plate in 100 µL of complete media. Cells were transduced with 100 µL of each viral serial dilution to give a final dilution range of 1/2 to 1/32. Mock transduction containing only complete media was used as the negative control to confirm the absence of any endogenous fluorescent signal.

At 24 hrs post-transduction, growth media was aspirated and replaced with 100 µL of pre-warmed, complete MCF10A media. 48 hrs post-transduction, 5 images per well were captured at 10x magnification using 570 nm filter/GFP channel on the Eclipse Ti Inverted Microscope System. For further titration analysis, only images from 1/32 final dilution were used as it was determined that this dilution gave approximately 50% transduction efficiency. ~50% transduction efficiency was used to increase counting accuracy, as lower titres increase relative error and higher titres increase multiple particle uptake by cells.
Counting of transduced, GFP-expressing cells was performed using ImageJ with the Cell Counter plugin. The average number of transduced cells per field was determined across 5 fields. This number was extrapolated to estimate the average number of transduced cells per well by adjusting with a factor of well:field size (~46.7; figure 2.6). Back calculation using transduced cells/well, correcting for dilution and volume, was used to determine the number of transducing units (TU)/mL.

![Figure 2.6. Cytell field surface area to well surface area adjustment.](image)

One field captured at 10x on Cytell 10x image field fits ~46.7 times into a well from a Costar 3603 96-well plate.

2.5.5. Multiplicity of infection

Optimal multiplicity of infection (MOI) needs to achieve high transduction efficiency while minimising cell death. The optimal MOI was determined by performing a dilution series using virus that had previously been titred. At 48 hrs post-transduction, viability by total nuclei count (chapter 2.7.3) and transduction efficiency were assessed. An initial indication of optimal MOI was up to 15, while any higher lead to early cell death.

2.5.6. Lentiviral transduction

MCF10A WT and MCF10A $CDH1^{−/−}$ cells were seeded at 1,000 cells per well in a black-walled, clear-bottom 96-well plate in 100 µL of complete media 24 hrs prior to transduction. Immediately prior to transduction, lentivirus stock was thawed in a 37°C water bath and diluted in complete media to adjust the titre. Cells were transduced with 100 µL of
lentivirus diluted in complete media at an MOI of 15 per seeded cell. Each isogenic cell line included mock (cell only) transduction and non-silencing shRNA controls.

At 24 hrs post-transduction, growth media was aspirated and replaced with 100 µL pre-warmed, complete media containing 1 µg/mL of the selective agent puromycin. Puromycin was omitted from cell only negative control. At 72 hrs post-transduction, cell viability was assessed as described in chapter 2.7.2-4. Following viability assessment, mRNA knockdown was confirmed by qPCR (chapter 2.3.3).

2.6. Drug inhibition of Aurora kinases

2.6.1. Drug suspension

Drugs were reconstituted in DMSO at 80 mM, aliquoted and stored at -80˚C. Aliquots were thawed prior to use and resuspended to 1 mM with complete media. Each aliquot was only used once.

2.6.2. EC\textsubscript{50} curve

EC\textsubscript{50} is the effective concentration of a drug that produces the half-maximal (ie, 50%) response. MCF10A WT and MCF10A CDH1\textsuperscript{-/-} cells were seeded at 4,000 cells per well in a black-walled, clear-bottom 96-well plate (Costar 3603) in 100 µL of complete media. 24 hrs post-seeding, cells were treated with 10 µL of 11X final drug concentration to yield a 1X drug concentration. An 8-point, 10-fold dilution series was performed for each drug, ranging between 100 µM and 10 pM. At 48 hrs post-treatment, viability was assessed by nuclei counting (chapter 2.7.3). EC\textsubscript{50} was calculated using non-linear regression in Prism 6.0.

DMSO concentration-matched controls were included, except where DMSO concentration
was under 0.05% as the effects are negligible (data not shown). A drug free negative control and high concentration cytotoxic (100 nM taxol) positive control was included in all drug experiments.

2.6.3. IncuCyte time course confluence assay

MCF10A WT and MCF10A CDH1−/− cells were seeded at 4,000 cells per well in a black-walled, clear-bottom 96-well plate (Costar 3603) in 100 µL of complete media 24 hrs prior to treatment and treated with 10 µL of 11X final drug concentration to yield a 1X drug concentration. Following treatment, plates were transferred to the IncuCyte Imaging System, which measures total cell surface area as a proxy measure of viability. Imaging was performed every 2 hrs over 48 hrs from treatment to end-point nuclei counting (chapter 2.7.3). Data was analysed using IncuCyte software. At 48 hrs post-treatment, end-point cell viability was assessed (chapter 2.7). This imaging does not require dyes or additional reagents, and thus the same experimental plate can be further assessed with end-point metabolic assay or total nuclei count.

2.6.4. Cell cycle analysis

Cell cycle analysis was performed at 10x magnification on the cell cycle BioApp on the Cytell Cell Imaging System. Gating thresholds were optimised as required for each experiment and cell cycle stage data was output as .csv files.

2.7. Viability

2.7.1. Manual cell counting

For optimisation of Neon transfection, total and GFP-positive cells were manually counted from 4x images captured with Eclipse Ti Inverted microscope. Images were counted in ImageJ with assistance from the CellCounter plugin.
2.7.2. Metabolic assay
At 72 hrs post-transduction, viability was assessed by oxidation/reduction-based indication of total ATP/metabolic capacity. Resazurin is a blue, weakly fluorescent dye that is irreversibly reduced to a pink/red, highly fluorescent resorufin in the presence of ATP/reducing power. 10% well-volume of Resazurin dye (appendix 5.1.5) was added to cells in a 96-well plate and incubated in the dark at RT for 30 mins. Following incubation, plates were scanned using the Optima POLARStar according to the manufacturer's instructions. Gain was adjusted to maximise the sensitive range for the experiment.

2.7.3. Total nuclei counting
48 hrs post-treatment or 72 hrs post-transduction, viability was assessed by measure of total nuclei counts. 100 µL of staining mixture containing 1 µg/mL Hoechst 33342 and 0.5 µg/mL propidium iodide (PI) in PBS was added to each well and incubated in the dark at RT for 30 mins. At least 10 fields of view were automatically imaged at 10x magnification using the cell viability BioApp on the Cytell Cell Imaging System. DAPI channel was used to count cells stained by Hoechst. PI-stained cells were counted in the Cy3 channel. Parameters for nuclei detection were optimised as required for each experiment.

2.7.4. Viability analysis
Experimental conditions were normalised to their respective controls (non-silencing for lentiviral transduction and concentration-matched DMSO for drug treatment only if DMSO > 0.05%) within each cell line. Standard deviation was calculated across technical replicates. Standard error was calculated across biological replicates.

2.8. Statistical analysis
Because the effects of silencing candidate genes in both cell lines was unknown, a test of statistical significance was performed using a Student's two-tailed T-test.
3. Results

The primary aim of this research was to validate the presence of synthetic lethal interactions between \textit{CDH1} and cytoskeletal-associated genes identified via high-throughput screening (Telford \textit{et al.}, 2015). The approach is summarised in four steps: characterisation of isogenic MCF10A cell lines, optimisation and testing of Neon Transfection System, lentiviral knockdown of candidates, and drug inhibition of Aurora kinases.
3.1. Characterisation of isogenic MCF10A cell lines

Our laboratory has previously published on the characteristics of the isogenic MCF10A cell line with and without $CDH1$ expression (Chen et al., 2014). This chapter describes additional validation and characteristics carried out by the candidate in isogenic MCF10A cell lines. MCF10A has normal epithelial characteristics including anchorage-dependent growth and has minimal chromosomal arrangements and high genomic stability (Soule et al., 1990). The normal genetic background is an important factor for an early cancer model, as cell lines derived from advanced cancers have often acquired additional genetic changes.
3.1.1. Morphology

Loss of E-cadherin leads to visible phenotypic change in MCF10A morphology. While wild-type (WT) cells grow to full confluence in monolayer culture, $CDH1^{-/}$ cells never reach confluence but rather form islands that never fully close (figure 3.1). Other changes include extended lamellipodia in $CDH1^{-/}$ cells. (figure 3.1.c). Because E-cadherin indirectly interacts with integrins to form focal adhesions, the observation is likely a result of altered migration, as has been previously described (Chen et al., 2014).

![Figure 3.1. Differential growth morphology between isogenic MCF10A. Cell monolayers are approximately 50% confluent 48 hrs after passage. (a) 4x WT, (b) 10x WT, (c) 20x WT; (d) 4x $CDH1^{-/}$, (e) 10x $CDH1^{-/}$, (f) 20x $CDH1^{-/}$. $CDH1^{-/}$cells grow in subconfluent clumps and show larger lamellipodia (arrow) than their WT counterparts.](image-url)
3.1.2. Western blot

To confirm the absence of E-cadherin in the CDH1\(^{-/-}\) cell line, a western blot (chapter 2.3.1) was carried out using a specific primary antibody (Ab) against E-cadherin (sc-7870) and a secondary Ab conjugated to a peroxidase enzyme which allows detection by chemiluminescent methods. Western blot analysis showed a 135 kDa E-cadherin protein in the MCF10A WT lysate, while the same band was not observed in the CDH1\(^{+}\) lysate (figure 3.2a), confirming the absence of E-cadherin in the CDH1\(^{-/-}\) cell line. β-actin was used as a loading control to show even loading across all the wells (figure 3.2.b).

Figure 3.2. Western blot of E-cadherin in isogenic MCF10A. (A) E-cadherin (135 kDa), and (B) β-actin (40 kDa). CDH1\(^{+}\)cell line lacks any detectable E-cadherin protein, while it is present in WT. Chemiluminescent exposure time was 30 s for both probes.
3.1.3. Immunofluorescence

To confirm the absence of E-cadherin in the $CDH1^{-/-}$ cell line, immunofluorescent imaging (chapter 2.3.2) was carried out using a specific primary Ab against E-cadherin (sc-7870). The secondary Ab is conjugated to a fluorophore to allow imaging of the sub-cellular presence and localisation of E-cadherin).

![Immunofluorescent imaging of isogenic MCF10A cells.](image)

**Figure 3.3. Immunofluorescent imaging of isogenic MCF10A cells.** E-cadherin is present in WT cells and absent in $CDH1^{-/-}$ cells. Primary E-cadherin antibody (sc-7870) targets histidine-108. (a) WT DAPI, (b) WT E-cadherin sc-7870, (c) a & b overlay; (d) $CDH1^{-/-}$ DAPI, (e) $CDH1^{-/-}$E-cadherin sc-7870, (f) d & e overlay;

As determined by Western blotting, $CDH1^{-/-}$ cells have no E-cadherin present (figure 3.3). E-cadherin is predominantly localised at the cell membrane, consistent with its role as a transmembrane adhesion protein. Furthermore, immunofluorescence targeting $\alpha$-tubulin
was performed to investigate any differences in cytoskeletal organisation between the isogenic MCF10A cell lines.

**Figure 3.4. Immunofluorescence imaging of MCF10A microtubules.** Image f shows \( CDH1^{-/-} \) cells lacking microtubule integrity. Primary α-tubulin-specific antibody (T6199) targets and is detected by secondary fluorophore conjugated antibody. (a) DAPI WT, (b) WT E-cadherin sc-7870, (c) WT α-tubulin T6199; (d) \( CDH1^{-/-} \) DAPI, (e) \( CDH1^{-/-} \) E-cadherin sc-7870. (f) \( CDH1^{-/-} \) α-tubulin T6199.

While the WT cell line displays coherent microtubule (MT) organisation at the centrosome and poles, \( CDH1^{-/-} \) cells have less organised MTs (figure 3.4; figure 1.12). This is consistent with similar work observing disrupted microtubule organisation following E-cadherin disruption (Yap et al., 2009), and provides early evidence that the cytoskeleton may be a synthetic lethal vulnerability in \( CDH1 \)-deficient cells.
3.1.4. qPCR

In conjunction with direct protein detection methods, qPCR (chapter 2.3.3) was used to confirm E-cadherin knockout at the mRNA level. \( CDH1 \) mRNA was significantly \((p < 0.0001)\) reduced in \( CDH1^{-/-} \) cells when compared to WT cells (figure 3.5).

Unlike the E-cadherin protein in \( CDH1^{-/-} \) cells, expression of E-cadherin mRNA is not null. The mRNA is still transcribed, although a truncated peptide is translated and probably rapidly degraded. As the transcript contains a frameshift mutation, it will be rapidly degraded, through what is termed nonsense-mediated mRNA decay. mRNA decay may be influenced and maintained via epigenetic modification (Anderson et al., 2009). This qPCR data is supported by exome sequencing data (Beetham et al., 2014), showing the \( CDH1^{-/-} \) cell line retains transcription of \( CDH1 \), but at low abundance.

**CDH1 mRNA expression**

**Figure 3.5. qPCR of CDH1 expression in isogenic MCF10A cells.** \( CDH1 \) mRNA expression in the MCF10A WT and \( CDH1^{-/-} \) cell lines \((p < 0.0001)\). Error bar indicates +/- 1 standard deviation, as derived from 3 technical replicates.
3.2. Optimisation and testing of Neon Transfection System

The primary aim of this research was to validate the presence of synthetic lethal interaction between \textit{CDH1} and the cytoskeletal genes \textit{MAST2} and \textit{MAP1B}. This was performed by introducing gene silencing constructs against candidate genes into isogenic MCF10A cells and observing the effect on viability.

In order to validate the presence of synthetic lethal interactions, candidate genes were transiently silenced by constitutive shRNA vectors. The first objective was to establish optimal conditions for the delivery of shRNA expression vectors into MCF10A cells by electroporation. A balance between transfection efficiency and the maintenance of sufficient cell survival rates was important. The approach taken can be summarised in two steps: optimisation of conditions for delivery of shRNA plasmid by electroporation, and determining the cause of the observed lethality in isogenic cells.

Different electroporation conditions were tested in isogenic cell lines and the electroporation efficiency was evaluated at 24, 48 and 72 hrs. Optimisation involved performing parallel transfections and acquiring data on cell morphology, monolayer confluence, viability and transfection efficiency. All together, 14 conditions were tested for transfection efficiency and cell viability (see appendix 5.6).

3.2.1. Optimisation of electroporation conditions

To determine the best parameters for transfection, a range of voltages, pulse widths and pulse numbers were tested (appendix 5.6). Three conditions were initially tested: T1 (1200 v; 30 ms; two pulses), T2 (three 1600 v, 10 ms pulses), and T3 (1700 v; 10 ms; three pulses). Condition T2 used was selected based on earlier work by colleagues (B Telford, pers. comms., 2013) and published research (Kim \textit{et al.}, 2008). Conditions T1 and T2
showed the highest transfection efficiency at 24 hrs, while T3 showed markedly decreased transfection efficiency, as observed by GFP expression (figure 3.6). T1 was better-tolerated by the cells than T2, though T2 showed a higher transfection efficiency. As maximum transfection efficiency was sought, T2 was selected for further iterations.

![Image](figure_3.6.png)

**Figure 3.6. Initial optimisation of Neon Transfection System conditions.** 24 hrs post-transfection, condition T2 was determined to give rise to the greatest transfection efficiency and iterated further. Condition parameters listed in appendix; initially optimised with 5 µg pDNA, 2x10^5 cells with three 10 ms pulses of 1600 v.

### 3.2.2. Optimisation of plasmid titre

The optimal plasmid titre for maximum transfection efficiency was then determined. Condition T2 was used with a plasmid (pDNA) titre of 5, 10 and 20 µg (T2, T9, and T10, respectively). 20 µg is the maximum recommended plasmid titre and, as expected,
increasing pDNA titre correlated with increased transfection efficiency (figure 3.6). Condition T10 achieved the highest transfection efficiency and was further iterated.

**Figure 3.7. Optimisation of plasmid titre for Neon.** 24 hrs post-transfection, increasing plasmid titre correlated with increased transfection efficiency. Though effective in WT cells, an increased titre is lethal to CDH1⁻/⁻ cells. Conditions as per T2 (5 µg pDNA); T9 – 10 µg pDNA, T10 – 20 µg pDNA.

3.2.3. Optimisation of cell density

To determine optimum cell seeding density, three different cell numbers were tested. Using the T10 condition, 2, 4, and 6 x 10⁵ cells were tested (T10, T11, and T12, respectively). At 24 hrs post-transfection, T11 and T12 both showed a high number of positively transfected cells (figure 3.6). Though the highest transfection efficiency, 75%, was achieved in condition T12 using 6 x 10⁵ cells. As the increasing cell density correlated...
positively with transfection efficiency and reduced lethality, the optimal condition may have used an even higher cell number.

**Figure 3.8. Optimisation of cell density for Neon.** Increasing cell number lead to more transfected cells, though the transfection efficiency remained stable. Conditions as per T10 with 20 µg pDNA (figure 3.5); T11 with $2 \times 10^5$ cells, T12 with $4 \times 10^5$ cells, T13 with $6 \times 10^5$ cells.

### 3.2.4. Attribution of lethality

Despite sufficient transfection efficiency, considerable lethality was observed early post-transfection. Accordingly, the factors that were contributing to the decrease in cell viability were investigated. First, the effects of electroporation and plasmid uptake were investigated next. Conditions with and without 20 µg plasmid and conditions with and without electroporation were tested, using both silencing and non-silencing inserts.
Figure 3.9. Attribution of lethality in Neon transfection. 24 hrs post-transfection and normalised to untreated (no plasmid or zap) cells, the electroporation alone has minor effect on lethality, while uptake of the plasmid coupled with electroporation leads to significant reduction of viability. Error bars show +/- one standard deviation, derived from at least 2 technical replicates.

The condition with plasmid and electroporation, when normalised to a sample with plasmid that did not undergo electroporation, demonstrates what reduction in viability is attributable to the electroporation itself. 24 hrs post-transfection, cells electroporated without plasmid showed a 20% reduction in viability compared to those that were not electroporated (figure 3.7). This reduction represents the effect of electroporation alone on the viability of the cells.

In the same experiment, the effect of plasmid uptake on early post-transfection cell viability was assessed. In conditions with plasmid and electroporation, any further reduction in viability would be attributed to uptake of the plasmid. 24 hrs post-transfection, cells electroporated with plasmid showed a ~37% reduction in viability compared to those cells electroporated without plasmid. This indicates that uptake of 20 µg of plasmid has the largest contribution to early reduction of cell viability.
It was not expected that non-silencing control shRNA would cause any reduction in viability. Because similar reductions in viability were observed with both silencing and non-silencing plasmids (figure 3.9; also over more than 24 hrs, data not shown), it was conceived that the non-silencing control plasmid was affecting cell viability via an unknown method. The non-silencing control pGIPZ was Sanger sequenced to ensure it was not contaminated or harbouring a silencing insert (chapter 2.4.3). The presence of the correct non-silencing insert was confirmed (chapter 3.3.1).

Puromycin is an inhibitor of 30S ribosomes, and halting protein translation is lethal to the cell. The shRNA expression vector, pGIPZ, harbours a gene encoding puromycin N-acetyl transferase (PAC) that confers resistance to puromycin (Puro\textsuperscript{R}; see chapter 2.4). A puromycin kill curve for MCF10A indicated that 1 µg/mL eliminated ~all cells after 72 hrs of treatment, while 2 µg/mL eliminated ~all cells after 24 hrs (data not shown). Puromycin selection (1 µg/mL) at 24 hrs post-transfection was used to kill non-transfected cells.

Selection was applied at 24 hrs to allow cell adhesion and a recovery period from electroporation. As electroporated cells are already vulnerable, early selection may have an overly damaging effect on cell viability. Perhaps most relevant, key pGIPZ genes including the shRNA, tGFP and Puro\textsuperscript{R} (PAC) genes are co-transcribed with an internal ribosomal entry site (IRES) to allow expression from a single transcript. GFP expression is evident, though far from maximal, at 24 hrs and does not peak until at least 40 hrs (data not shown). GFP requires post-translational modification for maturation and may thus take more time to reach a relevant threshold/maximum than PAC. Nonetheless, 24 hrs may be early enough that puromycin is compounding the effect of the electroporation and plasmid uptake on cell viability, particularly at higher doses.
It is worth noting that even without puromycin, the early effects on cell viability were so great that experimental data from full-length (72 hr) experiments was poor. Together, these results indicate that the early reduction in cell viability is most likely to be due to plasmid uptake rather than the electroporation itself, and the administration of puromycin is compounding the effect. As the uptake of plasmid is crucial to this research, transfection by electroporation was abandoned in favour of lentiviral transduction.
3.3. Lentiviral knockdown of MAST2 and MAP1B

Transfection via the Neon system was deemed sub-optimal for MCF10A due to its toxic effects and was succeeded by a second generation lentiviral packaging system. Lentiviral packaging allows the generation of replication-incompetent lentivirus that can be used to deliver and express a gene of interest (Shimada et al., 1995). pGIPZ lentiviral shRNA plasmid harbours an shRNA insert based on the miR-30 primary transcript for the efficient silencing of target genes via the endogenous RNA interference (RNAi) pathway. The pGIPZ lentiviral vector also contains psi (Ψ) signal for lentiviral packaging, 3' and 5' long terminal repeats (LTRs) for integration, GFP marker and puromycin resistance (Puro\(^R\); PAC) for selection. For detailed vector map see chapter 2.4.1, and for an overview of lentiviral packaging and use in this context see appendix 2.5.1.

3.3.1. Confirmation of shRNA insert sequence

In the Neon experiments (chapter 3.2), non-silencing control shRNA led to decreased viability similar to that observed in synthetic lethal candidate gene conditions. To ensure the control shRNA sequence had not acquired a mutation or been mislabeled, sequencing was carried out (chapter 2.4.3). Sequence quality was sufficiently high despite the tertiary structure of the hairpin providing interference and low read quality around the shRNA sequence (data not shown). Analysis confirmed the presence of the correct non-silencing shRNA insert (figure 3.12).

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**Figure 3.10. pGIPZ sequencing analysis.** Non-silencing shRNA sequence annotation confirms the correct shRNA insert sequence. Reads produced by Sanger sequencing at Genetic Analysis Services, Otago.
3.3.2. Viral titration

Following production of lentiviral particles, a viral titration was performed to determine viral titre as transducing units (TU)/mL (chapter 2.5.4). Viral titres were consistent, ranging between ~7x10^5 and ~9.5x10^5 TU/mL (table 3.1). These concentrations were sufficient for subsequent experiments and did not require further concentrating.

<table>
<thead>
<tr>
<th>Lentiviral package</th>
<th>Functional viral titre (TU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-silencing control shRNA</td>
<td>8.54x10^5</td>
</tr>
<tr>
<td>No insert control shRNA</td>
<td>7.98x10^5</td>
</tr>
<tr>
<td>MAST2 D10 shRNA</td>
<td>8.71x10^5</td>
</tr>
<tr>
<td>MAST2 D11 shRNA</td>
<td>7.01x10^5</td>
</tr>
<tr>
<td>MAST2 D12 shRNA</td>
<td>9.06x10^5</td>
</tr>
<tr>
<td>MAP1B H5 shRNA</td>
<td>9.54x10^5</td>
</tr>
<tr>
<td>MAP1B H6 shRNA</td>
<td>9.04x10^5</td>
</tr>
<tr>
<td>MAP1B H7 shRNA</td>
<td>9.45x10^5</td>
</tr>
</tbody>
</table>

Table 3.1. Range of functional viral titres. Titres range from 7.01x10^5 to 9.45x10^5.

3.3.3. Multiplicity of infection

Creating optimal experimental conditions that maximise transduction efficiency while minimising toxicity is crucial. The multiplicity of infection (MOI) is the number of viral particles administered per cell. Optimal MOI for synthetic lethal experiments was determined by assessing transduction efficiency and total nuclei count over a range of viral titres. Transduction efficiency was determined from the number of transduced cells over the total cell count.

Next, the relationship between MOI and viability was assessed. Predictably, increasing MOI was paralleled by increasing transduction efficiency, up until a point where too many integration events disrupt cell function and begin to affect viability. At MOI = 15 the viability was not reduced compared to mock cells, while an MOI = 37.5 lead to some toxic effects (figure 3.11). Although MOI = 37.5 yielded higher transduction efficiency, reducing early
cell death was a priority following the Neon experiments. An MOI of 15 lead to at least 50% transduction efficiency (figure 3.12), and together with the little observed lethality was deemed sufficient for subsequent experiments.

**Figure 3.11. Multiplicity of infection and viability.** Up to an MOI of 15, cell viability is unaffected; As MOI increases (MOI > 15) the cell viability decreases, most likely attributable to gene disruptions by multiple integrations and an increase in pro-death signals. Error bars represent +/- 1 standard deviation as derived from at least 2 technical replicates.

**Figure 3.12. Multiplicity of infection and transduction efficiency.** As MOI increases, the transduction efficiency increases in a non-linear manner. At the highest MOI (MOI = 75), transduction efficiency reaches 80% in \( CDH1^- \) cells and 90% in WT cells. Error bars represent +/- 1 standard deviation, derived from at least 2 technical replicates.
However, similar studies have not used MOI > 10 (Hao et al., 2008). Although transduction varies considerably based on cell type (S Hughes, pers. comms., 2015), such a high MOI yielding such a low transduction efficiency is not supported by mathematical models (figure 3.13). According to the statistical process, at an MOI = 1 the probability of not being infected \( P(0) = 36.79\% \), the probability of being infected by a single particle \( P(1) = 36.79\% \), two particles \( P(2) = 18.39\% \), three particles \( P(3) = 6.13\% \). As above, at MOI 1 the probability of a cell being infected by at least one particle \( P(>1) = 63.2\% \), at MOI 2 = 86.5\%, at MOI 3 = 95\%, at MOI 4 = 98.2\%, up to MOI 8 = ~100\%.

\[
P(n) = \frac{m^n \cdot e^{-m}}{n!}
\]

Figure 3.13. Multiplicity of infection as a statistical process. \( m \) is the MOI, \( n \) is the number of infectious agents entering the target, and \( P(n) \) is the probability that a target cell will be infected by \( n \) infectious agents. As MOI increases, the percentage of cells infected with at least one particle (\( P(>1) \)) will increase. This application of Poisson’s distribution was adapted from an interpretation by Ellis & Delbrück (1939).

In this investigation, a multiplicity of infection (MOI) of 75 only yielded 80 and 90\% transduction for the \( CDH1^- \) and WT cell lines, respectively (figure 3.12). This highlights the gap between biology and attempts to model it. There are several possible explanations that explain the effects of multiplicity (see chapter 4.2.4). Most likely, the viral titre we derived may be overestimated because cells are transduced and counted 48 hrs later so GFP signal can mature. During this time, the cells, including transduced cells, are dividing and leading to overestimate of initial transducing unit/lentiviral particle concentration. It is likely that the true MOI is much lower than the values in table 3.1. Possible explanations
for this observation are discussed in chapter 4.2.5. Regardless, the investigation continued with an MOI of 15 as this method yielded data (chapter 3.3.4-6).

**Figure 3.14. Theoretical distribution of lentiviral copy number.** Fractions of cells infected with none, one or multiple particles. As MOI increases, the percentage of cells infected with multiple particles will increase. Multiple particles per cell result in multiple integration events, increasing background noise due to disrupted genes. Adapted from Ellis & Delbrück (1939).
3.3.4. Knockdown of candidate genes

To confirm the presence of synthetic lethal interactions between CDH1 and cytoskeletal candidate genes, isogenic cells (WT and CDH1\(^{+/−}\)) were transduced with replication-incompetent lentiviral particles containing unique shRNA sequences from pGIPZ. For a complete list of shRNA sequences, see appendix 5.5.

**Figure 3.15. MAST2 shRNA binding sites.** MAST2 (NM_015112) targets. Each shRNA clone targets a unique sequence of the mRNA, preventing translation and marking the transcript for degradation. Binding position is designated in brackets.

**MAP1B shRNA targets**

**Figure 3.16. MAP1B shRNA binding sites.** MAP1B (NM_005909) targets. Each shRNA clone targets a unique sequence of the mRNA, preventing translation and marking the transcript for degradation. Binding position is designated in brackets.

This work aimed to assess the effect of shRNA knockdown on isogenic cell line viability. Figures 3.15 and 3.16 show the position on the transcript that the shRNA sequence targets on MAST2 and MAP1B, respectively. First, the temporal effects of shRNA knockdown were investigated at an MOI of 15 as had previously been determined to be optimal (chapter 3.3.2). For this experiment, control shRNA targeting GAPDH was used, which is supported by RNA sequencing data showing consistent expression across both cell lines.
mRNA expression was assessed by qPCR at 48, 72 and 96 hrs post-transduction to assess the rate of RNAi knockdown at an MOI of 15. At 48 hrs post-transduction, knockdown across the isogenic cell lines reached ~55%, and reached the maximum 80% knockdown at 72 hrs (figure 3.17). There was little additional knockdown between 72 and 96 hrs.

**Figure 3.17. Temporal effect of lentiviral shRNA silencing.** Maximum knockdown is reached at 72 hrs post-transduction, though preexisting protein product may persist. Error bars represent +/- 1 standard deviation, derived from at least 2 technical replicates.

**Figure 3.18. Relative levels of MAST2 expression.** Values normalised to non-silencing shRNA control, 72 hours following treatment with pGIPZ lentivirus containing unique shRNA sequences targeting MAST2 transcript. Error bars indicate +/- 1 standard deviation, derived from at least two technical replicates.
It was considered that 96 hrs post-transduction would be optimal to allow full RNAi effects to be reached, as well as allowing time for residual protein degradation and/or recycling. However, WT cells were reaching full confluence by 96 hrs (plated as described in chapter 2.5.6). Attempts to circumvent this by reducing initial seeding density lead to new errors as seeding became less consistent at low numbers. For subsequent experiments, mRNA was measured at 72 hrs post-transduction in line with end-point assays. This is discussed further in chapter 4.2.5.

![MAP1B mRNA expression](image)

**Figure 3.19. Relative levels of MAP1B expression.** Values normalised to non-silencing shRNA control, 72 hours post-transduction with pGIPZ lentivirus containing unique shRNA sequences targeting MAP1B transcript. Error bars indicate +/- 1 standard deviation, derived from at least 2 technical replicates.

Figures 3.18 and 3.19 summarise the level of mRNA silencing for candidate target clones at 72 hrs post-transduction. For MAST2, clones D10-12; and for MAP1B, clones H5-7 resulted in the greatest level of knockdown (figures 3.18-9), and were thus used for validation experiments.
3.3.5. Assessment of viability following knockdown

To assess the effect of shRNA knockdown of candidate genes, cell viability was measured over 72 hr time-course experiments with end-point metabolic assay and total nuclei counts. It is expected that a reduction in fitness caused by a synthetic lethal interaction will manifest as reduced viability in \( CDH1^{-/} \) cells over time.

3.3.5.1. Metabolic assay

Assessing cell metabolic activity may, under predefined conditions, reflect the viability or number of viable cells. Resazurin is a blue dye used as a colorimetric assay for cell viability by measuring total reducing power (ATP content within a sample). Because the dye irreversibly binds DNA, this metabolic assay is end-point.

![Figure 3.20. Metabolic assay of MAST2-silenced cells.](image)

**Figure 3.20. Metabolic assay of MAST2-silenced cells.** Values normalised to non-silencing shRNA control, 48 and 72 hrs following treatment with pGIPZ lentivirus containing unique shRNA sequence targeting synthetic lethal candidate gene mRNA. At 48 hrs there is no reduction in viability compared to control, while at 72 hrs the synthetic lethal effect presents itself. Error bars indicate +/- 1 standard deviation, derived from at least 3 technical replicates.

Following silencing of either \( MAST2 \) and \( MAP1B \) with two different shRNA clones, there is no discernible difference between WT and \( CDH1^{-/} \) cell viability at 48 hrs post-transduction. The synthetic lethal effect becomes apparent at 72 hrs post-transduction. For \( MAST2 \), knockdown in the WT cells confers a mild protective effect, while \( CDH1^{-/} \) viability is
reduced by 10-20% compared to control (figure 3.20). For MAP1B, there is a similar increase in WT viability at 72 hrs while CDH1\(^{-}\) cell viability is reduced by 20-40% (figure 3.21). Interestingly, the protective effect of MAST2 silencing on WT cells is not supported by preliminary siRNA screening data, nor by validation data derived from total nuclei counting.

**Figure 3.21.** Metabolic assay of MAP1B-silenced cells. Values normalised to non-silencing shRNA control, 48 and 72 hrs following treatment with pGIPZ lentivirus containing shRNA sequence targeting synthetic lethal candidate gene mRNA. At 48 hrs there is no reduction in viability, while at 72 hrs the synthetic lethal effect presents itself. Error bars indicate +/- 1 standard deviation, derived from at least 3 technical replicates.

**3.3.5.2. Total nuclei and dead cell counting**

To build a more accurate representation of viability, total nuclei were counted as a direct measure of cell numbers 72 hrs post-transduction. For each candidate gene, three shRNA sequences were investigated for synthetic lethal effect. For MAST2, clones D10-D12 were used, while for MAP1B, clones H5-7 were used.

72 hrs following MAST2 silencing, WT viability is unaffected while CDH1\(^{-}\) viability is reduced by ~20% on average (figure 3.22). Following MAP1B-silencing, WT viability is also unaffected while CDH1\(^{-}\) viability is reduced by 30% on average (figure 3.23).
Figure 3.22. Nuclei counts of *MAST2*-silenced cells. Values normalised to non-silencing shRNA control, 72 hrs following treatment with pGIPZ lentivirus containing unique shRNA sequences targeting *MAST2* mRNA. Error bars indicate +/- 1 standard deviation, derived from at least 2 technical replicates.

Figure 3.23. Nuclei counts of *MAP1B*-silenced cells. Values normalised to non-silencing shRNA control, 72 hrs following treatment with pGIPZ lentivirus containing unique shRNA sequences targeting *MAP1B* mRNA. Error bars indicate +/- 1 standard deviation, derived from at least 2 technical replicates.

After the initial qPCR and knockdown studies on multiple shRNA constructs, the experiments were repeated on selected shRNAs in an attempt to reduce the errors associated with each analysis. For final nuclei counting experiments, only one shRNA
clone was used per candidate gene, MAST2 D11 and MAP1B H6. For MAST2 and MAP1B, mRNA expression was, on average, reduced by at least 85 and 80%, respectively (figure 3.24).

**Figure 3.24. Relative levels of synthetic lethal candidate gene expression.** Normalised to non-silencing control, 72 hrs following treatment with pGIPZ lentiviral shRNA. Error bars indicate +/- standard error, derived from at least 2 biological replicates.

Final validation experiments used only one shRNA for each candidate gene. WT viability was unchanged while CDH1−/− viability was reduced by 20 and 30% in MAST2- and MAP1B-silenced cells, respectively (figure 3.25).
Figure 3.25. Combined nuclei counts of candidate-silenced cells for validation. Values normalised to non-silencing control, 72 hrs following treatment with pGIPZ lentiviral shRNA. Error bars indicate +/- 1 standard error, derived from 3 biological replicates.
3.3.6. Cytell field of view coverage comparison

Reproducibility between experimental replicates of high-throughput cell line work can be affected by inconsistent seeding density and the distribution of cells within the well. While the former is readily overcome by good experimental technique, there is less control over the latter. For total nuclei counts, a representative sample of fields is captured to reflect the conditions of each experimental replicate. The most accurate assessment of cell viability would cover 100% of surface area of the well for each replicate. However, this would require some ~50 fields per well of a 96-well plate which renders the approach unfeasible.

![Cytell (n) field of view comparison](image)

**Figure 3.26. Comparison of Cytell coverage and accuracy.** The dataset comes from an HDACi experiment (entinostat) provided by A Chen as described in chapter 2.6.

To investigate the optimal representative number of fields, a dataset of 25 fields per technical replicate provided by colleague A Chen was broken down into subsets of 5, 10, 15 and 20 fields. These subsets were then compared to the 'most representative' method of 25 fields. While the relative viability was the same across all subsets, the standard error increased as the number of fields was decreased (figure 3.26; table 3.2). This showed that while few fields (ie, 5 or 10) were sufficient in representing the trend of the experiment, it
would be a barrier in reaching statistical significance and perhaps discredit valid findings. It was determined that 20 fields of view was sufficiently representative.

Figure 3.27. Spread of Cytell viability data. Values are normalised to the value derived from 25 fields of view. Decreasing the number of fields of view decreases the accuracy and consistency of the method and increases the deviation and error from the 25 field approach. Dataset comes from HDACi (entinostat) experiment provided by A Chen.

<table>
<thead>
<tr>
<th>Cytell (n) fields</th>
<th>20 fields</th>
<th>15 fields</th>
<th>10 fields</th>
<th>5 fields</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max. norm. value</td>
<td>1.089</td>
<td>1.085</td>
<td>1.169</td>
<td>1.288</td>
</tr>
<tr>
<td>Min. norm. value</td>
<td>0.894</td>
<td>0.793</td>
<td>0.785</td>
<td>0.628</td>
</tr>
<tr>
<td>Mean value</td>
<td>0.991</td>
<td>0.984</td>
<td>0.970</td>
<td>0.939</td>
</tr>
<tr>
<td>N &lt; 0.90</td>
<td>2</td>
<td>4</td>
<td>10</td>
<td>21</td>
</tr>
<tr>
<td>N &gt; 1.10</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 3.2. Cytell field of view comparison data.

More subtle fold-changes are less likely to reach statistical significance, reducing strength and validity of the results. For the most informative results, the maximum practical number of fields should be used for representative imaging.
3.4. Drug treatment with Aurora kinase inhibitors

The purpose of this chapter was to evaluate known drugs for potential synthetic lethal effect. While shRNA knockdown of candidate genes will demonstrate the validity of a gene target, drugs against these targets are often not yet available. Drugs that are already approved or under clinical investigation for different conditions may be repurposed and fast-tracked into the clinic and thus make a positive impact in the short-term.

Aurora kinases are a family of protein kinases that play critical roles in mitosis. Disruption of mitotic machinery is a proven anticancer strategy that is used by multiple chemotherapeutic agents. Mitotic regulators and cytoskeletal proteins were over-represented as synthetic lethal candidates in the primary siRNA screen (Telford et al., 2015), likely stemming from the role E-cadherin plays in cytoskeletal maintenance.

<table>
<thead>
<tr>
<th>siRNA knockdown</th>
<th>MCF10A WT viability</th>
<th>MCF10A CDH1⁻/⁻ viability</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAST2</td>
<td>0.65</td>
<td>0.38</td>
<td>0.58</td>
</tr>
<tr>
<td>MAP1B</td>
<td>0.84</td>
<td>0.65</td>
<td>0.77</td>
</tr>
<tr>
<td>AURKA</td>
<td>0.64</td>
<td>0.58</td>
<td>0.91</td>
</tr>
<tr>
<td>AURKB</td>
<td>0.38</td>
<td>0.32</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Table 3.3. MCF10A viability in preliminary siRNA screen (Telford et al., 2015).

In the primary siRNA screen, both AURKA and AURKB siRNAs showed modest levels of reduced viability in CDH1⁻/⁻ cells (table 3.3). However, neither met the inclusion criteria for further validation. This inclusion criteria following candidate knockdown was at least 15% more reduction in CDH1⁻/⁻ viability compared to WT and no more than 50% reduction in WT viability (ie, too toxic for reliable viability measurement). AURKA knockdown lead to only 9% reduction in relative CDH1⁻/⁻ viability, while AURKB knockdown lead to 62% reduction in WT viability. These data suggest that Aurora kinase inhibition in an E-cadherin-deficient context was worthy of further investigation, given the availability of Aurora kinase inhibitors.
3.4.1. Determining optimal drug concentration and EC\textsubscript{50}

First, the EC\textsubscript{50} concentrations of two Aurora kinase inhibitors, alisertib and danusertib, were determined to measure the potency of each drug after a specified duration of exposure. Using this result, the optimal experimental dosing range could be determined.

![Figure 3.28. EC\textsubscript{50} curves for Aurora kinase inhibitors alisertib and danusertib. Alisertib (A & B, WT & CDH1\textsuperscript{-/-}) and danusertib (C & D, WT & CDH1\textsuperscript{-/-}). EC\textsubscript{50}, the effective concentration of drug that gives the half-maximal response, is calculated from a drug serial-dilution dose-response, utilising the curve fitting method of least squares. Experiments included three technical replicates, but no biological replicates were performed.](image)

The EC\textsubscript{50} was determined by treating cells with an 8-point, 10-fold dilution series of each drug, 24 hrs post seeding. At 48 hrs post-treatment, viability was assessed via total nuclei count (chapter 2.7.4). EC\textsubscript{50} was calculated using non-linear regression with a curve fitted
by least squares. For alisertib, the EC$_{50}$ was calculated as 11.38 nM for WT and 8.66 nM for CDH1$^{-/-}$; for danusertib, the EC$_{50}$ was calculated as 45.45 nM for WT and 36.03 nM for CDH1$^{-/-}$ (figure 3.28; table 3.4).

<table>
<thead>
<tr>
<th></th>
<th>MCF10A WT</th>
<th>MCF10A CDH1$^{-/-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alisertib (MLN8237)</td>
<td>11.38 nM</td>
<td>8.66 nM</td>
</tr>
<tr>
<td>Danusertib (PHA-739358)</td>
<td>45.45 nM</td>
<td>36.03 nM</td>
</tr>
</tbody>
</table>

**Table 3.4. EC$_{50}$ values for Aurora kinase inhibitors alisertib and danusertib.**

The EC$_{50}$ of both drugs is lower for CDH1$^{-/-}$ cells than WT cells, indicating the potential of Aurora kinase inhibition in a CDH1-deficient context. This suggests there is a vulnerability in CDH1$^{-/-}$ MCF10A cells that may be amenable to inhibition of Aurora kinases in a synthetic lethal manner.

### 3.4.2. IncuCyte time course confluence assay

To gather further evidence of a synthetic lethal effect for alisertib, the effect of drug inhibition on isogenic cell growth was measured in real-time. The IncuCyte live cell imaging system tracks the confluence of a cell culture monolayer as a proxy measure of viability in real-time. Cell confluence was measured over 45 hrs post-treatment. Over the first 24 hrs, WT cells exhibit little decrease in viability across the experimental dosage range. In contrast, CDH1$^{-/-}$ cells show a dose-dependent inhibition of growth (figure 3.29); 80 nM alisertib shows the greatest growth inhibition, followed by 26.7 nM.

However, between 24 and 48 hrs post-treatment, WT cells began to show inhibited growth similar to CDH1$^{-/-}$ cells (figure 3.29); cell proliferation was inhibited in a dose-dependent manner. This suggests that WT cells can only tolerate Aurora kinase A inhibition for around 24 hrs. Together these data suggest there may be a short therapeutic window for Aurora kinase inhibition in a CDH1-deficient context, although the difference between the
two cell lines was insufficient to be confident that there would significant clinical utility.

Figure 3.29. Time-course confluence assay of alisertib-treated cells. Cells were drugged at t = 0 hrs and readings taken at 2 hr intervals. While there is marked growth inhibition only in \(CDH1^{-/-}\) cells over the first 24 hrs, both cell lines show a dose-dependent growth inhibition between 24 and 48 hrs.

A real-time confluence assay was not performed for danusertib-treated cells due to time constraints and varying drug potency despite all attempts to correctly aliquot and store the drug.
3.4.3. End point nuclei counting

For a more accurate measure of end-point viability, total nuclei counting was performed at 48 hrs post-treatment. For alisertib, all drug dosages lead to preferential reduction in CDH1⁺ viability, though only 25 nM reached statistical significance (p = 0.048). At 25 nM, WT cells show a 20% reduction in viability compared to untreated, while CDH1⁻ cells show 35% reduction compared to untreated. This equates to an 18% reduction in CDH1⁺ viability compared to WT. This confirms Aurora-A is a valid synthetic lethal target in a E-cadherin-deficient context, but its additional clinical utility in treating CDH1-deficient cancers would be marginal.

![Alisertib treated MCF10A](image)

**Figure 3.30. Total nuclei count of alisertib-treated cells.** Viability was assessed 48 hrs post-treatment. Cells were drugged at t = 0 hrs and an end-point staining cocktail was added at t = 47.5 hrs. CDH1⁺ cells show reduced viability compared to WT, though the effect is the strongest at 25 nM.

Treatment with danusertib also leads to selective reduction in CDH1⁺ cell viability. At 25 nM, WT cells show almost unchanged viability (<5%), while CDH1⁺ cell viability is reduced by 25%. This equates to a fold change of about 20% (within inclusion criteria) and confirms Aurora-B as a synthetic lethal partner of CDH1.
Figure 3.31. Total nuclei count of danusertib-treated cells. Viability was assessed 48 hrs post-treatment. Cells were drugged at t = 0 hrs and an end-point staining cocktail was added at t = 47.5 hrs. CDH1⁻/⁻ cells show reduced viability compared to WT, though the effect is the strongest at 25 nM.

These results support the existence of a synthetic lethal relationship between E-cadherin and components of the cytoskeleton. Although Aurora kinase inhibitors themselves may not provide a clinical 'advantage', this work encourages further examination of drugs that target cytoskeletal components and processes.
4. Discussion

This study investigated the validity of putative synthetic lethal partner genes to E-cadherin gene *CDH1*. Candidate genes were identified in a genome-wide siRNA screen (Telford *et al.*, 2015), performed by PhD student Bryony Telford. Putative synthetic lethal candidates were validated with shRNA knockdown and drug inhibition of target genes and their proteins, respectively.
4.1. Importance of developing a novel strategy for HDGC management

4.1.1. HDGC outlook

Familial cases represent 1-3% of nearly one million annual diagnoses of gastric cancer, and HDGC attributable to E-cadherin inactivation has been reported in over 155 families worldwide (Vogelaar et al., 2015; Hansford et al., 2014). A recent study evaluating lifetime risk for cancer found that by the age of 80 years, the cumulative incidence for gastric cancer was 70% for males and 56% for females, with an additional 42% risk for breast cancer (Hansford et al., 2015).

Despite the further understanding of the biology of these tumours and the availability of increasingly effective drug therapies, late detection results in an overall 5-year survival rate not exceeding 30%. The survival rates for individuals treated for early gastric cancer are best, but advanced cancers carry very poor prognoses. The difficulty of early stage detection is compounded by the lack of luminal perforation in early gastric cancer and difficulty distinguishing between gastric cancer and benign ulcers or gastritis (Okabayashi & Shima, 2013). As a result, endoscopic surveillance for HDGC cancer foci is imperfect and highlights the need for better screening and/or diagnostic tools.

4.1.2. Emerging technologies

The highlights of emerging technologies for the surveillance of gastrointestinal pathology include blue laser imaging, narrow-band imaging and confocal laser endomicroscopy (Meining, 2009; Togashi et al., 2016). Blue laser and narrow-band imaging utilises monochromatic lasers that allow visualisation of the microarchitecture of specific features and structures based on powerful colour contrast discrimination. Assessment of changes to microvasculature and microsurface patterning by magnifying endoscopy with blue laser and/or narrow-band imaging, allow for in situ diagnoses of foci that are undetectable with
conventional white-light endoscopy (Togashi et al., 2016).

Confocal laser endomicroscopy (CLE) is perhaps the most promising technological development and would allow submucosal in vivo imaging at a cellular and subcellular level. Depth-focused lasers increase the spatial resolution and permit the reconstruction of highly-resolved 3D images. However, because CLE relies upon fluorescence, there are concerns over mutagenic potential of the required contrast agents (Wallace et al., 2010), and it is unlikely that CLE will allow practical imaging of the entire stomach as required for HDGC. It is likely that future surveillance will consist of a functionally complementary set of image-enhanced endoscopic tools, biopsies and histopathological examination.

4.1.3. Chemoprevention

While early detection is an important prognostic factor, prevention is the most effective management modality. To date, there has been little focus on chemoprevention and the clinical recommendation for HDGC families is prophylactic total gastrectomy in preference to annual endoscopic surveillance. Although total gastrectomy is curative in >95% of HDGC-affected individuals there is significant long-term morbidity, highlighting a need for a targeted therapy that can efficiently clear early lesions. Eliminating pre-clinical cancer foci may help prevent or delay tumour progression while reducing the need for surgery.

Loss of function events in tumour suppressor genes are difficult to target because the encoded protein is lost from the cancer cell. However, loss of CDH1 expression is predicted to create vulnerabilities that may be exploited in a synthetic lethal manner. Efficient treatment modalities that can eliminate pre-clinical lesions will both improve patient outcomes and reduce the public health burden. Further, any synthetic lethal drugs developed for HDGC may have application across other cancer types. Most pertinently,
these drugs may be utilised in sporadic E-cadherin-deficient DGC and LBC, but also other cancers where E-cadherin is silenced, including prostate, endometrial, ovarian carcinomas and medulloblastoma, among others (Perl et al., 1998).

4.2 Experimental protocol

4.2.1. Cell line selection
The investigation of synthetic lethal partnerships required a cell line model of CDH1 loss. Isogenic (CDH1+/+ and CDH1-/-) MCF10A non-transformed, mammary epithelial cell line derived from human fibrocystic tissue were selected (Sigma, St. Louis, USA). The latter features a homozygous 4 base-pair deletion in CDH1 achieved by zinc-finger nuclease excision. The model was chosen because breast cancer is part of the HDGC spectrum and is near to genetically normal, and there are no equivalent gastric cell lines.

MCF10A has the characteristics of normal breast epithelium with anchorage-dependent growth, minimal chromosomal rearrangements and high genomic stability (Soule et al., 1990). With a stable genome and minimal alterations to pathways and functions, MCF10A closely reflects the biology of the non-malignant cells in vivo. Whereas more advanced cancers acquire additional genetic changes, the lack of genetic abnormalities in MCF10A is desirable for its use as part of a chemoprevention model (Chen et al., 2014). Although MCF10A does have some abnormalities associated with in vitro cultured epithelial cells, such as MYC amplification and p16 deletion (Debnath et al., 2003), it is considered genetically 'normal'.

4.2.2. Plasmid selection
Investigation of synthetic lethal interactions requires a method to silence candidate gene expression. While siRNA was used for preliminary screening, transient and/or stable long-
term RNAi requires an expression vector. To perform specific and efficient silencing of candidate genes, lentiviral shRNA vector pGIPZ was selected. pGIPZ features microRNA-adapted shRNA based on miR-30 that the manufacturers claim delivers efficient silencing of gene expression with minimal cytotoxicity. The availability of extensive pGIPZ shRNA libraries (Dharmacon) that specifically target candidate synthetic lethal genes also supported the selection of this silencing modality.

MicroRNA-adaptors on shRNA sequence increase processing efficiency of shRNA into siRNA by the endogenous RNAi machinery and promote degradation of target transcript. Selectable GFP and puromycin markers allow easy identification and selection of cells harbouring the shRNA vector. Under control of a strong CMV promoter, the shRNA and reporting markers (turboGFP; tGFP and puromycin N-acetyl transferase; PuroR) are constitutively expressed in a polycistronic transcript. The single transcript means that the strength of GFP signal mirrors the level of shRNA expression, acting as a visual indicator of gene silencing effects. For a vector map of pGIPZ, see chapter 2.4.1.

4.2.3. Electroporation

The simplest method for RNAi is the cytosolic delivery of siRNA, as was performed in primary screening by liposome transfection (lipofection; Telford et al., 2015). Although rapid and simple for high-throughput work, caveats including short-term efficacy and lack of selectable markers underpin the insufficiency of this method for robust validation of synthetic lethal genes. Transient delivery of a replicative shRNA-vector such as pGIPZ allows for long-term silencing of the targeted gene and easy visualisation of activity. MCF10A are notoriously difficult to transfect (P Guilford, pers. comms., 2014) and consequently, this project explored multiple transfection conditions.
At the commencement of this project a novel/commercial electroporation method/package – the Neon transfection system – based on Kim et al., (2008) was tested. The electroporation itself and the uptake of the plasmid each contributed to significant cell death early post-transfection (0 – 24 hrs; chapter 3.2.4). Although it was unclear why plasmid uptake was so poorly tolerated, one explanation was that the plasmid titre is too high, although lower plasmid titres were ineffective (figure 3.12; chapter 3.3.3), highlighting the difficulty of MCF10A transfection. It remains possible that additional treatment of cells with electrocompentence protocols prior to electroporation could have increased transduction efficiency and decreased cell death.

In untreated MCF10A, it takes between 1-2 hrs for adherence/adhesion and for 2-6 hrs for anchorage-dependent growth to resume following trypsin-mediated loss of adhesion (Chen et al., 2014). Although it was not thoroughly investigated, the effects of electroporation and plasmid uptake together may have delayed the recovery time. It is likely that delayed recovery, together with rapid puromycin selection 24 hrs post-transfection, may have also contributed to early cell death (24 hrs+). Standard selection protocols recommend for puromycin selection to follow a low-dose 7-10 day workflow including serial passage where necessary. As a lethal phenotype is being sought, it is not possible to run an experiment of this length. Furthermore, attempts to passage transfected cells were unsuccessful and therefore the use of short-acting, higher-concentration puromycin is supported.

Although early transfection efficiency was promising, a significant majority of cells did not recover from the combined effects of the transfection/electroporation and selection agents/puromycin, even in non-silencing control conditions (figure 3.9; figure S.1; chapter 3.24). The compromising effects of electroporation, plasmid uptake and early selective
agents on cell viability produced poor experimental data, leading to the method being abandoned (data not shown). Although cost did not contribute to the decision to abandon the method, it is a factor to consider. While good experimental technique would encourage Neon pipette tip replacement for each technical replicate, it has been demonstrated that tips may be used up to 10 times before the protocol is compromised (H Beetham, pers. comms., 2015).

For experimental controls, no insert and non-silencing pGIPZ plasmids were initially tested. No insert pGIPZ has no miR-30 shRNA sequence and does not activate endogenous RNAi pathways. Non-silencing pGIPZ produces a scrambled shRNA sequence with no known targets/homology. It was noted that non-silencing control leads to some reduction in viability compared to no insert control, but is a more appropriate control so its continued use was supported. Given the reduction in viability it is likely that the strong activation of endogenous RNAi machinery has an early impact on cell viability, rather than just uptake of the plasmid. Higher cell densities should have also been investigated; while the maximum plasmid titre was used, the kit allows for up to $1 \times 10^6$ cells and an increased cell density may have reduced toxicity.

The factors contributing to early cell death might have been mitigated with use of an inducible shRNA-expression vector. To elaborate, an inducible system would remove the temporal constraints arising from investigating a lethal phenotype because cells could be transfected, allowed to recover, seeded, and then induced and observed. Furthermore, mixed or even clonal populations of transfected cells could be propagated and preserved for later use. Therefore, with an inducible system, in principle, endless replicates of each experimental condition could be performed from a single electroporation.
4.2.4. Lentiviral transduction

To mitigate the cytotoxic effects of vector uptake, validation of synthetic lethal interactions was performed by lentiviral transduction. For an overview of this process, see chapter 2.5.1. Lentiviral delivery and integration of expression vectors is often followed by establishment of a stable, clonal populations. However, as the synthetic lethal phenotype is lethal, surviving stable populations may have confounding integrations/disruptions in the genome. Instead, experimental protocols were performed on non-clonal, mixed populations without serial passage following transduction. While this method was successful, the fold-changes in viability were not as great as those generated in primary screening, and some candidates did not validate whatsoever.

Factors affecting the lentiviral protocol may stem back in the experimental pipeline to as early as viral titration. Here, some possible improvements to the experimental protocol are discussed. First, the use of polybrene for increased transduction efficiency is standard in viral transduction protocols (S Hughes, pers. comms., 2015), although it was not used in this investigation. Future experiments involving viral transduction would likely benefit from addition of this reagent, as it would decrease the use of viral particles and lead to a more even distribution throughout a cell population.

It was retrospectively determined that the multiplicity of infection (MOI) was very high – similar work only rarely exceeded an MOI of 10 (Hao et al., 2008). Theoretically, at MOI = 1, it is expected that ~63% of cells will take up a viral particle, up to MOI = 15 where it is expected that ~100% of cells will have at least one viral particle (figure 3.14). However, in the experiments of this investigation, MOI = 15 only resulted in approximately 75% transduction, representing at least one viral particle for each of those cells. The following ideas may explain this observation: (1) factors in the media may affect viral particles, (2)
receptor expression follows uneven distribution, or (3) the initial viral titration was highly overestimated.

Viral particles are produced in serum-free DMEM media, and it is possible that transduction of cells in complete media reduces viral potency as the particles interact with serum. Lentiviral particles are internalised following docking with low density lipoprotein receptor (LDLR; Finkelstein et al., 2013). Transcriptome data suggests the expression of this protein is not altered following E-cadherin loss (Chen et al., 2014), and both cell lines are expected to be equally amenable to viral transduction.

In pGIPZ, shRNA and reporters (tGFP and PAC/PuroR) are transcribed together as one polycistronic transcript, processed, and then translated separately. Therefore, a strong GFP signal infers a high level puromycin resistance and shRNA expression and, by extension, target RNAi. 24 hrs following transduction, no GFP was visible and it takes at least 48 hrs for a strong GFP signal to develop (figure S.2). The delayed end-point for viral titration may have lead to an over estimation of functional viral titre as cells continue to divide. This could have been mitigated by using anti-GFP antibodies before the signal had developed to full strength (~24 hrs), or by serum-starving or otherwise inhibiting growth with low doses of drugs. It is necessary to count GFP-positive colonies or use antibodies as described above because the lentivirus used here is replication-incompetent and therefore cannot be titred with plaque-forming assays or focal forming assays.

Given that the GFP signal does not reach maximum until 48 hrs post-transduction, it is likely that application of puromycin at 24 hrs is having an effect on cell viability. Meanwhile, strong GFP signal is already observed 24 hrs following transfection by electroporation, suggesting electroporation/cytosolic shRNA vector leads to more rapid RNAi and more
rapid synthetic lethal phenotype than lentiviral transduction/integration. This delay is due to the lentiviral pGIPZ genome being reverse transcribed and integrated while pGIPZ plasmid will be immediately functional. Given the delay in activity in lentiviral RNAi it is probable that any synthetic lethal phenotype is delayed, evident by metabolic assay data (chapter 3.3.6.1), where there was no effect at 48 hrs but a detectable effect did present by 72 hrs post-transduction. Indeed extending experimental duration may lead to a stronger effect – like those observed in siRNA screening.

However, extending experimental duration leads to different issues. In the current experimental conditions cells become fully confluent at 96 hrs, and stop dividing due to contact inhibition. The obvious solution of reducing seeding density is difficult for the 96-well plate layout that was used. As the density is already relatively low, attempts to further decrease it introduced higher error.

To better control density and confluence, amino acid or serum starvation could be used for growth stasis. This would allow a higher density of cells to be used, and only those that die are attributed to synthetic lethal effect. This model has drawbacks including that epithelial cells are dividing in vivo, and some mechanisms of lethality require division for a stress threshold to be reached. However, cells could be transduced, allowed to recover, and then returned to complete media to initiate the experiment.

Changing the plate format for larger wells is an option, though would require reoptimisation and increased reagent use. While all published work, including early screens, was performed in 96-well plates, the use of 24- or 12-well plates would likely reduce seeding error and allow for longer experiment duration. Again, these potential improvements would be redundant if an inducible shRNA-expression system were to be used. Replacement of
the constitutive pGIPZ with an inducible shRNA expression system would allow for the same improvements/features as discussed for Neon (chapter 4.2). The lentiviral experiments in this investigation commence at transduction (t = 0 hrs), while an inducible system would allow for the fine tuning of condition parameters (cell density, cell recovery, copy number, etc) prior to commencement. Inducible expression vector pTRIPZ is similar to pGIPZ, though the expression of key genes is controlled by the addition of doxycycline.

The pGIPZ shRNA library used for this investigation is not available in pTRIPZ format, although the presence of multiple cloning sites would allow for shRNA inserts to be cloned between these vectors. My principle recommendation for improving this experimental system is to clone pGIPZ shRNA inserts into pTRIPZ and for work to be carried out from stable cell lines. However, recent attempts in our laboratory to clone fragments of pGIPZ into pTRIPZ have been very inefficient (A bin Aiderus, pers. comms., 2015).

4.3. Synthetic lethal target validation

4.3.1. MAST2

MAST2 had previously been identified in the primary siRNA screen, where knockdown elicited a strong synthetic lethal effect (WT 0.65, CDH1−/− 0.38, fold-change 0.58). Using lentiviral shRNA knockdown to further investigate putative synthetic lethal interactions in isogenic MCF10A cell lines, MAST2 has been validated as a synthetic lethal partner of CDH1 (figure 3.25). Four of the seven shRNA clones, D9-11, E2, induced a synthetic lethal effect, as observed by total nuclei counts (figure 3.22). One shRNA clone, D10, gave rise to the strongest phenotype and was used for most of the data shown.

MAST2 (aka MAST205, MTSSK) encodes its namesake protein: microtubule-associated serine/threonine kinase 2 that plays a part in modulating microtubule interactions and
dynamics (Clay et al., 2013). MAST2 is expressed in all tissues at low to moderate levels, where dystrophin and utrophin networks facilitate interaction between the cell membranes and microtubules to stabilise the cytoskeleton and thus the structure of the cell (Terrien et al., 2012). MAST2 phosphorylates dystrophin/utrophin to modulate their affinities for associated proteins, including microtubules.

In epithelial cells, utrophin interacts with adherens junctions proteins for the role of modulating microtubule interactions and stabilising the cytoskeleton (Belkin & Burridge, 1995). Loss of E-cadherin-dependent cytoskeletal anchorage leads to a disarrayed cytoskeleton (figure 1.12). A potential mechanism for synthetic lethality of MAST2 posits that further destabilisation of an already unstable cytoskeleton will be intolerable and thus lethal to the cell.

To date, there has been little work characterising the role of MAST2 in cancer. Functional fusions of MAST2 and NOTCH1 with strong driving/tumourigenic roles have been found in breast carcinoma and associated pre-invasive lesions (Clay et al., 2013, Robinson et al., 2011). These fusions drive a subset of breast cancers, albeit rarely (McCarthy 2012, Robinson et al., 2013) and exhibit unique phenotypic effects in breast epithelial cells. Overexpression of MAST1 or MAST2 gene fusions has a proliferative effect both in vivo and in vitro (Robinson et al., 2011). However, MAST2 expression is barely affected by CDH1 downregulation (Chen et al., 2014).

Protein homologue of tensin (PTEN) is a dual function phosphatase with tumour suppressor function. PTEN is a negative regulator or PI3K/AKT signalling, and its downregulation is associated with increased PI3K/AKT signalling (Fournier et al., 2010). MAST2 interacts with PTEN as negative regulators of cell survival pathways such as
PI3K/AKT and silencing of either leads to increased survival (Terrien et al., 2012; Delhommel et al., 2015).

Synthetic lethal targeting of PTEN-deficient cancers identified MAST2 as a target (Mereniuk et al., 2013), meaning simultaneous silencing of both genes is selectively lethal. If either PTEN or MAST2 are lost, increased survival signalling is expected to lead to increased survival. In a PTEN-deficient context, MAST2 silencing leads to selective cell death via a synthetic lethal phenotype. MAST2 silencing alone is possibly tolerated as it is rescued by increased survival signalling involving PTEN. However, PTEN co-localises with E-cadherin at cell-cell contacts, so it is expected E-cadherin loss will displace PTEN. It has also been shown that E-cadherin expression is positively linked to PTEN expression (Fournier et al., 2009). In an E-cadherin-deficient context, it is possible that a mechanism for MAST2 synthetic lethality involves decreased PTEN activity leading to insufficient activation of PI3K/AKT signalling.

Recently, MAST2 knockout mice were demonstrated as viable with a normal phenotype (Gardin, 2010) This suggests knockdown in humans may be relatively well tolerated. Indeed, the WT (CDH1+/+) MCF10A cells showed very little reduction in viability (~5%), while CDH1−/− cells showed a ~20% reduction in viability. This indicates that drugs targeting MAST2 may produce little side effects in normal cells, while directly targeting E-cadherin-deficient cells.

Drug inhibition of MAST2 was considered for this project. However, the initial drug of interest, RB478952, was found to be a pan-kinase inhibitor with insufficient specificity for MAST2 and not further followed. The ubiquitous nature and conserved catalytic domains of kinases reflect the difficulty in achieving drug specificity with small molecules. However,
given the similar functions of some synthetic lethal candidates, the drug may have been worth investigating.

Inconsistent gene silencing effects between shRNA clones may have been explained by the mRNA secondary structure around the target sequence preventing the shRNA from binding the transcript. The lack of access may explain the shRNA clones that had no effect, while the shRNA clones killing both cell lines may be explained by off-target effects. Interestingly, one clone, E3, lead to (relatively highly) decreased viability in both isogenic cell lines. While MAST2 mRNA was reduced by E3 shRNA knockdown, it is possible that the shRNA is also targeting other genes whose inactivation is not synthetic lethal, but simply lethal. This is supported because clones E3 and D9 target the identical location on MAST2 transcript (1381 – 1399; figure 3.15) but have different sequences so one or both may have off-target effects.

Investigation of synthetic lethal kinase partners of p53 identified MAST2 as a potential target (Wang & Simon, 2013), though MAST2 expression was significantly increased in tumours with functional mutations of p53. In p53-deficient tumourigenesis, tumour suppressor roles including cell cycle arrest, DNA repair and apoptosis become defective and an aggressive phenotype is promoted (Aylon & Oren, 2011)

Gene ontology analysis of the synthetic lethal partners of p53 showed a strong representation of genes involved in cell cycle regulation, cellular growth, polarity and microtubule dynamics including PLK1, PLK4, CDK1, CDK16, MAST2, MAP3K4, MARK2 and Aurora-A (Wang & Simon, 2013). These candidates show significant crossover with those identified in CDH1-deficient MCF10A and reflect similar vulnerabilities in tumourigenesis that are driven by defective cell cycle and microtubule regulation.
4.3.2. MAP1B

MAP1B was also identified in the primary siRNA screen, with knockdown leading to a good synthetic lethal effect (WT 0.84, CDH1⁻/⁻ 0.65, fold-change 0.77). In this study, lentiviral shRNA knockdown has validated MAP1B as a synthetic lethal partner of CDH1. All three shRNA clones targeting MAP1B (H5, H6, H7) led to a synthetic lethal phenotype. H5 produced the strongest phenotype and was used for final validation experiments. 72 hrs post-transduction with H5 shRNA, CDH1⁻/⁻ cells showed a 30% reduction in viability compared to WT.

MAP1B encodes a protein belonging to the microtubule-associated protein family that influences cytoskeletal dynamics and cell fate (Bialik & Kimchi, 2006; Esser et al., 1997) MAP1B is highly expressed in neuronal regions, and shows a median expression pattern in the rest of the body (Protein Atlas, 2016). There is a paucity of new research on MAP1B outside of a neuronal context, where it regulates microtubule organisation, mediates axon guidance for receptor neurons and contributes to transport of cargo on the microtubules (Conde & Caceres, 2009; Aoki et al., 1995).

MAP1B plays a role in polarised growth and maintenance of plasma membrane domains in epithelial biogenesis (Spiliotis et al., 2008) by coupling microtubules to post-Golgi vesicle transport. Though not essential for this process, MAP1B silencing may affect polarised epithelial morphogenesis and downregulation has been associated with epithelial transition to hyperplasia and dysplasia (Pedrero et al., 2004). Conversely, ectopic MAP1B expression leads to increased microtubule stability and increased α-tubulin acetylation (Takemura et al., 1992). Increased microtubule stability can bolster tolerance against microtubule depolymerising agents such as vinblastine.
MAP1B interacts with DAPK-1 (death-activated protein kinase), a protein with wide ranging functions in cell growth control. These two proteins cooperate to induce growth inhibition, and together form a synthetic lethal interaction (Harrison et al., 2008). MAP1B and DAPK-1 co-localise in two groups – one with tubulin, the other with cortical F-actin. Reduction of MAP1B function in this context resulted in attenuated DAPK-1-stimulated autophagy.

MAP1B shows a maturation- and differentiation-dependent expression in retinal epithelium (Esser et al., 1997). In the same work, MAP1B expression was shown to increase during pathologic conditions and in cell culture (following multiple passages) resulting in cells losing epithelial characteristics and altering morphology to more resemble fibroblasts (Esser et al., 1997). Similarly, MAP1B has been shown to have a similar expression pattern to vimentin (VIM; also vitronectin (VTN) and fibronectin (FN1)) – associated with neuronal/mesenchymal/fibroblast (ie, long) characteristics (Aoki et al., 1995). Loss of epithelial characteristics and increased expression of vimentin is associated with epithelial-mesenchymal transition (EMT). It is possible MAP1B plays a role in EMT, a de-differentiation process involving cytoskeletal remodeling that is associated with invasiveness and poor prognoses. Thus, MAP1B may make an attractive target in advanced epithelial cancers, where increased MAP1B expression may be involved in lost epithelial characteristics and/or EMT events.

MAP1B expression is decreased 10- to 20-fold in CDH1−/− MCF10A compared to CDH1+/+ (Chen et al., 2014). The reduction in expression suggests MAP1B downregulation is well tolerated in an E-cadherin-deficient context, and makes it difficult to rationalise MAP1B as a synthetic lethal drug target. It is possible that decreased MAP1B expression and function contributes to E-cadherin-deficient tumourigenesis through reduced microtubule stability, and may contribute to the differences in microtubule architecture between the pair of cell
Interestingly, treatment with low dose of taxol 3 µM lead to a 7- to 8-fold increase of MAP1B expression in CDH1⁺⁺ cells, likely in response to the pressures of taxol targeting microtubule stability (unpublished, Telford, Beetham, Chen, Guilford).

Because ectopic expression leads to increased microtubule stability, it is likely that targeting/silencing MAP1B will reduce microtubule stability. The mechanism for synthetic lethality between MAP1B and CDH1 is not clear, though it is likely that dysregulation of microtubule dynamics and decreased stability with an already defective cytoskeleton is not tolerated by the cell.

To date, no drugs have been described that specifically target MAP1B. The absence of a solved crystal structure or absence from compound screening assays do not aid specific drug development targeting MAP1B. However, MAP1B and CDH1 are synthetic lethal, and silencing MAP1B is likely to lead to reduced microtubule stability. Therefore, in an E-cadherin-deficient context, all microtubule destabilising/depolymerising drugs could be investigated for effect. In isogenic MCF10A, there were no obvious phenotypic changes following silencing of MAP1B. Due to the role played by MAP1B in controlling microtubule dynamics, immunofluorescent staining of tubulin or actin may have highlighted some more subtle phenotypic changes.

Reduced protein content in the cell following shRNA knockdown was inferred from reduced mRNA measured by qPCR. Logically, as mRNA is translated into protein, reduced precursor should lead to reduced product. However, the rate of turnover/recycling varies greatly between proteins and some persist for days. Viability was assessed at 72 hrs post-transfection (in line with the preliminary screen), though the synthetic lethal effect may have increased with time. Ideally, MAP1B (and MAST2) knockdown would have been
validated at the protein level, but could be due to a lack of suitable antibodies.

4.3.3. Aurora kinase inhibition

Drug inhibition of Aurora-A and -B kinases (encoded by AURKA and AURKB) in isogenic E-cadherin-deficient cells found a synthetic lethal effect. (chapter 3.4). The Aurora kinase family is a collection of related serine/threonine kinases that function as key mitotic regulators. There are three members of the family, Aurora-A, -B and -C, but only Aurora-A and -B are expressed at detectable levels in somatic cells undergoing mitosis. Aurora-A and Aurora-B regulate centrosome function and act by controlling chromatid segregation and microfilament polymerisation (Ding et al., 2015; Bolanos-Garcia, 2005). Expression and activity of Aurora kinases is tightly controlled, and dysregulation leads to genetic instability, aneuploidy and tumourigenesis (Bolanos-Garcia, 2005; Fu et al., 2007). Overexpression of Aurora kinases is associated with malignancy (Carvajal et al., 2008) and chromosomal instability and thus the development of Aurora kinase inhibitors has gained attention.

Aurora-A localises primarily at the spindle poles and transiently along spindle microtubules, with key roles in bipolar spindle organisation, centrosome formation and mitotic entry (Fu et al., 2007; Ding et al., 2015). Inhibition of Aurora-A leads to unseparated chromosomes, and mitosis checkpoint activation, thereby impairing mitotic progression. (Hirota et al., 2003). Death can occur in mitosis through activation of apoptosis before escaping mitotic arrest. (Hilton & Shapiro, 2014).
Figure 4.1. Effects of Aurora kinase inhibition on cell cycle. While Aurora-A inhibition leads to mitotic arrest and defective spindle assembly, Aurora-B inhibition leads to bypass to mitotic checkpoints with dysregulated chromosomal segregation leading to polyploidy. Adapted from Komlodi-Pasztor (2011).

Aurora-B plays important roles in chromosome condensation, spindle-kinetochore attachment/regulation and cytokinesis (Komlodi-Pasztor, 2011; Hilton & Shapiro, 2014). Aurora-B inhibition leads to loss of normal chromosomal alignment, chromosomal segregation and cytokinesis (Hilton & Shapiro, 2014). In mitosis, Aurora-B inhibition results in failure to activate mitotic checkpoint proteins and rapid exit from mitosis. This leads to bypass of the checkpoint and cytokinesis failure, which contribute to polyploid cells and genomic instability (figure 4.1).

Both Aurora kinase inhibitors alisertib and danusertib have demonstrated efficacy in cancer cell lines, tumour xenograft models and clinical trials (Friedberg et al., 2014; Ding et al., 2015). Alisertib is a small-molecule inhibitor of Aurora-A, with 200-fold selectivity over Aurora-B (Manfredi et al., 2011) Danusertib is a small molecule inhibitor of all Aurora
kinases but more so isotype B (IC$_{50}$ of 13, 79 and 61 nmol/L for A, B, C, respectively; Carpinelli et al., 2007), as well as other kinases pertinent to cancer such as Bcr-Abl, FGFR-1 and TrkA tyrosine kinases (Gontarewicz & Brummendorf, 2010; Meulenbeld et al., 2012).

4.3.3.1. Aurora-A inhibition

Alisertib has demonstrated potent anticancer effects in epithelial cancer cell lines with strong growth-inhibiting, proapoptotic, proautophagic and EMT-inhibitory effects (Ding et al., 2015). Alisertib blocks the cell cycle with G2/M phase arrest and induced mitochondria-mediated apoptosis and autophagy. Alisertib suppresses PI3K/AKT, mTOR and p38 MAPK pathways but activates 5'-AMPDK, also contributing to proautophagic activity (Ding et al., 2015). In a tumour xenograft model, alisertib decreased bipolar and aligned chromosomes and produced strong growth inhibition (Manfredi et al., 2011).

Elevated levels of Aurora-A are found in about half of colorectal, ovarian and gastric cancers, and up to 95% of invasive breast duct carcinoma (Fu et al., 2007). Alisertib clinical trials are currently underway for several uses including gastrointestinal tumours, metastatic triple-negative breast cancer, urothelial cancer and advanced solid tumours (clinicaltrials.gov, 2016) and AK inhibitors have demonstrated clinical activity in B- and T-cell aggressive lymphomas (Friedberg et al., 2014) advanced and/or metastatic solid tumours (Cohen et al., 2009; Meulenbeld et al., 2013), and platinum-resistant or refractory epithelial carcinomas (Matulonis, 2012). Chromosomal instability is more common in advanced tumours, and underpins the use of Aurora kinase inhibition as an advanced anticancer strategy. In alisertib-treated MCF10A cells, chromatids appear misshapen and are incorrectly segregated, likely due to defective mitotic spindle machinery.
For reference, in the primary siRNA screen, Aurora-A showed a relative viability decrease of 10% (WT viability 0.64; CDH1\(^{-/-}\) viability 0.58; Telford et al., 2015). This did not meet the criteria of 15% viability reduction of CDH1\(^{-/-}\) compared to WT. In the same screen, Aurora-B gave a 15% reduction in CDH1\(^{-/-}\) viability (WT 0.39, CDH1\(^{-/-}\) 0.32; Telford et al., 2015), but did not meet the initial inclusion criteria because WT viability was decreased by more than 50% (ie, it is too toxic).

4.3.3.2. Aurora-B inhibition

Danusertib, the pan Aurora kinase inhibitor, has previously shown strong antiproliferative effects in a wide range of tumour cell lines (Meulenbeld et al., 2012), particularly those with polyploidy or other anomalies of endoreplication. It has also been used in a small number of clinical studies including leukaemia, prostate cancer and multiple myeloma (clinicaltrials.gov, 2016). Danusertib has been used in patients with advanced solid tumours, generating partial responses and instances of prolonged stable disease in a wide variety of advanced refractory cancers (Cohen et al., 2009). However, dose-limiting toxicity is reported with neutropenia, nausea and fatigue as the primary side effects (Steeghs et al., 2009).

Because cell cycle progression remains unperturbed, Aurora-B-inhibited cells undergo continued DNA replication, eventually reaching a degree of polyploidy at which death occurs from massive genome instability (Kops et al., 2005). The instability is intolerable and cells undergo apoptosis in mitochondria-mediated manner. Aneuploidy, polyploidy and multinucleation phenotypes make Aurora-B a less attractive therapeutic target, and this is reflected by the side effects and lower interest in clinical trials. Danusertib reduced viability more than siRNA treatment, perhaps due to functional overlap of Aurora-C providing rescue to Aurora-B-silenced multinucleation phenotypes (Balboula et al., 2014).
Danusertib-treated cells show obvious ploidy and effects on chromosome segregation. This target is likely not attractive for early DGC prevention or treatment because of massive genomic instability associated with Aurora-B inhibition (figure S.3).

Tozasertib (VX-680) is a potent inhibitor of Aurora kinases with particular affinity for isotype B. This compound did not produce any synthetic lethal effects in a known drug screen of the MCF10A isogenic cell lines (B Telford, unpublished). While danusertib does preferentially inhibit Aurora kinase B, it does have activity against other isotypes and perhaps different kinases altogether, so the mild synthetic lethal effect observed may be attributable to additive or synergistic effects of multiple interactions.

4.3.3.3 Aurora inhibition in HDGC

Reduced cytoskeletal stability in E-cadherin-deficient cells, together with chromosomal instability caused by defective mitosis following Aurora kinase inhibition may together be fatal to the cell. Both Aurora-A and Aurora-B are upregulated in CDH1-deficient MCF10A cells (Chen et al., 2014), though only by ~10%. It is likely that Aurora kinase inhibition would be well suited for targeting of genomically unstable tumours, a characteristic of more advanced disease or different subtype altogether (TCGA, 2014). The strategy of targeting mitosis has drawbacks for HDGC chemoprevention because early SRCC divide slowly. However, there is evidence that Aurora kinases may help maintain stem cell characteristics in carcinomas (Yi-Chao Hsu et al., 2016), possibly contributing to the poorly-differentiated phenotype observed in HDGC.

Alisertib is particularly interesting as it has been used in clinical combination with histone deactylase (HDAC) inhibitors (HDACi) and microtubule stabilising drugs such as paclitaxel (Do et al., 2014). HDACs and microtubule-associated genes were well represented in the
primary screen, suggesting this combination could be effective in E-cadherin-deficient cells. Targeting microtubules may be a better strategy for HDGC chemoprevention because tubulin has crucial roles in both mitotic and non-mitotic cells (Komlodi-Pasztor et al., 2011). Overall, mitosis-specific agents have had less clinical success than microtubule-targeting agents. While the efficacy of targeting microtubules is usually attributed to inducing mitotic arrest, other cellular mechanisms are also targeted during interphase. Targeting mitosis allows interference/inhibition during certain cell cycle stages, while targeting microtubules impairs functions during all stages. While targeting Aurora kinases may be a good strategy for advanced carcinoma, it is likely that targeting microtubules is more effective for early HDGC chemoprevention/chemotherapy.

Aurora kinase inhibition may be more useful in advanced DGC than the early stages of the disease. Early DGC cells are indolent, so inhibition of Aurora-A leading to attenuated cell division may not be a good mechanism for HDGC chemoprevention. As well as suppressing tumour growth, silencing of Aurora-A has been demonstrated to sensitise cells to more advanced-stage treatment modalities such as conventional chemotherapy and radiotherapy (Verheij et al., 2010; Shao et al., 2012). Conversely, inhibition of Aurora-B forces cells through the mitotic checkpoint and cell division, leading to genomic instability and polyploid cells. This mechanism is less useful for an early genomically stable tumour, although may have potential for advanced or genomically unstable tumours. As discussed above for p53, Aurora-A was identified as a synthetic lethal partner. Inhibition of Aurora-A leads to cell cycle arrest, a process that is ablated in p53-deficient tumourigenesis. This highlights the utility of Aurora-A as an amenable target in different genetic contexts and underscores the therapeutic benefits that may come from further development of Aurora kinase inhibitors.
4.3.3.4. Experimental

Treatment with Aurora-A and Aurora-B inhibitors alisertib and danusertib demonstrated synthetic lethal effect (chapter 3.4). Initial EC$_{50}$ experiments could have benefited from a tighter dilution series. This would cause more data points to fall on the curve, leading to a more accurate result. Despite this, a higher EC$_{50}$ concentration in WT than CDH1$^{-/-}$ hints at a synthetic lethal effect and the optimal concentrations for testing were determined.

Aurora kinase inhibitors led to distinct, aberrant nuclear morphologies with some ploidy in danusertib (figure S.3). Viability assessment of drugs that affect nuclear morphology can be compromised when nuclei counts are used to measure viability. Dose-dependent effects of the Aurora kinase inhibitors includes a spectrum of nuclear morphologies between normalising DMSO control and the maximum drug concentration. The principal methods of nuclei counting, utilising CellProfiler and/or Cytell analysis package, rely on cell size/shape parameters defined for each experiment to identify individual nuclei. These parameters were kept constant within each experiment (though optimised between each biological replicate), which introduces errors that grow in line with the dose-dependent effects of the drug.

Assessment of AKi-treated cell viability may therefore be more accurate with assays that don't rely on morphology, such as metabolic assays (resazurin, MTT, etc). It is possible that this experiment may have benefited from using two sets of parameters – one for DMSO control and one for drug-treated. Manual counting is also an option, although obviously undesirable. Given the role of Aurora kinases in mitosis, it may have been informative to undertake cell-cycle analysis. Again, the morphological effects of Aurora kinase inhibiton on the nucleus makes it difficult to perform cell cycle analysis using the Cytell.
4.4. Future directions

This research validated the presence of synthetic lethal interactions identified in preliminary siRNA screening (Telford et al., 2015). This supports the validity of the screen and indicates that there is merit in further investigating other candidates. For example, genes related to PI3K/AKT signalling are enriched for synthetic lethal interactions with CDH1, suggesting this network is a valuable target.

Although none of the candidate drugs or siRNA produce true synthetic lethality, the reductions in viability are more reflective of a synthetic sick phenotype, where cells are selectively impaired or have their fitness reduced. In BRCA1/2-deficient cell lines, the silencing of PARP leads to a selective ~80% reduction in viability, a much stronger effect than the 30% reduction observed in this investigation. However, it is worth noting that simply delaying or slowly progression and development is therapeutically beneficial.

This work warrants the development of targeted therapies against validated synthetic lethal genes, including serine/threonine kinases MAST2, Aurora-A and Aurora-B kinases AURKA and AURKB, and microtubule-associated protein MAP1B. The findings also warrant the investigation of known and approved compounds that target E-cadherin-deficient vulnerabilities such as cytoskeletal processes or PI3K/AKT signalling, even if they are not necessarily highly specific.

The function of both MAST2 and MAP1B is to stabilise microtubules, so it is likely that microtubule-destabilising agents will elicit strong responses. Investigation of drug activity in E-cadherin-deficient context provides valuable evidence for combinations of drugs to increase efficacy while mitigating side effects. Prediction of synthetic lethality and synergy would be well supported by mapping of gene networks and functional redundancy.
Although this investigation was primarily focused on chemoprevention of HDGC, findings may support synthetic lethal targeting in a wider range of sporadic cancers with reduced \textit{CDH1} expression. Most cancers with reduced \textit{CDH1} expression are carcinomas, and include ovary, prostate, lobular breast and colorectal cancers, as well as medulloblastomas.

Recently, Medsafe-approved drug denosumab has been demonstrated to halt early breast cancer cells and delay the growth of tumours harbouring \textit{BRCA1} mutations (Nolan, 2016). Denosumab is an antibody drug that is used in the treatment of osteoporosis via targeting of a surface protein that is also expressed in \textit{BRCA1}-deficient cells. Although this study was performed in mice and cell lines, the work has future implications for cancer chemoprevention in high-risk individuals and aptly highlights three things: (1) the therapeutic potential of repurposing existing drugs into novel clinical contexts; (2) the value of targeted therapy with specific antibody-based drugs; and (3) the possibility of reducing surgical reliance through chemopreventive strategies.

4.4.2. Future research

Although silencing the synthetic lethal partners of \textit{CDH1} do not yield viability losses likely to lead to a revolutionary new therapy, there is value in further investigating the cellular effects. To better characterise the effects of targeting mitosis or microtubules, some simple assays such as tetrazolium dye assays or cytostatic activity assays could be used to assess cytotoxicity or shifts in proliferative capability. Further cell cycle analysis and immunofluorescent imaging following treatment would provide good evidence on the effect of targeting mitosis or microtubules.

More biologically relevant models would benefit the further development of synthetic lethal
drugs or validation of further targets. To represent early in situ development, 3D cultures/spheroids would be a useful tool for further drug and shRNA experiments. Cultivation of cells on ECM protein-containing gel or in a suspended/hanging media droplet are possible options for achieving 3D cultures in MCF10A (A Chen, pers. comms., 2015). Xenografting and engineered or gastric cancer-prone mouse models are also powerful tools for studying synthetic lethality in vivo.

The results of this investigation highlight the need to explore further drugs that target functions and processes of mitosis and the cytoskeleton, especially those identified and validated in this work. Although antibody-based drugs can achieve the greatest specificity, they cannot enter a cell and only bind targets on the cell surface. Therefore, specificity of drugs targeting MAST2 or MAP1B is best achieved with small molecules to allow entry into the cell. However, the development of these drugs is costly, and it is unlikely that these findings warrant new drug development.

Investigation of drugs targeting the cytoskeleton, although unlikely to be a targeted therapy, are likely to show synthetic lethal activity in E-cadherin-deficient cells. There is a wide variety of compounds that target the cytoskeleton, and are classed as stabilising or destabilising agents. Taxol and its derivatives are stabilising agents that impair the dynamic nature of microtubules by preventing depolymerisation, while destabilising agents such as vinblastine cause rapid depolymerisation and derangement of microtubules. The results of this investigation suggest destabilising agents may provide the best clinical effects, and these drugs should be further explored in more advanced HDGC models. Despite being a stabilising agent, treatment with taxol leads to selective reduction in CDH1−/− viability (A Single, pers. comms., 2015). There have been few advancements in microtubule-targeting drugs in the last decade, although there are many drugs, such as
pseudolaric acid B, are worth testing (Wong *et al.*, 2005). However, given the clear vulnerabilities in the cytoskeleton and mitosis, more drugs that target these functions should be investigated.

Identifying complementary compounding effects of drugs (ie, synergy) is important for clinical use and as well as building a more complete understanding of the early cancer biology. HDACs were identified as targets in siRNA screening, and combined treatment of taxol and vorinostat/SAHA (high HDAC class I and II specificity) shows an additive effect, though no synergy (A Single, pers. comms., 2015). Synergistic relationships between drugs can be rationalised like synthetic lethal effects (ie, related/functional redundancy, convergent pathways), where targeting two things together elicits a greater response. Given the success of simultaneous Aurora kinase and microtubule inhibition in the clinic (Do *et al.*, 2014), this combination should be investigated for further in a CDH1-deficient context. There is good arsenal of HDAC inhibitors, and may translate well for chemoprevention because second somatic allele loss of CDH1 is often caused by hypermethylation.

Novel drug screening is one method that may yield further useful compounds for HDGC chemoprevention. Compounds derived from flora have serendipitously evolved to interact with many facets of biology, and this underpins the value of sourcing novel drugs from plants where it is estimated some 95% have yet to be investigated (Balunas, 2005). High-throughput screening of New Zealand flora for useful compounds has uncovered many molecules predicted to have relevant bioactivity that warrant further investigation. Methods of screening for compounds can include mass spectrometry, or functional predictions based on sequencing data in the case where a reference genome is available, among others. Screening drugs in isogenic cell lines – as was performed in this investigation –
may uncover further E-cadherin-deficient vulnerabilities and targets for therapy or chemoprevention.

Other approaches to the clinical management of HDGC include options outside of drug therapy. In assisted reproduction therapy (ART), pre-implantation genetic diagnosis (PGD) is the genetic profiling and screening for specific diseases. Embryos are evaluated and those with certain genetic characteristics may be selected against, while those without the risk variant are selected for implantation and gestation. In HDGC families, the offspring of an affected individual carry a 50% chance of inheriting a defective CDH1 allele – higher if both parents carry a defect in CDH1. In HDGC families, CDH1+/− embryos can be discarded while CDH1+/+ embryos can be implanted, effectively eliminating inherited cancer incidence. This approach has been proven (Rechitsky et al., 2002) and would reduce the public health burden and increase individual wellbeing by effectively and permanently eliminating the predisposition to highly penetrant cancer for an entire lineage.

Along the same lines, genome editing with precision techniques can be used to reduce disease penetrance. Engineering of the human genome is a controversial issue that has generated heated debate, although the practice will inevitably become more prevalent. The grey area between ‘fixing’ a pathogenic genome and transhumanist enhancements will need to be clearly defined. Methods of disease proofing includes creating functional overlap/redundancy, removing pathogenic gene variants, reducing DNA polymerase error/mutation rate and/or telomerase activity to delay senescence. For example, cell cycle regulator p53 is mutated/affected in some 90% of human cancers and it is possible that engineering functional redundancy could reduce cancer incidence. Recently, approval has been granted for human studies using the CRISPR/Cas genome editing system to engineer T-cells for the targeting of immune-evading cancer cells.
For now, continued effective management of HDGC requires multicentre efforts due to the rarity of the syndrome. However, this work has shown that CDH1-deficient cells can be selectively targeted. By further refinement of the synthetic lethal drugs used, particularly in combination, it is likely that HDGC chemoprevention will become a reality.
5. Appendix

5.1. Reagent preparation

5.1.1. PBS
One PBS tablet was added to 100 mL of mQH$_2$O and autoclaved as per the manufacturers instructions.

5.1.2. Trypsin
0.5% trypsin was diluted in PBS 1/2 and 1/10 for 0.25% and 0.05% trypsin, respectively, and stored at 4°C.

5.1.3. Lysogeny broth (LB)
For 500 mL LB, 5 g tryptone, 2.5 g yeast extract, 5 g NaCl were added to 500 mL mQH$_2$O and autoclaved. For LB agar plates, 7.5 g agar was added prior to autoclaving. Following autoclaving, agar was allowed to cool before being poured into 100 mm plates. For
selective plates 100 µg/mL ampicillin was added just prior to plate pouring. With asceptic technique, LB was stored at RT and used for up to one month, while LB plates were stored at 4°C and had a usable time of two weeks.

5.1.4. Super optimal broth with catabolite repression (SOC)

For 500 mL of SOC, 10 g tryptone, 2.5 g yeast extract, 0.292 g NaCl, 0.093 g KCl, 0.476 g MgCl2, 1.233 g MGSO4, 1.8 g glucose were added to a 1 L bottle, made up to 500 mL with mQH2O and autoclaved. With asceptic technique, SOC was stored at RT and used for up to one month.

5.1.5. Resazurin metabolic assay reagent

For 400 mL of dye, 0.044 g of resazurin was dissolved with prolonged stirring in 400 mL of 1X PBS pH 7.4 for a final stock concentration of 440 µM. Dye was filtered through 0.22 µm polyethersulfone filter and aliquoted. Dye was stored at 4°C for use or -20°C for long-term storage.

5.1.6. Western blot buffers

5.1.6.1. Lower buffer

For 1 L of 4X lower buffer (1.5 M Tris-Cl pH 8.8, 0.4% SDS): some number g Tris and 40 mL of 10% SDS was dissolved in 800 mL mQH2O, made up to 1 L, and adjusted to pH 8.8 with 1 M NaOH and HCl. Buffer was stored at RT and used within one year.

5.1.6.2. Upper buffer

For 1 L of 4X upper buffer (0.5 M Tris-Cl pH 6.8, 0.4% SDS): some g Tris and 40 mL of 10% SDS was dissolved in 800 mL mQH2O, made up to 1 L, and adjusted to pH 6.8 with 1 M NaOH and HCl. Buffer was stored at RT and used within one year.
5.1.6.3. Running buffer

For 1 L of 10X running buffer: 135.2 g glycine, 24.16 g Tris, and 8 g SDS was dissolved in 800 mL mQH\(_2\)O and made up to 1 L. For 1 L of 1X buffer: 50 mL of 20X buffer was mixed with 950 mL mQH\(_2\)O. Buffer was stored at RT and used within one year.

5.1.6.4. Transfer buffer

For 1 L of 20X transfer buffer without methanol: 150.1 g glycine and 24.16 g Tris was dissolved in 800 mL mQH\(_2\)O and made up to 1 L. For 1 L of 1X buffer: 50 mL of 20X buffer was mixed with 850 mL mQH\(_2\)O followed by 100 mL methanol. Buffer was made fresh from 20X concentrate on the day of protein transfer.

5.1.6.5. Tris-buffered saline with tween-20 (TBST)

For 1 L of 10X TBST: 24.23 g Tris, 87.66 g NaCl, and 10 mL tween-20 was dissolved in 200 mL mQH\(_2\)O and made up to 1 L, adjusted to pH 7.5, and then made up to 1 L. For 1X TBST: 10X TBST was diluted 1/10 with mQH\(_2\)O. Buffer was stored at RT and used within one year.

5.1.6.6. Protein loading Laemmli buffer

For 100 mL of 5X protein loading Laemmli buffer (250 mM Tris-Cl pH 6.8, 10% SDS, 50% glycerol, 25% 2-mercaptoethanol, 0.05% bromophenol blue): 25 mL Tris-Cl (pH 6.8; upper buffer), 10 g SDS, and 0.5 g bromophenol blue were combined in 50 mL glycerol. 25 mL 2-mercaptoethanol was added immediately prior to use (for solution longevity, 750 µL buffer without 2-mercaptoethanol was combined with 250 µL 2-mercaptoethanol at the commencement of each experiment). 5X protein loading buffer was diluted with 4 parts protein lysate for use at 1X.
5.1.6.7. Stripping buffer

For 100 mL of harsh stripping buffer (2% SDS, 60 mM Tris-HCl, 0.8% 2-mercaptoethanol):
20 mL of 10% SDS, 12.5 mL of 0.5 M Tris-Cl (pH 6.8), and 0.8 mL of 2-mercaptoethanol
was added to 66.7 mL of mQH$_2$O/made up to 100 mL with mQH$_2$O. Buffer was stored at
RT and used within one year.

5.1.7. SDS-PAGE gel

For one 10% resolving gel: a mixture of 4.2 mL ddH$_2$O, 2.5 mL lower buffer, 3.3 mL 30%
acrylamide (37.5:1 bis:acrylamide) and 200 μL of 10% sodium dodecyl sulfate (SDS) was
polymerised by addition of 20 μL tetramethylethlenediamine (TEMED) and 150 μL of 10%
ammonium persulfate (APS) and rapidly cast. To prevent desiccation, resolving gel was
gently covered with isopropanol and prior to the casting of the stacking gel the isopropanol
was carefully removed.

For one stacking gel, a mixture of 2.35 mL ddH$_2$O, 1 mL upper buffer, 0.65 mL 30%
acrylamide (37.5:1 bis:acrylamide) and 80 μL of 10% SDS was polymerised by addition of
20 μL TEMED and 60 μL of 10% APS and rapidly cast atop the resolving gel and the stack
comb carefully added. Once set, gel was used immediately or otherwise stored airtight
inside a moist tissue at 4°C until use within two weeks.

5.1.8. Immunofluorescent imaging buffers

5.1.8.1. Fixing buffer

For 100 mL fixing buffer, 3.6 g paraformaldehyde (PFA) was slowly added to 90 mL of
PBS with stirring. 1M NaOH was added dropwise until PFA was dissolved. pH was
adjusted to 6.9 with 6M HCl. Fixing buffer was then filtered through 0.22 μm
polyethersulfone filter and frozen in 10 mL aliquots at -20°C. Aliquotes were thawed at RT
on day of use, and were used within one week.

5.1.8.2. Permeabilisation buffer

For 100 mL permeabilisation buffer, 5 mL of 10% Triton (final 0.5%) was added to 95 mL PBS and dissolved with agitation. Bubbles from agitation were allowed to settle before use, and stored at room temperature under no specific conditions. Buffer was stored at RT and used within one month.

5.1.8.3. Blocking buffer

For 100 mL of blocking buffer, 5 mL of secondary antibody-matched serum and 1 mL of fetal bovine serum was added to 94 mL of PBS. Blocking buffer was made fresh on the day of immunofluorescent imaging.

5.1.8.4. Antibody diluting buffer

For 100 mL of antibody diluting buffer, 1 mL of FBS and 0.5 mL of 10% Triton (final 0.05%) was added to 94.5 mL PBS. Buffer was stored at RT and used within one month.

5.1.9. Plasmid extraction kits

Zyppy MidiPrep & Zyppy MiniPrep (Zymo, PureLink MaxiPrep & PureLink MidiPrep (Invitrogen); NucleoSpin MiniPrep & NucleoBond Xtra MidiPrep (Machery-Nagel); and PureYield MidiPrep (Promega) plasmid extraction kits as per the manufacturers instructions.

5.2. Antibody dilutions
<table>
<thead>
<tr>
<th>Western blot</th>
<th>1˚</th>
<th>2˚</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin (CDH1)</td>
<td>Sc7870 Pc rabbit anti-E-cadherin 1:200</td>
<td>NA934 Pc α-rabbit HRP-conjugate 1:5000</td>
</tr>
<tr>
<td>α-tubulin (TUBA)</td>
<td>T6199 Mc mouse anti-α-tubulin 1:2500</td>
<td>NA931 Pc α-mouse HRP-conjugate 1:5000</td>
</tr>
</tbody>
</table>

Table 5.1. Western Blot antibody dilutions.

<table>
<thead>
<tr>
<th>Immunofluorescence</th>
<th>1˚</th>
<th>2˚</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin (CDH1)</td>
<td>Sc7870 Pc rabbit anti-E-cadherin 1:200</td>
<td>AlexaFluor 488 goat α-rabbit 1:500</td>
</tr>
<tr>
<td>F-actin (ACTA/ACTB)</td>
<td>A2066 Pc rabbit anti-actin 1:400</td>
<td>AlexaFluor 647 goat α-rabbit 1:1000</td>
</tr>
<tr>
<td>α-tubulin (TUBA)</td>
<td>T6199 Mc mouse anti-α-tubulin 1:1000</td>
<td>AlexaFluor 547 rabbit α-mouse 1:4000</td>
</tr>
<tr>
<td>Pericentrin (PCNT)</td>
<td>A4448 Pc mouse anti-pericentrin 1:1000</td>
<td>AlexaFluor 555 rabbit α-mouse 1:1000</td>
</tr>
<tr>
<td>Nucleus (DAPI)</td>
<td>Vectashield GOLD</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 5.2. Immunofluorescence antibody dilutions.

5.3. PCR primer sequences

**MAST2** fwd 5’ – TTG CCC TCT TCA GGA TAT GG – 3’
**MAST2** rev 5’ – GTG CTG AAA TGC TTC GTC AA – 3’
**MAP1B** fwd 5’ – CCT CGA GAC GTG ATG AGT GA – 3’
**MAP1B** rev 5’ – AAT CCG TTG AGC GGT GTA AC – 3’
**GAPDH** fwd 5’ – GCG CCC AAT ACG ACC AA – 3’
**GAPDH** rev 5’ – GCT CTC TGC TCC TCC TGT T – 3’
**PPIA** fwd 5’ – TCT TTC ACT TTG CCA AAC ACC – 3’
**PPIA** rev 5’ – CAT CCT AAA GCA TAC GGG TCC – 3’
**RPL13a** fwd 5’ – CTG GCC TCG CTG GTA TT – 3’
**RPL13a** rev 5’ – GAT GAA CAC CAA CCC TCC CC – 3’
**CDH1** fwd 5’ – CTG AGG ATG GTG TAA GCG ATG – 3’
**CDH1** rev 5’ – GTC TGT CAT GGA AGG TGC TC – 3’
**pGIPZ miR** fwd 5’ – GCA TTA AAG CAG CGT CTG ATC – 3’
5.4. PCR reaction mixtures

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X PS buffer</td>
<td>2</td>
</tr>
<tr>
<td>RT enzyme</td>
<td>0.5</td>
</tr>
<tr>
<td>Oligo dT primer</td>
<td>0.5</td>
</tr>
<tr>
<td>Random 6mers</td>
<td>0.5</td>
</tr>
<tr>
<td>RNA</td>
<td>6.5</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>10</strong></td>
</tr>
</tbody>
</table>

Table 5.3. Reaction mixture for 10 µL PrimeScript cDNA synthesis.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL) for 384-well</th>
<th>Volume (µL) for 96-well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evagreen master mix</td>
<td>3</td>
<td>6.5</td>
</tr>
<tr>
<td>F/R primer (300 nM)</td>
<td>0.0975</td>
<td>0.195</td>
</tr>
<tr>
<td>Template cDNA</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Water</td>
<td>1.9025</td>
<td>4.305</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>6</strong></td>
<td><strong>13</strong></td>
</tr>
</tbody>
</table>

Table 5.4. Reaction mixture for 6 and 13 µL Evagreen qPCR.

5.5. shRNAmir-30 sequences

*MAST2 D9* – CCGAGACTGCCTGGATAAA  
*MAST2 D10* – TCTTTGTAACATGATTCA  
*MAST2 D11* – TTGCTTTAAAGCTCATCT  
*MAST2 D12* – TACTTGTCCGACAAAAGCT  
*MAST2 E1* – TGATCACTTTGTTGACAGG  
*MAST2 E2* – TGGACAGTCCAAAGTCCGT  
*MAST2 E3* – TTTATCCAGGCAGTCTCGG  
*MAP1B H5* – CCCTTGCCAGCTTCACCA  
*MAP1B H6* – GACTCCAGTTATCACATA  
*MAP1B H7* – CAAGGACTTTGAAGAGTTA  
*GAPDH* – TGGACAAAGTTGTCATTGAG  
Non-silencing – CTTACTCTCGCCCAAGCGAG  
No insert – no shRNAmir
5.6. Neon Transfection System conditions

Conditions were initially tested with 5 µg of plasmid and 2 \times 10^5 cells.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Voltage (v)</th>
<th>Pulse length (ms)</th>
<th>Number (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>1200</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>T2</td>
<td>1600</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>T3</td>
<td>1700</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>T4</td>
<td>1600</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>T5</td>
<td>1600</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>T6</td>
<td>1500</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>T7</td>
<td>1500</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>T8</td>
<td>1600</td>
<td>30</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 5.5.a. Initial Neon transfection optimisation conditions.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Plasmid (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>5</td>
</tr>
<tr>
<td>T9</td>
<td>10</td>
</tr>
<tr>
<td>T10</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 5.5.b. Neon transfection plasmid titre conditions.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>T10</td>
<td>2 \times 10^5</td>
</tr>
<tr>
<td>T11</td>
<td>4 \times 10^5</td>
</tr>
<tr>
<td>T12</td>
<td>6 \times 10^5</td>
</tr>
</tbody>
</table>

Table 5.5.c. Neon transfection cell density optimisation conditions.

5.7. Factor well size calculation

Costar 3603 well size (from catalogue) 0.3165 cm²  
Eclipse Ti Inverted Microscope 10X field size (derived) 0.00684 cm²  
0.3165 / 0.00684 = ~46.7 fields/well
Supplementary
Figure S.1. Early death following Neon transfection. Centre of well-plate (for dead cell accumulation) for non-silencing pGIPZ condition T12. Rounded/extra bright cells are undergoing apoptosis and necrosis in response to cellular stressors such as electroporation, puromycin selection, and plasmid uptake and RNAi. Note, no puromycin is added until 24 hrs, so all prior death is due only to electroporation and plasmid activity.
Figure S.2. Expression delay in gene silencing modalities. While transfection by electroporation leads to strong expression of GFP at 24 hrs, the signal is delayed in the lentiviral method, likely due to extra processing (ie, reverse transcription, integration).
Figure S.3. Effects of Aurora kinase inhibition on MCF10A nuclear morphology. Alisertib (Aurora-A) treatment leads to improper chromosome condensation and "sickle cell" morphology, while danusertib (Aurora-B) treatment leads to ploidy and genomic instability.
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Synthetic lethal screens identify vulnerabilities in GPCR signalling and cytoskeletal organization in E-cadherin-deficient cells

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Running title: Druggable vulnerabilities in E-cadherin-deficient cells

Keywords: E-cadherin, synthetic lethality, Hereditary Diffuse Gastric Cancer, cytoskeleton, chemoprevention

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**Conflicts of interest:** No conflicts of interest

Word Count: 5108

Four figures and one table. Four Supplementary Figures and Four Supplementary Tables.
Abstract

The \textit{CDH1} gene, which encodes the cell-to-cell adhesion protein E-cadherin, is frequently mutated in lobular breast cancer (LBC) and diffuse gastric cancer (DGC). However, because E-cadherin is a tumor suppressor protein and lost from the cancer cell, it is not a conventional drug target. To overcome this, we have taken a synthetic lethal approach to determine whether the loss of E-cadherin creates druggable vulnerabilities. We first conducted a genome wide siRNA screen of isogenic MCF10A cells with and without \textit{CDH1} expression. Gene ontology analysis demonstrated that G-protein coupled receptor (GPCR) signalling proteins were highly enriched amongst the synthetic lethal candidates. Diverse families of cytoskeletal proteins were also frequently represented. These broad classes of E-cadherin synthetic lethal hits were validated using both lentiviral-mediated shRNA knockdown and specific antagonists including the JAK inhibitor LY2784544, Pertussis toxin and the aurora kinase inhibitors Alisertib and Danusertib. Next, we conducted a 4,057 known drug screen and time course studies on the \textit{CDH1} isogenic MCF10A cell lines and identified additional drug classes with linkages to GPCR signalling and cytoskeletal function that showed evidence of E-cadherin synthetic lethality. These included multiple histone deacetylase inhibitors including Vorinostat and Entinostat, phosphoinositide 3-kinase inhibitors, and the tyrosine kinase inhibitors Crizotinib and Saracatinib. Together, these results demonstrate that E-cadherin loss creates druggable vulnerabilities that have the potential to improve the management of both sporadic and familial LBC and DGC.
**Introduction**

E-cadherin is a cell-to-cell adhesion protein that is localized at the adherens junction of all epithelial cells (1). Other than its roles in cell adhesion, E-cadherin is involved in establishing and maintaining cell polarity and differentiation, the organization of cell migration and architecture and the mediation of signalling through various proliferation and survival pathways including WNT and EGFR (2, 3).

Abrogation of expression of the E-cadherin gene (*CDH1*) by mutation, deletion or promoter hypermethylation is a feature common to many epithelial tumors and its downregulation is the hallmark of both diffuse gastric cancer (DGC) and lobular breast cancer (LBC) (1, 4-6). Disrupting E-cadherin’s expression or localization has a pronounced impact on a cell’s cytoskeletal structure, with changes including misalignment of the microtubule and actin cytoskeletons, defects in cell migration and irregularities in the orientation of the mitotic spindle (7-9).

Germline *CDH1* mutations are responsible for Hereditary Diffuse Gastric Cancer (HDGC), a cancer syndrome characterized by the highly penetrant, early onset of multifocal DGC and an elevated rate of LBC. In HDGC, *CDH1* inactivation is an initiating event that may be related to abnormal mitotic spindle orientation resulting in daughter cells being displaced into the *lamina propria*, outside the epithelial plane (10-12). In other cancer types its downregulation is considered to be a late event that promotes increased invasive capacity, frequently through association with the epithelial-mesenchymal transition (13).

Although E-cadherin is a tumor suppressor protein that is lost from the cancer cell and therefore not a conventional drug target, the downregulation of such a multifunctional protein during tumorigenesis would be predicted to create vulnerabilities in the cell which are targetable using a synthetic lethal approach. In the context of drug development, synthetic lethality can be defined as a drug which reduces cell viability or fitness only in cells carrying a
specific mutation. The utility of synthetic lethal targeting of tumor suppressor genes is well illustrated clinically by Olaparib, an inhibitor of the DNA repair enzyme poly-ADP ribose polymerase (PARP). Olaparib elicits strong clinical responses in breast and ovarian cancer patients who harbour inactivating mutations in the homologous recombination dsDNA repair genes BRCA1/2 (14, 15).

In addition to providing new therapeutic avenues for the treatment of sporadic epithelial cancers, synthetic lethal targeting of E-cadherin-deficient cells also has the potential to improve the clinical management of HDGC. In order to identify druggable synthetic lethal vulnerabilities in E-cadherin-deficient cells, we have conducted both a genome-wide siRNA synthetic lethal screen and a four thousand compound known drug screen on isogenic breast MCF10A cells with and without CDH1 expression. Together, these screens have identified multiple druggable targets which suggest new therapeutic strategies for the treatment of E-cadherin-deficient cancers and the chemoprevention of HDGC.

**Materials and Methods**

**Cell lines and Media**

The MCF10A breast cell line and its paired isogenic MCF10A CDH1<sup>−/−</sup> line (here designated CDH1<sup>−/−</sup>) were obtained from Sigma Aldrich in 2011 (parental line ATCC® CRL-10317) and had been authenticated using short terminal repeat analysis. CDH1<sup>−/−</sup> had been created by homozygous deletion of 4bp from exon 11 of the CDH1 gene. The lines were resuscitated within one week of receipt and early passage cells (passage 3-7) were aliquoted and frozen. All experiments were conducted with cells between passages 6-15 in DMEM F12 media with glutamate, 5% horse serum, 20 ng/mL EGF, 0.5 µg/mL hydrocortisone, 100 ng/mL cholera toxin and 10 µg/mL insulin. Characterization of the cell line pair is described elsewhere (7).
siRNA high throughput screen

Cells were transfected with siRNAs from the Dharmacon SMARTpool whole genome protein-coding siRNA library (RefSeq 27) housed in the Victorian Center for Functional Genomics. Each SMARTpool contained four siRNAs that targeted different regions of each gene in one well. Each reaction, in a white walled, clear bottom 384 well plate format, contained 0.125% DharmaFECT 3 (0.05 µL), 27.4% OptiMEM (Invitrogen) and 40 nmol/L of the siRNA SMARTpool (total volume 37.5 µL). Cells were reverse transfected and seeded onto the siRNA cocktail at a density of 700 MCF10A cells/well and 900 CDH1⁻/⁻ cells/well to enable the two cell lines to reach confluence at the same time point (72 hours after seeding). The following controls were included in each plate: the death controls siEGFR and siPLK1 (4 wells each), a synthetic lethal control siCTNNB1 (6 wells) and two negative controls, siRISC free and mock (lipid only, 9 wells each). After 24 hours, the media was replaced and at 72 hours 10 µL CellTiter-Glo® was added to each well (final concentration 1/5), shaken on an orbital shaker for 2 minutes and incubated for a further 30 minutes at room temperature before measuring luminescence using a Synergy H4 microplate reader (Biotek). siRNA was dispensed using a Caliper Sciclone ALH3000 (PerkinElmer). All other liquid handling steps were performed using a Biotek406 liquid handling workstation. Primary screen analysis was performed by normalizing genes in each cell line individually to the average mock value (across all screen plates) for the respective cell line. The level of increased kill was determined by the ratio of CDH1⁻/⁻ viability to MCF10A viability. Candidates with MCF10A viability ≥50% and a fold change ratio of ≤0.85 were considered synthetic lethal candidates. Selection of the final 500 genes chosen for secondary screening was based on further analysis of druggability and biological relevance. Secondary siRNA screening was performed using four individual siRNAs targeting each gene arrayed in individual wells, separately using the same transfection conditions described above with a final siRNA concentration of 25 nmol/L. The secondary screen was analysed using the same normalization strategy and cut offs as the primary screen.
Viral knockdown

Dharmacon pGIPZ lentiviral shRNA mir30 plasmids were prepared from cultures using the Machery Nagel EasyPure Miniprep kit. 293FT cells were co-transfected 24hr after seeding with 18.6 µg pGIPZ, 9.6 µg PAX2 and 4.8 µg VSVG plasmids using 55.7 µL Lipofectamine 2000 (Invitrogen). Media was changed at 24 hours, and after a further 24 hours viral particles were harvested by aspirating media, centrifugation at 3,000 rpm for 15 minutes to remove cellular debris and filtration through a 0.45 µM PVDF filter. Virus was aliquoted and snap frozen for subsequent use.

Viral titer was determined by seeding MCF10A cells at 4000 cells/well and transducing with a 1/32 dilution of virus 24 hours later. Media was changed after 24 hours, and after a further 24 hours GFP expressing cells were quantitated over 5 fields at 10x magnification. The average number of transduced cells/well was used to determine the number of transducing units per mL.

For knockdown experiments, 1000 MCF10A and 2000 CDH1−/− cells/well were seeded in black walled, clear bottomed 96 well plates and allowed to adhere overnight. The following day, virus was added at a multiplicity of infection (MOI) of 10. Media was changed at 24 hours and 1 µg/mL puromycin added. 72 hours after transduction, media was aspirated and 1 µg/mL Hoechst 33342 and 0.5 µg/mL propidium iodide in PBS added. After 30 minutes incubation plates were imaged with 4 fields/well on the Cytell (GE) at 4x magnification and 10 fields/well at 10x magnification using the ‘Cell Viability BioApp’. CellProfiler (16) was used to quantitate the total nuclei, as well as the proportion stained with propidium iodide.

RNA was extracted at 72 hours to determine gene knockdown using the RNAgem-PLUS kit (ZyGem). cDNA was synthesized using the Primescript cDNA synthesis kit (Takara), and RT-qPCR performed using Sybr Fast kit (KAPA) on an ABI7900HT with an enzyme activation step of 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds, 57°C for 15 seconds
and 72°C for 15 seconds. GAPDH and PPIA were used as reference genes and results were analysed using the efficiency method as described by Pfaffl (17).

**Known drug screen**

Assay ready plates containing 20 nL of 4,057 compounds diluted in DMSO at four concentrations (2 μmol/L, 1 μmol/L, 0.5 μmol/L and 0.25 μmol/L) were prepared by the Walter and Eliza Hall Institute (WEHI) High-Throughput Chemical Screening Facility. Cells were seeded directly onto these plates in a volume of 50 μL at a density of 1000 cells/well for MCF10A and 1,200 cells/well for CDH1−/− in 384 well clear bottom plates. After 48 hours, 20 μL of CellTiter-Glo® (Promega) was added to each well, shaken for 2 minutes on an orbital shaker and incubated for 30 minutes before reading. Values were normalised to the average DMSO control value of the whole screen for each cell line at each concentration.

The secondary drug screen was performed on 316 compounds selected from the primary screen. These were provided at 5 μmol/L and 11 1:1 dilutions were made with DMSO to create a 5 μmol/L to 5 nmol/L concentration range. Cells were seeded at a density of 700 cells/well for MCF10A and 900 cells/well for CDH1−/− for 24 hours before 100 nL of each compound was added to the plates robotically. 32 DMSO control wells were included in each plate. Plates were incubated for a further 48 hours before assaying with CellTiter-Glo®.

**Drug titrations and time course assays**

To determine drug EC50, cells were seeded in white walled, clear bottom 384 well plates at a density of 800 cells/well in a volume of 45 μL. After 24 hours incubation, 5 μl of drug (rehydrated in DMSO to an 80 mM stock and diluted in complete media) was added to each well. A DMSO control and a cell only control were used on each plate. The 11 drug concentrations used were dependent on the individual drug. After 48 hours of treatment, cell viability was assayed using either the CellTiter-Glo® (Promega) or Alamar blue assay. All results were normalized to the average DMSO control for individual cell types. EC50 values
were calculated by plotting viability against the log drug concentration and fitting a nonlinear regression curve using Prism version 6.0 for Mac (GraphPad Software).

Drug time course assays were carried out by seeding equal numbers of MCF10A or CDH1−− at 4000 cells/well in xCELLigence plates (Roche), except for Crizotinib which was seeded at 2000 cells/well. The xCELLigence system measures relative changes in electrical impedance in a well (‘cell proliferation index’) which can be used as a measure of cell number. After 24 hours, three concentrations of each drug were added and growth followed in real time for a further 72 hours. Assays were carried out in duplicate and the data averaged. Time course assays were also performed on the IncuCyte imaging system (Essen Bioscience). Cells were seeded at 4000 cells/well in black walled clear bottom 96 well plates. After 24 hours, five concentrations of each drug were added and 3 fields were imaged/well at 4x magnification every 2 hours for 48 hours. Plates were then removed and cell numbers determined using metabolic assays and nuclei imaging as described above.

For drug synergy studies, the combination index was determined using CompuSyn software (ComboSyn Inc) and the Chou-Talalay method (18). EC50 was estimated for both drugs and cells treated with each drug alone and in combination at this concentration and concentrations two and four fold higher and lower. Viability was calculated by nuclei counting and normalized values imported into CompuSyn.

Results

Genome-wide siRNA screen of CDH1 isogenic MCF10A cells

To identify genes potentially involved in a synthetic lethal interaction with E-cadherin, we conducted a genome-wide functional screen using the Dharmacon siGENOME SMARTpool library targeting 18,120 genes in a pooled format, with 4 individual siRNAs targeting each gene. Isogenic MCF10A cells with and without CDH1 expression were screened in parallel, and viability was assayed 72 hours after transduction using CellTiter-Glo®. siPLK1 and
siEGFR were used as positive death controls (Fig. 1A) and siCTNNB1 used as a positive synthetic lethal control, after showing a mild synthetic lethal effect in a pilot screen (Supplementary Figure S1). Mock (lipid only) and RISC-free control (Dharmacon) were included as non-targeting controls. Little variability was observed between these controls; consequently we normalized the values of each gene to the average screen-wide mock value within each cell line. Hits were selected based on the ratio of cell viability between the MCF10A and CDH1<sup>-/-</sup> cells 72 hours after transduction (Fig. 1B). We rejected siRNAs that were highly toxic to the MCF10A cells (a decrease in viability of ≥50%) and selected those for which the viability of CDH1<sup>-/-</sup> cells decreased by ≥15% more than the corresponding knockdown in MCF10A cells. These targets were classed as CDH1 synthetic lethal candidate genes (List SL1, 2,437 genes; Supplementary Table S1). From this set, 501 genes were manually selected for secondary screening using criteria including predicted druggability of the encoded proteins and biological significance. The secondary screen was performed by deconvoluting the four individual siRNAs that constitute the SMARTpool. Using the same stringent threshold as the primary screen (ie. MCF10A viability ≥50% and ≥15% more death in CDH1<sup>-/-</sup>), 21 genes (5%) had 3/4 or 4/4 of the individual siRNAs show a synthetic lethal effect. 183 genes (44%) had 1/4 or 2/4 siRNAs validate. 51% of genes were not validated (0/4) by this approach. Division of the selected genes into groups based on the strength of the synthetic lethal phenotype in the primary screen demonstrated that candidates which showed a greater viability differential between MCF10A and CDH1<sup>+/+</sup> cells were more likely to be validated in the secondary screen (Fig. 1C-D). Of the candidates that reduced CDH1<sup>+/+</sup> cell number by ≥25% more than MCF10A cells, 12% validated with 3/4 or 4/4 individual siRNAs, a validation rate comparable to other genome-wide RNAi studies (19, 20).

**Functional Diversity: Gene Ontology analysis**

To search for functional enrichment in the 2,437 synthetic lethal candidates identified in the primary screen (List SL1), we conducted a gene ontology analysis using DAVID (21). Using the Functional Annotation Clustering tool, the most enriched functional cluster was a group of
ten terms associated with G-protein coupled receptor (GPCR) signalling (Enrichment Score=10.01; Supplementary Table S2A). Accordingly, the two most significant biological process terms (Supplementary Table S2B) were ‘G-protein coupled receptor protein signalling pathway’ (Benjamini-adjusted p value= 4.1x10⁻⁸) and ‘cell surface receptor linked signal transduction’ (adj. p value=1.7x10⁻⁵). These cell signalling processes were strongly reflected in the DAVID gene ontology molecular function terms (Supplementary Table S2C) which included peptide, neuropeptide and purinergic nucleotide receptor activity and protein kinase activity (adj. p value 3.5x10⁻⁷ to 3.2x10⁻²).

Because our ultimate goal is to identify targeted drugs for E-cadherin-deficient tumors that have minimal toxicity against non-malignant tissues, we also performed gene ontology analysis on a subset of genes from List SL1 whose corresponding siRNA SMARTpools had little or no impact on MCF10A cells (MCF10A viability ≥0.85 mock). 1,136 genes met this revised threshold (List SL2; Supplementary Table S3). Notably, this more stringent cut-off led to further enrichment of both the GPCR-associated functional cluster (Enrichment Score 12.14) and the biological process terms ‘G-protein coupled receptor protein signalling pathway’ (adj. p value= 9.0x10⁻¹⁶) and ‘cell surface receptor linked signal transduction’ (adj. p value=4.8x10⁻¹⁰; Supplementary Table S2D).

Our earlier observation of abnormal cytoskeletal organization in E-cadherin-deficient MCF10A cells (7) prompted us to look for specific cytoskeletal functions associated with synthetic lethality. Although the adjusted p-values for cytoskeletal-like terms in List SL1 and SL2 did not reach significance, DAVID functional clusters associated with each of cell motility, cell polarity and cell adhesion were amongst the top five clusters observed in the group of synthetic lethal candidate genes which reduced MCF10A viability to <0.85 of the mock controls (Enrichment Scores 2.18, 1.97 and 1.84, respectively; Supplementary Table S2E). This greater representation of cytoskeletal genes amongst synthetic lethal candidates which impact seriously on MCF10A cell viability presumably reflects the essential role of many
cytoskeletal proteins. Cyto- or nucleoskeletal functions that were represented in List SL1, often by multiple family members, included microtubule nucleation, organization and function (eg. TUBA1C, TUBG1, TUBB2A, MZT2A, ARPC3, NME4, NME7, MAST1, MAST2, MAST3, NEK1, NEK3, NEK4, NEK10, CLIP1, CLIP2, TEKT3, TEKT4, TEKT5), Rho-mediated motility (eg. RHOB, RHOC, RHOH, RAC1, PAK2, TIAM1), linkages between the cytoskeleton and nucleoskeleton (eg. SUN1, SUN2, SUN5, NSUN3, NSUN6, SYNE1), polarity (eg. DLG1, DLG2, DLG4, DLG5, CELSR1, CELSR3) and actin filament organization and remodelling (eg. AVIL, ARF6, CYTH2, CTYH3, CYTH4).

E-cadherin-deficient MCF10A cells are sensitive to downregulation of microtubule associated genes and the aurora kinase A inhibitor Alisertib

To validate the apparent synthetic lethal interaction between E-cadherin and the microtubule cytoskeleton, we selected three microtubule associated genes, MAST2, MAP1B and MAPRE3 for confirmation using lentiviral shRNA knockdown. MAST2 is a microtubule-associated serine-threonine kinase (22); MAP1B is a microtubule binding and stabilizing protein that can also interact with actin microfilaments (23) and MAPRE3 is a microtubule plus end binding protein involved in regulating the dynamics of microtubules and their interactions with intracellular structures such as the cell cortex or mitotic kinetochore (24). These three genes had previously been validated in the secondary screen, with 3/4, 2/4 and 1/4 siRNAs, respectively, decreasing the viability of CDH1-/- cells by at least 15% more than the MCF10A (Fig. 2A). shRNAs targeting these three genes resulted in 51-86% mRNA knockdown in both cell lines (Fig. 2B). 72 hours after transduction, cell viability was measured using nuclei counting (Fig. 2C). Knockdown of each of MAP1B, MAST2 and MAPRE3 resulted in 15-29% more cell death in the CDH1-/- cells compared to the MCF10A cells, confirming the siRNA data.

To determine whether the synthetic lethal phenotype observed with downregulation of microtubule associated genes could be recapitulated using known inhibitors of microtubule
function, we treated the isogenic MCF10A cell line pair with the microtubule stabilising drug Taxol and inhibitors of the microtubule-associated proteins aurora kinase A and aurora kinase B. Increasing concentrations of Taxol (1-16 nmol/L) led to a small increase in cell death in \(CDH1^{-/-}\) cells compared to MCF10A cells (Supplementary Figure S2). The aurora kinase A inhibitor Alisertib and the aurora kinase B inhibitor Danusertib both showed a minor synthetic lethal effect (Fig. 2D-E), with 18% (at 25 nmol/L) and 12% (50 nmol/L) more death in the \(CDH1^{-/-}\) line, respectively. Together, the RNAi and aurora kinase inhibitor data demonstrate that E-cadherin-deficient MCF10A cells are more vulnerable to disruption of specific microtubule-related functions than E-cadherin expressing MCF10A cells.

**E-cadherin-deficient MCF10A cells show vulnerabilities in GPCR signalling**

Gene ontology analysis (21) identified >200 genes from our list of synthetic lethal candidates (List SL1) that were associated with GPCR protein signalling pathways. These candidates included several proteins involved in signal transduction from activated GPCRs such as the G protein subunits GNAS, GNAT1, GNG2 and GNG5, the membrane bound adenyl cyclase ADCY7 and the downstream signalling protein JAK2. ADCY7 validated in the secondary screen with 4/4 siRNAs recapitulating the SMARTpool phenotype (Fig. 3A). The synthetic lethal effect of the \(JAK2\) siRNA SMARTpool was confirmed using lentiviral-mediated shRNA transduction. Both the siRNA SMARTpool and lentiviral shRNA knocked down JAK2 mRNA by >44% (Fig. 3B). Normalized cell numbers post transduction were significantly lower in the \(CDH1^{-/-}\) cells compared to the MCF10A cells for both the siRNA pool and the shRNA (Fig. 3C). To determine whether the synthetic lethality of \(JAK2\) downregulation could be mimicked using a \(JAK2\) antagonist, we treated the isogenic \(CDH1\) MCF10A cell line pair with the \(JAK\) inhibitor LY2784544. Using the xCELLigence system to monitor cell growth in real time, LY2784544 had only a modest effect on MCF10A cells at 0.32, 0.63 and 1.25 μmol/L concentrations, however the cell proliferation index was reduced in the \(CDH1^{-/-}\) cells in a concentration-dependent manner. 48 hours after drug addition, the three concentrations of LY2784544 resulted in a 15%, 29% and 51% reduction in \(CDH1^{-/-}\) cell number, respectively.
(Fig. 3D). In contrast, the three drug concentrations reduced MCF10A cell number by 0%, 11% and 10%, respectively. A second JAK2 inhibitor, AG490, showed a similar synthetic lethal response in xCELLigence assays (data not shown). Further real time assays using the IncuCyte replicated this effect (Supplementary Figure S3). Nuclei counting confirmed a significant synthetic lethal effect at LY2784544 concentrations of 1.25 μmol/L (p=0.005) and 0.63 μmol/L (p=0.03) (Fig. 3E). LY2784544 resulted in cells becoming more spindle shaped, with an increased number of extended filopodia; this effect was particularly marked in the

CDH1<sup>−/−</sup> cells (Fig. 3F).

The presence of G protein subunits in list SL1 prompted us to examine CDH1 synthetic lethality using the Gα<sup>q</sup> and Gα<sub>o</sub> subunit inhibitor Pertussis toxin (25). Treatment of MCF10A and CDH1<sup>−/−</sup> cells with 10, 100 and 200 ng/ml of Pertussis toxin over a period of 48 hours resulted in growth inhibition of both MCF10A and CDH1<sup>−/−</sup> cell lines in a concentration dependent manner (Fig. 3G). However, the MCF10A cells recovered and reached the same confluence as the PBS control after 48 hours, whereas the CDH1<sup>−/−</sup> cells showed 20-30% less confluence at that time point for the three drug concentrations.

**E-cadherin loss sensitises MCF10A cells to HDAC inhibitors and other drug classes**

To explore how E-cadherin loss alters sensitivity of MCF10A cells to other known drugs, we screened 4,057 compounds against the CDH1 MCF10A isogenic cell line pair. The compounds comprised the Walter and Eliza Hall Institute known drug library (3,600 compounds from the Tocriscreen™ Total library, the Prestwick Chemical Library and the ‘Lopac 1280’ library), the Selleck Chemistry inhibitor library (326 compounds consisting of approximately half known drugs and half kinase inhibitors) and a kinase inhibitor library (131 compounds supplied by SYNthesis Medicinal Chemistry). The initial screen covered four drug concentrations ranging from 0.25 μmol/L to 2 μmol/L, with cell viability measured at 48 hours after drug addition using the CellTiter-Glo® assay. Potentially synthetic lethal drugs were selected for further characterization if they met two criteria: (i) modest toxicity to
MCF10A cells (a decrease in viability of no more than 30%) and (ii) a minimum of 15% greater reduction in $CDH1^+$ viability compared to the MCF10A cells at one or more concentrations. 316 compounds were selected for secondary analysis using an 11 point serial dilution from 5 mmol/L to 5 nmol/L. 21/316 compounds in this secondary screen had EC50 values that were 10-50% lower in the $CDH1^+$ cells compared to the MCF10A cells (Table 1). These included multiple histone deacetylase (HDAC) and phosphoinositide 3-kinase (PI3K) inhibitors, Crizotinib (an inhibitor of receptor tyrosine kinases c-MET, ALK and ROS1), CGP 71683 hydrochloride (an inhibitor of the neuropeptide receptor NPY5R) and the guanine nucleotide exchange factor inhibitor Brefeldin. The synthetic lethality of the majority of drug classes shown in Table 1 was supported by the siRNA primary screen data with one or more targets (or associated proteins) for each being included in List SL1 (Supplementary Table S4).

The E-cadherin synthetic lethal effects of Crizotinib and several HDAC inhibitors were further characterized in time course and direct cell counting studies. Saracatinib, a c-SRC kinase inhibitor not included in the 11 point screen was also further examined because of a borderline effect in the original four point screen. Crizotinib had little effect on the growth of MCF10A cells at 0.63, 1.25 and 2.50 μmol/L up to 48 hours after drug addition, as observed using the xCELLigence system. The same concentrations, however, reduced the growth of $CDH1^+$ cells to 86%, 76% and 46% of mock (Fig. 4A). Nuclei counting at 48 hours confirmed the synthetic lethal effect, although an inhibitory effect was observed on the MCF10A cells at all three concentrations (Fig. 4B). The difference between the nuclei counting method and the IncuCyte and xCELLigence methods is primarily due to cell density differences at full confluency which can only be determined by direct nuclei counting.

Treatment with Saracatinib caused greater growth inhibition in the $CDH1^+$ cells compared to the MCF10A cells at three different concentrations in two different assay systems, cell confluence (IncuCyte) and direct nuclei counting. In the IncuCyte system, a dose dependent
inhibition was observed in both isogenic cells with the \( \text{CDH1}^{-/-} \) cells demonstrating greater susceptibility (Fig. 4C). At 0.63 \( \mu \text{mol/L} \), Saracatinib had negligible effect on the confluence of MCF10A cells but caused a 26\% inhibition of \( \text{CDH1}^{+/+} \) cells (relative to DMSO) after 48 hours. Similarly, differentials of 0.17 (p value=0.06), 0.30 (p value=0.02) and 0.15 (p value=0.10) were observed in normalized cell counts of the \( \text{CDH1}^{+/+} \) cells compared to the MCF10A cells at Saracatinib concentrations of 0.63, 1.25 and 2.5 \( \mu \text{mol/L} \), respectively (Fig. 4D).

Entinostat selectively targets class I HDACs, in particular HDAC1-3 (26). 0.63, 1.25 and 2.5 \( \mu \text{mol/L} \) Entinostat had a negligible effect on cell proliferation of MCF10A as determined using the xCELLigence system. In contrast, \( \text{CDH1}^{-/-} \) cells showed 18\%, 67\% and 78\% growth inhibition at these concentrations 48 hours after drug addition (Fig. 4E). Comparable results were obtained using the IncuCyte (Supplementary Figure S3). Nuclei counting also showed a significant synthetic lethal effect across the three Entinostat concentrations, although, as observed previously for Crizotinib, reduced nuclei count was observed in both cell lines with increasing drug concentration using this more direct method. At concentrations of 0.63, 1.25 and 2.5 \( \mu \text{mol/L} \), a cell viability differential of 0.13 (p-value=0.04), 0.23 (p-value=0.004) and 0.14 (p-value=0.02) was observed between the \( \text{CDH1}^{-/-} \) cells and the MCF10A cells (Fig. 4F).

Vorinostat (SAHA) is a pan HDAC inhibitor, acting on both class I and class II HDACs (26). Assays using the IncuCyte system showed preferential inhibition of \( \text{CDH1}^{+/+} \) cells over 48 hours of drug treatment at 0.63, 1.25 and 2.5 \( \mu \text{mol/L} \), with little effect on the MCF10A cells (Fig. 4G). This effect was confirmed on the xCELLigence system (Supplementary Figure S3). Similarly to Entinostat, direct nuclei counting showed a greater effect of Vorinostat on the MCF10A cells than was observed using the real-time proliferation assay platforms. A significant cell viability differential was still observed between the \( \text{CDH1}^{+/+} \) and MCF10A cells with cell viability differentials of 0.21 (p-value=0.02), 0.29 (p-value=0.01) and 0.17 (p-value=0.10) between \( \text{CDH1}^{+/+} \) and MCF10A at concentrations of 0.63, 1.25 and 2.5 \( \mu \text{mol/L} \) (Fig. 4H). The HDAC inhibitors Mocetinostat and Pracinostat showed comparable synthetic
lethal effects when assayed at 0.63, 1.25 and 2.5 µmol/L on the xCELLigence system (Supplementary Figure S4A-B). The class I/II HDAC inhibitor valproic acid also showed a minor synthetic lethal effect when assayed by direct nuclei counting (Supplementary Figure S4C).

Previous reports of synergy between HDAC inhibitors and Taxol (27-30) prompted us to test combinations of Taxol and each of Vorinostat and Entinostat in our isogenic cell line pair. CompuSyn (ComboSyn Inc) was used to calculate EC50 concentrations, and to determine the Combination Index. In contrast to other studies, we found no evidence for synergy between Taxol and these HDAC inhibitors (Supplementary Figure S2).

Discussion

Synthetic lethality provides a potential method to target cancers carrying inactivating mutations in tumor suppressor genes such as the E-cadherin gene, \textit{CDH1}. To provide an initial survey of E-cadherin’s synthetic lethal interactions, we conducted a genome-wide functional screen of non-malignant, isogenic MCF10A cells with and without E-cadherin expression. 13% of the 18,120 genes in the siRNA screen met our threshold for synthetic lethality of at least 15% more death in the \textit{CDH1}⁻/⁻ cells than the wildtype MCF10A cells. Although this threshold is low stringency and subsequently not highly specific, it is clear that E-cadherin-deficiency creates large numbers of vulnerabilities in non-malignant cells which are exposed by genetic knockdown of additional genes.

Gene ontology analysis identified a striking enrichment for GPCR signalling proteins amongst the synthetic lethal candidates. Notably, this enrichment was greater for hits which showed minimal impact on the viability of the E-cadherin-expressing MCF10A cells, suggesting that drug targeting of GPCR signalling in \textit{CDH1} mutant tumors may be a means to obtain clinical gain while minimizing collateral damage to normal tissues. The nature of the synthetic lethal relationship between E-cadherin and GPCR signalling is not yet known, although the
functional diversity of the candidate synthetic lethal GPCR signalling proteins would suggest that the interaction involves a common downstream mechanism such as interplay with the actin and microtubule cytoskeletons (31-33). The importance of cytoskeletal functions to the E-cadherin synthetic lethal phenotype is supported by the abundance of cytoskeletal genes associated with synthetic lethality in our primary siRNA screen. These genes were involved in all aspects of cytoskeletal function including the nucleation, organization and function of microtubules mitotic spindle organization and control, linkages between the cytoskeleton and nucleoskeleton, polarity, actin filament organization, vesicle transport, focal adhesion kinase signalling and Rho-mediated motility (8, 34-36). The increased vulnerability of E-cadherin-deficient MCF10A cells to knockdown of so many diverse but inter-related functions is consistent with the widespread disorganization of cytoskeletal networks observed in the CDH1-/- cells (7). Notably, after CDH1 and TP53, RHOA is the most commonly mutated gene in DGC (37-39), emphasizing the importance of dysregulated cytoskeletal function to development of the diffuse phenotype.

The increased vulnerability of E-cadherin-deficient MCF10A cells to RNAi knockdown of cytoskeletal and GPCR signalling genes was supported by the increased sensitivity of these cells to antagonists of multiple protein families associated with GPCR signalling and cytoskeletal function including HDACs (40, 41), JAK (42), aurora kinases (43), c-SRC tyrosine kinase (44), G-protein subunits and PI3K (45). The enrichment for GPCR signalling genes amongst our synthetic lethal candidates does not, however, exclude the possibility that other signalling functions, such as the cytokine responses of JAK2, may also be associated with synthetic lethality. EC50 differences between MCF10A and CDH1-/- cells for these known drugs were on average only approximately 25% less in the CDH1-/- cells. This small differential is to be expected since neither MCF10A or CDH1-/- cells are tumorigenic and therefore these lines cannot be distinguished by the presence/absence of addiction to the targeted pathways. Synthetic lethal drugs which are significantly more potent in E-cadherin-deficient cells will be more readily identified using fit-for-purpose, high-throughput compound screens across the MCF10A and other CDH1 isogenic cell line pairs. Differences
between the E-cadherin-expressing and E-cadherin-deficient cells may also be more pronounced in phenotypes other than cell viability, such as invasive capability.

This research demonstrates for the first time that loss of the tumor suppressor protein E-cadherin creates druggable vulnerabilities in cells. It remains to be determined whether any of the observed drug sensitivities in CDH1−/− cells will be robust to the genetic dysregulation of advanced tumors, and therefore able to provide additional clinical benefit in the treatment of sporadic CDH1-mutant tumors. Instead, the observed sensitivities may have more near term application to HDGC chemoprevention. The natural history of cancer development in CDH1 germline mutation carriers involves the development of multifocal lobular carcinoma in situ (46, 47) and tens to hundreds of gastric stage T1a signet ring cell carcinomas prior to the onset of advanced disease (11, 48, 49). The high multiplicity of these early stage foci argues against additional genetic hits being required for their initiation. These early breast and gastric cancers are therefore relatively genetically homogenous and distinguished from normal tissue predominantly by the cellular changes associated with deficiency of E-cadherin. As a consequence, the E-cadherin synthetic lethal interactions identified in the non-malignant breast MCF10A cells provide strong leads for drugs that may eliminate early stage disease in germline CDH1 mutation carriers, potentially providing a new clinical management option for HDGC families.

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References


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<th>Drug Name</th>
<th>drug class</th>
<th>MCF10A EC50 (µM)</th>
<th>CDH1&lt;sup&gt;+/−&lt;/sup&gt; EC50 (µM)</th>
<th>CDH1&lt;sup&gt;+/−&lt;/sup&gt; to MCF10A ratio</th>
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<tr>
<td>Mocetinostat</td>
<td>HDAC inhibitor</td>
<td>1.76</td>
<td>1.02</td>
<td>0.58</td>
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<tr>
<td>Entinostat</td>
<td></td>
<td>4.31</td>
<td>2.50</td>
<td>0.58</td>
</tr>
<tr>
<td>Quisinostat</td>
<td></td>
<td>0.05</td>
<td>0.04</td>
<td>0.72</td>
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<tr>
<td>Pracinostat</td>
<td></td>
<td>0.73</td>
<td>0.54</td>
<td>0.74</td>
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<tr>
<td>LAQ824</td>
<td></td>
<td>0.09</td>
<td>0.06</td>
<td>0.76</td>
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<tr>
<td>Panobinostat</td>
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<td>0.08</td>
<td>0.07</td>
<td>0.84</td>
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<tr>
<td>Crizotinib</td>
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<td>3.98</td>
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<td>PI103</td>
<td>PI3K inhibitor</td>
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<td>0.59</td>
<td>0.79</td>
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<tr>
<td>GSK2126458</td>
<td>PI3K inhibitor</td>
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<td>0.04</td>
<td>0.79</td>
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<tr>
<td>PIK-75 hydrochloride</td>
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<tr>
<td>CGP 71683 hydrochloride</td>
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<td>0.89</td>
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<td>Tyrphostin A9</td>
<td>PDGFR and EGFR inhibitor</td>
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<td>0.65</td>
<td>0.50</td>
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<td>mTOR inhibitor</td>
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<td>0.12</td>
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<tr>
<td>Obatoclax Mesylate</td>
<td>BCL2 inhibitor</td>
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<td>0.47</td>
<td>0.74</td>
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<td>Brefeldin A</td>
<td>Guanine nucleotide exchange factor inhibitor</td>
<td>0.20</td>
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<td>0.76</td>
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<td>LY2784544</td>
<td>JAK family inhibitor</td>
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<td>4.70</td>
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<td>FCCP</td>
<td>uncoupler of mitochondrial oxidative phosphorylation</td>
<td>3.74</td>
<td>2.90</td>
<td>0.78</td>
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<tr>
<td>JNJ-7706621</td>
<td>CDK and aurora kinase inhibitor</td>
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<td>2.77</td>
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<td>Danusertib</td>
<td>Inhibitor of aurora kinases, Bcr-Abl, c-RET and FGFR</td>
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<td>1.07</td>
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<td>PD-166285 hydrate</td>
<td>broad spectrum tyrosine kinase inhibitor</td>
<td>0.66</td>
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<td>10-DEBC hydrochloride</td>
<td>AKT/protein kinase B inhibitor</td>
<td>8.10</td>
<td>7.26</td>
<td>0.90</td>
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</table>

**Table 1.** Known drugs with greater inhibitory effect on MCF10A cells compared to CDH1<sup>+/−</sup> cells. EC50 values were obtained from 11 point dilution curves carried out at least in duplicate. Cell viability was determined using the CellTiter-Glo® assay 72 hours after drug addition. The CDH1<sup>+/−</sup> to MCF10A ratio is a measure of the reduced viability of the CDH1<sup>+/−</sup> cells in the presence of drug.
Figure Legends

Figure 1. siRNA screening overview of outcomes and analysis strategy

(A) The resulting effect on cell viability after knockdown of each protein-coding target in MCF10A and CDH1−/− cells. The positive death controls siPLK1 and siEGFR both consistently cause death in both cell lines. (B) The analysis workflow used to select synthetic lethal candidates based on MCF10A and CDH1−/− viability. (C) Correlation between primary and secondary screens. The dotted line marks where CDH1−/− has 15% less viability than MCF10A. The primary screen identified 500 candidates that were selected for validation in a secondary screen. 5% of these validates as with 3/4 or 4/4 individual siRNA. (D) Candidates tested in the secondary screen were split into two categories, based on the decrease in viability of CDH1−/− compared to MCF10A. Candidates with a greater differential were more likely to be validated in the secondary screen.

Figure 2. Disruption of microtubule associated proteins induces synthetic lethal effect.

(A) Bar graphs with primary and secondary screen viability normalized to mock for MAST2, MAP1B and MAPRE3. Each value is the average of two technical replicates. The arrowheads mark siRNAs that reached the synthetic lethal criteria (15% less viability in CDH1−/− cells). (B) Level of mRNA knockdown 72 hours after transduction with shRNA lentivirus constructs. Error bars show standard error of two independent experiments, except for MAST2 which was only assayed once. (C) Nuclei count normalized to non-silencing control after knockdown with MAP1B, MAST2 and MAPRE3 shRNA. Error bars show standard error of two independent experiments. * = p-value < 0.05 by 1 tailed, equal variance Students T-test. (D) Nuclei count normalized to DMSO control 48 hours after Alisertib treatment. Error bars show standard error of the mean of two independent experiments. (E) Nuclei count normalized to DMSO control 48 hours after Danusertib treatment. A single experiment is shown.
**Figure 3.** Disruption of GPCR associated proteins causes synthetic lethality

(A) Bar graphs with primary and secondary screen viability normalized to mock for ADCY7. Each value is the average of two technical replicates. All siRNA reached the synthetic lethal criteria. (B) Normalized mRNA levels after knockdown by JAK2 shRNA and siRNA pool. (C) Nuclei counts normalized to non-silencing (shRNA) or mock (siRNA) controls after knockdown by JAK2 RNAi. (D) Representative xCELLigence experiment for cells treated with LY2784544. Arrow marks time when compound was added. (E) Nuclei count normalized to DMSO 48 hours after LY2784544 treatment. (F) Images taken 48 hours after LY2784544 treatment showing reduced confluence and morphological changes. (G) Representative IncuCyte assay showing confluence over 48 hours after Pertussis toxin treatment. Error bars show standard error of at least two independent experiments. * = p-value < 0.05 ** = p-value < 0.01 by 1 tailed, equal variance Students T-test.

**Figure 4.** Treatment with various known drugs causes CDH1 synthetic lethality

(A, E), Representative xCELLigence assay of cells treated with Crizotinib (A) and Entinostat (E). Cells were seeded at 4000 or 2000 (Crizotinib) cells/well and drug was added at 24 hours. Cells were grown for an additional 72 hours. (C, G), Representative IncuCyte assays of cells treated with Saracatinib (C) and Vorinostat (G). Cells were seeded at 4,000 cells/well and drug was added at 24 hours. Cells were then grown for an additional 48 hours. (B, D, F, H), Nuclei counts normalized to DMSO after treatment with Crizotinib (B), Saracatinib (D), Entinostat (F) and Vorinostat (H). Error bars show standard error of at least two independent experiments. * = p-value < 0.05 ** = p-value < 0.01 determined by 1 tailed, equal variance Students T-test.
PLK1, EGFR

0
1.0
0

CDH1-/- viability
MCF10A viability
0.5
1.5
0.5 1.0 1.5
MCF10A CDH1-/- 
>15% less viable 
than MCF10A
Viability < 50%
Viability > 50%
>15% less viable 
than MCF10A
<15% less viable 
than MCF10A
Exclude
Synthetic lethal candidates (SL1)
Exclude
Secondary screening

CDH1-/- viability difference
4/4 and 3/4
2/4 and 1/4
0/4
34%
54%
12%
55%
43%
3%
Proportion validated (%)

15% differential

0/4
1/4 and 2/4
3/4 and 4/4

Figure 1
Figure 2
Figure 3
Figure 4