

**THE DETERMINANTS OF SENSITIVITY AND
MECHANISM OF ACTION OF EPRENETAPOPT
(APR-246)**

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For my parents, Kymme and Eiji

The coupon did say "*7 kids gets you at least one PhD*"

You are forever my inspiration.

ABSTRACT

The tumour suppressor gene *TP53* is mutated in over half of all cancers, resulting in mutant p53 protein accumulation and poor patient survival. Decades after the discovery that p53 plays a central role in the human body's defence against cancer, the development of effective therapeutic strategies to target mutant p53 cancers still remains an unmet need in oncology. Eprenetapopt (also known as APR-246 and PRIMA-1^{MET}) is currently in clinical development as a mutant p53 reactivator. The reported mechanism of action of eprenetapopt is to restore wild-type p53 functionality to mutant p53 in cancer cells, triggering p53-dependent cell cycle arrest and caspase-dependent apoptosis. As a result, clinical investigation of eprenetapopt has focused on *TP53*-mutated cancers, relying solely on *TP53* mutation status for patient selection into clinical trials. However, the mechanism of action of eprenetapopt remains contentious, as evidence demonstrates that eprenetapopt maintains strong potency in cancer cells with no mutant p53. Herein, utilising novel unbiased approaches, including CRISPR perturbation screening, metabolomics, proteomics and pan-cancer cellular-feature profiling, this thesis demonstrates two major innovations regarding the determinants of sensitivity and mechanisms of action of eprenetapopt. First, the expression of *SLC7A11*, which encodes the functional subunit of the cystine-glutamate antiporter, not *TP53*-mutation status, is the major determinant of sensitivity to eprenetapopt. Second, eprenetapopt triggers a caspase-independent, iron-dependent form of cell death known as ferroptosis, following glutathione depletion and inhibition of iron-sulfur cluster biogenesis. Together, this thesis provides a roadmap for broadening the clinical utility of eprenetapopt as a ferroptosis inducer in haematological and solid malignancies, beyond *TP53*-mutant cancers.

DECLARATION

This is to certify that:

1. The thesis comprises only my original work towards the Ph.D. with contributions of others indicated in the Preface section.
2. Due acknowledgement has been made in the text to all other material used.
3. The thesis is less than 100,000 words in length, exclusive of tables, bibliography and appendices.

Kenji Mark Fujihara

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PREFACE

The majority of the work described in this thesis was performed by the candidate under the supervision of A/Prof. Nicholas Clemons, Dr. Sue Haupt and Prof. Wayne Phillips. Other members of the Peter MacCallum Cancer Centre as well as national and international collaborators performed work that contributed to this thesis as detailed below.

Chapter 3 – SLC7A11 is a superior determinant of APR-246 (Eprexapopt) response than TP53 mutation status

Dr. Carlos S. Cabalag performed circulating tumour DNA analysis from the APROC trial presented in **Figure 3.1**. Dr. Hyun S. Ko analysed the CT scans presented in **Figure 3.1**. Dr. Lara Lipton recruited patients for the APROC trial presented in **Figure 3.1**. Ms. Bonnie Z. Zhang performed the western blot and dose response experiments presented in **Figure 3.3**. Ms. Mariana Corrales-Benitez performed the siRNA screen presented in **Figure 3.4**. Dr. David S. Liu performed a western blot and total glutathione level assay presented in **Figure 3.5**.

Chapter 4 – Eprexapopt (APR-246) triggers ferroptosis and inhibits iron-sulfur cluster biogenesis

Dr. Iva Nikolic and Dr. Simon J. Hogg aided in the design and analysis for the CRISPR perturbation screens presented in **Figure 4.1**. Dr. Brunda Nijagal aided in the design and analysis of the metabolomics experiments presented in **Figure 4.1** and **Figure 4.2**. Dr. Ching-Seng Ang aided in the design and analysis of the proteomics experiment presented in **Figure 4.1**. Ms. Bonnie Z. Zhang performed the cystine uptake experiment presented in **Figure S4.2**. Dr. Carlos S. Cabalag, A/Prof. Nicholas

J. Clemons, Ms. Bonnie Z. Zhang and Mr. Asuka T. Fujihara aided in the completion and enabled the blinding of *in vivo* experiments presented in **Figure 4.2**. Dr. Conor J Kearney generated the apoptosis-deficient Mc38 cells used in **Figure 4.3**. Dr. Sally Watts performed the western blot presented in **Figure 4.3**. Mr. Moses O. Ogunkola performed the *in vitro* and in cell NFS1 activity assays and western blot presented in **Figure 4.4** and **Figure S4.4**. Dr. David A. Sallman performed the analysis of the phase II clinical trial data presented in **Figure 4.4**.

Chapter 5 – Opportunities for ferroptosis in cancer therapy

Ms. Bonnie Z. Zhang aided in the generation of diagram presented in **Figure 5.1**.

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Awards

- 2018, Victorian Comprehensive Cancer Centre PhD Symposium, Poster Prize
- 2018, European Molecular Biology Laboratory Australia, Course Scholarship
- 2020, Lorne Cancer Conference, Poster Prize
- 2021, Victorian Comprehensive Cancer Centre and Picchi Foundation, Excellence in Cancer Research Award

SCIENTIFIC PRESENTATIONS

Some of the work in this thesis has been presented at scientific conferences by the candidate or a collaborator.

2018

- Victorian Comprehensive Cancer Centre PhD Symposium, Melbourne, Australia
- Lorne Cancer Conference, Lorne, Australia
- Garvan Signalling Symposium, Sydney, Australia
- EMBLA PhD Symposium, Brisbane, Australia

2019

- Lorne Cancer Conference, Lorne, Australia
- Australian Cancer and Metabolism Meeting, Sydney, Australia
- International Mutant p53 Meeting, Lyon, France
- Functional High Throughput Technologies Australia Meeting, Canberra, Australia

2020

- Lorne Cancer Conference, Lorne, Australia
- Tumor Metabolism Keystone Conference, Banff, Canada (*Cancelled*)

PUBLICATIONS

All of the work in this thesis has been published, or is in preparation for submission.

Their references are as follows:

- **Fujihara KM**, Corrales-Benitez C, Cabalag CS, Zhang BZ, Ko HS, Liu DS, Simpson KJ, Haupt Y, Lipton L, Haupt S, Phillips WA, Clemons NJ. *SLC7A11 is a superior determinant of APR-246 (Eprexetapopt) response than TP53 mutation status*. Mol Cancer Ther. 2021 Jul 26:molcanther.0067.2021
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ABBREVIATIONS

5-FU.....	5-Fluorouracil
AML.....	Acute myeloid leukaemia
APROC.....	APR-246 in oesophageal cancer
ATO.....	Arsenic trioxide
CCL.....	Cancer cell line
CDX.....	Cancer cell line-derived xenograft
CDK.....	Cyclin-dependent kinase
CESTA.....	Cellular thermal shift assay
ChIP.....	Chromatin immunoprecipitation
CPX.....	Ciclopirox olamine
CR.....	Complete remission
CT.....	Computed tomography
ctDNA.....	Circulating tumour DNA
CRISPR.....	Clustered regularly interspaced short palindromic repeats
CRISPRa.....	CRISPR activation
CRISPRko.....	CRISPR knockout
CTRPv2.....	Cancer therapeutics response portal version 2
DMSO.....	Dimethylsulfoxide
DepMap.....	Cancer Dependency Map
EMT.....	Epithelial-to-mesenchymal transition
Fer-1.....	Ferostatin-1
GI50.....	50% maximal growth inhibition
GPX4.....	Glutathione peroxidase 4
GSH.....	Reduced glutathione
GSSG.....	Oxidised glutathione
HSP90.....	Heat shock protein 90
HGSOC.....	High-grade serous ovarian carcinoma
HL.....	Haematopoietic and lymphoid
ICI.....	Immune checkpoint inhibitor
ISC.....	Iron-sulfur cluster
LC-MS.....	Liquid chromatography tandem mass spectrometry
MDS.....	Myelodysplastic syndrome

MDS-RS.....	MDS with ring sideroblasts
Mito-1C.....	Mitochondrial one-carbon
MoA.....	Mechanism of action
MOMP.....	Mitochondrial outer membrane permeabilisation
MQ.....	2-methylene-3-quinuclidinone
Mut-p53.....	Mutant p53
NAC.....	N-acetyl-cysteine
NCI.....	National Cancer Institute
NGS.....	Next generation sequencing
NMR.....	Nuclear magnetic resonance
NSG.....	NOD-SCID IL-2R γ ^{KO}
NTC.....	NTC
p53RE.....	p53 responsive elements
PI.....	Propidium iodide
PDX.....	Patient-derived xenograft
PMCC.....	Peter MacCallum Cancer Centre
PNS.....	Peripheral nervous system
PonA.....	Ponasterone A
PRIMA-1.....	p53 reactivation and induction of massive apoptosis
PUFA.....	Polyunsaturated fatty acid
RFP.....	Red fluorescent protein
ROS.....	Reactive oxygen species
SG.....	Serine and glycine
STS.....	Staurosporine
TAD.....	Transactivation domain
TCGA.....	The Cancer Genome Atlas
US-FDA.....	United States Food and Drug Administration
VAF.....	Variant allele frequency
Wt-p53.....	Wild-type p53

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“You never change things by fighting the existing reality. To change something, build a new model that makes the existing model obsolete.”

- Buckminster Fuller

CHAPTER 1

LITERATURE REVIEW

Introduction

In 2021, 49 thousand Australians and over 10 million people worldwide will die from cancer (1,2). Even as the COVID-19 pandemic rages on throughout the world, cancer still remains an enormous healthcare burden, imbued with a level of consistent societal trepidation unparalleled in these polarising times. In Australia, one in three women, and one in two men will develop cancer in their lifetime (1). Cancer begins and ends with people – individuals – some struggling to fight against its inevitable lethality from within and others attempting to understand it from the sidelines. In the midst of the abstraction of cancer as a complex scientific investigation, we sometimes forget this simple fact: cancer is as devastating as it is complex.

How scientists and clinicians come to name phenomena rings through time, and can both aid and hinder future investigation and discovery. Hippocrates (460-375 BC) is credited with the origin of the word cancer, first describing malignant tumours as *karkinoma* from the Greek word for crab, *karkinos*. Descriptions of cancer, and attempts to treat it, track back to Ancient Egypt in the medical texts of the *Edwin Smith Surgical Papyrus* from 3,000 BC (3). Surgical resection by cauterisation was attempted to remove superficial breast lesions with the author noting, “There is no cure” (3). Ancient Egyptians also used arsenic paste, which is still in clinical use as arsenic trioxide (ATO) for a subtype of acute myeloid leukaemia (AML) (4), and a recent study indicated that ATO acts as a cysteine reactive allosteric activator of one of the most potent drivers of cancer, mutant p53 (mut-p53), which is a central focus of this thesis (5).

How far have we really come in over 5,000 years? With all the technology that would have seemed like magic to the Ancient Egyptians – is it possible to eradicate cancer? Are we destined to reach this goal in this century?

Cancer: A disease of mutations

Cancer is a catch-all term for over 200 different diseases characterised by the limitless potential for somatic cell division. *Hallmarks* are attributed to the inherent characteristics of the cells inside of tumours: enabling replicative immortality, resisting cell death, sustaining proliferative signalling, evading growth suppressors, avoiding immune destruction, deregulating cellular energetics, inducing angiogenesis, activating invasion and metastasis, tumour-promoting inflammation – all brought about by the final *hallmark*: genome instability and mutation (6,7). As cancer develops, the cancer cells within a tumour accumulate mutations in order to co-opt normal cellular pathways as well as gain neomorphic functions to sustain unchecked proliferation (8).

The mutations in endogenous genes that promote cancer are bifurcated into two categories: genes that suppress the formation of cancer, tumour suppressor genes, and genes that promote the formation of cancer, oncogenes. Over the past two decades, the Cancer Genome Atlas (TCGA) has profiled tens of thousands of patient samples in order to reveal the complexity of the mutational landscape of cancer using massively paralleled exome-wide and even genome-wide next-generation sequencing (NGS) (9,10). From these datasets, the median number of mutations found in cancers was 59 (**Figure 1.1A**). Meanwhile, in the Memorial Sloan Kettering Cancer Centre IMPACT (MSK-IMPACT) study applying a targeted NGS panel covering 450 cancer-associated genes to samples of patient with metastatic disease found a median

mutation count of 4 (11) (**Figure 1.1B**). Analyses of these massive datasets confirm over a century of experimental and clinical observations: a typical cancer develops through a step-wise accumulation of a few “driver mutations” (in 2-8 genes) but also sustains a large number of “passenger mutations” that confer limited or no growth advantage (12-15). The number of driver mutations sustained within a tumour is correlated with age of cancer onset (16,17). Driver mutations appear in the early steps of tumourigenesis and form “trunks” of single-tumour evolutionary trees, highlighting their importance for tumour development and maintenance (18,19). Given that the mutations in tumours drive major changes to the cellular structures and functions, identifying the most common driver mutation in cancers and developing therapeutic strategies to target this mutation would provide a powerful therapeutic avenue.

***TP53* mutations in human cancers**

Mutations in *TP53* are the most frequent genetic alteration in human cancer (**Figure 1.2**), occurring in up to 50% of all cases of cancer (20), although the remaining proportion almost certainly harbour other aberrations within the p53 signalling network – making abnormalities in p53 signalling an immutable hallmark of tumourigenesis (6,21-23). As a result, *TP53* is arguably the most important gene in human cancer. Furthermore, *TP53* mutations are prognostically significant and are associated with increased metastatic potential, chemotherapy and radiotherapy resistance, and poorer overall survival (**Figure 1.3**) (24-26). The frequency of *TP53* mutations across cancer types is diverse with rates of up to 90% detected in oesophageal cancers whilst mutations are seldom detected in thyroid carcinoma (**Figure 1.4**). Of note, the rate of *TP53* mutation in AML patients is less than 9%.

The clear majority of *TP53* somatic mutations lead to missense substitutions clustered within the central DNA binding domain of p53, where several “hotspots” have been identified (**Figure 1.5A,B**) (27). Six hotspots (R175, G246, R248, R249, R273 and R282) constitute ~30% of all *TP53* mutations in human cancers (28). These mutations result in the formation of mut-p53 proteins that lack wild-type p53 (wt-p53) tumour suppression activities, primarily due to loss of sequence-specific DNA binding (29). These mutations in the DNA binding domain can either be DNA contact mutations (eg. R248 and R273), which alter the amino acid residues that interact with DNA, or conformational mutations (e.g. R175) that distort the structure of the p53 protein thereby preventing its binding to the canonical p53 DNA response elements (**Figure 1.5C**) (30). At the organismal level, inherited germline mutations in *TP53* have also been reported and reflect a similar mutational profile to somatic mutations found in cancer (31). Carriers of *TP53* germline mutations, resulting in the condition referred to as Li-Fraumeni syndrome, have a lifetime risk of cancer of ~75% for males and 100% for females (32,33).

p53: The master tumour suppressor

The p53 protein was first discovered in 1979 as a protein bound to the SV40 large T antigen in SV40-transformed cells (34), and simultaneously identified independently by two other laboratories (35,36) (reviewed in (37)). p53 was named after the apparent molecular mass of the protein, 53 kDa. Initial characterisation of cancer cell lines (CCLs) and non-transformed cells using anti-p53 monoclonal antibodies revealed that cancer cells had elevated levels of p53 protein compared to non-transformed cells, which only expressed minute amounts (38). This was attributed to the stabilisation observed in cancer cells of this usually short-lived protein (39). Early experiments

overexpressing p53 in non-transformed cells, utilising molecular cloning of p53 from cancer cells, found that p53 acted to drive immortalisation and cooperated with mutant *Ras* transduction (40,41). Furthermore, mutagenesis of the *TP53* gene was found to enhance malignant transformation (42). As a result, p53 was initially defined as a cellular proto-oncogene.

These data contradicted other investigations at the time, which noted that functional loss of the *TP53* gene was frequently observed in malignant transformation (43,44). Eventually, the p53 cDNA cloned out of cancer cells was found to be mutated and acting in a dominant-negative fashion over endogenous wt-p53 (37). Recloned wt-p53 was instead found to inhibit the transformation process and block tumour formation *in vivo* (45,46). Moreover, overexpression of wt-p53 in cancer cells with no endogenous p53, led to a complete block of cell proliferation and induction of cell death (47). As a result, p53 was redefined as a tumour suppressor gene. In concert with these experimental findings, a flurry of papers quickly reported the high frequency of *TP53* mutations across a variety of cancers (48,49).

p53 is a master regulator of the genome, directly activating the transcription of hundreds of genes involved in DNA repair, proliferation, apoptosis, cell-cycle arrest, senescence, metabolism and cell migration, whilst indirectly regulating the repression and activation of potentially thousands more genes (50-53). In unstressed, non-transformed cells, wt-p53 protein levels are kept under tight control by the E3 ubiquitin ligase, MDM2, which targets p53 for proteasomal degradation (54,55). This is potentiated by MDM4, which aids MDM2 in mediating wt-p53 degradation (56), as well as inhibiting wt-p53 DNA binding (57). In response to cellular stress stimuli, including

DNA damage, oncogene activation, hypoxia, nucleotide depletion, glucose withdrawal and oxidative stress, p53 is activated through a complicated network of post-translational modifications (reviewed in (58)). Generally, phosphorylation and acetylation events result in the stabilisation, nuclear accumulation and activation of p53 (59). Phosphorylation of serine 15 is often utilised as a biomarker for p53 activation and is known to alleviate inhibition by MDM2 (60). The list of key protein kinases that directly regulate p53 includes ATM, ATR, CHK1, CHK2 and DNA-PK (60,61). The convergence of events leading to p53 activation results in a strongly context-dependent output, which is both a function of the post-translational modifications conferred on p53 and the nature of the cell state.

The predominant function of p53 is transcriptional regulation and this is enacted in a homotetrameric form mediated by interactions within the tetramerisation domain of p53. This then allows p53 to then bind to the promoters of its target genes in a sequence-specific manner through its DNA binding domain (62-64). p53 responsive elements (p53RE) contain two decameric half-sites with the consensus sequence, RRRCWWGYYY, that confer selectivity of p53 binding (65). Broadly, p53 transcriptional activation results in three outcomes: DNA damage repair, cell cycle arrest or ultimately, if the cellular insult is significant enough, cell death via apoptosis. p53 binds to and recruits general transcriptional machinery, including TATA-binding protein-associated factors and RNA polymerase II, to the promoter region and transcriptional start site of p53 target genes to both initiate transcription and promote elongation (66-69) (Reviewed in (70)). The p53 protein contains two N-terminal transactivation domains (TADs) that mediate protein-protein interactions with transcriptional machinery (70). Importantly, p53 directly acts solely as a transcriptional

activator (70), although p53 indirectly downregulates the expression of genes through downstream effectors such as p21 (*CDKN1A*), E2F7 and various microRNAs (71,72).

p53 induction of cell cycle arrest

p21 (encoded by *CDKN1A*) was one of the first genes identified to be transcriptionally activated by p53 (73), and is essential for the repression of cell cycle promoting genes (74). Following wt-p53 activation, a suite of genes regulating the progression through the cell cycle are downregulated, including several cyclins (*CCNA1*, *CCNB1*, *CCNB2*), cyclin-dependent kinase 1 (*CDK1*), and cell cycle phosphatases (*CDC25A*, *CDC25C*) (75-79). This transcriptional downregulation is facilitated by p21, a potent cyclin-dependent kinase (CDK) inhibitor, which prevents the phosphorylation of pocket proteins, Rb (retinoblastoma), p130 and p107 (80). These hypophosphorylated pocket proteins then bind E2F transcriptional factors leading to the formation of the DREAM and Rb-E2F transcriptional repressor complexes (Reviewed in (81)).

p53 induction of apoptosis

Wt-p53 was first shown to induce cell death via apoptosis in p53-null leukaemic cells utilising a conditional temperature sensitive mut-p53^{A135V} construct (82,83). Wt-p53 stabilisation led to the loss of cell viability, triggered apoptotic morphology and characteristic DNA fragmentation (83). Wt-p53-induced cell death occurs through a distinct pathway from p53-induced cell cycle arrest. Notably, blocking the cell death induced by p53 still results in cell cycle arrest (84). Subsequent papers demonstrated the requirement for endogenous wt-p53 to mediate radiation-induced apoptosis (85,86). Furthermore, loss of p53-mediated apoptosis in transgenic mice leads to tumour progression (87), highlighting the role of p53 induced apoptosis to aid in tumour

suppression. However, more recent studies have also pointed to p53-mediated regulation of ferroptosis (an iron-dependent, non-apoptotic form of cell death) as a major contributor to its capacity to suppress tumour development (88) (discussed below in detail). Mechanistically, wt-p53 has been shown to induce apoptosis through two distinct pathways: a transcriptional-dependent and a transcriptional-independent pathway. However, there is disagreement in the field as to the degree to which either of these pathways contributes to the capacity of wt-p53 to elicit cell death and thus tumour suppression. This is discussed further below.

The central axis by which wt-p53 induces apoptosis is through modulating the balance between the expression and localisation of anti-apoptotic and pro-apoptotic BCL-2 homology proteins, which regulate the release of cytochrome c from the mitochondria of mammalian cells, leading to the activation of caspases that carry out the destruction of cells from the inside out. Wt-p53 directly upregulates the expression of pro-apoptotic genes, *PUMA* (89), *NOXA* (90), *BID* (91) and indirectly upregulates *BIM* (92). PUMA, NOXA, BID and BIM exert their pro-apoptotic effects by displacing anti-apoptotic BCL-2 family proteins, BCL-2, BCL-XL and MCL-1, releasing BAX and BAK, which are required for mitochondrial-mediated apoptosis (93). BAX and BAK form heterodimers that oligomerise leading to the formation of pores triggering mitochondrial outer membrane permeabilisation (MOMP) (**Figure 1.6**). Of note, no single p53 target gene acting alone has been identified to be capable of mediating p53-dependent apoptosis nor tumour suppressive capacity (94).

Wt-p53 also demonstrates the capacity to induce transcription-independent apoptosis (95). Furthermore, wt-p53 can translocate from the nucleus to the mitochondria to elicit

direct effects on anti-apoptotic BCL-2 family member proteins and stabilise BAX and BAK (96,97). Importantly, wt-p53 translocation to the mitochondria occurs rapidly following cytotoxic insults (within 30 minutes) and precedes the transcriptional activation of p53 target genes (98). Intriguingly, several mutant p53 proteins (e.g. R237H and C277F) still retain the capacity to interact with BAX and BAK in cells, however they have no capacity to induce apoptosis in a transcription-independent fashion (99,100). As a result, it is probable that transcription-dependent and independent induction of apoptosis by wt-p53 operates on a fulcrum and interacts in complex ways that are difficult to ascertain using reductionist approaches.

The role of ferroptosis in p53-mediated tumour suppression

Beyond its role in regulating apoptosis in response to stress, wt-p53 also plays a critical role in regulating non-apoptotic forms of cell death (101) (see detailed review on cell death nomenclature (102)). Notably, studies have demonstrated that mouse wt-p53 and acetylation-defective p53 (p53^{3KR}) mediates transcriptional suppression of *SLC7A11*, which regulates cellular cystine uptake, promoting ferroptosis sensitivity in cancer cells (88,103,104). Ferroptosis is a form of non-apoptotic cell death described as iron-dependent, lipid peroxidation-induced, non-apoptotic cell death (105). Ferroptosis can be triggered by glutathione (GSH) depletion via pharmacological inhibition of cystine-glutamate antiporter, system x_c⁻ (encoded by *SLC7A11* and *SLC3A2*), or by direct inhibition of the unique GSH peroxidase 4 (GPX4), the major regulator of lipid peroxidation (106) (**Figure 1.7**). Mechanistically, Jiang et al. (88) demonstrated mouse wt-p53 and p53^{3KR} suppress *SLC7A11* expression directly through binding to the promoter region of *SLC7A11*. Importantly, mouse p53^{3KR} is unable to induce p53-dependent apoptosis, cell-cycle arrest or senescence, however

it retains the capacity to suppress tumour formation in mice (107), suggesting that wt-p53-mediated sensitisation to ferroptosis contributed to tumour suppression. Mutation of a fourth acetylation site on mouse p53^{3KR} (p53^{4KR98}), which retains the capacity to induce the p53-MDM2 negative feedback loop, loses the ability to suppress SLC7A11 and sensitisation to ferroptosis (104). Interestingly, Wang et al. (104) demonstrated that p53^{4KR98} still bound the *SLC7A11* promoter region that was identified as a p53RE (88), suggesting that wt-p53 binding to the *SLC7A11* promoter may be insufficient to explain the capacity of wt-p53 to transcriptionally suppress *SLC7A11*. Meanwhile, more recent studies have indicated that wt-p53 regulation of SLC7A11 was context dependent, where loss or suppression of wt-p53 also resulted in diminished SLC7A11 expression (108,109). Mechanistically, wt-p53-mediated p21 induction leads to conservation of GSH in response to oxidative stress, suggesting wt-p53 plays a protective role against ferroptosis (109,110). Moreover, wt-p53 also regulates other genes involved in ferroptosis sensitivity, including *SAT1* (111), *FDXR* (112) and *GLS2* (113), further highlighting the complex role of wt-p53 in ferroptosis and this remains an active area of research in the field.

Mutant p53 drives tumourigenesis

Not all *TP53* mutations elicit equivalent effects in cancer cells, however several generalisable functional cellular outcomes occur as a result of *TP53* mutations (114). *TP53* mutations most frequently occur in the DNA binding domain of p53 resulting in the expression of mut-p53 protein, which often, although not always (115), exerts dominant-negative effects over the remaining wt-p53 protein (116,117), or the second allele of *TP53* is genetically deleted through loss of heterozygosity (118). Mut-p53 in cancer cells abrogates most, if not all, of the normal cellular responses mediated by

wt-p53 in response to cellular stress or other stimuli that trigger wt-p53 activation (119). The clustering of *TP53* mutations indicates that DNA binding is a critical function in p53-mediated tumour suppression. Mut-p53 loses the capacity to bind in a sequence specific manner to DNA and as a result loses the control exerted by wt-p53 over gene expression (114). Mut-p53 is able to accumulate as it lacks the capacity to activate the canonical MDM2-mediated negative feedback mechanism (54,120). Furthermore, even when MDM2 is abundant, mut-p53 can be protected from the E3 ligase activities of MDM2 due to coating by the chaperone Heat shock protein 90 (HSP90) (121). Critical to the development of cancer, in the absence of wt-p53 functionality, cells are able to accumulate substantial DNA damage without triggering apoptosis. This increases genomic instability and accelerates the rate of mutations sustained in cells, potentiating malignant transformation (122,123). This provides an explanation for the prevalence of *TP53* mutations in pre-cancerous lesions even prior to tumour initiation. For example, *TP53* mutations and mut-p53 protein accumulate in cells of patients with Barrett's oesophagus, the pre-malignant metaplasia observed prior to oesophageal adenocarcinoma (124,125). Mut-53 also drives resistance to chemotherapeutic agents and ionising radiation, which damage DNA, and rely on signals mediated by wt-p53 to kill cells effectively (126,127).

The spectra of mutations that *TP53* sustains in cancer development is unique for a tumour suppressor gene and mimics, more or less, that of an oncogene. Why retain a defective tumour suppressor protein rather than deleting it completely? In addition to dominant-negative effects over wt-p53 protein (128-130), mut-p53 acquires neomorphic functions that are oncogenic and promote tumourigenesis – so called “gain-of-function” (131). Pivotal work demonstrating that ectopic mut-p53 expression

in p53-null cells enhanced their oncogenic potential both *in vitro* and *in vivo*, established the tumourigenicity associated with mut-p53 protein (131-133). Knock-in mut-p53 mouse models also demonstrated more aggressive characteristics and propensity to metastasise compared to a p53-null background (134,135). Mechanistically, accumulated mut-p53 drives cancer cell survival through aberrant protein-protein interactions, transrepression of tumour suppressor genes and transactivation of oncogenes – altering chromatin structures and co-opting gene expression globally (131,136,137). Similar to its dominant-negative effects on wt-p53, mut-p53 is able to bind and inhibit the tumour suppressive capacity of other p53 family members, p63 and p73 (138-140). Specifically, in epithelial cancer cells, mut-p53 abrogates p63 control over epithelial gene expression, leading to epithelial-to-mesenchymal transition (EMT) (140), a characteristic attribute of metastatic cells.

Mut-p53 also binds to NRF2 (encoded by *NFE2L2*) to elevate the 26S proteasome gene expression and function, leading to increased degradation of tumour suppressors, including p21, p27 and NOXA (141). NRF2 is a master regulator of anti-oxidant defence, regulating the expression of over 500 genes including *de novo* GSH synthesis genes (*SLC7A11*, *GCLC*, *GCLM*) (142). Furthermore, we have previously demonstrated that mut-p53 entraps NRF2 to hinder its capacity to upregulate the expression of anti-oxidant response genes in response to oxidative stress (143). Others have also demonstrated that mut-p53 “fine tunes” NRF2 anti-oxidant target gene programs, upregulating some target genes whilst downregulating others (144,145).

These gain-of-function attributes of mut-p53 appear to be context-dependent and restricted to epithelial-derived tumours (146), whilst in haematological malignancies, no evidence of mut-p53 conferring oncogenic gain-of-function has been found and mut-p53 only exerts dominant-negative effects and wt-p53 loss-of-function (147). This work has prompted debate regarding how to determine the criteria for what “gain-of-function” is in the context of haematological malignancies. This differs from solid tumours, which have a clear and observable gain-of-function parameter in metastatic spread (148). Importantly, p63 is expressed at lower levels in haematological tissues and tumours (149), and NRF2 deficiency drives the expansion of haematopoietic stem and progenitor cells (150). Therefore, neomorphic protein-protein interactions between mut-p53 and p63 or NRF2 may not occur in haematological settings, resulting in no apparent gain-of-function. Alternatively, analysis of patient samples supports the notion that *TP53* mutation neither drives gain-of-function phenotypes nor exerts dominant-negative effects, and instead the mutational bias for hotspot mutations in the *TP53* gene is determined by the susceptibility of certain nucleotides in specific codons to become mutated (115,151,152).

Targeting mutant p53 for cancer therapy

Given the reported frequency of *TP53* mutation and the potent role mut-p53 plays in driving tumourigenesis, one might expect that developing therapeutic strategies to selectively target mut-p53 would be the highest priority of academic and pharmaceutical laboratories around the world. Currently, there are no clinically implemented, approved treatment regimens that require identification of *TP53* mutations in a patient’s tumour prior to treatment stratification. *TP53* mutation, unlike other tumour suppressor genes, most frequently leads to an accumulated mut-p53

protein that is theoretically targetable. However, mut-p53, like other transcription factors, was largely viewed to be undruggable due to the inherent difficulty of targeting protein-DNA interactions to elicit conformations and desired functional outputs that match the complex, sequence-specific and context-specific nature of wt-p53 target gene regulation (153).

Specifically targeting transcription factors in cancer cells is possible through targeting protein-protein interactions in order to direct a specific transcriptional program for anti-cancer benefit (154). A relevant example is targeting the activation of wt-p53 through inhibiting its oncogenic MDM2-mediated degradation, which is frequently amplified in human cancers (155). Immunoprecipitation experiments mapped the p53-MDM2 interaction to the N-terminus transactivation domain of wt-p53 protein (156,157), and later nuclear magnetic resonance (NMR) and X-ray crystallography experiments more precisely mapped the interaction to an α -helix of p53₁₅₋₂₉ (158). The interaction between p53 and MDM2 is mediated by three hydrogen bonds (Phe19, Trp23, Leu26 of p53) that are disrupted following the phosphorylation of Thr18 leading to p53 stabilisation (159,160).

This led to the identification of small molecule inhibitors of the p53-MDM2 interaction utilising a high-throughput protein competition assay to screen for synthetic compounds that liberated p53₁₋₃₁₂ from immobilised MDM2 (161). These compounds, termed Nutlins (from Nutley inhibitor; Nutley is the location of Roche Research Centre), were found to bind and occupy the p53₁₅₋₂₉ binding pocket on MDM2 and, in effect, mimic the hydrogen bonds that p53₁₅₋₂₉ usually coordinates (161). Importantly, treatment with Nutlins led to the accumulation of wt-p53 protein (without affecting

mRNA levels), induced p53 target gene expression (p21 and MDM2), blocked cell cycle progression and induced apoptosis in wt-p53 CCLs (161). None of these effects was detected in mut-p53 CCLs and any effects on proliferation and apoptosis at high doses were p53 and MDM2 independent (161). Nutlins also demonstrated *in vivo* efficacy (161). 2nd and 3rd generation derivatives have been generated with improved potency, pharmacodynamics and kinetics profiles (161-165), and are being tested in clinical trials in haematological malignancies, specifically AML, where pertinently, >90% of cases are wt-p53. The most clinically advanced MDM2 inhibitor, idasanutlin (RG7388), underwent a placebo-controlled phase III clinical trial in relapse or refractory AML in combination with cytarabine (MIRROS Trial; NCT02545283), following positive signals from a phase I/Ib study (166). However, interim analysis failed to meet the primary endpoint of increased overall survival, and as a result Roche withdrew Idasanutlin from consideration for United States Food and Drug Administration (US-FDA) approval in this indication in combination with cytarabine (167,168). Roche is still pursuing the utility of idasanutlin in AML patients in a phase I/Ib trial in combination with the BCL-2 inhibitor, venetoclax (NCT02670044) (169).

Can a similar approach be applied to directly reactivate mut-p53? Is it possible to reactivate mut-p53 to restore its wt-p53 capabilities to selectively kill mut-p53 cancer cells? Certainly genetically restoring wt-p53 in p53-null tumours has been demonstrated to effectively induce cancer cell death and tumour regression in various mouse models (170-172), but is mut-p53 reactivation feasible?

Based on how Nutlins and other MDM2 inhibitors lead to the activation of wt-p53 to selectively kill wt-p53 cancer cells, below is a list of criteria for pharmacological mut-p53 reactivation:

1. Selective killing of mut-p53 cancer cells over wt-p53 cancer cells
2. Functional restoration of wt-p53 activities:
 - a. p53-DNA sequence-specific interactions
 - b. Upregulation of p53 target genes
 - c. p53-mediated cell cycle arrest and/or apoptosis
3. No activity in p53-null cancer cells

At the turn of the century, several protein-based and cell-based screens were attempted to identify small molecules that restored sequence-specific DNA binding and transactivation of p53 target genes in mut-p53 cancer cells (reviewed in (114,173,174), **Table 1.1**). The majority of the compounds identified to date reported a mechanism of action (MoA) wherein mut-p53 protein is thermostabilised, adopting a wt-p53-like structure (as determined by conformation-specific p53 antibodies), and confer sequence-specific binding via *in vitro* DNA oligonucleotide assays. Eprenetapopt is the most clinically advanced compound that claims to be a mut-p53 reactivator (besides ATO which is clinically approved but not originally developed as a mut-p53 reactivator), and has been the most extensively studied over the past two decades.

Discovery of APR-017 (PRIMA-1)

APR-017 (or PRIMA-1: p53 reactivation and induction of massive apoptosis), the lead compound of eprenetapopt (also known as PRIMA-1^{MET} or APR-246), was identified

in 2002 through a phenotypic drug screen of the National Cancer Institute (NCI) Diversity library of ~2,000 low molecular weight compounds. This screen aimed to identify compounds that induced growth suppression selectively dependent upon mut-p53 protein expression (175). Saos-2 osteosarcoma cells expressing a tetracycline-regulated mut-p53^{R273H} construct (Tet-Off) were utilised and compounds were screened at a single dose of 25 µM in the presence and absence of doxycycline (175). Cell growth was measured 48 h after dosing using the WST-1 proliferation detection reagent. Subsequent experiments reported APR-017 induced p53-dependent cell death, restoration of wt-p53 conformation to structural mut-p53^{R175H} and transcriptional transactivation activity (175). Importantly, APR-017 was demonstrated to suppress tumour growth *in vivo*, in human CCL-derived xenograft (CDX) models. Mice exposed to up to 100 mg/kg twice daily through intraperitoneal injection for 3 days exhibited no reported adverse effects. A follow-up study analysed the NCI Developmental Therapeutic Program database (known as the NCI60) containing p53 mutation status and protein levels and response to APR-017 across 60 CCLs (176). This study further supported that notion that CCLs with mut-p53 were more sensitive to APR-017 compared to p53-null or wt-p53 expressing CCLs. Of note, the authors of this study selected only 34 CCLs for these analyses, which is a relatively small number compared to more recently developed datasets. Together, these studies claimed that APR-017 was a mut-p53 reactivator that selectively targeted mut-p53 CCLs with little activity in p53-null and wt-p53 CCLs. Following these studies, Aprea Therapeutics was founded in 2003 by the discoverers of APR-017, including Klas Wiman and Vladimir Bykov, to develop APR-017 into a commercialisable treatment for cancer patients.

Upon detailed reflection and interrogation of the data presented in these seminal papers characterising APR-017, one can highlight several points of contention in the conclusion that APR-017 induces mut-p53 reactivation to trigger apoptosis. First, the deduction that APR-017 induces apoptosis relies on cell cycle analysis by flow cytometry using propidium iodide (PI) and TdT-mediated dUTP nick-end labelling (TUNEL) analyses, which alone are not definitive in determining apoptosis (175). The authors report in the manuscript that "...pre-treatment with the caspase inhibitor zDEVD-FMK reduced PRIMA-1-induced cell death 3-fold and the caspase inhibitor BOC-D-FMK completely abolished PRIMA-1-induced cell death". However, disappointingly, no data are presented in the figures to support this claim, nor are zDEVD-FMK or BOC-D-FMK mentioned in the methods sections of the manuscript (175). Furthermore, whilst immunofluorescent images of cancer cells treated with APR-017 are presented throughout the manuscript, no phase images are provided, which would have proven helpful to assess the manifestation of macroscopic morphological alterations inherent to apoptotic cell death.

Second, conformation-specific p53 antibodies (PAb240 and PAb1620 (177)) were used to determine the shift between mut-p53 conformation and wt-p53 (175). Indeed, APR-017 treatment of recombinant p53 and endogenously expressed mut-p53 in cancer cells leads to an increase in the level of wt-p53 conformation and a decrease in the mut-p53 unfolded conformation detected, compared to untreated conditions. APR-017 was dissolved in dimethylsulfoxide (DMSO) for this study. Interestingly, DMSO has previously been shown to induce a mut-p53 to wt-p53 conformation shift in murine cancer cells as determined by conformational antibodies (178). Furthermore, APR-017 as a pro-drug requires heat and time to undergo non-enzymatic hydrolysis

into its active compound (179) (see below for details). This process occurs over several hours at 37°C (179), however, the experiments utilising recombinant p53 protein exposed the p53 protein for only 30 minutes (175). This could raise the possibility that the induced p53 conformational shift is due to exposure to DMSO instead of APR-017 specifically.

Third, the reported MoA (i.e. APR-017 shifting mut-p53 conformation to wt-p53 conformation, leading to induction of p53 target genes and apoptosis) would not explain the comparable activity demonstrated in CCLs expressing mut-p53 contact mutations (e.g. R273H), wt-p53 or those that are p53-null. Furthermore, APR-017 also demonstrated activity in CCLs expressing wt-p53 at relatively low concentrations, especially in the haematological CCLs tested (175). Isogenic systems were utilised to show mut-p53 selectivity by comparing p53-null with ectopic overexpression of mut-p53; however, the increase in sensitivity to APR-017, based on changes to GI50 values (concentration required for inhibition of maximal cell growth by 50%), showed a shift of only $\sim 1 \log_2$ fold (e.g. GI50 in H1299 cells: 43 μM in p53-null to 20 μM in p53^{R175H} expressing) (175). This is a clear contrast to Nutlin-3, the MDM2 inhibitor introduced above, which targets wt-p53 CCLs selectively, by inducing wt-p53 stabilisation to trigger apoptosis (161). Previous studies of isogenic systems comparing wt-p53 and p53-null cells have illustrated $\sim 4 \log_2$ fold change in GI50 values for Nutlin (e.g. GI50 in HCT116 cells: 2 μM in wt-p53 expressing to 33 μM in p53-null (180)). What is clear, with regards to APR-017 activity in CCLs, is that mut-p53 overexpression influences the sensitivity of cancer cells to APR-017, however, the absence of mut-p53 protein does not preclude CCL sensitivity to APR-017. Thus,

the explanation that APR-017 reactivates mut-p53 to drive apoptosis is insufficient to explain its therapeutic activity.

Early evidence of mutant p53 reactivation-independent activity

Evidence quickly emerged contradicting the model of mut-p53 reactivation leading to wt-p53 target gene expression and apoptosis as the MoA of APR-017. This was most strongly demonstrated in a study utilising CCL cytoplasts, cells lacking a nucleus (181). Here, CCLs treated with APR-017 underwent cell death in the absence of functional nuclei and even when co-treated with cycloheximide to preclude any *de novo* protein synthesis from residual RNA. However, pre-treating CCL cytoplasts with the pan-caspase inhibitor, zVAD-FMK, suppressed the apoptosis induced by APR-017 as measured by Annexin V staining (181). As a result, the authors concluded that APR-017 was eliciting transcription-independent apoptosis through the mitochondrial pathway.

APR-017 demonstrated high potency against patient-derived chronic lymphocytic leukaemia cells *ex vivo* in both wild type and hemizygous *TP53* deletion patient samples (182). Chronic lymphocytic leukaemia cells appeared to be far more sensitive to APR-017 compared to the previously reported sensitivity profiles in the NCI60 database and investigations in other solid tumour CCLs (175,176). Furthermore, healthy donor lymphocytes treated with cytotoxic levels of APR-017, determined in chronic lymphocytic leukaemia cells, were resistant to APR-017. This provided the first evidence that haematological malignancies may provide a future avenue for the utility of this drug in the clinic. This study also demonstrated synergy between APR-017 and the purine analogue, fludarabine. A similar study in patient-derived AML cells *ex vivo*

also demonstrated the efficacy of APR-017 against cells with both normal and complex karyotypes (183). APR-017 was more efficacious in AML cells with chromosomal 17p abnormalities resulting in hemizygous *TP53* deletion. It would appear that the authors were misled in their interpretation that APR-017 was restoring “p53 protein” as an explanation for this differential sensitivity (183).

Given the early promise of this drug, a synthetic chemistry approach was subsequently undertaken to develop novel compounds that resemble the structure of APR-017 (184). Forty-six compounds were synthesised starting from 2-aminoacetophenone hydrochloride. The compounds were tested in isogenic Saos-2 cells (p53-null) overexpressing R175H or R281G mut-p53 to look for selective toxicity in mut-p53 expressing cells. Compound 7d-cis was found to selectively eliminate mut-p53 expressing cancer cells and was more potent than APR-017 (184). It is important to note here that Compound 7d-cis contains an α/β -unsaturated carbonyl capable of Michael addition. Importantly, the authors report that they were unable to detect any evidence of the restoration of wt-p53 in cells treated with either Compound 7d-cis or APR-017. In this more advanced study, the authors concluded that mut-p53 may not be the target of APR-017 and the mut-p53 selectivity may instead be explained by other mechanisms.

Early evidence of non-apoptotic cell death

Evidence of non-apoptotic mechanisms of cell death induced by APR-017 also emerged. Specific inhibitors of caspase-3, caspase-8 and caspase-9 failed to protect cancer cells against APR-017 induced cell death (185). Furthermore, caspase activation was not detected upon APR-017 treatment in this study. A more rigorous

investigation in murine leukaemia L1210 cells determined, by flow cytometry analysis, that APR-017 could induce both apoptotic and necrotic cell death (186). Caspase-3 activity remained unchanged in the lysates of L1210 cells treated with 100 μ M APR-017 for up to 6 hours.

Development of more potent analogues for clinical development

Eprenetapopt, a more potent methylated analogue of APR-017, was identified in 2005 (187). An explanation for the increase in efficacy with eprenetapopt is likely due to its increased stability and cell permeability (188), however a definitive explanation is yet to be established. Eprenetapopt is the lead clinical compound being developed by Aprea Therapeutics. APR-017 and eprenetapopt were found to be pro-drugs that undergo significant decomposition in a time- and temperature-dependent fashion (179). 2-methylene-3-quinuclidinone (MQ) is the reported active compound. Eprenetapopt undergoes non-enzymatic hydrolysis to MQ in cell-free conditions over 24 hours, however cell culture and *in vivo* experiments indicate more rapid decomposition over 6 hours (179). MQ is a thiol-reactive electrophile, or Michael acceptor, that is able to covalently bind to nucleophiles including the sulfhydryl group of cysteine and GSH (**Figure 1.8**). To date, APR-017 and eprenetapopt have demonstrated micromolar anti-cancer efficacy in pre-clinical models of solid and haematological malignancies, including bladder cancer (189,190), breast cancer (191-199), colorectal cancer (175,179,194,200-203), glioblastoma (204), head and neck cancer (205), leukaemia (182,183,206-208), liver cancer (209,210), lung cancer (143,175,179,181,187,194,200,201,211-216), melanoma (216-219), myeloma (220-223), oesophageal cancer (143,224), ovarian cancer (175,225-228), pancreatic cancer (210,229), prostate cancer (230,231), sarcoma

(175,179,187,200,201,214,232,233), thyroid cancer (234-236) and Waldenström macroglobulinaemia (237). More recently, APR-548 is a next generation analogue with high oral bioavailability and further enhanced potency *in vitro* relative to eprenetapopt (www.aprea.com, accessed April 14, 2021).

Efforts to demonstrate mutant p53 as the target for eprenetapopt

Eprenetapopt, through MQ, has been shown to covalently modify the core domain of both recombinant wt-p53 and mut-p53 and thermostabilise these proteins: a property critical for maintaining wt-p53 activity (238,239). There are 10 cysteine residues in the core domain of p53 (240). C124 and C277 have been identified as the key residues targeted by MQ, and both of these residues were found to be critical for eprenetapopt-mediated thermostabilisation and cell death (238,239). Conformation-specific antibody-based assays have been used to detect the conformational shift of structural mut-p53 (e.g. R175H) to wt-p53 in cancer cells (175). In cell-free transcription factor DNA binding assays, APR-017 has been shown to increase the binding affinity of mut-p53 to p53 consensus sites in promoter DNA (175,179). Concordantly, APR-017 and eprenetapopt have been shown to induce the expression of canonical p53 target genes, *CDKN1A* (p21), *MDM2*, *PUMA* and *NOXA* – implying activation of p53 transcriptional pathways (179,200).

Of note, thermostabilisation through covalent modification of recombinant p53 proteins has been detected with other thiol-binding compounds (e.g. *N*-ethylmaleimide (241)). Furthermore, we have shown that the oesophageal CCL FLO-1, which expresses mut-p53^{C277F}, is sensitive to eprenetapopt (143,224). OE19 cells, which express a truncated form of mut-p53 (*310fs) that contains no tetramerisation domain (a feature

required for wt-p53 transcriptional activation), still undergo cell death and upregulate p53 target gene expression after eprenetapopt treatment (224). To date, no study has demonstrated that eprenetapopt induces sequence-specific mut-p53-DNA interactions under physiological conditions through, for example, chromatin-immunoprecipitation coupled to genomic sequencing (ChIP-seq). Moreover, *in vitro* incubation of eprenetapopt (pre-heated to liberate MQ) with bovine serum albumin (BSA) demonstrated that MQ-modification of cysteine residues occurs on BSA performed under similar conditions attempting to demonstrate specific cysteine modification on recombinant mut-p53 (**Figure 1.9**) (Fujihara and Clemons, *Unpublished*). Together, these studies and data provide counterpoints to the determination that eprenetapopt (and APR-017) selectively targets mut-p53.

Unbiased approaches to probe eprenetapopt mechanisms of action

To date, only a few studies have attempted to apply unbiased strategies to probe the MoA of eprenetapopt and its analogues (191,200,207,242). First, the proteins bound by mut-p53 following APR-017 were co-immunoprecipitated with subsequent proteomics analyses performed using mass spectrometry. This approach was applied to selected protein bands excised from a denaturing gel (191). HSP90 was found to be bound to mut-p53 following APR-017 treatment, and the authors note this may aid in the nuclear localisation of mut-p53 and refolding of mut-p53 to wt-p53 (191). Previous investigations have also shown that HSP90 interacts with wt-p53 to promote its DNA binding capacity (243).

Three studies have applied unbiased analysis of changes to global gene expression following eprenetapopt treatment in CCLs: two used microarrays, one used RNA

sequencing (200,207,242). All studies failed to detect changes in canonical p53 target genes (p21, MDM2 and BAX) across several CCLs of varied tissue origins (both epithelial and haematopoietic) and mut-p53 status (200,207,242). Instead, upregulation of oxidative stress-induced and NRF2 target genes was detected, including upregulation of *SLC7A11* (207,242). Studies have yet to profile changes in the proteome or metabolome induced in cells in response to eprenetapopt exposure or correlate the cellular profile of CCLs to their sensitivity to eprenetapopt to identify pharmacogenomic/transcriptomic/proteomic/metabolic relationships.

Investigations of p53-independent mechanisms of action of eprenetapopt

Following early findings that APR-017 and eprenetapopt induced cell death in wt-p53 and p53-null CCLs, investigation began to provide explanations for the mut-p53-independent MoA of eprenetapopt. First, the p53-independent activity of eprenetapopt was attributed to reactivation of other p53 family member proteins, p63 and p73 (201), which, although they are seldom mutated in human cancers (244), are expressed in some CCLs. However, p53, p63, p73-null cancer cells (e.g. HL-60 leukaemic cells) can still undergo cell death in response to eprenetapopt treatment (245).

Eprenetapopt also depletes the major cellular anti-oxidant, GSH, in cancer cells both *in vitro* and *in vivo* (143,221,226). GSH is a tripeptide, comprising glutamate, cysteine and glycine, and is synthesised in cells through a step-wise pathway of *de novo* synthesis (reviewed in (246), **Figure 1.10**). Cysteine availability controls the rate of *de novo* GSH synthesis (246-248) and is predominantly supplied in the form of an oxidised dimer, cystine, through extracellular import via the system x_c^- transporter (encoded by *SLC7A11* and *SLC3A2*). Co-treatment with N-acetyl-cysteine (NAC) or

GSH completely blocks the activity of eprenetapopt in CCLs (141,143,144,179,221,249). MQ forms adducts directly with NAC and GSH through Michael addition (143,179,226). Eprenetapopt also exerts effects on other key components of the cellular redox balance machinery, including thioredoxin reductase 1 (TrxR1), inhibiting the anti-oxidant activity of TrxR1 and turning it into a pro-oxidant NADPH oxidase (250), and inhibiting thioredoxin 1 and glutaredoxin 1 (251). Preceding the onset of cell death, eprenetapopt induces the generation of reactive oxygen species (ROS) and accumulation of lipid peroxides, especially in the mitochondria of cancer cells (143,221). Furthermore, eprenetapopt-induced cell death can be blocked by co-treatment with lipophilic anti-oxidants, including ferrostatin-1, trolox, and 2-mercaptoethanol (143) – all of which are, incidentally, inhibitors of ferroptosis (105). Whether eprenetapopt triggers ferroptosis remains unclear.

Clinical development of eprenetapopt

Eprenetapopt has been and is currently being evaluated in several clinical trials, across several clinical indications in oncology, both alone and in combination with various standard-of-care and novel therapies (**Table 1.2**). Interestingly, whilst pre-clinical studies into eprenetapopt focused on solid tumour types with high rates of *TP53* mutations, such as high-grade serous ovarian carcinoma (HGSOC) and oesophageal cancers (143,224,226,227), the majority of the clinical trials that remain underway as of the 25th July, 2021, are in patients with haematological malignancies that have relatively low rates of *TP53* mutations (eg. AML: ~8% (252); myeloid dysplastic syndromes (MDS): ~7% (253)). Furthermore, the majority of these clinical trials include the detection of at least one *TP53* mutation as inclusion criteria, with no specification of the type of *TP53* mutation (ie. homozygous deletion of *TP53* are

included in this criteria). Eprenetapopt was first tested in a phase I clinical trial in patients with advanced haematological and prostate cancers ineligible for other therapies (254). Generally, eprenetapopt monotherapy was well tolerated and this study established a predictable pharmacokinetic profile for eprenetapopt delivery via intravenous infusion with no aberrant accumulation noted (254). The dose escalation study demonstrated the safety of up to 60 mg/kg eprenetapopt (delivered over two hours) as the maximum tolerated dose with reversible neurological toxicities detailed at 90 mg/kg (e.g. headache, sensory disturbance) (254). At 60 mg/kg, the maximum plasma concentration of eprenetapopt was $286.57 \pm 27.9 \mu\text{M}$ (mean $\pm$ standard deviation) across six patients (254). A follow-on open-label phase I trial in AML patients indicated that a dosing regimen of 67.5 mg/kg eprenetapopt, delivered over six-hour infusions on four consecutive days was also well tolerated (255).

Following these trials, and based on promising pre-clinical investigations (245,256), two phase II clinical trials investigating the combination of eprenetapopt and standard-of-care, azacitidine, in patients with *TP53*-mutated MDS were initiated and the results from these studies were recently published (257,258). Both of these trials reported strong rates of complete remission (CR) (257,258), leading to the instigation of a randomised phase III clinical trial in *TP53*-mutated MDS comparing the efficacy of eprenetapopt and azacitidine versus azacitidine alone. However, early results from this phase III trial, reported by Aprea (259), disappointingly failed to meet the primary endpoint of CR rate (33.3% in eprenetapopt + azacitidine, 22.4% in azacitidine alone, $P=0.13$). Together, these results provide a strong motivation to re-examine the requirement for *TP53* mutation as a criterium for eprenetapopt utility in oncology and

identify a robust therapeutic biomarker to aid in patient selection. Moreover, a re-evaluation of the MoA of eprenetapopt is in order to reinform its clinical utility.

Objectives

Dysregulation of the p53 target gene transcriptional network is a cornerstone of tumourigenesis and *TP53* mutation is the most frequent aberration detected in human cancers. *TP53* mutations are a cancer risk if they accumulate as dysfunctional mut-53 protein. Therapeutically, however, accumulated p53 protein provides a potential anti-cancer target, with broad-spectrum clinical applicability. Eprenetapopt is the most clinically advanced compound marketed as a mut-p53 reactivator. However, its MoA remains contentious as it maintains strong efficacy against cancer cell lines without mut-p53. Currently, clinical investigations of eprenetapopt rely on *TP53* mutation status for patient selection even though this approach may be hindering the clinical translation of eprenetapopt. An unbiased examination of what cellular features determine cancer cell sensitivity to eprenetapopt, and its MoA is yet to be performed.

Therefore, the objectives for this thesis were:

1. To identify a robust therapeutic biomarker for eprenetapopt to aid in patient selection.
2. To determine the MoA of eprenetapopt utilising unbiased strategies.

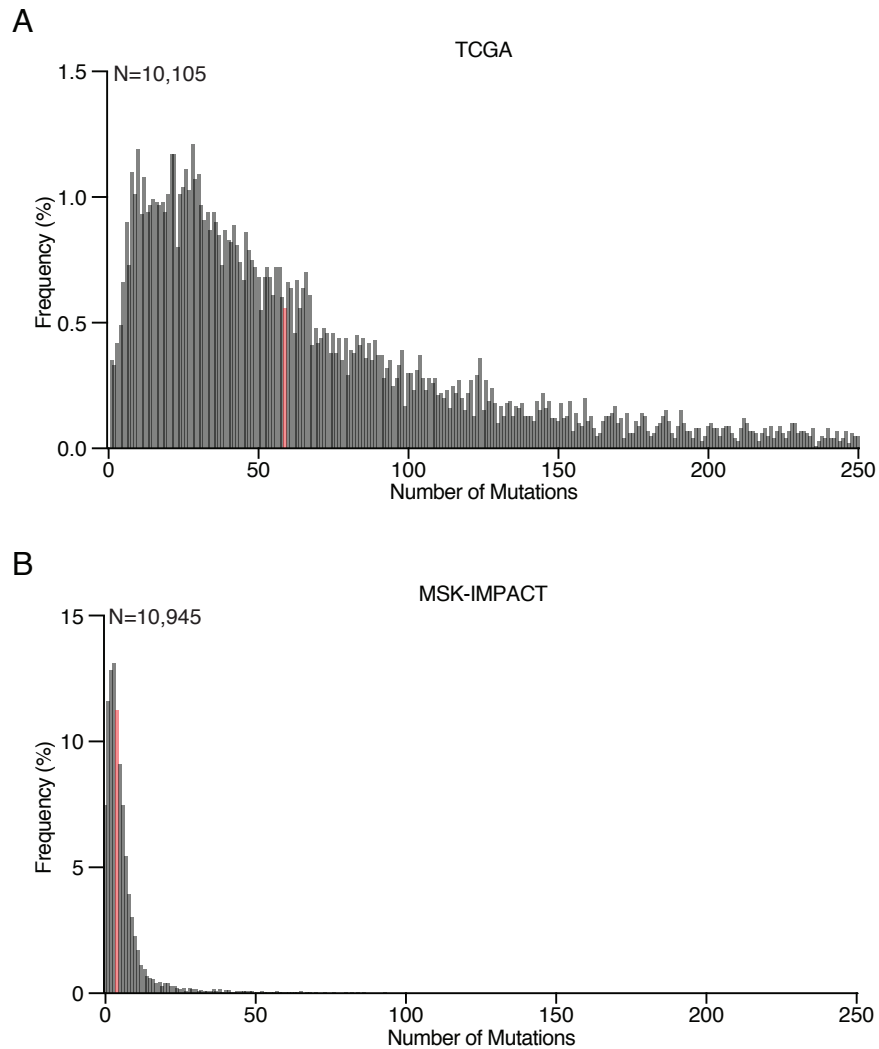


Figure 1.1 Number of somatic mutations in cancer tissue per patient from **(A)** The Cancer Genome Atlas (TCGA) pan-cancer project (exome-wide) and **(B)** the Memorial Sloan Kettering (MSK) IMPACT project (450 targeted cancer-related gene panel). Red bar indicates the median number of mutations. Refs: (9-11).

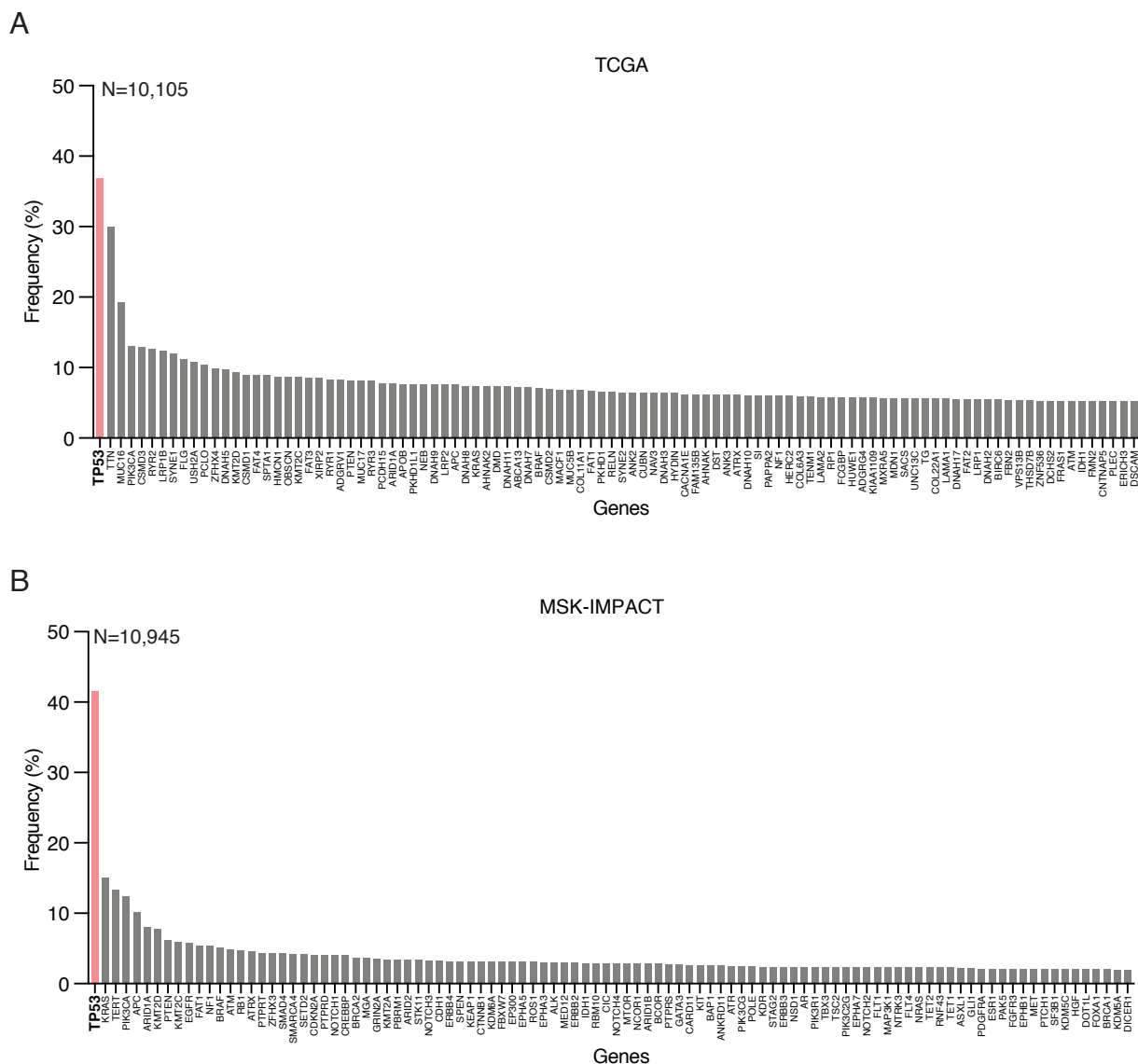


Figure 1.2 Frequency of the top 100 genomic mutations detected in the **(A)** TCGA and **(B)** MSK-IMPACT pan-cancer studies. *TP53* is the most frequently detected mutation across both studies.

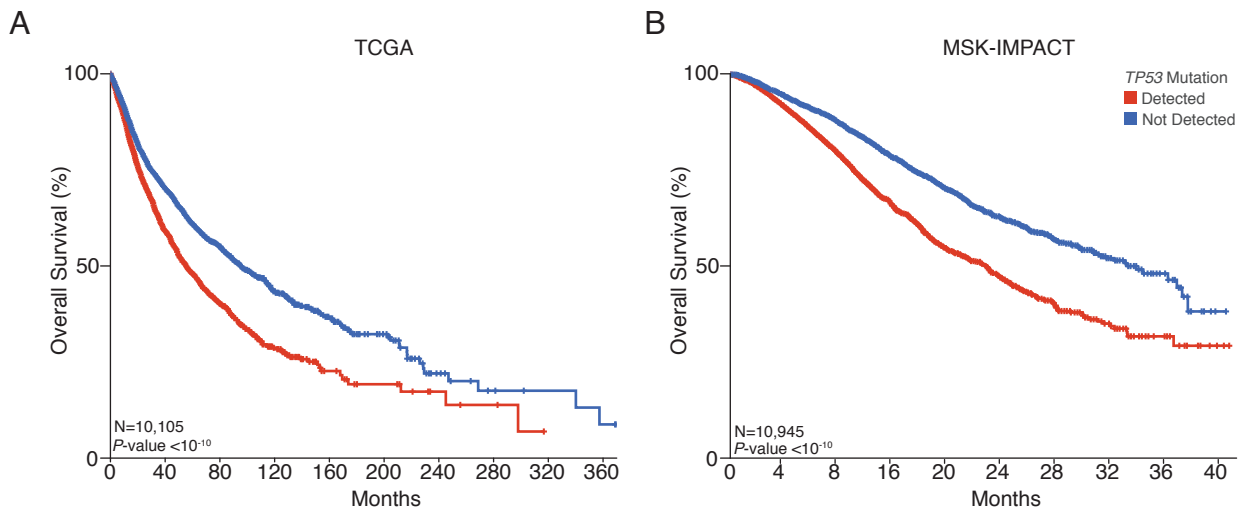


Figure 1.3 Overall survival comparing patients with detected *TP53* mutations and those without from **(A)** TCGA and **(B)** the MSK-IMPACT pan-cancer studies. Note: All MSK-IMPACT pan-cancer study patients have metastatic burden, explaining their rapid decline compared to the TCGA cohort. **(A,B)** Logrank test performed on cBioportal (Accessed 10 May 2021).

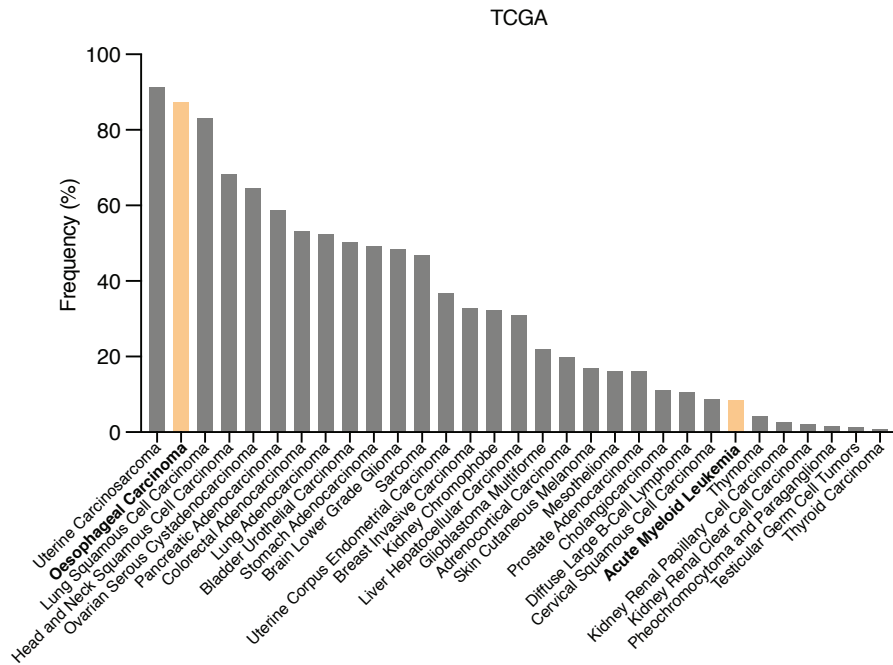


Figure 1.4 Rate of *TP53* mutation across the different cancer studies in TCGA cohort. Notably, oesophageal cancers have high rates of *TP53* mutation, whilst patients with acute myeloid leukaemia have low rates of *TP53* mutation.

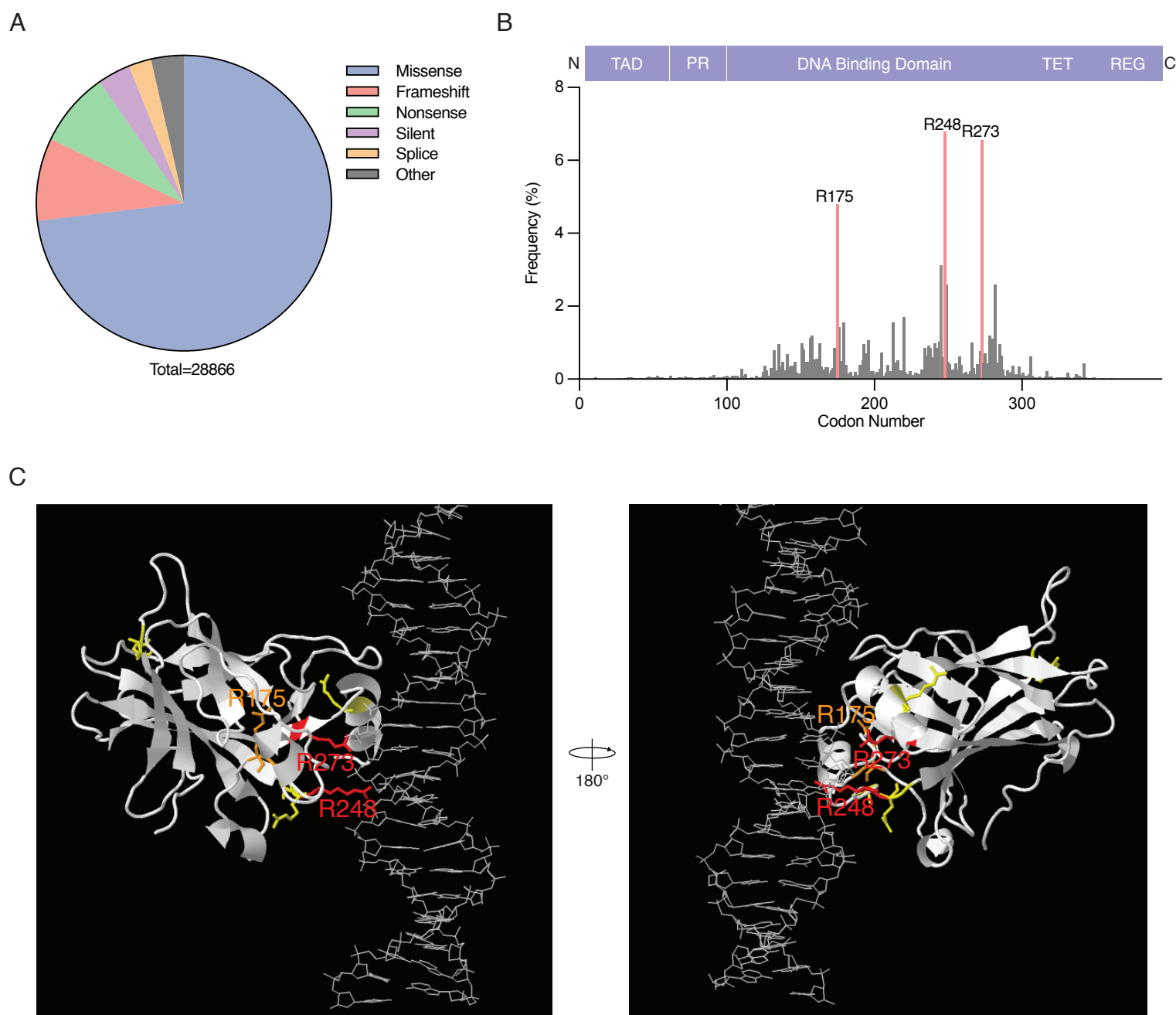


Figure 1.5 (A) Mutation effect and **(B)** distribution of *TP53* mutations across the amino acid coding sequence of p53 protein. The three most common hotspot mutations are highlighted in red (R248, R273) or orange (R175) and the location of these residues is depicted in **(C)**. Data was extracted from the International Agency for Research on Cancer (IARC) TP53 database (www.p53.iarc.fr/TP53somaticmutations.aspx). (TAD, transactivation domain; PR, proline rich domain; TET, tetramerisation domain; REG, regulatory domain.)

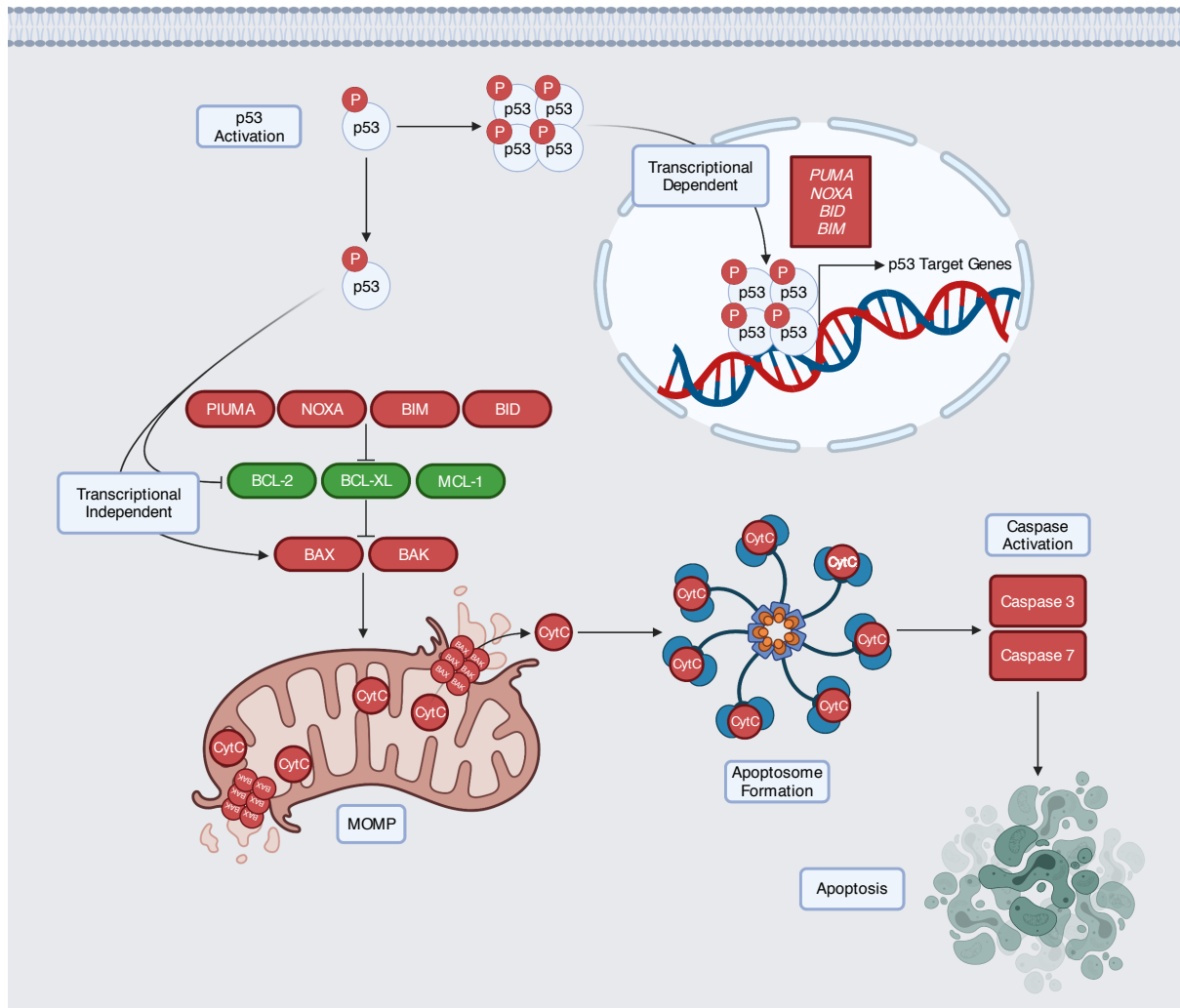


Figure 1.6 p53 triggers apoptosis through transcription-dependent and -independent mechanisms, converging on the formation of BAX/BAK oligomers, which leads to mitochondrial outer membrane permeabilisation (MOMP). Cytochrome-c (CytC) is then released, leading to the formation of the “apoptosome”, which contains Apaf-1, CytC and caspase-9. Finally, caspases 3 and 7 are activated through cleavage, triggering apoptosis.

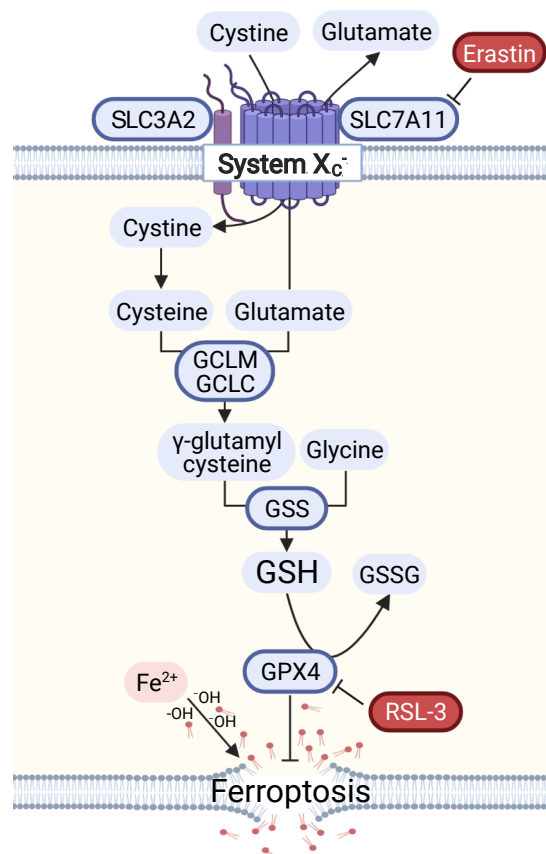


Figure 1.7 Schematic diagram of the system x_c^- /GSH/GPX4/ferroptosis axis and examples of ferroptosis inducers, erastin (cystine uptake inhibitor) and 1S,3R-RSL-3 (GPX4 inhibitor). (GSH, glutathione; GPX4, GSH peroxidase 4; Fe^{2+} , iron(II), RSL-3, 1S,3R-RSL-3). Also see *Perspective in Chapter 5* for detailed discussion on ferroptosis.

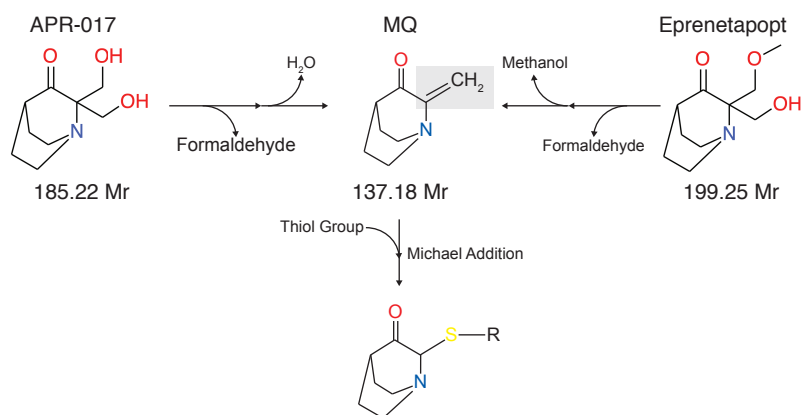


Figure 1.8 Eprenetapopt and APR-017 undergo non-enzymatic degradation into the active molecule, 2-methylene-3-quinuclidinone (MQ), which contains a reactive α/β -unsaturated carbonyl. This carbonyl group can undergo Michael addition to form a covalent thioether with a donor thiol group, such as the sulfhydryl in cysteine or GSH. (Mr, molecular weight).

A

Peptide Sequence	Position	Mass	+ MQ
ECCHGDLLECADDR	267-280	1578.5981	1716.6821
TCVADESHAGCEK	76-88	1349.546	1486.63

B

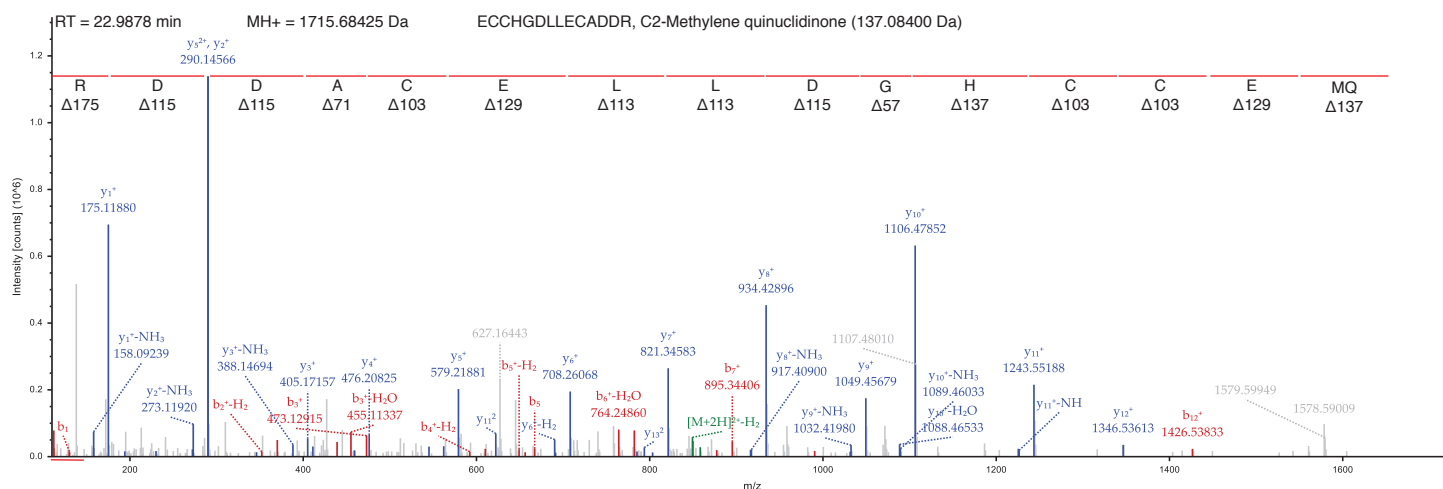


Figure 1.9 MQ-modification detection by mass spectrometry on bovine serum albumin (BSA). One mg of BSA was incubated with 50 μ M eprenetapopt (pre-heated to generate MQ) for 5 min, then analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Peptides were prepared following standard tryptic digest protocol following the addition of Tris(2-carboxyethyl)phosphine (TCEP) and iodoacetamide (IAA) to reduce and alkylate proteins prior to digestion. **(A)** Table of two peptides determined to be modified by MQ. Given that proteins have been processed with TCEP and IAA, the abundance of MQ-modifications would likely be suppressed. **(B)** Mass spectra from MQ-modified peptide.

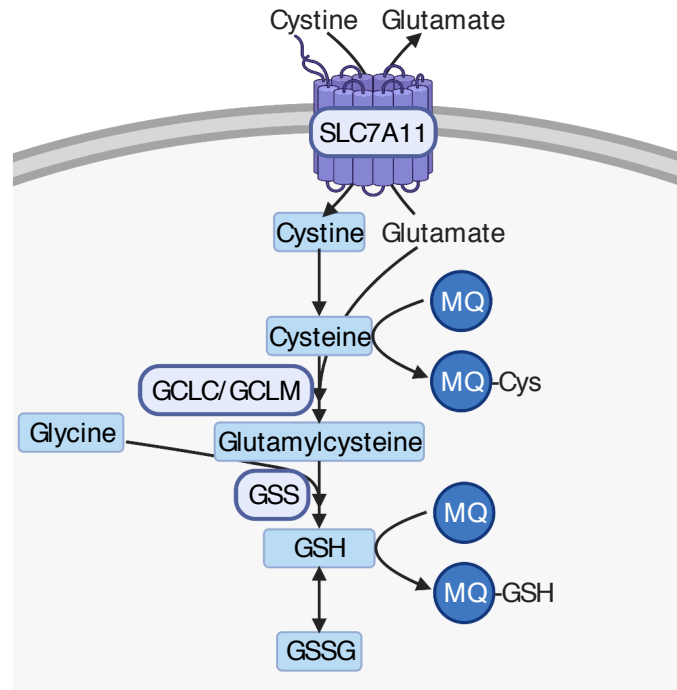


Figure 1.10 Schematic diagram of *de novo* GSH synthesis. GCLC (catalytic) and GCLM (modifier) subunits form glutamate-cysteine ligase. GSS is glutathione synthase. GSSG is oxidised GSH. MQ can form adducts with both cysteine and GSH.

Table 1.1 List of mutant p53 targeted therapeutics (Adapted from (174)).

	Compound	Mode of Discovery	Chemical Class	Mechanism of action	Clinical Development	Year	Refs
Thiol-Targeting Compounds	CP-31398	Protein Screen	Styrylquinazoline	Michael Acceptor	Experimental	1999	(260)
	APR-017	Cellular Screen	Quinuclidinone	Michael Acceptor	Experimental	2002	(175)
	Eprenetapopt	Cellular Screen	Quinuclidinone	Michael Acceptor	Phase III	2005	(187)
	Compound 7d-cis	Rational Design	Aminoacetophenone	Michael Acceptor	Experimental	2005	(184)
	MIRA-1	Cellular Screen	Maleimide	Michael Acceptor	Experimental	2005	(261)
	STIMA-1	Cellular Screen	Styrylquinazoline	Michael Acceptor	Experimental	2008	(262)
	3-Benoylacrylic acid	Protein Screen	Benzoylacrylate	Michael Acceptor	Experimental	2010	(263)
	KSS-9	Rational Design	Piperlongumine	Michael Acceptor	Experimental	2016	(264)
	PK11007	Protein Screen	Sulfonylpyrimidine	Nucleophilic Aromatic Substitution	Experimental	2016	(265)
	Arsenic Trioxide	Database Analysis	Arsenic	Cysteine-binding metal	Approved	2021	(5)
Zn2+ Chelator	ZMC1	Database Analysis	Thiosemicarbazone	Zn2+ Chelator	Experimental	2012	(266)
	COTI-2	<i>In Silico</i>	Thiosemicarbazone	Zn2+ Chelator	Phase I	2016	(267)
Peptides	pCAPs	Phase Display	Peptide	Binds p53	Experimental	2016	(268)
	Reacp53	Rational Design	Peptide	Disrupts mut-p53 aggregates	Experimental	2016	(269)
Other	RETRA	Cellular Screen	Dianhydrogalactitol	Disrupts mut-p53/p73 complexes	Experimental	2008	(270)
	PK083	<i>In Silico</i>	Carbazole	Binds mut-p53Y220C	Experimental	2008	(271)
	P53R3	Protein Screen	Quinazoline	Restores DNA bindings to mut-p53	Experimental	2008	(272)
	SCH529074	Protein Screen	Piperazinylquinazoline	Restores DNA bindings to mut-p53	Experimental	2010	(273)
	PK7088	Rational Design	Pyrazole	Binds mut-p53Y220C	Experimental	2013	(274)
	Stictic Acid	<i>In Silico</i>	Depsidone	Binds p53 <i>in silico</i>	Experimental	2013	(238)
	Chetomin	Cellular Screen	Epidithiodioxopiperazine	HSP40-mediated refolding of mut-p53R175H	Experimental	2015	(275)

Table 1.2 Clinical trials involving eprenetapopt on clinicaltrials.gov. Accessed: 25th January 2021.

Study title	Conditions	TP53 mut?	Interventions	Ph	Status	Year	Idents	Refs
An open-label phase I dose escalating study of APR-246 for infusion in patients with refractory haematologic malignancies or prostate carcinoma	Haematologic neoplasms Prostatic neoplasms	No	eprenetapopt	I	Completed	2009	NCT00900614	(254,255)
PiSARRO: p53 suppressor activation in recurrent high grade serous ovarian cancer, a phase Ib/II study of systemic carboplatin combination chemotherapy with or without APR-246	HGSOC	Yes (IHC)	eprenetapopt + carboplatin + PLD	I/II	Completed	2014	NCT02098343, NCT03268382	
A phase Ib/II study to evaluate the safety and efficacy of APR-246 in combination with azacitidine for the treatment of TP53 mutant myeloid neoplasms	MDS AML CML	Yes	eprenetapopt + azacitidine	I/II	Active, not recruiting	2017	NCT03072043, NCT03588078	(257,258)
A phase Ib/II study evaluating the efficacy of APR-246, a first-in-class agent targeting mutant p53 in the treatment of platinum resistant advanced and metastatic oesophageal or gastro-oesophageal junction cancers	Oesophageal cancer	No	eprenetapopt + 5-FU + cisplatin	I/II	Suspended	2016	NCT02999893	
A Phase Ib/II Study to investigate the safety and clinical activity of APR-246 in combination with dabrafenib in patients with BRAF V600 mutant unresectable and/or metastatic cutaneous melanoma resistant to dabrafenib/trametinib combination	Melanoma	No	eprenetapopt + dabrafenib	I/II	Terminated	2018	NCT03391050	
A phase III multicenter, randomized, open label study of APR-246 in combination with azacitidine versus azacitidine alone for the treatment of (tumor protein) TP53 mutant myelodysplastic syndromes	MDS	Yes	eprenetapopt + azacitidine	III	Active, not recruiting	2018	NCT03745716	
Phase II trial of APR-246 in combination with azacitidine as maintenance therapy for TP53 mutated AML or MDS following allogeneic stem cell transplant	MDS AML	Yes	eprenetapopt + azacitidine	II	Active, not recruiting	2019	NCT03931291	
Phase I study of APR-246 in combination with venetoclax and azacitidine in TP53-mutant myeloid malignancies	AML	Yes	eprenetapopt + azacitidine + venetoclax	I	Recruiting	2020	NCT04214860	
Phase I and dose expansion study of APR-246 in combination with ibrutinib or venetoclax-based therapy in subjects with TP53-mutant R/R NHL including chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL)	Lymphomas	Yes	eprenetapopt + ibrutinib OR venetoclax	I	Recruiting	2020	NCT04419389	
Study of APR-246 in combination with pembrolizumab in subjects with solid tumor malignancies	Advanced solid tumours	No	eprenetapopt + pembrolizumab	I/II	Recruiting	2020	NCT04383938	

(TP53 mut?, Criteria for inclusion requiring TP53 mutation; Ph, Phase; Idents, identifiers; Refs, references of publications; IHC, immunohistochemistry; HGSOC, high-grade serous ovarian carcinoma; MDS, myeloid dysplastic syndrome; AML, acute myeloid leukaemia; CML, chronic myelogenous leukaemia).

CHAPTER 2 *TP53* MUTATION AND CANCER CELL RESPONSE TO MUTANT P53 TARGETED THERAPEUTICS

Introduction

Identifying genetic alterations that prime cancer cell sensitivity to an anti-cancer agent is pivotal for the development of precision cancer medicines, particularly for drugs with an uncertain mechanism of action (MoA). Previous efforts have demonstrated the power of large-scale cancer cell line (CCL) screening to systematically relate the genetic alterations in CCLs to compound efficacy (276-279). To date, several major screening attempts have been undertaken, all with varied approaches, number of CCLs and compounds profiled (**Figure 2.1**). Generally, due to obvious feasibility limitations, there exists a trade-off between the number of compounds and the number of CCLs screened.

Enormous efforts to provide detailed annotations of the > 1,000 available CCLs using every “omics” approach, including DNA and RNA sequencing, proteomics, metabolomics, and genome-wide RNA inference and CRISPR knockout for gene dependency have been completed and are continually updated on the Broad Institute’s Cancer Dependency Map (www.depmap.org). Combining these pharmacogenomic data allows for both biomarker identification for response to anti-cancer agents and aids in the identification or verification of compound MoA.

The current landscape of mutant p53 (mut-p53) targeted therapeutics is dominated by eprenetapopt, and its pre-clinical and clinical development have been central to the focus on mut-p53 reactivation through covalent cysteine modification as the predominant MoA for mut-p53 targeted therapeutics. Previous reports have

demonstrated that the efficacy of eprenetapopt in CCLs is agnostic to specific type of *TP53* mutation and instead is more related to the accumulation of mut-p53 protein (143). As a result, *TP53* mutation has been the presumptive biomarker for response to mut-p53 targeted therapeutics. Here, these assumptions are challenged, instead finding that the mut-p53 targeted therapeutics included in the Cancer Therapeutics Response Portal version 2 (CTRPv2) do not rely on *TP53* mutation for efficacy. Furthermore, CCL lineage plays a role in determining sensitivity to this class of compounds.

Materials and Methods

Datasets

Compound activity (CTRPv2), binary *TP53* mutation status and CCL lineage data were obtained from the Cancer Dependency Map portal at www.depmap.org.

TP53 Mutation Binary Analysis

To determine *TP53* mutation specific compound sensitivity, CCLs were pre-annotated as either *TP53* wild-type or *TP53*-mutated and the activity of each compound in the CTRPv2 dataset was compared between these two designated groups. R was used to perform Student's t-test assuming equal variance. Data are resented using GraphPad Prism version 9.

Delta-median AUC Calculation

In order to determine lineage specific sensitivity to compounds, first the overall median area-under-the-curve (AUC) was calculated across all CCLs for a given compound. This was then subtracted from the median AUC in a given lineage of CCLs. For example, overall median AUC for APR-017 was 12.844, median AUC in leukaemia CCLs for APR-017 was 11.083. Therefore, delta-median AUC in leukaemia CCLs for APR-017 was -1.761.

Results

Performance of mutant p53 targeted therapeutics in Cancer Therapeutics Response Portal v2 (CTRPv2)

Four compounds described as mut-p53 targeted therapeutics (APR-017, eprenetapopt, MIRA-1 and Compound 7d-cis, **Table 1.1**) were included within the CTRPv2 small-molecule sensitivity profiling effort (276,277) generated by the Broad Institute (**Figure 2.2A**). CTRPv2 profiled 481 compounds against ~800 CCLs in 16-point log₂ dose responses and viability was determined by CellTitreGlo (which measures ATP). AUC analysis was performed based on the 16-point log₂ dose response data with a higher AUC reflecting compound resistance, and a lower AUC reflecting compound sensitivity (**Figure 2.2B**). To be clear, an AUC of 15 for a given cell line indicates that the cell line was completely unresponsive at the doses assayed. AUC analysis indicates that only APR-017 was profiled at a sufficient concentration across the majority of CCLs to ensure a lethal dose at the time the assay was measured. However, eprenetapopt, MIRA-1 and Compound 7d-cis were not (**Figure 2.2C**). Generally, however, compound activity across the four mut-p53 target therapies showed strong co-correlations (**Figure 2.2D**).

TP53 mutations do not predict sensitivity to mutant p53 target therapeutics

To answer the question of whether *TP53* mutation drives sensitivity to mut-p53 targeted therapeutics in the CTRPv2 dataset, differential compound activity between *TP53*-mutated CCLs and wild-type *TP53* CCLs was computed across all ~500 compounds (**Figure 2.3A**). Nutlin-3, the MDM2 inhibitor, was found to be selective for wild-type *TP53* CCLs in keeping with the MoA of Nutlin-3 to induce wt-p53 stabilisation through inhibiting MDM2 mediated p53 ubiquitination and degradation (161) (**Figure**

2.3A,B). Importantly, *TP53* mutant CCLs did not display increased sensitivity to any of the mut-p53 targeted therapeutics (**Figure 2.3C**). In fact, in the case of APR-017, wild-type *TP53* CCLs were more sensitive than *TP53* mutant CCLs. Interestingly, out of the 481 compounds in the CTRPv2, no compound displayed a robust mutant *TP53* CCL selectivity.

Haematopoietic, lymphoid and peripheral nervous system lineages are sensitive to mutant p53 target therapeutics

Next, whether CCL lineage plays a role in the sensitivity to mut-p53 targeted therapeutics was surveyed. To do this, delta-median AUC for each lineage was computed for each compound (see *materials and methods*). This analysis revealed that CCLs derived from haematopoietic and lymphoid (HL; multiple myeloma, leukaemia, lymphoma) and peripheral nervous system (PNS) are highly sensitive to mut-p53 targeted therapeutics (**Figure 2.4**). Conversely, oesophageal and other solid CCLs displayed resistance to mut-p53 targeted therapeutics. Of note, all oesophageal CCLs evaluated harbour *TP53* mutations compared to HL CCLs which harbour *TP53* mutation rates of ~68%. This likely accounts for the statistically significant difference in sensitivity to APR-017 between wild-type and mutant *TP53* CCLs (**Figure 2.3C**).

Discussion

The data presented above demonstrates that *TP53* mutation does not predict sensitivity to presumptive mut-p53 targeted therapeutics, eprenetapopt, APR-017, MIRA-1 and Compound 7d-cis across hundreds of CCLs of diverse lineage. This is in keeping with numerous studies that report potent anti-cancer efficacy of eprenetapopt and APR-017 in a *TP53* status-independent fashion (199,203,204,207,222,228). However, the MoA is implied by the nomenclature ascribed to this family of therapeutics. This nomenclature connotes assumptions that have led to an inverse direction to the logic being applied to elucidate the MoA of eprenetapopt and other mut-p53 targeted therapeutics. For example, eprenetapopt, through MQ, is a soft electrophile that conjugates thiols, and p53 protein contains 10 cysteine residues in its core DNA binding domain. Therefore, cysteine conjugation in the core domain of mut-p53 by MQ is studied to determine that MQ conjugates to mut-p53 cysteine residues (179,239). Mut-p53 reactivation is then confirmed as a *fait accompli*.

More egregious still, pre-defining eprenetapopt as a mut-p53 reactivator has led to the continued dismissal of the redox perturbations induced by eprenetapopt as being auxiliary to its MoA rather than central. Eprenetapopt and all other mut-p53 targeted therapeutics have activity in p53-null CCLs, where studies conclude GSH depletion and redox perturbations are responsible for eliciting cell death (143,221,280). Here, simply applying *Occam's razor* would lead to the conclusion that GSH depletion and the effects on cancer cell redox status determine the MoA of eprenetapopt.

Reflecting on the discovery of eprenetapopt and MIRA-1, both were identified in the same high-throughput compound screen comparing differential compound activity in

an isogenic mut-p53 overexpressing and p53-null CCL at a single dose (175,261). These results utilising isogenic systems demonstrated a consistent phenotype wherein mut-p53 overexpression led to increased susceptibility to eprenetapopt and MIRA-1 (175,261). However, if we do not make the assumption that mut-p53 reactivation by eprenetapopt and other mut-p53 targeted therapeutics is their primary MoA, other rationalisations can explain the increase in cell death induced by these compounds in mut-p53 overexpressing cancer cells.

With respect to the importance of lineage-specific sensitivity to mut-p53 targeted therapeutics, we found that HL and PNS CCLs were amongst the most sensitive to these compounds. In the first-in-human phase I trial, patients with haematological malignancies demonstrated response to eprenetapopt monotherapy, whilst in refractory prostate cancer patients, limited efficacy was observed (254). Currently, eprenetapopt is under clinical investigation in *TP53*-mutated MDS and AML (257,258). Furthermore, both neurological and haematological toxicities have been observed during eprenetapopt monotherapy treatment (254,257,258). As a result, mut-p53 targeted therapeutics may be targeting a vulnerability inherent to both neurological and haematological tissues, potentially in both healthy and neoplastic tissues.

Together, these data strengthen the rationale to identify a more robust biomarker that predicts response to eprenetapopt in cancer cells. Furthermore, these results add to the argument as to whether it is appropriate to refer to eprenetapopt, and other compounds like it, as mut-p53 targeted therapeutics.

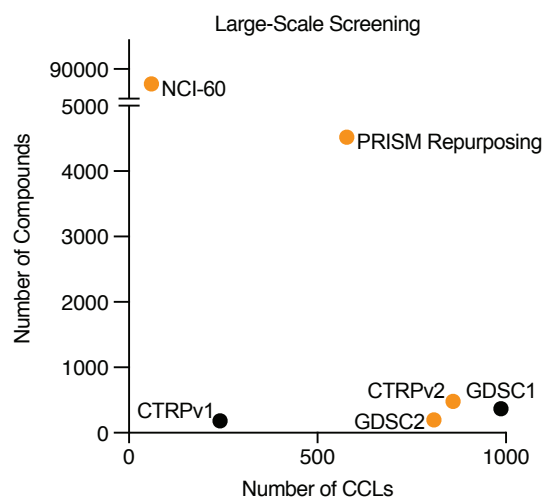


Figure 2.1 Dimensionality of publicly available pharmacogenomic drug screening experiments. Orange dots indicate the inclusion of eprenetapopt and/or APR-017. (NCI-60, National Cancer Institute 60; CTRPv1/2, Cancer Therapeutics Response Portal version 1/2; GDSC1/2, Genomics of Drug Sensitivity in Cancer 1/2).

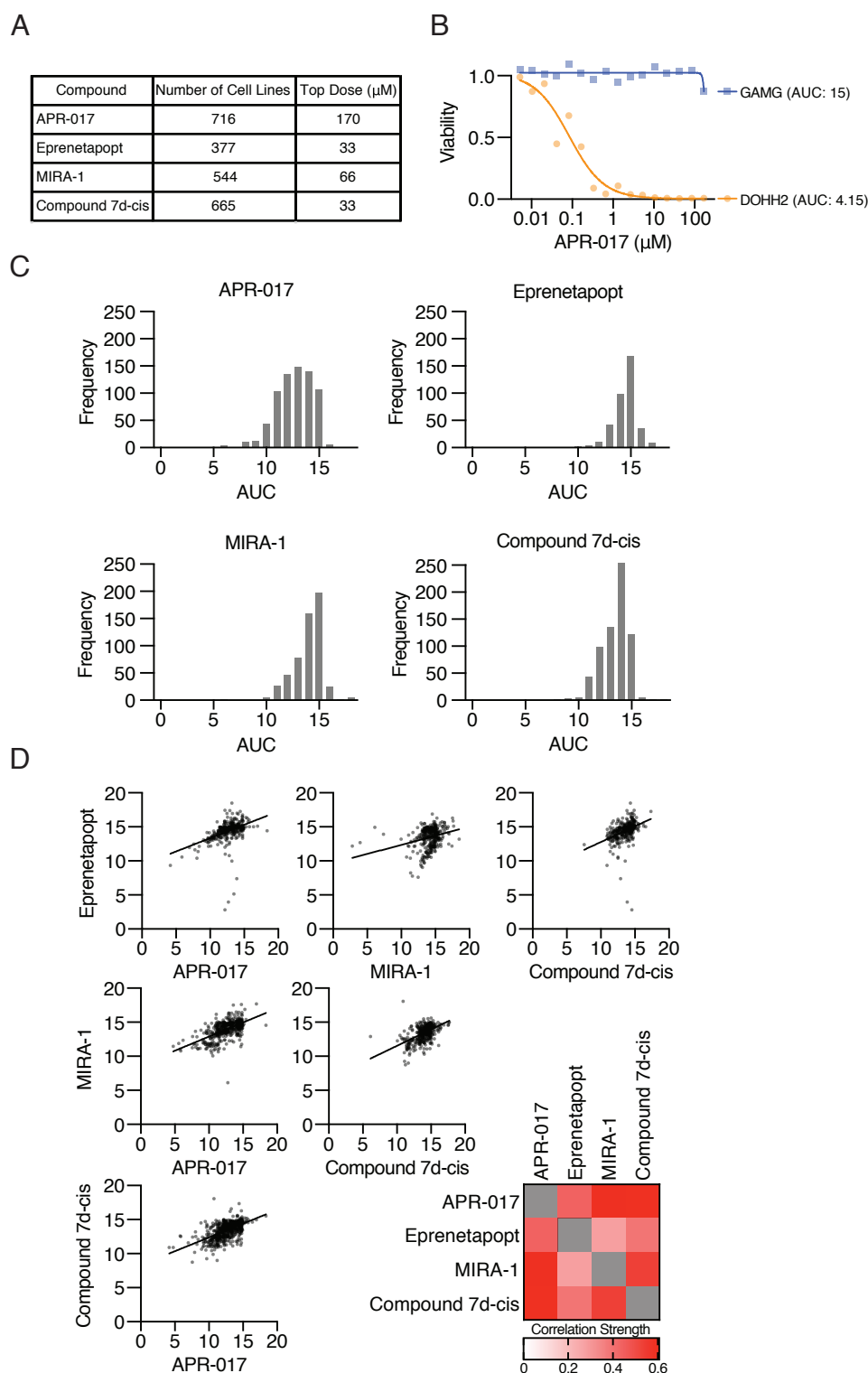


Figure 2.2 Performance of mutant p53 targeted therapeutics in Cancer Therapeutics Response Portal v2 (CTRPv2).

(A) Number of cancer cell lines (CCLs) and top doses utilised for profiling compounds. **(B)** Example of dose response data generated in CTRPv2 to determine area-under-the-curve (AUC). GAMG and DOHH2 are CCLs. **(C)** Histograms of compound activity (AUC) vs. number of CCLs. **(D)** Scatterplots correlating compound activity by AUC and heatmap of Pearson's correlation strengths.

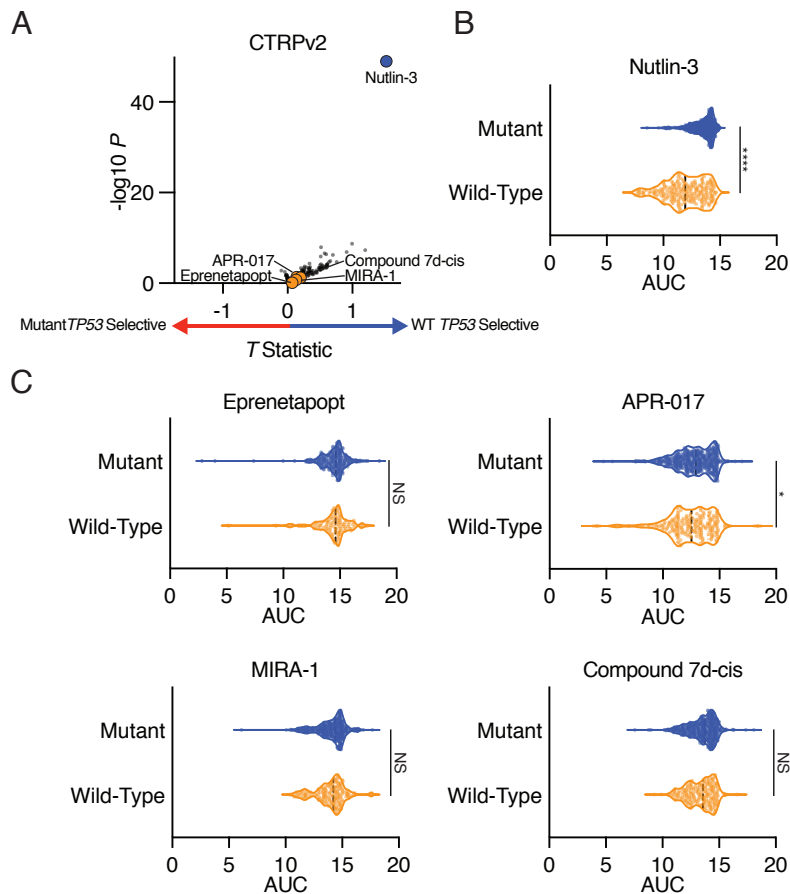


Figure 2.3 *TP53* mutation does not predict sensitivity to mutant p53 targeted therapeutics.

(A) Volcano plot displaying the *TP53* mutation status of all the compounds in the CTRPv2 selectivity. CCLs were designed as *TP53* mutant or wild-type and compound activity between these two binary groups was compared by a student *t*-test. Mut-p53 p53 target therapeutics are denoted by orange dots, Nutlin-3 is denoted by a blue dot. Violin plots demonstrating the efficacy, as determined by AUC, of **(B)** Nutlin-3 and **(C)** mut-p53 targeted therapeutics between mutant *TP53* and wild-type *TP53* CCLs. Dotted line indicates average AUC. **(A,B,C)** Two-tailed *t*-tests. * $P < 0.05$, **** $P < 0.0001$, NS, not significant.

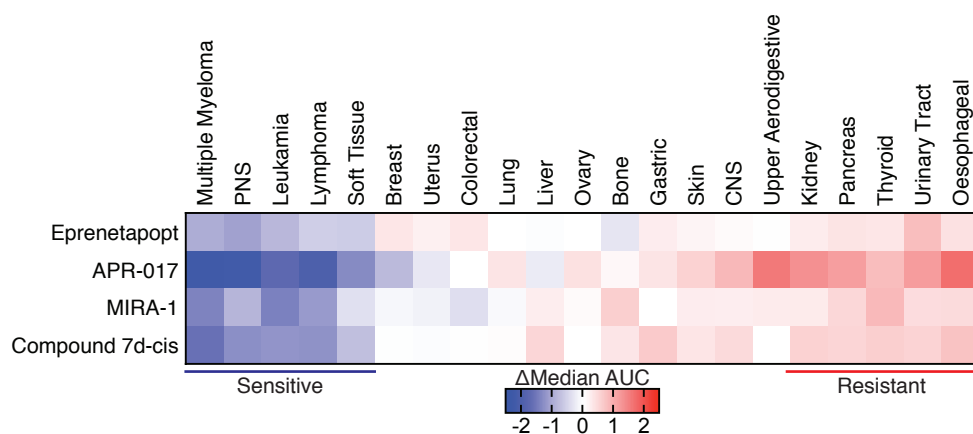


Figure 2.4 CCLs of haematopoietic, lymphoid and peripheral nervous system lineages are sensitive to mut-p53 targeted therapeutics. The order of lineages displayed in was determined by the average delta-median AUC across all four mut-p53 targeted therapeutics.

CHAPTER 3 IDENTIFICATION OF THE MAJOR DETERMINANT OF EPRENETAPOPT SENSITIVITY

3.1 Preface

The aim of this chapter is to identify a biomarker for eprenetapopt to aid in the clinical advancement of eprenetapopt. Building on the literature discussed in **Chapter 1** and data presented in **Chapter 2**, this chapter moves beyond the focus on *TP53* mutation status as a presumptive biomarker for eprenetapopt response, and instead leverages analyses of the cellular features (DNA mutations, gene and protein expression, and metabolite abundance) of hundreds of cancer cell lines to identify biomarkers that predict response to eprenetapopt in an unbiased fashion.

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SLC7A11 is a superior determinant of eprenetapopt (APR-246) response than TP53 mutation status

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Abstract

Eprenetapopt (APR-246, PRIMA-1^{MET}) is in clinical development with a focus on haematological malignancies and is promoted as a mutant p53 reactivation therapy. Currently, the detection of at least one *TP53* mutation is an inclusion criterion for patient selection into most eprenetapopt clinical trials. Preliminary results from our phase Ib/II clinical trial investigating eprenetapopt combined with doublet chemotherapy (cisplatin and 5-Fluorouracil) in metastatic oesophageal cancer, together with previous pre-clinical studies, indicate that *TP53* mutation status alone may not be a sufficient biomarker for eprenetapopt response. This study aims to identify a robust biomarker for response to eprenetapopt. Correlation analysis of the APR-017 activity (lead compound to eprenetapopt) with mutational status, gene expression, protein expression and metabolite abundance across over 700 cancer cell lines was performed. Functional validation and a boutique siRNA screen of over 850 redox-related genes were also conducted. *TP53* mutation status was not consistently predictive of response to eprenetapopt. The expression of SLC7A11, the cystine/glutamate transporter, was identified as a superior determinant of response to eprenetapopt. Genetic regulators of SLC7A11, including ATF4, MDM2, wt-p53 and c-Myc were confirmed to also regulate cancer cell sensitivity to eprenetapopt. In conclusion, SLC7A11 expression is a broadly applicable determinant of sensitivity to eprenetapopt across cancer and should be utilised as the key predictive biomarker to stratify patients for future clinical investigation of eprenetapopt.

Keywords

Eprenetapopt, APR-246, *TP53*, SLC7A11, pan-cancer, biomarker

Introduction

Detailed understanding of drug mechanism of action (MoA) is vital for assessing on-target efficacy *in vivo*, to facilitate the translation of efficacious, novel anti-cancer therapies into the clinic. Strong predictive biomarker identification can then follow, allowing for rational patient selection for clinical trials, which in turn dictates appropriate application in the clinic. Eprenetapopt is proposed to be a mutant p53 (mut-p53) reactivator and is currently under clinical investigation in *TP53*-mutated myeloid dysplastic syndrome (MDS) and acute myeloid leukaemia (AML) (174). However, the MoA of eprenetapopt remains contentious. Eprenetapopt, like its lead compound APR-017, is a pro-drug that is converted to the active compound 2-methylene 3-quinuclidinone (MQ) that conjugates to thiols through Michael addition (179). The predominant reported MoA involves reactivation of wild-type p53 (wt-p53) activity through covalent modification of cysteine residues in the core domain of mut-p53 protein (179,239). However, more recent work links the anti-neoplastic capacity of eprenetapopt to targeting anti-oxidant pathways, causing glutathione (GSH) depletion and direct conjugation and inhibition of enzymes involved in resistance to oxidative stress (143,214,251).

Currently, the vast majority of eprenetapopt clinical trials include the detection of at least one *TP53* mutation as inclusion criteria. The variable mut-p53 dependence for efficacy of eprenetapopt, observed in some studies (217,218), has been rationalised as cancer-type specific, however no unequivocal predictors of response to eprenetapopt in a pan-cancer setting are known. More recently, *ARID1A* mutation was also shown to drive sensitivity to eprenetapopt and other GSH depleting agents (249,281). Previous cellular profiling efforts to identify unique and robust biomarkers

for small molecule activity have succeeded by combining small-molecule sensitivity data with cancer cell line omics datasets (282). Identification of a strong predictive biomarker for this first-in-class compound eprenetapopt, which may also be indicative of its MoA, will allow appropriate patient selection for ongoing clinical development.

By using a pan-cancer, cellular-feature, correlative approach to incorporate transcriptomic, metabolomic and proteomic data, we identified the cystine-glutamate antiporter, SLC7A11, to be a robust biomarker for eprenetapopt response. Remarkably, the predictive capacity of SLC7A11 proved to be superior compared to *TP53* mutation status, highlighting its value as a prognostic indicator for patient stratification of eprenetapopt administration.

Materials and methods

Clinical Trial

Signed informed consent explaining the investigational nature of the clinical trial was obtained in accordance with Peter MacCallum Cancer Centre Human Ethics policies and guidelines. Patients were recruited from Peter MacCallum Cancer Centre, Monash Medical Centre and Western Health. All 5 patients enrolled in the study received Dose Level 1, which included 75 mg/kg (Lean body mass) eprenetapopt, 750 mg/m²/day 5-FU and 25 mg/m² cisplatin. Eprenetapopt was given for 4 days via intravenous infusion over 6 hours starting day 1, 5-FU was administered as a continuous infusion over 96 hr commencing day one, and cisplatin was commenced on day 2 via intravenous infusion over 1 hr following the infusion with eprenetapopt. Patient blood was collected before administration of the treatment regimen (Baseline) and between successive cycles of treatment after two cycles. Oesophageal cancer specific ctDNA panel was performed following previously established protocols (283). Further details of the inclusion/exclusion criteria, protocols used and outcome measures for the phase Ib/II study can be found here:

<https://www.clinicaltrials.gov/ct2/show/NCT02999893>

Boutique siRNA screen

A detailed description of the methods used in the siRNA screen have been previously described (284). Briefly, working in 384 well format, H1299 parental and H1299 overexpressing p53^{R273H} were reverse transfected in quadruplicate on day 1, media changed and replaced on day 2 with 10 µM eprenetapopt or water as vehicle (duplicate plates for each condition), and on day 5 cells were fixed and stained with DAPI. siGENOME SMARTpool siRNAs (Dharmacon, Horizon Discovery) were used at 40

nM final concentration in complex with DharmaFECT lipid (0.08 uL per well) and Opti-MEM. Negative (siON-TARGETplus non-targeting control) and positive (siPLK1) technical controls were included in multiple replicates on each plate. A BioTek EL406 personal liquid handling workstation was used for dispensing cells in high throughput and a Caliper Sciclone ALH3000 robot dispensed the siRNA:lipid:opti-MEM complexes. Cells were imaged using the ArrayScan VTI high-content system (10X objective lens, 16 fields imaged per well, ThermoFisher Scientific) and Cellomics Morphology V4 Bioapplication was used to determine cell number based on DAPI staining. Cell numbers were normalised to the average of the vehicle + siON-TARGETplus non-targeting control wells. Eprenetapopt Growth Inhibition was calculated as follows:

$$\text{Relative cell number}_{\text{siRNA} + \text{Eprenetapopt}} - \text{Relative cell number}_{\text{siRNA} + \text{Vehicle}}$$

Complete set of results for siRNA screens detailed in **Dataset S3.1**.

Cell proliferation and dose-repose assays

For dose-response assays, 10-point log₂ serial dilutions of eprenetapopt and PX-12 were added to 96-well plates containing cells. After 72 h incubation, cell viability was determined using AlamarBlue reagent (Life Technologies) and fluorescence was read at 550 nm/590 nm on a Cytation 3 Imaging Reader (BioTek). For proliferation assays, confluency in 96-well plates was tracked over 72 h using an Incucyte FLR (Essen BioScience) following manufacturer's guidelines.

Cellular Thermal Shift Assay (CETSA)

Performed as per established protocols (285). Briefly, in 15 cm plates, H1299 p53^{R273H} cells were plated overnight and dosed for 2 h with pre-heated (90°C for 20 mins in

water) 50 μ M eprenetapopt and 25 μ M PX-12 or DMSO control. Cells were collected in ice-cold PBS and evenly distributed into strip tubes. Cells were subjected to three freeze-thaw cycles and then the lysates were subjected to indicated temperatures for 3 minutes. Lysates were centrifuged, and supernatants were resolved by SDS-PAGE and analysed western blot (see below).

Western blot analysis

As per established protocols (143,224), cells were lysed at 4°C in RIPA buffer (1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 1 mM sodium pyrophosphate in PBS) mixed with protease and phosphatase inhibitors (Roche). Equal amounts of protein were boiled, resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were incubated in blocking buffer (5% skim milk in 0.05% TBS-T) for 1 h at room temperature and probed overnight in primary antibody at 4°C. Blots were washed thrice in 0.05% TBS-T, followed by incubation with peroxidase-conjugated secondary antibody (Dako) for 1 h at room temperature. Protein levels were detected using Amersham ECL Western Blotting Detection reagents (GE Life Sciences) or ECL Plus Western blotting substrate kit (ThermoFisher Scientific). Antibodies are detailed in **Table S3.1**.

Quantitative RT-PCR

As per established protocols (143,224), following RNA extraction with NucleoSpin RNA kit (Macherey-Nagel), reverse transcription reaction with Transcriptor First Strand cDNA Synthesis kit (Roche) and SYBR-green qPCR Lightcycler 480 (Roche), gene expression was normalised to GAPDH and ACTB and determined using the $\Delta\Delta C_t$ method. Primer sequences are detailed in **Table S3.2**.

Chromatin immunoprecipitation

1.25×10^6 PonA-inducible wt-p53 H1299 cells were seeded in 15 cm plates and wt-p53 was induced for 48 h before proceeding to chromatin immunoprecipitation (ChIP). As per established protocols (143), protein-DNA conjugates were cross-linked by the addition of methanol-free 1% formaldehyde (Life Technologies) for 10 min. The cross-linking reaction was quenched by the addition of glycine to a final concentration of 0.05 M for 5 min. Cells were collected in ice-cold PBS and sequentially lysed in buffer 1 (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% TritonX-100), buffer 2 (10 mM Tris pH 8, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA) and buffer 3 (10 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, 0.5% N-lauroylsarcosine) with protease inhibitors to enrich for the nuclear fraction. Lysates were sonicated for 35 min (S220 Focused-ultrasonicator, Covaris), followed by precipitation of cellular debris with 10% TritonX-100. An aliquot of lysate was removed for input sample prior to immunoprecipitation. Protein A-Sepharose beads (Invitrogen) were pre-blocked with BSA and sonicated salmon testes DNA. Immunoprecipitations were performed with p53 antibody-agarose conjugate (Santa Cruz Biotechnology, clone sc-6243, Dilution: 1:20) or Protein A-Sepharose only at 4°C overnight. Beads were washed five times with LiCl buffer (50 mM HEPES pH 7.5, 500 mM LiCl, 1 mM EDTA, 50 mM NaCl). Protein-DNA conjugates were eluted by incubation in elution buffer (50 mM Tris pH 8, 10 mM EDTA, 1% SDS) at 65°C for 15 min. Reverse cross-linking was performed by the addition of 0.2 mg ml⁻¹ RNaseA (Qiagen) and 0.2 mg ml⁻¹ Proteinase K (Promega). Samples were analysed by qPCR with primers specific for the promoters of *SLC7A11* and *CDKN1A*. Primers are detailed in **Table S3.2**.

Radioactive cystine uptake assay

Following c-Myc knockdown, cells were washed twice in pre-warmed cystine-free DMEM (high glucose, no glutamine, no methionine, no cystine) to deplete cystine. At this point, in each well the cystine-free-media was replaced with 300µL uptake buffer containing 0.04 µCi of L-[1, 2, 1', 2'-¹⁴C]-cystine (PerkinElmer) for 5 minutes at room temperature. Cells were then washed thrice with ice-cold cystine-free media and lysed in 400 µL 0.2M NaOH with 1% SDS. This lysate was added to 4mL of scintillation fluid, and radioactive counts per minute were obtained using a scintillation counter. To control for differences in the absolute counts of radioactivity between replicates, data were normalised to non-targeting control within each replicate.

Intracellular glutathione assay

Total intracellular GSH level was assayed using the Cayman Chemicals Glutathione kit as per the manufacturer's instructions. GSH concentration was calculated from an internal standard curve and normalised to total cell number as determined from parallel plates.

Statistical analysis

Data were presented as mean $\pm$ SEM and analysed by Student's *t*-test unless otherwise indicated. GI50 dose of single agents was determined by fitting the Hill equation. Statistical analyses and data presentation were performed using Prism 8 (GraphPad). No power analyses were performed to determine sample size prior to experimentation. No blinding was undertaken during experiments. For correlation analysis, the cancer cell line compound activity profiles, binary mutational calls, gene

expression, proteomics and metabolomics data were obtained from Cancer Dependency Map (<https://www.depmap.org>) to determine potential associations. Pearson correlations between compound AUC and gene expression were calculated using *cor.test* function in R (Version 3.6.0) for ~800 cancer cell lines and 481 compounds. Correlation strengths were determined by adjusting the Pearson correlations with a Fisher's z-transformation in order to account for the differences in cell lines analysed.

Results

Clinical investigation of eprenetapopt in advanced and metastatic oesophageal and gastro-oesophageal junction cancer

The translation of our laboratory's previous findings, demonstrating efficacy of eprenetapopt in combination with chemotherapy in pre-clinical models of oesophageal cancer (224), was launched as an investigator-led phase Ib/II clinical trial (APROC, NCT02999893) to investigate the safety and efficacy of eprenetapopt in combination with cisplatin and 5-fluorouracil (5-FU), in advanced and metastatic oesophageal and gastro-oesophageal junction cancer (286). Five patients completed enrolment for the phase I trial, before its suspension (due to funding cessation). All patients received Level 1 dose regimen. Given the difficulties inherent in accessing patient tumour materials directly in the metastatic setting, analysis of efficacy and biomarker investigations were undertaken using radiographic imaging and circulating tumour DNA (ctDNA) (**Figure 3.1A**). Somatic mutations were detected in four out of five patients by ctDNA at baseline, using a targeted amplicon panel of nine genes that are commonly mutated in oesophageal cancer (283). Of importance to eprenetapopt, *TP53* mutations were detected in 3 patients, whilst *ARID1A* mutations were identified in two patients. Intriguingly, compared to those patients with detectable *TP53* and/or *ARID1A* mutations who progressed within the first 2 cycles, patient PMC002 showed stable disease over 5 trial cycles, yet did not have any detectable *TP53* or *ARID1A* mutations by ctDNA analysis. PMC002's tumour burden, as determined by serial computed tomography (CT) scans of target metastatic lesions, was stable – exemplified by their posterior lower paraoesophageal node (**Figure 3.1B,C**). Meanwhile, another patient (SUN002), with both detected *TP53* and *ARID1A* mutations, progressed on trial after only two cycles, as evident by both a rise in ctDNA

and in their CT scans (**Figure 3.1D,E**). Although the number of patients is small, these clinical results emphasise the need to establish a sensitive and robust biomarker for eprenetapopt response, other than *TP53* mutation status.

Performance of eprenetapopt in the Cancer Therapeutics Response Portal v2

In order to identify a robust biomarker for response to eprenetapopt, we interrogated the pan-cancer therapeutic response and cancer cell line omics datasets from the Broad Institute's DepMap portal (www.depmap.org). Both eprenetapopt and APR-017 were included in the Broad Institute's Cancer Target Response Portal version 2 (CTRPv2) small-molecule sensitivity profiling effort (276,277). However, only APR-017 was profiled at a sufficient concentration to ensure a lethal dose was achieved across a majority of cancer cell lines (CCLs) (**Figure 3.2A**). As a result, APR-017 and eprenetapopt showed only a moderate similarity in their sensitivity profiles based on area-under-the-curve (AUC) analysis, however single dose effects, at similar concentrations, highlighted remarkable concordance between sensitivity to eprenetapopt and APR-017 (**Figure 3.2B**). Therefore, APR-017 sensitivity profile was utilised for further analyses.

CCL response data to APR-017 across 24 tissue lineages highlighted higher sensitivity amongst haematopoietic and lymphoid malignancies (multiple myeloma, lymphoma and leukaemia) compared to solid malignancies (**Figure 3.2C,D**). In keeping with previous studies (277,287), the 481 compounds in CTRPv2 were generally more efficacious in haematopoietic and lymphoid (HL) cancer cell lines compared with those from solid cancers (**Figure 3.2C-bottom**). We also confirmed that eprenetapopt has increased efficacy in representative leukaemia cell lines

compared to oesophageal CCLs (**Figure 3.2E**). Of note, oesophageal CCLs were the most resistant to APR-017 in this analysis, meanwhile peripheral nervous system (PNS) CCLs were the most sensitive.

Next, analysis of the binary mutational association with APR-017 sensitivity across all CCLs was performed (**Figure 3.2F**). *TP53* or *ARID1A* mutation status were not predictive of response to APR-017, whilst *KEAP1* mutation was associated with resistance (**Figure 3.2F,G**). KEAP1 is an E3 ubiquitin-ligase known to control the expression of the master transcriptional regulator of anti-oxidant response, NRF2 (encoded by *NFE2L2*) (288,289). Importantly, *TP53* mutation status alone is not predictive of favourable response across solid, HL, lung or breast cancer cell lineages, even if subset into missense or nonsense *TP53* mutations compared to no mutation detected (**Figure S3.1A**). Additionally, use of a recently published *TP53* mutation signature (290) based on cancer cell gene expression profiles showed no correlation with APR-017 activity (**Figure 3.2G**). Finally, whilst we previously demonstrated that mut-p53 protein accumulation correlated with increased sensitivity to Eprenetapopt in oesophageal CCLs (143,224), no comparable correlation was evident in a pan-cancer CCL analysis, based on p53 protein expression as determined by quantitative proteomics (**Figure S3.1B**) (291).

Correlation of APR-017 activity to the other 480 compounds in the CTRPv2, revealed that PX-12, a thioredoxin inhibitor (292), shares a similar activity profile with APR-017 among CCLs (**Figure 3.2H, Figure S3.2A**). Interestingly, whilst overexpression of mut-p53 (R273H) led to an increase in sensitivity to eprenetapopt in p53-null H1299, as we have shown previously (224), there was no change in sensitivity to PX-12

(**Figure S3.2B**). Given that eprenetapopt has been previously demonstrated to thermostabilise mut-p53 through direct MQ conjugation (239), we assessed the effect of eprenetapopt and PX-12 incubation on mut-p53 thermostability in H1299 cells through cellular thermal shift assay (285). Our results show that PX-12 maintained mut-p53 thermostability to a greater extent than eprenetapopt at comparable doses in terms of growth inhibitory activity (**Figure 3.2I, Figure S3.2C**). In conclusion, our data confirm that *TP53* mutation status alone is insufficient to determine sensitivity to eprenetapopt across multiple lineages of CCLs.

SLC7A11 is the major determinant of eprenetapopt sensitivity

To look beyond association with *TP53* mutation status, we performed a range of analyses correlating CCL response to APR-017 with cell line omics features, including gene transcript levels, protein expression and metabolite abundance (291,293,294). To avoid potential confounding effects of increased sensitivity to APR-017 in HL lineages, solid and HL CCL lineages were separated for these analyses. SLC7A11 mRNA and protein and reduced GSH were the strongest predictive biomarkers for response to APR-017 in solid CCLs (**Figure 3.3A**). A consistent pattern was also found across analyses of HL, lung and breast lineages for GSH and SLC7A11 mRNA, while SLC7A11 protein was less pronounced, likely due to the smaller number of CCLs available in the proteomics dataset (**Figure S3.3A**). SLC7A11, expression of which is regulated predominantly by NRF2 (295), is the key functional subunit of the system x_c^- antiporter, which imports extracellular cystine in exchange for intracellular glutamate (247). The import of cystine through system x_c^- provides the predominant source of intracellular cysteine, which is the rate limiting substrate required for *de novo* GSH synthesis (246). Furthermore, mRNA and protein expression of SLC7A11 is

consistently higher in solid CCLs compared to HL CCLs (**Figure 3.3B**), likely driven by higher levels of NRF2 mRNA (**Figure S3.3B**). These results are also consistent with the finding that functional deactivation of KEAP1, the negative regulator of NRF2, through its mutation is predictive of resistance to APR-017 in CCLs (**Figure 3.2F**). There are also significant correlations between *SLC7A11* expression and APR-017 activity in both solid and HL CCLs (**Figure 3.3C**). Furthermore, *TP53* mutation status does not influence this correlation between *SLC7A11* expression and APR-017 activity (**Figure S3.3C**).

We previously demonstrated that *SLC7A11* levels correlate with sensitivity to eprenetapopt in oesophageal cancer models through the capacity of mut-p53 to bind and inhibit NRF2 transcriptional activation of *SLC7A11* (143). Broadening these findings, eprenetapopt resistance is induced by the ectopic overexpression of *SLC7A11* in H1299 p53^{Null} *KEAP1* wild-type cells (**Figure 3.3D**). In contrast, acute knockdown of *SLC7A11* prior to eprenetapopt treatment, increased sensitivity to eprenetapopt. Altogether, these results illustrate that *SLC7A11* is the major determinant of eprenetapopt sensitivity across multiple cancer lineages with varying *TP53* mutational status.

Extending the clinical validity of these findings, we analysed patient tumour expression levels of *SLC7A11* across all tumour lineages in The Cancer Genomic Atlas (TCGA) (**Figure 3.3E**) and found that the *SLC7A11* expression across the majority of tumour lineages was generally consistent with the cell line lineage sensitivity to APR-017 (**Figure 3.2C**). For example, leukaemia samples express low levels of *SLC7A11*,

whilst oesophageal tumours have high expression – reflecting sensitivity to eprenetapopt corresponds with low SLC7A11 levels across these lineages.

siRNA screen reveals modulators of SLC7A11 as key genetic regulators of eprenetapopt sensitivity

To complement our biomarker investigation, we performed a boutique siRNA synergy screen with a library targeting 853 genes involved in redox metabolism and/or NRF2 target genes (**Figure 3.4A**). In order to account for mut-p53-dependent and independent effects of eprenetapopt and redox perturbation synergy, parallel screens were performed in isogenic p53-null and mut-p53 overexpression cancer cells. To this point, there was significant concordance between the two isogenic cell lines across two independent siRNA screen runs (**Figure 3.4B**). Consistently, *SLC7A11*, as well as *SLC3A2* (another component of system x_c^-), were amongst the top gene knockdowns synergistic with eprenetapopt in both cell lines (**Figure 3.4B**). Interestingly, *MDM2* and *ATF4* gene ablation were also synergistic with eprenetapopt, independent of mut-p53 expression (**Figure 3.4B,C**). MDM2 is the major negative regulator of p53 protein expression – acting as an E3 ligase that targets p53 for proteasome-mediated degradation (54), however it also has p53-independent activities. Previous efforts investigating its p53-independent functions revealed that MDM2, together with ATF4, directly regulate cellular redox status through transcriptional regulation of *SLC7A11* (296). In keeping with this model, siRNA ablation of *ATF4* or *MDM2* decreased the expression of *SLC7A11* (**Figure 3.4D**), likely explaining the synergy with eprenetapopt. Importantly, these findings, further demonstrate the importance of SLC7A11 in determining eprenetapopt sensitivity and highlight that multiple mechanisms can modulate SLC7A11 in cancer cells.

Wild-type p53 regulation of SLC7A11 is indirect and dictates sensitivity to eprenetapopt

Given that the canonical negative regulator of p53, MDM2 positively regulates SLC7A11, we next focused on examining the relationship between wt-p53 and SLC7A11 in relation to eprenetapopt sensitivity. Using p53-null H1299 cells engineered with a ponasterone A (PonA) inducible wt-p53 construct, we characterised the effects of p53 on SLC7A11 expression. Consistent with previous studies (88,104), ectopic overexpression of wt-p53 resulted in decreased SLC7A11 mRNA and protein levels (**Figure 3.5A, Figure S3.4A**). Functionally, this resulted in decreased total GSH levels and increased sensitivity to eprenetapopt (**Figure 3.5B,C**). Interestingly, whilst no effect was observed on ATF4 protein levels, wt-p53 overexpression led to stabilisation of NRF2 protein and upregulation of other NRF2 target genes, *HMOX1* and *KEAP1* (**Figure S3.4A,B**). Furthermore, as expected, MDM2 protein level increased following p53 induction – likely indicating that wt-p53 inhibition of SLC7A11 is either likely to be oscillatory, or a balanced outcome between the negative impacts of p53 and the positive impacts of MDM2 (**Figure 3.5A**).

Previous studies indicated that wt-p53 regulation of SLC7A11 was context dependent, where loss or suppression of wt-p53 also resulted in diminished SLC7A11 expression (108,109). To this point, we found that p53 knockdown decreased SLC7A11 protein in two CCLs with endogenous wt-p53 expression, HCT116 and U2OS (**Figure 3.5D**). *SLC7A11* mRNA was decreased following p53 ablation, whilst other NRF2 targets remained unchanged (**Figure S3.4C**). This resulted in increased sensitivity to eprenetapopt, however this response was more pronounced in the HCT116 cells

(**Figure 3.5E**). Interestingly, whilst p53 ablation resulted in diminished SLC7A11 expression in both cell lines, *MDM2* mRNA levels only decreased in the U2OS cells and remained unchanged in the HCT116 cells (**Figure S3.4C**). We further tested whether MDM2 inhibition with known MDM2 antagonist, Nutlin-3a, in wt-p53 cells led to SLC7A11 suppression. Nutlin-3a treatment led to p53 protein stabilisation, however no change in SLC7A11 protein levels up to 24 hours of exposure in both HCT116 and U2OS cells (**Figure S3.4D**). Combination of eprenetapopt with Nutlin-3a produced an additive effect on cell viability across the doses tested (**Figure S3.4E**).

Probing deeper into the mechanism underlying wt-p53 regulation of SLC7A11, in contrast to previous studies (88,104), chromatin-immunoprecipitation (ChIP) studies in our hands showed no enrichment of p53 at the *SLC7A11* promoter, but was enriched at the promoter of the well-established target gene, p21 (encoded by *CDKN1A*) (**Figure 3.5F**). This finding was supported by analysis of 26 publicly available genome-wide p53 chromatin binding profiles that found no p53 occupancy at the *SLC7A11* promoter (www.targetgenereg.org (297)). We also investigated other possible mechanisms of *SLC7A11* suppression relating to the p53 transcriptional network. A comprehensive meta-analysis of the p53 transcriptional program suggested that p53-dependent gene repression relies on the p21-DREAM axis (72). However, siRNA-mediated ablation of p21 (*CDKN1A*) did not restore *SLC7A11* mRNA expression following p53 induction (**Figure 3.5G**). Collectively, these results highlight that wt-p53 regulation of *SLC7A11* is most likely indirect and cell context dependent, however by altering wt-p53 levels and thus causing suppression of SLC7A11, this results in increased sensitivity to eprenetapopt.

Finally, analysis of the *SLC7A11* promoter region and previously published c-Myc ChIP-seq data revealed a putative c-Myc binding site and c-Myc occupancy at the *SLC7A11* promoter (**Figure S3.5A**). Furthermore, knockdown of c-Myc in HCT116 and U2OS cells resulted in ablation of SLC7A11 protein expression and concomitantly diminished cystine uptake (**Figure S3.5B,C**), although this did not decrease the total basal levels of intracellular GSH (**Figure S3.5D**). Nevertheless, knockdown of c-Myc resulted in marked increased sensitivity to eprenetapopt in HCT116 and U2OS (**Figure S3.5E**). This suggests that capacity for cystine uptake through SLC7A11 may be the important predictor of eprenetapopt sensitivity and not necessarily intracellular basal GSH levels *per se*. This could indicate that cellular capacity to regenerate GSH through *de novo* synthesis pathways may be inhibited by eprenetapopt, which would be further stressed by lowered capacity to uptake cystine when SLC7A11 levels are diminished (**Figure 3.5H**). These results highlight that depleting SLC7A11 through silencing c-Myc leads to increased sensitivity to eprenetapopt in wt-p53 cancer cell lines. It also further demonstrates that SLC7A11 expression is a consistent determinant of sensitivity to eprenetapopt – independent of *TP53* mutation.

Discussion

Evaluation of eprenetapopt is currently underway in a phase III clinical trial in *TP53*-mutated MDS in combination with azacitidine (259), with preliminary phase II results reporting strong efficacy (298,299). While previous reports have defined *TP53* mutation status as the presumptive clinical biomarker for eprenetapopt patient selection (224), we propose that the major predictor for response to eprenetapopt is expression levels of the cystine-glutamate antiporter, SLC7A11: where low levels correlate with greater sensitivity. This is in agreement with the active drug formed from eprenetapopt being the thiol-reactive compound MQ, and GSH being the first line of defence against this type of reactive compound, but also a functionally important direct therapeutic target in cancer cells with aberrant redox homeostasis. This is furthermore supported by our results showing that by altering the level of expression of four key proteins involved in cancer biology, MDM2, ATF4, wt-p53 and c-Myc, we were able to diminish SLC7A11 levels and therefore demonstrate increased sensitivity to eprenetapopt in p53-null/wt-p53 CCLs (**Figure 3.5H**). Furthermore, whilst loss of ARID1A (249,281) and mut-p53 protein accumulation (143) have been shown to suppress SLC7A11 through NRF2 regulation, only *KEAP1* mutation was found to be significantly predictive of resistance to eprenetapopt across CCLs. This is consistent with functional deactivation of KEAP1 through its mutation, to in turn relieve its suppression of NRF2 and drive *SL7A11* expression. The role of MDM2 in modulating SLC7A11 levels and eprenetapopt sensitivity also recontextualise the synergy seen between eprenetapopt and MDM2 agonists, which was previously to be explained as mut-p53-dependent (229). Instead, our data hints that eprenetapopt and MDM2 inhibition should be explored in wt-p53 cancers with MDM2 amplification, as MDM2 inhibition here may both trigger p53-mediated SLC7A11 downregulation and

simultaneous loss of the MDM2-ATF4-SLC7A11 axis (296). Equally, using of the demonstration of synergy with MDM2 inhibitors as further evidence for mut-p53 reactivation (174) by eprenetapopt or other mut-p53 targeted therapy should be cautioned. As a result, our study critically demonstrates the potential for broadening the clinical investigation of eprenetapopt therapy beyond *TP53* mutant malignancies – in both solid and haematological settings.

On the point of the proposed mut-p53 reactivation by eprenetapopt, notably, Cys277 on p53 was recently identified to be a key residue in the DNA binding domain of mut-p53 for MQ docking to drive thermostabilisation (239). However, concurrent with and in previous studies (143,224), we have shown that the oesophageal CCL FLO-1, which expresses mut-p53^{C277F}, is sensitive to eprenetapopt. In our investigation into the capacity of eprenetapopt and PX-12 to induce mut-p53 thermostability in cells, eprenetapopt did not thermostabilise mut-p53 to a greater degree than PX-12 (**Figure 3.2I**). Additionally, it should be noted that other compounds proposed to be mut-p53 reactivators, including CP-31398, MIRA-1 and STIMA-1, are also Michael acceptors that have demonstrated effects on cellular redox balance (174). As such, it could be hypothesised that mut-p53 thermostabilisation could be a by-product of the cellular response to redox disruption. Therefore, the similarity between eprenetapopt and PX-12 activity in CCLs could be explained by the capacity of these compounds to perturb cellular redox balance: PX-12 through thioredoxin inhibition (292), and eprenetapopt through direct conjugation via MQ to GSH as we and others have previously demonstrated (143,226,300) (**Figure 3.5H**). Furthermore, previous investigations have also shown that eprenetapopt inhibits the thioredoxin anti-oxidant system pathways (214,251) and that inhibition of this enzymatic activity can lead to ER stress

(242) and downstream p53-independent apoptosis (222). However, more research should be performed to examine the MoA of eprenetapopt in light of the growing evidence of the importance of these p53-independent effects.

Pan-cancer lineage analysis highlighted PNS tumours as a lineage of interest for eprenetapopt, as they express low levels of *SLC7A11* and are highly sensitive to APR-017 (**Figure 3.2C, 3.3E**). This is supported by pre-clinical investigations of eprenetapopt in neuroblastoma models, which demonstrated caspase-independent, p53-independent cell death through GSH depletion (280). Given that neuroblastoma is a heterogeneous childhood tumour, with limited therapeutically targetable genomic alterations and poor prognosis, more work investigating the clinical utility of eprenetapopt in neuroblastoma should be undertaken.

On the strength of our new findings of the value of *SLC7A11* as a prognostic marker for eprenetapopt efficacy, validation is now warranted in patient samples from clinical trials of this drug. Determining *SLC7A11* expression in clinical samples by immunohistochemistry (301), or *in situ* tumour system x_c^- activity by PET imaging using (4S)-4-(3-[^{18}F]Fluoropropyl)-L-glutamate (^{18}F -FSPG) (302,303), is achievable and rapidly translatable into the clinic. Given our evidence suggests that *TP53* mutation status is not adequate to comprehensively predict eprenetapopt responses across a breadth of cancers, clinical investigation and utility of eprenetapopt should expand beyond *TP53* mutation status as the sole genetic inclusion criteria.

Acknowledgements

We thank Aprea Therapeutics for providing the eprenetapopt used in this study. Thanks to Lars Abrahmsen from Aprea Therapeutics for critical discussion of the manuscript. The diagrams in Figure 4 and 5 were created using www.Biorender.com.

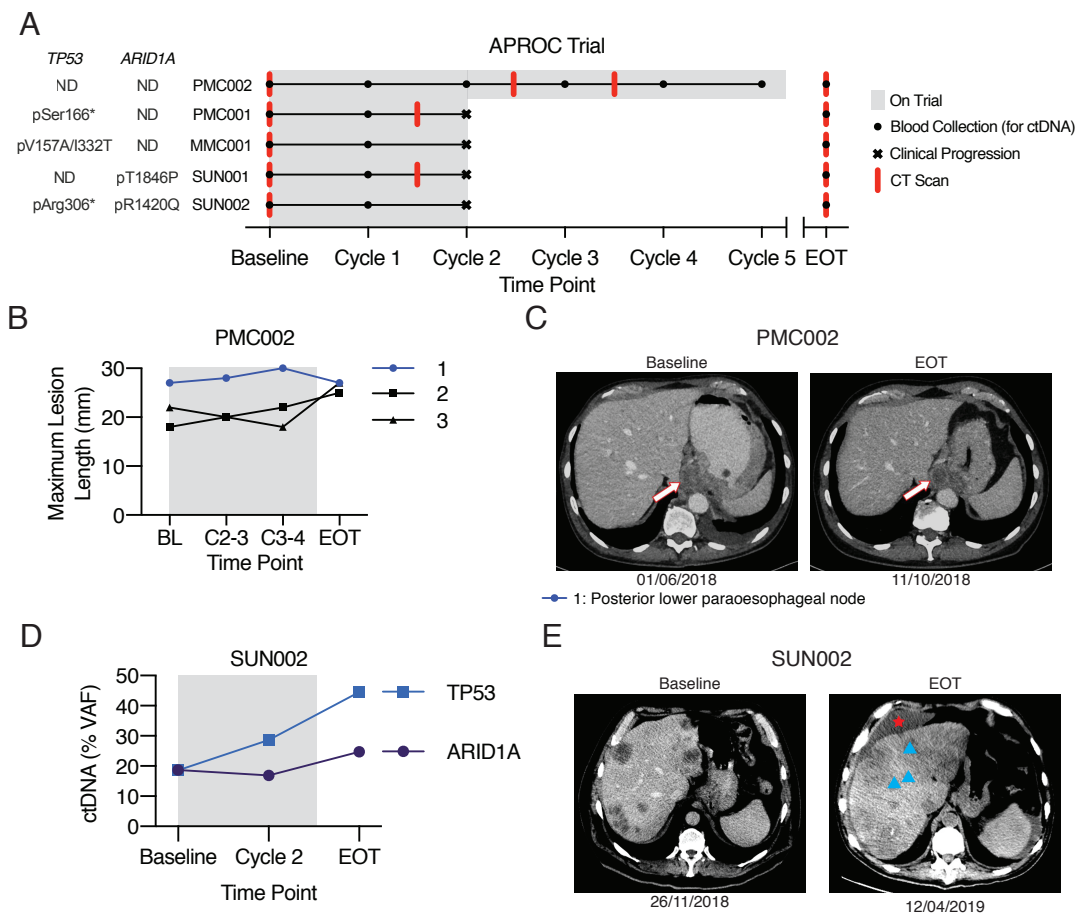


Figure 3.1 Clinical investigation of eprenetapopt in advanced and metastatic oesophageal and gastro-oesophageal junction cancer

(A) Schematic representing the patients recruited into the APR-246 (eprenetapopt) in Oesophageal Cancer (APROC) Phase Ib/II trial indicating number of cycles of eprenetapopt received in combination with chemotherapy (5-FU and cisplatin), when computer tomography (CT) scans and blood was taken for circulating tumour DNA (ctDNA) to track progression, and indicates the presence of *TP53* and *ARID1A* mutations as detected by ctDNA targeted amplicon sequencing (TAMseq). (B) Maximum dimension of patient target metastatic lesions (by RECIST 1.1) measured by CT scan timepoints and (C) representative CT scan images at indicated timepoints in patient PMC002. Arrow indicates nodal tumour (1: Posterior lower paraoesophageal node, 2: left lateral supraclavicular node, 3: Right level V lower cervical node). (D) Levels of ctDNA (variant allele frequency, VAF) and (E) CT scans at indicated timepoints in patient SUN002. Blue triangles indicate new metastatic lesions, star indicates liver ascites. Data in (B) represents mean of two technical replicates.

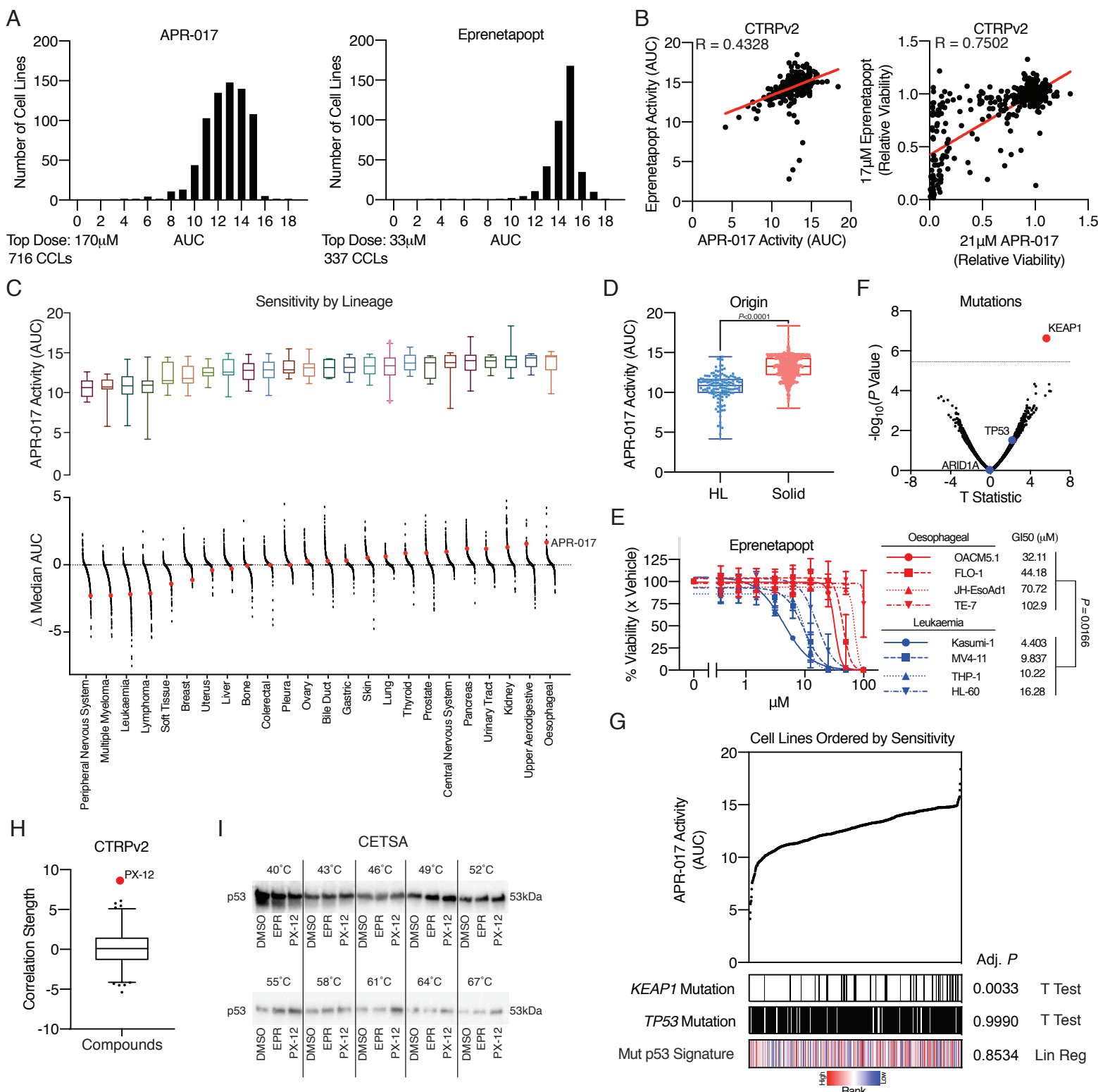


Figure 3.2 Performance of eprenetapopt and APR-017 in the Cancer Therapeutics Response Portal v2

(A) Histograms of APR-017 and eprenetapopt activity in cancer cell lines in the Cancer Therapeutics Response Portal v2 (CTRPv2) as measured by area-under-the-curve (AUC) of dose response curves. **(B)** Scatterplots of APR-017 and eprenetapopt AUC (left) and relative viability at comparable doses (right) in cancer cell lines. **(C)** Box-and-whisker plots (1st-99th percentile) of lineage specific sensitivity to APR-017 ordered by

median sensitivity (top), and relative activity of individual compounds by lineage – indicating APR-017 in red (bottom). Points represent the 481 compounds included in the CTPRv2. Points above the dotted line indicate preferentially inactive compounds in a specific lineage, whilst points below the dotted line are preferentially active. **(D)** Boxplots (min-max) comparing sensitivity to APR-017 in haematopoietic and lymphoma (HL) and solid tumour derived cell lines. **(E)** Cell viability following 72 h exposure with eprenetapopt at indicated doses in leukaemia (blue) and oesophageal (red) cancer cell lines. **(F)** Volcano plot comparing the effect of mutation status on APR-017 sensitivity across all cancer cell lines. Dotted line indicates Benjamin-Hochberg cut off for false discovery. *KEAP1* mutation (red) is significantly associated with resistance to APR-017, *TP53* and *ARID1A* mutation (blue) are not significantly associated with sensitivity or resistance to APR-017. **(G)** APR-017 activity and top correlated cancer cell line mutation status (indicated by black lines for mutations), *KEAP1*, as well as *TP53* mutation and mut-p53 transcriptional signature (see methods for details). **(H)** Box-and-whisker plot (1st-99th percentile) of Fischer's transformed z-scored Pearson correlation strength of APR-017 AUC and AUC of the other 480 CTRPv2 compounds, indicating PX-12 as the most significant outlier in red. **(I)** Cellular thermal shift assay (CETSA) performed in H1299 cells overexpressing mut-p53 (R273H). Cells were treated for 2 h with DMSO, pre-heated 50 μ M eprenetapopt or 25 μ M PX-12 before harvest, freeze-thaw and subject to increasing temperatures. Pearson's correlation **(B, G – Mut p53 Signature)**, two-tailed unpaired t-test **(D, E, F, G)**. Error bars = SEM. **(E)** *N*=2-3, **(I)** *N*=3, representative blot. See also *Supplementary Fig. 1 and 2*.

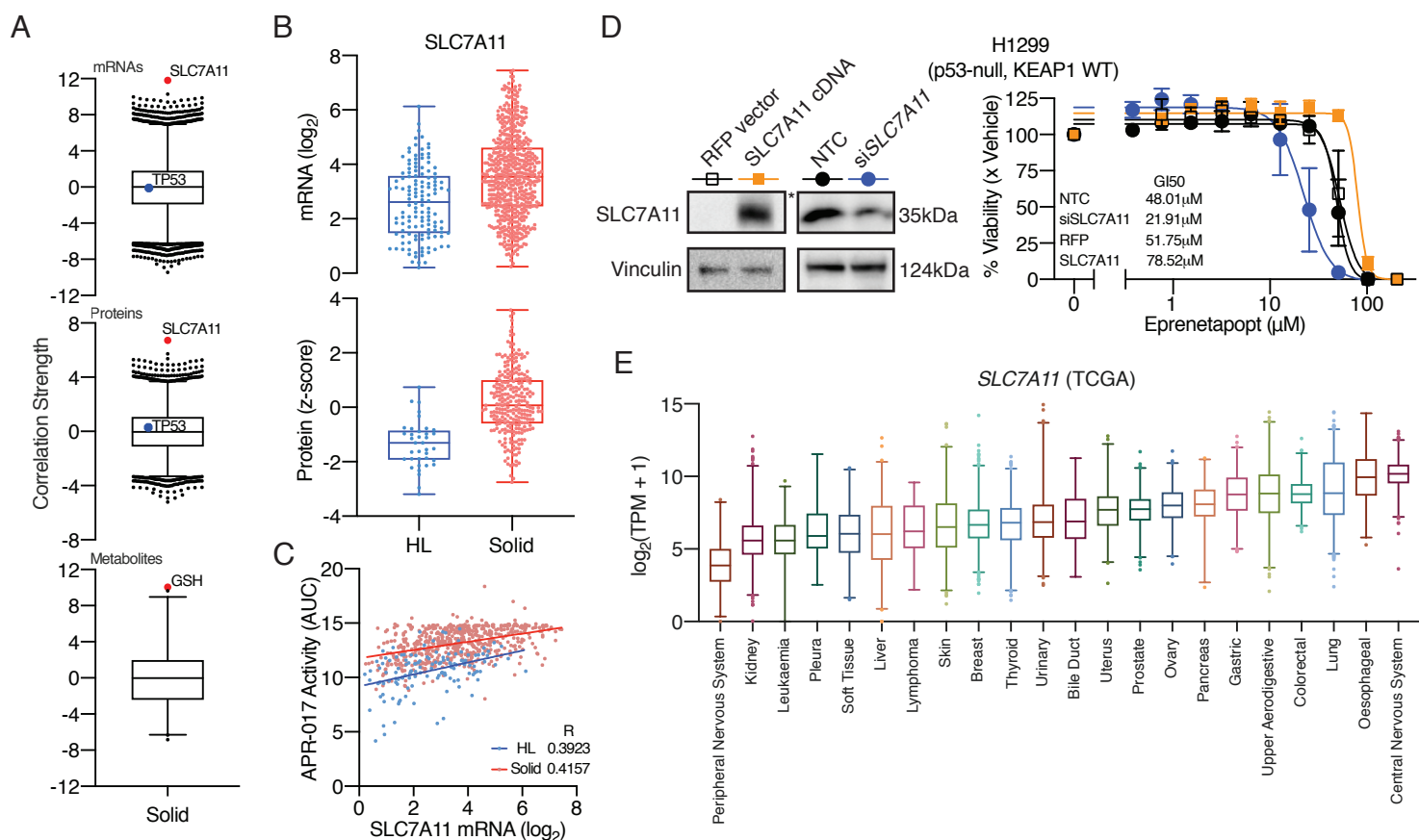


Figure 3.3 SLC7A11 expression is the major determinant of eprenetapopt sensitivity

(A) Box-and-whisker plots (1st-99th percentile) of Fischer's transformed z-scored Pearson correlation strengths of APR-017 AUC and gene transcripts (top), proteins (middle) and metabolites (bottom) in solid cancer cell lines. Red dots indicate SLC7A11 mRNA and protein, and glutathione (GSH) correlation strengths. Blue dots indicate TP53 mRNA and protein correlations. **(B)** Boxplots (min-max) comparing SLC7A11 mRNA (top) and protein (bottom) levels in HL and solid cancer cell lines. **(C)** Scatterplot correlating the expression of SLC7A11 with APR-017 AUC in HL (blue) and solid (red) cancer cell lines. **(D)** SLC7A11 and red fluorescent protein (RFP) stable overexpression or transient SLC7A11 knockdown in H1299 cells confirmed by western blot (left). * indicates short exposure time for SLC7A11 overexpression blot. Eprenetapopt was applied 24 h post transfection of SLC7A11 and non-targeted control (NTC) siRNA or plating of overexpression cells with viability measured at 72 h post addition of eprenetapopt (right). **(E)** Box-and-whisker plots (1st-99th percentile) of normalised expression of SLC7A11 across 23 tumour types by lineage in publicly available TCGA datasets. Pearson's correlation **(C)**. Error bars = SEM. **(D – western blot)** N=1. **(D – dose responses)** N=3. See also Supplementary Fig. 3.

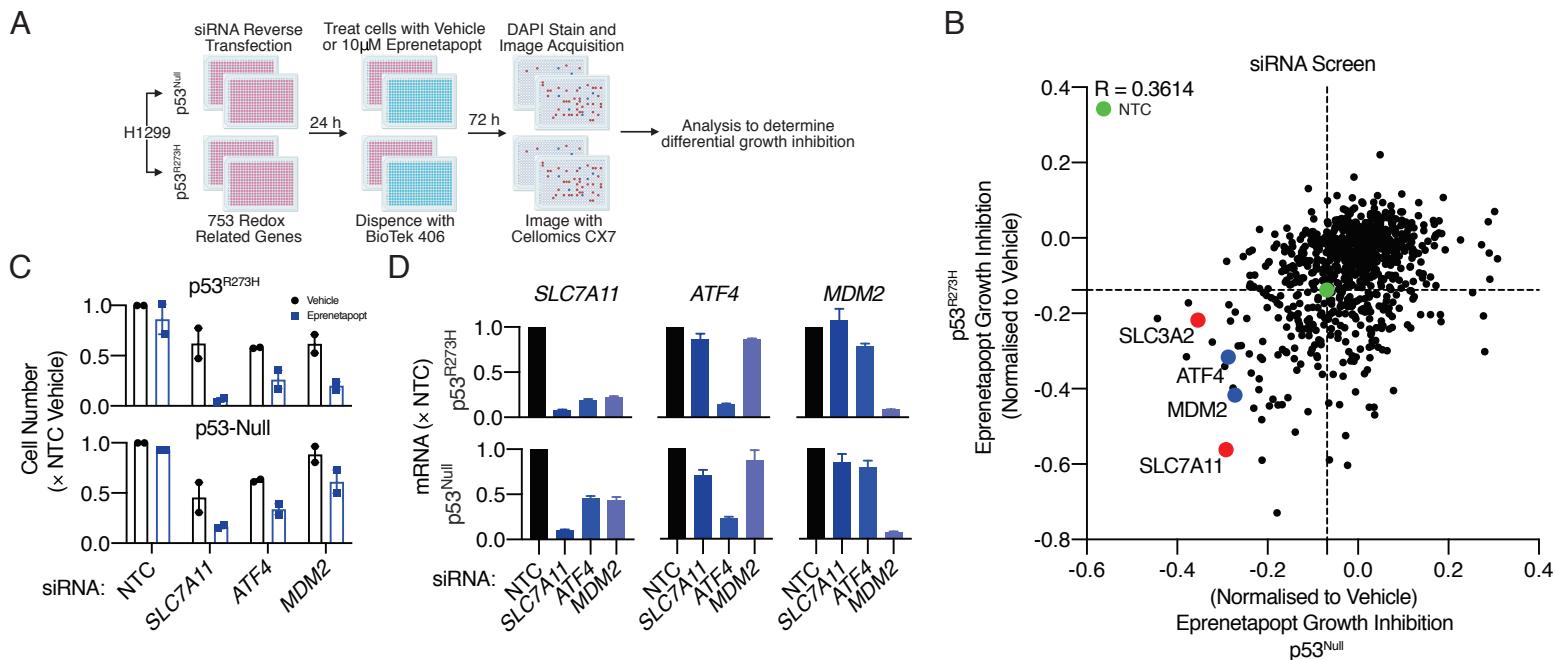


Figure 3.4 siRNA screen reveals modulators of SLC7A11 expression as key genetic regulators of eprenetapopt sensitivity

(A) Schematic representation of the boutique siRNA screen performed in H1299 parental cells (p53^{Null}) and cells overexpressing mut-p53 hotspot R273H (p53^{R273H}) (see methods for details). **(B)** Scatterplot indicating eprenetapopt-mediated growth inhibition following siRNA transfection. Dotted line indicates eprenetapopt (10 μ M) growth inhibition in cells with non-targeting control (NTC). **(C)** Relative cell number changes following 24 h siRNA-mediated knockdown of *SLC7A11*, *ATF4* and *MDM2* and 72 h treatment with 10 μ M eprenetapopt from the two independent siRNA screen runs. **(D)** Changes in *SLC7A11*, *ATF4* and *MDM2* expression by qPCR following 24 h knockdown. Pearson's correlation **(B)**. Error bars = SEM. **(B, C)** $N=2$. **(D)** $N=3$.

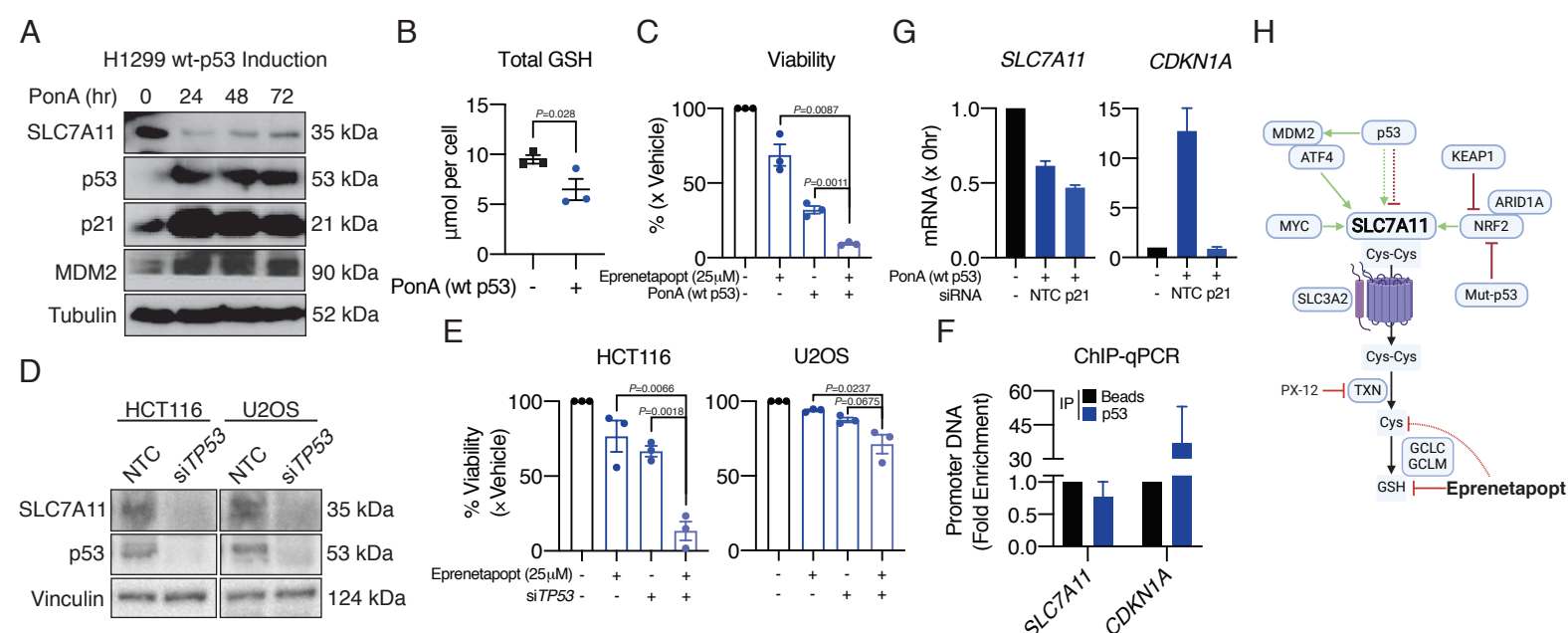


Figure 3.5 Wild type p53 indirectly regulates SLC7A11 expression and alters sensitivity to eprenetapopt

(A) Western blot of p53, SLC7A11 and p21 protein levels in H1299 cells with Ponasterone A (PonA) inducible wild type p53 (wt-p53) following treatment with 2.5 μg/mL PonA for indicated timepoints. **(B)** Total GSH levels following 48 h PonA treatment. **(C)** Viability following 48 h pre-treatment with PonA and subsequent treatment with 25 μM eprenetapopt or vehicle. **(D)** p53 and SLC7A11 protein levels following 48 h knockdown with *TP53* siRNA or non-targeting control (NTC) in wt-p53 HCT116 and U2OS cancer cell lines. **(E)** Viability following 24 h *TP53* knockdown and subsequent treatment with 25 μM eprenetapopt or vehicle in wt-p53 HCT116 and U2OS cancer cell lines. **(F)** Chromatin immunoprecipitation (ChIP) of the *SLC7A11* and *CDKN1A* promoters using p53 antibody or agarose control beads following 48 h treatment in H1299 wt-p53 cells. **(G)** *SLC7A11* and *CDKN1A* (p21) mRNA levels following 48 h knockdown with *CDKN1A* or NTC siRNA and co-induction of wt-p53 with PonA in H1299 cells. **(H)** Schematic representation summarizing the regulation of SLC7A11 in cancer cells and downstream control of sensitivity to eprenetapopt. Dotted arrows indicate indirect regulation or targeting. One-tailed unpaired t-test (B, G). Two-tailed t-test (C, E, F). Error bars = SEM. (A, D) N=1. (B, C, E, F, G) N=3. See also Supplementary Fig. 4 and 5.

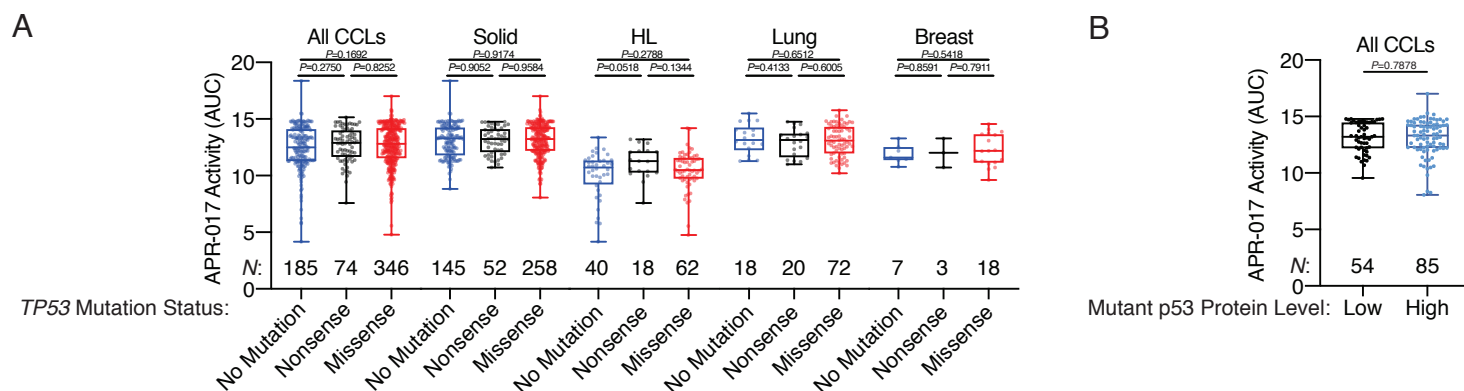


Figure S3.1 *TP53* mutation alone is not predictive of cancer cell response to APR-017

(A) Boxplots (min-max) of APR-017 AUC across the Cancer Therapeutics Response Portal v2 (CTRPv2) stratified by *TP53* mutation status in all cancer cell lines (CCLs), solid, haematopoietic and lymphoid (HL), lung and breast cancer cell lines. Lung and breast cancer line lines were analysed due to their high number of cell lines available. CCLs with frameshift and splice site mutations were excluded from these analyses.

(B) Boxplot of APR-017 AUC in all *TP53* mutant CCLs comparing low ($z < -1$) and high ($z > 1$) mut-p53 protein levels based on proteomics. Two-way unpaired t-test **(A, B)**.

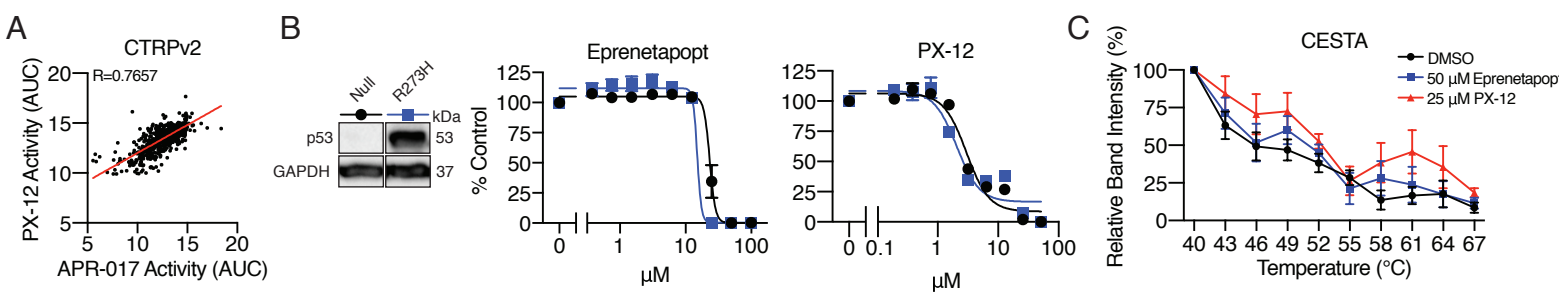


Figure S3.2 Correlation of eprenetapopt and PX-12 activity in CTRPv2 and their impact on mutant p53 thermostability

(A) Scatterplot correlating APR-017 and PX-12 AUC across all CCLs in CTRPv2. **(B)** Confirmation of mut-p53 overexpression in H1299 cells (left). Viability following 72 h treatment with eprenetapopt or PX-12 in p53^{Null} and p53^{R273H} H1299 cells (right). **(C)** Quantification of cellular thermal stability assay from Figure 2.2I. Pearson's correlation **(A)**. Error bars = SEM. **(B – western blot)** N=1. **(B – dose responses, C)** N=3.

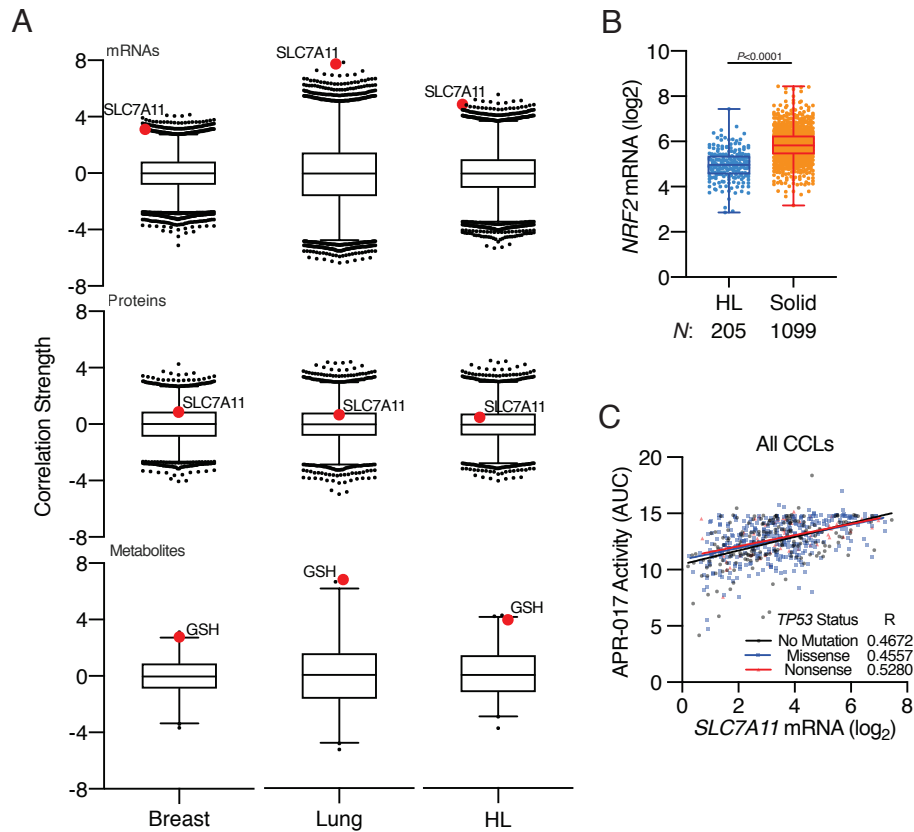


Figure S3.3. Extended correlation analysis in haematopoietic and lymphoid, breast and lung cancer cell lines

(A) Box-and-whisker plots (1st-99th percentile) of Fischer's transformed z-scored Pearson correlation strengths of APR-017 AUC and gene transcripts (top), proteins (middle) and metabolites (bottom) in breast, lung or haematopoietic and lymphoid (HL) cell lines as indicated. Red dots highlight SLC7A11 mRNA and protein, and glutathione (GSH) correlation strengths. **(B)** Boxplots (min-max) comparing NRF2 mRNA levels in HL and solid tumour derived cell lines. **(C)** Scatterplot correlating the expression of SLC7A11 with APR-017 AUC across all CCLs subset by TP53 mutation status. Two-way unpaired t-test **(B)**, Pearson's correlation **(C)**.

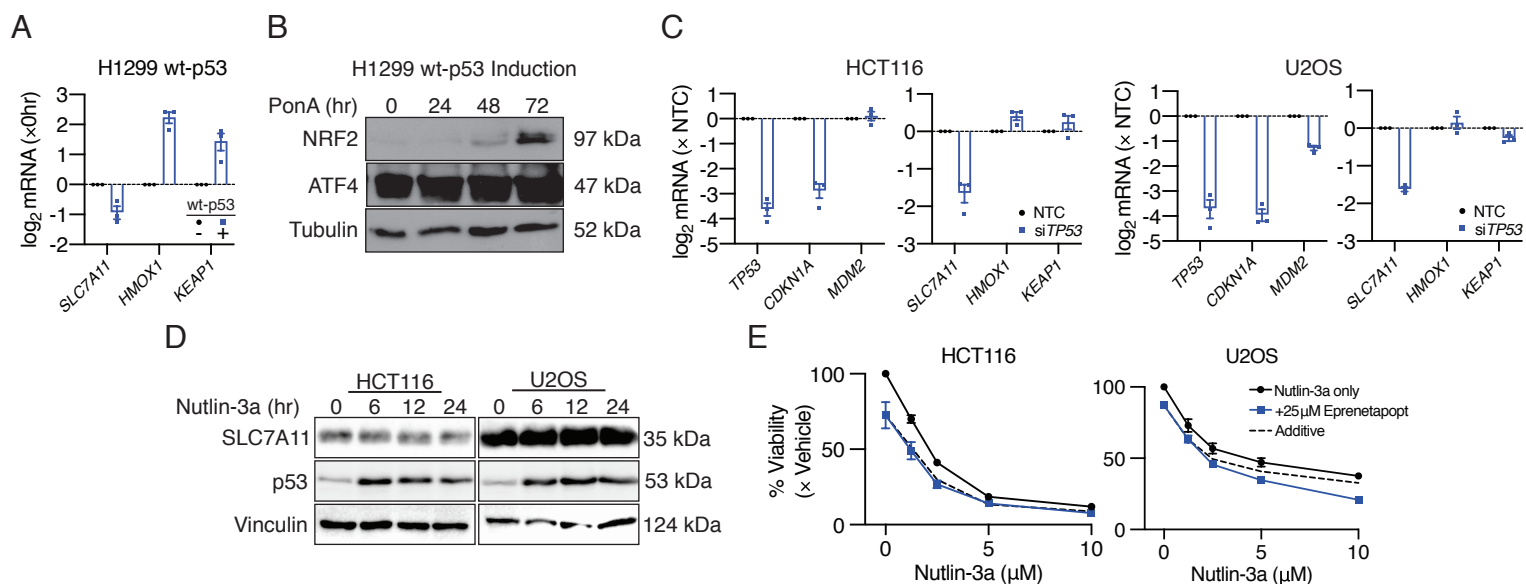


Figure S3.4 The effects of p53 overexpression and repression on *SLC7A11* mRNA expression

(A) *SLC7A11*, *HMOX1* and *KEAP1* mRNA levels in H1299 wt-p53 following 48 h of PonA treatment. **(B)** NRF2 and ATF4 protein levels following induction of wt-p53 with PonA over indicated timepoints. **(C)** mRNA expression of *TP53*, p53 target genes (*CDKN1A*/p21, *MDM2*) and NRF2 target genes (*SLC7A11*, *HMOX1*, *KEAP1*) following p53 knockdown compared to non-targeting control (NTC) in HCT116 (left) and U2OS (right) cells. **(D)** *SLC7A11* and p53 protein levels following treatment with 10 μM Nutlin-3a over indicated timepoints in HCT116 and U2OS cells. **(E)** Viability following 24 h pre-treatment with Nutlin-3a at indicated concentrations followed by 72 h co-treatment with eprentapopt in HCT116 and U2OS cells. Dotted line indicates predicted additive effect based on the product of single agents. Error bars = SEM. *N*=3 (**A**, **C**, **E**). *N*=1 (**B**, **D**).

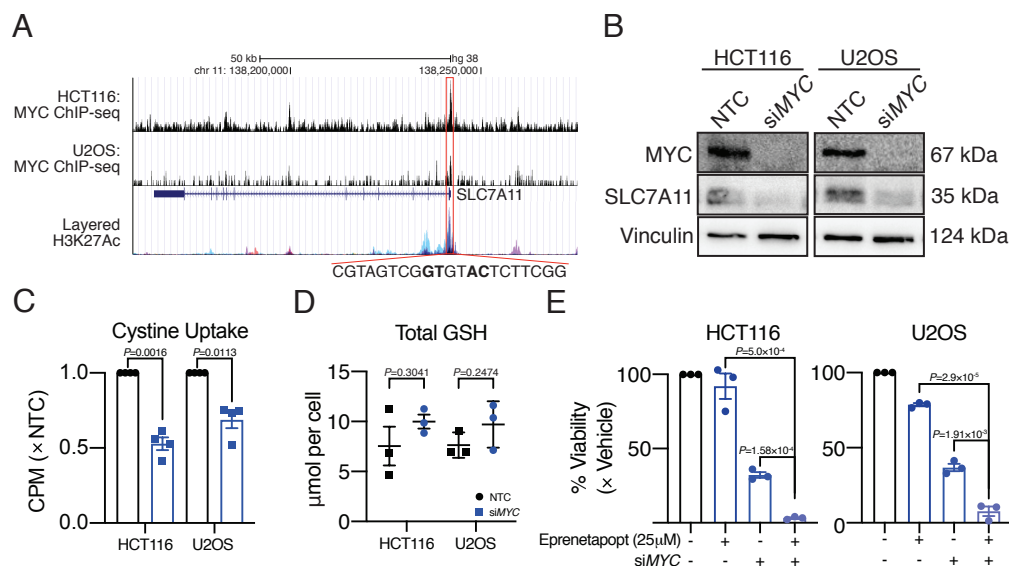


Figure S3.5 MYC directly regulates SLC7A11 levels and eprenetapopt sensitivity
(A) Association of MYC with *SLC7A11* promoter in HCT116 and U2OS shown according to the ENCODE ChIP-seq data overlaid with H3K27Ac. Indicates the predicted MYC consensus binding site based on Eukaryotic Promoter Database (EPD) in bold. **(B)** MYC and SLC7A11 protein, **(C)** cystine uptake and **(D)** total GSH levels following MYC knockdown in HCT116 and U2OS cells compared to NTC. **(E)** Viability following 24 h MYC knockdown and subsequent treatment with 25 μ M eprenetapopt or vehicle for 72 h. Two-way unpaired t-test **(C, D, E)**. Error bars = SEM. $N=1$ **(B)**. $N=3$ **(D, E)**. $N=4$ **(C)**.

SUPPLEMENTAL MATERIALS AND METHODS

Compounds and reagents

Eprenetapopt was provided by Aprea Therapeutics Inc. (Boston, MA). PX-12 and Nutlin-3a were from SelleckChem. Ponasterone A (PonA) was from Sigma-Aldrich.

Cell cultures

NCI-H1299 (RRID:CVCL_0060), OACM5.1 (RRID:CVCL_1842), FLO-1 (RRID:CVCL_2045), JH-EsoAD1 (RRID:CVCL_8098), TE-7 (RRID:CVCL_F785), HCT116 (RRID:CVCL_0291), U2OS (RRID:CVCL_0042) and HEK-293T (RRID:CVCL_0063) were from ATCC. Kasumi-1 (RRID:CVCL_0589), MV4-11 (RRID:CVCL_0064), THP-1 (RRID:CVCL_0006) and HL-60 (RRID:CVCL_0002) were provided by Prof Ricky Johnstone's laboratory (Peter MacCallum Cancer Centre). All cells were maintained at 37°C with 5% CO₂ and their identities were authenticated by short tandem repeat analysis using the PowerPlex 16 genotyping system (Promega) and confirmed mycoplasma free by PCR routinely every two months (Cerberus Sciences). Cells were thawed from early passage stocks and passaged for no more than 4 months. Unless otherwise specified, all culture media contained 10% foetal bovine serum supplemented with 50 U ml⁻¹ penicillin and 50 mg ml⁻¹ streptomycin (Life Technologies). FLO-1, HCT116, U2OS and HEK-239T cells were grown in DMEM containing 2.5 mM L-glutamine and 4.5 g l⁻¹ D-glucose (Life Technologies). H1299, OACM5.1, JH-EsoAD1, TE-7, MV4-11, Kasumi-1, THP-1 and HL-60 cells were grown in RPMI 1640 medium containing 2.5 mM L-glutamine. Haematologic cell lines were supplemented with in 20% foetal bovine serum (excepted for MV4-11) and 1× GlutaMAX (Life Technologies).

Inducible p53 expression

PonA-inducible wt-p53 H1299 cells were a gift from Dr. Paul Neilsen (CQ University Australia). Briefly, H1299 cells were stably transfected with pVgRXR aided by selection in 100 $\mu\text{g ml}^{-1}$ zeocin, followed by stable transfection of pl-TK-Hygro-p53-wt and subsequent selection of clones in 600 $\mu\text{g ml}^{-1}$ hygromycin-B. In all experiments, 5×10^5 cells were seeded in 6 cm plates and wt-p53 expression was induced by 2.5 $\mu\text{g ml}^{-1}$ PonA treatment.

Mutant p53 overexpression

p53^{Null} H1299 cells were transduced to stably express p53^{R273H} mutants as per established protocols (126). Single cell clones were generated following G418 selection and mut-p53 expression was confirmed by western blotting.

Genetic knockdown using siRNA

Cells were reverse transfected in 96-well plates for dosing experiments and 6-well plate for protein and RNA collection with 40 nM *SLC7A11*, *TP53*, *MYC*, *CDKN1A*, *ATF4*, *MDM2* or non-targeting control siRNA SMARTpools (Dharmacon, Horizon Discovery) using Lipofectamine RNAiMAX solution (Life Technologies) according to the manufacturer's guidelines. Knockdown efficiency was determined by qPCR or western blotting. siRNA IDs are detailed in **Table S3.3**.

SLC7A11 overexpression

SLC7A11 was ectopically expressed in H1299 using the GE Dharmacon Precision LentiORF pLOC downstream of the CMV promoter and contains turbo-GFP as a reporter gene. The turbo-RFP gene in place of *SLC7A11* was used as a control.

Following transduction, GFP-positive H1299 cells were sorted. The expression of *SLC711* was confirmed by qPCR and western blotting.

SUPPLEMENTAL TABLES AND DATASETS

Table S3.1 List of antibodies

Antibody	Origin	Clone	Dilution	Source
Anti-SLC7A11	Rabbit	D2M7A	1:1000	Cell Signaling Technology
Anti-NRF2	Rabbit	D1Z9C	1:1000	Cell Signaling Technology
Anti-p53	Mouse	DO-1, 1801	1:20	Hybridomas, gifts from David Lane (A*Star, Singapore)
Anti-p21	Rabbit	12D1	1:1000	Cell Signaling Technology
Anti-c-Myc	Rabbit	T69	1:1000	Abcam
Anti-MDM2	Mouse	4B11	1:1000	EMD Millipore
Anti-ATF4	Rabbit	D4B8	1:1000	Cell Signaling Technology
Anti-Tubulin	Mouse	B512	1:7000	Sigma-Aldrich
Anti-Vinculin-HRP	Mouse	7F9	1:2000	Santa Cruz Biotechnology
Anti-GAPDH-HRP	Rabbit	14C10	1:10000	Cell Signaling Technology
Swine anti-rabbit	Swine	P0217	1:7000	Dako
Goat anti-mouse	Goat	P0447	1:7000	Dako

Table S3.2 List of primers

Gene	Forward (5'-3')	Reverse (5'-3')
<i>SLC7A11</i>	ATGCAGTGGCAGTGACCTTT	GGCAACAAAGA TCGGAAGCTG
<i>ATF4</i>	GGTTCTCCAGCGACAAGG	TCTCCAACATCCAATCTGTCC
<i>MDM2</i>	TGTTGTGAAAGAAGCAGTAGCA	CCTGATCCAACCAATCACCT
<i>HMOX1</i>	GGGTGATAGAAGAGGCCAAGA	AGCTCCTGCAACTCCTCAAA
<i>KEAP1</i>	GGGAGGTGGCCAAGCAAGAGG	TCACCTGCGTGGGCTTGTGCAG
<i>TP53</i>	CGCTTCGAGA TGTTCCGAGA	CTTCAGGTGGCTGGAGTGAG
<i>CDKN1A</i>	GCTGCGTTCACAGGTGTTT	GTCGAAGTTCCATCGCTCAC
<i>GAPDH</i>	GGTGTGAACCATGAGAAG	CCACAGTTTCCCGGAG
<i>ACTB</i>	AGAAAATCTGGCACCACACC	GGGGTGTGTAAGGTCTCAAA
<i>SLC7A11</i> promoter	AGGCTTCTCATGTGGCTGAT	TGCATCGTGCTCTCAATTCT
<i>CDKN1A</i> promoter	GTCGTGGCTCTGATTGGCTTT	ACAGGCAGCCCAAGGACAAA

Table S3.3 siRNA IDs for siGENOME SMARTpools

Gene	ID
<i>TP53</i>	M-003329-03
<i>SLC7A11</i>	M-007612-01
<i>CDKN1A</i>	M-003471-00
<i>MYC</i>	M-003282-07
<i>ATF4</i>	M-005125-02
<i>MDM2</i>	M-003279-04

Dataset S3.1 Gene List from siRNA screen with eprenetapopt growth inhibition in H1299 p53-null and p53-R273H

	p53-Null			p53-R273H		
Gene	N=1	N=2	Average	N=1	N=2	Average
AADAT	-0.0849	-0.2857	-0.1853	-0.0490	-0.0020	-0.0255
AASS	0.0229	0.1518	0.0874	-0.1570	0.0594	-0.0488
ABCA2	0.0382	0.0670	0.0526	-0.0299	0.0025	-0.0137
ABCB11	-0.0423	-0.0840	-0.0631	-0.0119	0.0076	-0.0022
ABCB4	0.0682	-0.2911	-0.1115	-0.2197	-0.0693	-0.1445
ABCB6	-0.0156	-0.0649	-0.0402	-0.2347	-0.0406	-0.1376
ABCB9	0.0270	-0.1512	-0.0621	-0.0567	0.0164	-0.0201
ABCC1	-0.0507	-0.4063	-0.2285	-0.1716	-0.0251	-0.0984
ABCC5	-0.0212	0.2522	0.1155	-0.0304	0.0338	0.0017
ABHD12	0.1305	-0.0807	0.0249	-0.0700	0.0025	-0.0337
ABHD6	-0.0001	0.0092	0.0045	-0.1960	-0.0333	-0.1147
ABT1	0.0038	-0.0753	-0.0357	0.0548	0.0476	0.0512
ACO1	-0.0761	0.0220	-0.0271	-0.0998	-0.0569	-0.0784
ACTR10	0.0995	-0.1594	-0.0300	-0.2208	0.0074	-0.1067
ADA	-0.0200	-0.3018	-0.1609	-0.1445	-0.0894	-0.1170
ADAM9	0.0840	-0.2055	-0.0607	-0.3405	-0.1747	-0.2576
ADAMTS12	0.1697	-0.1234	0.0232	-0.0540	0.0918	0.0189
ADARB1	0.0804	-0.1813	-0.0504	0.0084	0.0486	0.0285
ADGRD2	-0.1600	-0.1103	-0.1352	-0.3945	-0.3078	-0.3511
ADIPOQ	0.0442	-0.1332	-0.0445	-0.1469	-0.0215	-0.0842
ADNP2	0.0191	-0.0813	-0.0311	0.0017	0.0482	0.0249
ADO	0.0692	-0.0743	-0.0026	-0.0938	0.0120	-0.0409
ADORA1	-0.0332	0.1797	0.0733	0.0605	0.0548	0.0577
ADORA2A	0.0046	0.0675	0.0360	0.0411	0.0625	0.0518
ADRB2	0.0145	0.0233	0.0189	-0.2492	0.0146	-0.1173
ADSSL1	-0.0039	-0.0416	-0.0228	-0.2440	-0.0823	-0.1631
AGAP3	-0.0203	0.2519	0.1158	-0.1097	-0.0416	-0.0756
AGMAT	-0.0226	-0.0438	-0.0332	-0.3639	-0.3690	-0.3665
AIFM1	-0.0406	0.0233	-0.0087	-0.1177	0.0939	-0.0119
AIFM2	-0.0747	-0.1566	-0.1156	0.0430	-0.0033	0.0198
AKAP5	-0.1494	-0.0944	-0.1219	-0.0090	0.0254	0.0082
AKAP8	-0.0296	-0.1906	-0.1101	0.1566	0.1051	0.1308
AKIRIN2	0.0178	-0.1341	-0.0582	-0.1453	-0.0478	-0.0966
AKR1C1	0.1337	-0.0745	0.0296	-0.1019	0.0107	-0.0456
AKR1C2	-0.0264	0.1373	0.0555	-0.4906	0.0253	-0.2326
AKR1C3	-0.0044	-0.1054	-0.0549	-0.0947	0.0027	-0.0460
ALDH18A1	0.0122	0.2000	0.1061	-0.0997	0.1838	0.0420
ALDH3B1	-0.1232	-0.0726	-0.0979	0.0890	0.0116	0.0503

ALDH4A1	0.0359	-0.2457	-0.1049	-0.4780	0.0211	-0.2284
ALDH5A1	-0.1168	-0.1611	-0.1389	-0.7078	-0.3228	-0.5153
ALKBH2	0.0052	-0.1507	-0.0728	-0.0638	-0.0882	-0.0760
ALOX12	0.1442	-0.0391	0.0525	-0.4949	0.0161	-0.2394
ALPI	-0.1147	0.1676	0.0264	-0.4383	-0.1969	-0.3176
ALS2	0.0058	0.1940	0.0999	-0.0143	0.0222	0.0040
AMDHD1	0.0374	-0.4311	-0.1969	-0.1289	0.0025	-0.0632
AMN1	0.0231	0.0149	0.0190	-0.1211	0.0123	-0.0544
ANGPTL7	-0.1243	0.0247	-0.0498	-0.1378	0.0919	-0.0230
ANKRD11	-0.0105	0.0279	0.0087	0.1115	0.0798	0.0957
ANO4	0.0490	0.2277	0.1383	-0.4912	0.0837	-0.2038
ANXA1	0.1714	-0.2363	-0.0325	-0.0823	-0.0133	-0.0478
ANXA6	0.0483	-0.1400	-0.0458	-0.0727	0.1496	0.0384
AOX1	-0.1751	-0.3015	-0.2383	-0.1918	-0.0526	-0.1222
APBA1	0.0425	-0.0411	0.0007	0.0104	0.0061	0.0082
APOA1	-0.0335	-0.0248	-0.0292	-0.1626	-0.0551	-0.1088
APOD	-0.0740	-0.3299	-0.2020	-0.1414	-0.1633	-0.1523
APOE	-0.0029	-0.0076	-0.0052	-0.0267	0.0546	0.0139
APP	0.1615	-0.1131	0.0242	-0.1448	0.0382	-0.0533
APTX	0.0751	-0.1743	-0.0496	-0.0719	0.1247	0.0264
AQP1	-0.0122	0.0045	-0.0039	-0.5115	-0.1197	-0.3156
ARC	0.0335	0.0177	0.0256	0.0426	0.0367	0.0396
ARG1	-0.0130	-0.1111	-0.0621	0.1224	-0.1229	-0.0003
ARG2	0.0186	-0.0026	0.0080	-0.0267	-0.0164	-0.0215
ARGLU1	-0.0800	-0.1263	-0.1031	0.0102	0.1287	0.0695
ARID3A	0.0642	0.0247	0.0444	-0.0708	-0.1208	-0.0958
ARL6IP1	0.0482	-0.2287	-0.0902	-0.5299	-0.1035	-0.3167
ARL6IP5	0.2137	-0.6152	-0.2007	-0.3252	-0.0440	-0.1846
ARMT1	0.1307	-0.2295	-0.0494	-0.0337	0.0313	-0.0012
ARNT	-0.0627	0.0900	0.0137	-0.0664	-0.0146	-0.0405
ARRB1	0.0794	0.0705	0.0749	-0.0656	0.0141	-0.0258
ARRB2	0.1467	0.2305	0.1886	0.1116	0.1033	0.1075
ARRDC3	0.1554	-0.1706	-0.0076	0.0713	-0.0153	0.0280
ART4	-0.0565	-0.0213	-0.0389	-0.3683	-0.0952	-0.2318
ASIC2	-0.0062	0.1299	0.0618	-0.1367	0.1178	-0.0095
ASL	0.0165	0.0006	0.0086	-0.1891	-0.0733	-0.1312
ASNS	0.0490	0.0942	0.0716	-0.2473	-0.0216	-0.1344
ASNSD1	-0.0223	0.0000	-0.0111	-0.0863	0.1198	0.0167
ASPG	-0.0504	0.1633	0.0564	-0.2432	0.0227	-0.1102
ASRGL1	-0.0832	-0.4465	-0.2648	-0.4320	-0.1410	-0.2865
ASS1	-0.1561	-0.1395	-0.1478	-0.3985	-0.1233	-0.2609
ATCAY	0.0393	-0.1887	-0.0747	-0.3603	0.0126	-0.1739

ATF3	0.0289	0.0739	0.0514	0.0156	0.0258	0.0207
ATF4	-0.2210	-0.3534	-0.2872	-0.4117	-0.2203	-0.3160
ATOX1	0.0662	-0.0978	-0.0158	-0.5436	-0.1079	-0.3258
ATP1A2	0.0160	-0.0410	-0.0125	-0.5435	-0.0818	-0.3126
ATP1A3	-0.0955	0.0543	-0.0206	-0.0052	0.0929	0.0438
ATP2B4	-0.1127	0.0200	-0.0464	-0.0204	-0.0626	-0.0415
ATP5E	-0.0902	0.2433	0.0766	-0.0971	0.1756	0.0392
ATP7A	0.0052	-0.0185	-0.0067	-0.0621	-0.0210	-0.0416
ATRN	0.0390	0.5217	0.2804	-0.4296	-0.1741	-0.3019
ATXN1	0.0132	-0.0490	-0.0179	-0.2279	-0.0832	-0.1556
ATXN3	0.0383	-0.1058	-0.0338	-0.2611	-0.0963	-0.1787
ATXN3L	-0.0683	0.0742	0.0029	-0.1047	-0.0199	-0.0623
AVP	0.0316	-0.0219	0.0049	0.0198	0.0251	0.0225
AVPR1A	0.1242	0.0582	0.0912	-0.2770	0.0535	-0.1117
AZIN1	-0.0911	-0.3451	-0.2181	-0.2615	-0.0067	-0.1341
B2M	-0.0103	-0.1288	-0.0696	-0.3239	0.1066	-0.1087
BAIAP2	0.0345	0.0119	0.0232	-0.0582	0.0739	0.0078
BCL10	-0.1176	-0.2191	-0.1684	-0.0865	-0.0919	-0.0892
BCL11A	-0.1997	-0.1162	-0.1580	-0.4210	-0.1880	-0.3045
BCL2	-0.0138	-0.1893	-0.1015	-0.0376	0.0524	0.0074
BCL2L11	-0.0027	-0.0123	-0.0075	-0.2228	-0.1157	-0.1692
BEND6	0.1448	-0.5115	-0.1833	-0.3905	0.0626	-0.1639
BICDL1	0.0017	0.0511	0.0264	-0.0970	0.1120	0.0075
BID	0.0471	-0.0601	-0.0065	-0.1359	-0.0608	-0.0983
BMP10	0.0647	0.0854	0.0750	0.0139	0.0604	0.0372
BMP7	0.0046	0.2326	0.1186	-0.0385	0.0167	-0.0109
BNIP3	-0.0122	-0.1868	-0.0995	0.0050	0.0289	0.0170
BOK	-0.0112	0.0261	0.0074	-0.3129	-0.0692	-0.1910
BRCC3	0.0793	-0.1347	-0.0277	-0.1895	0.1004	-0.0445
BRD2	-0.0270	-0.2196	-0.1233	-0.2952	0.0837	-0.1058
BTG1	-0.0611	-0.0025	-0.0318	-0.1844	-0.0831	-0.1337
BTG3	-0.0193	-0.1833	-0.1013	-0.5347	-0.0548	-0.2948
C6orf106	0.0354	0.0490	0.0422	0.0039	0.0525	0.0282
CA3	-0.1184	-0.3189	-0.2186	-0.3728	-0.3741	-0.3734
CACNG2	-0.0499	0.2123	0.0812	-0.0651	-0.0133	-0.0392
CACNG3	-0.0068	0.1069	0.0501	-0.1627	0.2057	0.0215
CACNG4	-0.0296	0.1465	0.0584	-0.0950	-0.0004	-0.0477
CACNG5	-0.0017	0.1460	0.0722	-0.2630	0.1192	-0.0719
CACNG7	0.0108	-0.1733	-0.0813	-0.0228	0.0142	-0.0043
CACNG8	0.0058	0.1484	0.0771	-0.1986	0.0833	-0.0577
CAD	0.0339	-0.1100	-0.0381	-0.0223	-0.0370	-0.0296
CAMK2A	-0.0006	-0.0805	-0.0406	-0.7678	-0.1160	-0.4419

CAMK2D	0.0283	0.1096	0.0689	-0.3996	0.0276	-0.1860
CAMK2G	-0.2238	-0.2441	-0.2339	-0.0397	-0.0258	-0.0327
CAND1	0.0652	-0.3369	-0.1359	-0.2106	-0.0955	-0.1530
CANX	-0.0546	-0.0394	-0.0470	0.0578	-0.0799	-0.0111
CAPN3	0.0571	0.1915	0.1243	-0.1133	-0.1991	-0.1562
CARF	-0.0640	0.1265	0.0312	-0.4494	-0.0974	-0.2734
CASC3	0.1654	-0.0953	0.0350	-0.1205	-0.0813	-0.1009
CASP10	0.1744	0.0059	0.0901	0.0079	-0.0323	-0.0122
CASP3	0.0289	0.2010	0.1150	-0.2879	0.0085	-0.1397
CASP6	0.1053	0.0206	0.0630	-0.2129	-0.1217	-0.1673
CASP8	0.1053	-0.1406	-0.0177	-0.0329	0.1615	0.0643
CAT	-0.1210	-0.0247	-0.0728	-0.1056	-0.0271	-0.0663
CBX8	-0.0015	0.0563	0.0274	-0.3733	-0.0178	-0.1955
CCER1	0.1156	0.2183	0.1670	0.0988	-0.0058	0.0465
CCK	-0.0263	-0.0265	-0.0264	0.1071	0.0403	0.0737
CCL19	-0.0063	0.0276	0.0107	0.1099	0.0738	0.0919
CCL5	0.1534	0.4012	0.2773	-0.2251	-0.1883	-0.2067
CCR7	-0.0825	0.0421	-0.0202	0.0396	-0.0420	-0.0012
CCS	0.0184	0.0264	0.0224	-0.0246	-0.0196	-0.0221
CD226	0.1059	0.0604	0.0831	0.0604	-0.0187	0.0209
CD27	-0.0091	-0.3253	-0.1672	-0.3521	-0.2386	-0.2953
CD36	-0.0275	-0.1535	-0.0905	-0.0219	0.0412	0.0097
CD38	-0.0036	0.1640	0.0802	-0.2309	0.0223	-0.1043
CD83	-0.0519	-0.0337	-0.0428	-0.2276	-0.0142	-0.1209
CDK1	0.0326	0.0228	0.0277	-0.1005	0.0592	-0.0207
CDK17	-0.0396	-0.0675	-0.0535	0.0646	-0.0300	0.0173
CDK2	0.1986	0.1692	0.1839	-0.4549	0.0116	-0.2217
CDK5R1	-0.1326	-0.0703	-0.1015	-0.4019	-0.0549	-0.2284
CDK6	0.0352	0.0374	0.0363	0.0425	0.1143	0.0784
CEBPA	-0.0771	0.1282	0.0255	-0.3500	-0.0785	-0.2142
CEP104	0.0673	-0.0051	0.0311	-0.1051	-0.0206	-0.0629
CES1	-0.0457	-0.1530	-0.0993	-0.1208	-0.0620	-0.0914
CFAP74	0.1306	0.0739	0.1022	-0.0180	0.0117	-0.0031
CHRNA4	-0.1240	0.1086	-0.0077	-0.2479	0.0557	-0.0961
CHST11	-0.0306	-0.2242	-0.1274	-0.0250	0.0178	-0.0036
CLIP4	0.1232	0.0213	0.0722	-0.0850	0.0387	-0.0232
CLLU1OS	0.1519	0.1167	0.1343	0.0506	0.1365	0.0936
CLN3	0.0614	-0.0610	0.0002	-0.1305	-0.0002	-0.0654
CLN8	0.1762	0.0059	0.0910	-0.0801	-0.0151	-0.0476
CLTC	0.0448	0.0228	0.0338	0.0092	-0.0158	-0.0033
CLU	0.1807	0.1221	0.1514	-0.1643	-0.0807	-0.1225
CLYBL	0.0313	-0.3332	-0.1509	-0.0777	0.0879	0.0051

CMIP	-0.2787	-0.4716	-0.3752	-0.2094	-0.1340	-0.1717
CMPK1	-0.1076	-0.0178	-0.0627	-0.2698	0.1043	-0.0827
CNIH2	0.0455	-0.0923	-0.0234	-0.2577	0.0611	-0.0983
CNIH3	-0.0103	0.1578	0.0738	-0.0633	0.0267	-0.0183
COL1A1	0.0403	-0.0877	-0.0237	-0.3951	0.0489	-0.1731
COL24A1	0.0697	-0.0363	0.0167	-0.0119	0.0931	0.0406
COQ7	0.0461	0.1435	0.0948	0.0968	0.0423	0.0696
CPEB2	0.0262	-0.1209	-0.0474	-0.0679	0.0297	-0.0191
CPEB3	0.2137	0.1338	0.1737	-0.0680	0.0864	0.0092
CPEB4	0.1502	-0.0061	0.0721	-0.0941	-0.0457	-0.0699
CPLX1	0.0276	-0.0137	0.0070	-0.0085	0.0564	0.0239
CPQ	0.0069	-0.1287	-0.0609	-0.0089	-0.0442	-0.0266
CPS1	0.0883	-0.0306	0.0289	-0.1154	0.0677	-0.0239
CPT1C	0.0048	-0.1515	-0.0733	-0.0145	0.0179	0.0017
CREB1	0.0662	0.0427	0.0544	-0.0145	0.0111	-0.0017
CREBZF	0.0142	0.0889	0.0516	-0.0373	0.0067	-0.0153
CRH	-0.1141	-0.1955	-0.1548	-0.1821	-0.1965	-0.1893
CRHBP	0.1312	-0.0809	0.0252	-0.0894	0.0051	-0.0421
CRYAA	0.0416	0.1057	0.0736	0.0020	-0.0215	-0.0098
CRYAB	0.0984	-0.0655	0.0165	-0.1520	-0.0228	-0.0874
CRYGD	0.1183	0.0551	0.0867	-0.1595	0.0300	-0.0647
CST3	-0.0018	-0.0470	-0.0244	-0.0922	0.0095	-0.0414
CTH	0.0014	0.1079	0.0546	-0.1886	0.0803	-0.0541
CTPS1	0.0220	-0.0231	-0.0006	-0.1958	-0.0652	-0.1305
CTPS2	-0.0754	-0.0560	-0.0657	-0.3762	-0.0543	-0.2152
CWC27	-0.0690	-0.0531	-0.0610	-0.1301	0.0386	-0.0458
CWH43	0.1139	-0.2052	-0.0456	-0.2698	0.0325	-0.1186
CXXC4	0.1305	-0.1161	0.0072	-0.0341	-0.0549	-0.0445
CYBA	-0.0526	-0.1929	-0.1228	-0.1728	0.0867	-0.0431
CYBB	-0.0119	-0.1283	-0.0701	-0.1664	0.0239	-0.0713
CYGB	0.1732	-0.0067	0.0833	0.0360	0.0652	0.0506
CYP11A1	-0.0529	-0.0804	-0.0667	-0.3002	0.0676	-0.1163
CYP2E1	0.0615	0.1580	0.1098	-0.3882	-0.0721	-0.2302
DAD1	0.0327	0.0227	0.0277	0.0187	0.0973	0.0580
DAGLA	0.1918	-0.0369	0.0774	-0.3053	-0.0355	-0.1704
DAO	-0.0157	0.0174	0.0009	-0.0624	0.0027	-0.0298
DAPK1	-0.0718	-0.0841	-0.0779	-0.1469	0.0259	-0.0605
DBH	-0.0162	-0.1165	-0.0664	-0.0704	0.0060	-0.0322
DCUN1D4	-0.0371	-0.0962	-0.0666	-0.0596	-0.1713	-0.1155
DDAH1	-0.0699	0.0920	0.0110	-0.1133	0.0020	-0.0557
DDAH2	-0.0209	0.1701	0.0746	-0.1815	-0.0175	-0.0995
DDIT3	0.0042	-0.0638	-0.0298	0.0523	0.0093	0.0308

DENND4C	-0.0160	-0.5657	-0.2908	-0.1072	-0.0159	-0.0615
DGCR6L	0.0464	0.0439	0.0452	0.0419	-0.0016	0.0202
DGLUCY	0.0750	-0.4171	-0.1711	-0.1582	-0.1491	-0.1536
DHCR24	0.0034	0.0151	0.0092	0.0225	-0.0143	0.0041
DHRS2	0.0239	0.1429	0.0834	-0.0721	0.0922	0.0101
DIABLO	0.0007	-0.0400	-0.0197	-0.0888	-0.0514	-0.0701
DISP2	0.0241	-0.0323	-0.0041	0.0495	0.0336	0.0416
DLG1	0.0114	0.0760	0.0437	-0.1716	0.0683	-0.0517
DLG2	-0.0419	-0.0171	-0.0295	-0.6179	0.1412	-0.2384
DLG3	-0.1778	0.1524	-0.0127	-0.3843	-0.0242	-0.2043
DLG4	-0.0943	0.0174	-0.0385	-0.2030	-0.0998	-0.1514
DNAJA2	0.0782	0.1359	0.1070	0.0096	0.0050	0.0073
DNM3	-0.0140	0.0116	-0.0012	-0.0789	0.1092	0.0151
DPEP1	0.0957	-0.1702	-0.0373	-0.0694	-0.0176	-0.0435
DPF3	0.1049	-0.1181	-0.0066	0.1084	-0.0566	0.0259
DRD2	-0.2780	-0.1606	-0.2193	-0.5609	-0.2438	-0.4024
DST	0.2431	0.0950	0.1690	-0.0097	0.0828	0.0365
DUOX1	-0.0165	-0.1550	-0.0858	-0.0691	0.0106	-0.0292
DUOX2	-0.0369	0.1210	0.0421	-0.1152	-0.1852	-0.1502
DUSP1	-0.2287	-0.0734	-0.1511	-0.1517	-0.0981	-0.1249
DUSP2	-0.0591	0.1187	0.0298	-0.4362	-0.0663	-0.2513
DUSP5	-0.0484	0.1853	0.0684	-0.1064	-0.0926	-0.0995
DYNC1LI1	-0.0451	-0.1276	-0.0863	-0.1760	0.1133	-0.0313
EARS2	0.2114	0.0801	0.1458	0.0989	-0.0600	0.0195
ECT2	-0.0355	-0.0161	-0.0258	-0.1115	-0.0859	-0.0987
EGLN1	-0.1318	-0.0711	-0.1015	-0.2159	-0.0245	-0.1202
EHMT1	0.0881	0.1184	0.1032	-0.0165	-0.0525	-0.0345
EIF4G3	-0.0851	-0.1024	-0.0938	-0.1912	-0.0412	-0.1162
ELN	0.1384	0.0826	0.1105	-0.0659	0.0194	-0.0233
EP300	0.0097	-0.0093	0.0002	-0.0786	0.0151	-0.0317
EPAS1	0.0233	-0.1527	-0.0647	-0.5716	-0.3044	-0.4380
EPB41	-0.0183	-0.2030	-0.1107	-0.3539	-0.0168	-0.1854
EPB42	0.0697	0.0857	0.0777	-0.0516	-0.0009	-0.0262
EPHB2	-0.1150	0.2440	0.0645	-0.3793	-0.2191	-0.2992
EPHX2	-0.1216	-0.3105	-0.2161	-0.4067	-0.2271	-0.3169
EPPK1	-0.0894	-0.0861	-0.0878	-0.2424	-0.0729	-0.1577
EPRS	0.0418	-0.0467	-0.0024	0.0207	-0.0192	0.0008
EPS8	0.0052	0.0444	0.0248	-0.2601	-0.3196	-0.2899
EPX	-0.0611	0.3493	0.1441	-0.1660	-0.0247	-0.0954
ERBB4	-0.1311	0.0428	-0.0441	-0.5046	-0.0277	-0.2661
ERC2	0.1023	-0.0045	0.0489	-0.0544	0.0912	0.0184
ERCC1	0.0118	0.0261	0.0189	-0.0423	0.0587	0.0082

ERCC2	-0.1564	-0.2679	-0.2121	-0.7691	-0.4102	-0.5897
ERCC3	0.0529	0.0037	0.0283	-0.3605	-0.1525	-0.2565
ERCC6	-0.2002	-0.0953	-0.1477	-0.1696	-0.0762	-0.1229
ERCC8	0.0565	-0.1268	-0.0352	-0.3324	0.0435	-0.1445
ERICH2	0.0530	-0.0078	0.0226	-0.0266	0.0358	0.0046
ETFDH	0.0497	0.1730	0.1114	-0.0216	0.0667	0.0225
ETS1	0.0613	0.0571	0.0592	-0.0563	-0.0054	-0.0309
ETV5	-0.0320	0.0767	0.0223	-0.1693	0.0058	-0.0817
EXOC7	0.0691	0.0343	0.0517	-0.0571	0.0189	-0.0191
F13A1	-0.2609	-0.2469	-0.2539	-0.0191	-0.0801	-0.0496
F3	-0.0284	0.0011	-0.0136	-0.0694	0.0628	-0.0033
FABP1	-0.0464	0.1273	0.0404	-0.0922	-0.0262	-0.0592
FABP2	0.0338	0.0531	0.0434	0.0565	0.1677	0.1121
FADD	0.0598	0.1817	0.1208	-0.0435	-0.0138	-0.0287
FAH	-0.1322	-0.0757	-0.1040	-0.0414	0.0284	-0.0065
FAM157A	-0.0265	0.0646	0.0190	-0.1927	0.0777	-0.0575
FAM219A	-0.0101	-0.0582	-0.0342	0.0356	0.0147	0.0252
FAM76B	0.1970	0.1620	0.1795	0.0768	0.0137	0.0452
FANCC	0.0859	-0.0382	0.0239	-0.2753	0.1354	-0.0700
FASTKD2	0.1331	0.0189	0.0760	-0.3242	-0.1503	-0.2372
FBL	0.0335	-0.0532	-0.0098	-0.0639	-0.0385	-0.0512
FBLN5	0.1295	-0.0011	0.0642	0.0349	0.0796	0.0572
FBXO28	0.0039	-0.0613	-0.0287	-0.1183	-0.0039	-0.0611
FCMR	0.0920	0.0464	0.0692	-0.0609	0.0533	-0.0038
FECH	0.0454	0.0273	0.0363	-0.0039	0.0273	0.0117
FER	-0.1657	-0.0258	-0.0957	-0.4353	-0.1329	-0.2841
FLNB	-0.0322	-0.0236	-0.0279	-0.2934	-0.1087	-0.2010
FLOT1	0.0367	-0.0219	0.0074	-0.1157	0.0005	-0.0576
FLOT2	0.2706	-0.0984	0.0861	-0.0856	0.0706	-0.0075
FMR1	0.0653	-0.1800	-0.0574	-0.1048	0.0001	-0.0524
FOLH1	0.0515	-0.0099	0.0208	-0.2324	-0.1240	-0.1782
FOS	-0.0313	-0.0942	-0.0628	-0.1293	0.0108	-0.0593
FOSL1	-0.0037	-0.1066	-0.0552	-0.1599	-0.0553	-0.1076
FOXO1	-0.0688	-0.1103	-0.0896	-0.0874	-0.0266	-0.0570
FOXO1	-0.0223	-0.2005	-0.1114	-0.1644	0.0151	-0.0747
FPGS	0.0896	0.0199	0.0547	0.0689	0.1194	0.0942
FRRS1L	0.0069	-0.2219	-0.1075	-0.3771	-0.0517	-0.2144
FSD1L	0.0173	-0.4958	-0.2393	-0.1844	-0.0147	-0.0995
FTCD	0.0458	-0.0663	-0.0103	-0.1508	0.0794	-0.0357
FTH1	-0.0268	-0.0774	-0.0521	-0.1195	-0.0797	-0.0996
FTL	0.0632	0.0402	0.0517	-0.2091	-0.0474	-0.1283
FUS	-0.0198	-0.2450	-0.1324	-0.2914	-0.1095	-0.2004

FXN	-0.0871	0.3229	0.1179	-0.3381	0.0566	-0.1407
FYN	0.0405	0.0408	0.0407	-0.5486	0.0726	-0.2380
G6PD	0.0238	0.0244	0.0241	-0.0182	-0.0041	-0.0112
GAB1	0.1130	-0.3292	-0.1081	-0.4779	-0.0094	-0.2436
GABBR2	0.0260	-0.0403	-0.0072	-0.2079	0.0163	-0.0958
GAD1	-0.0022	-0.0745	-0.0383	0.0144	0.0858	0.0501
GAD2	-0.0433	0.0191	-0.0121	-0.0837	-0.0555	-0.0696
GAPDH	-0.3601	-0.1561	-0.2581	-0.5058	-0.0656	-0.2857
GATB	0.0238	-0.1061	-0.0412	-0.1207	0.0356	-0.0425
GATC	0.0864	-0.1861	-0.0498	-0.3014	-0.1403	-0.2209
GATM	0.0165	-0.0099	0.0033	-0.0647	-0.0332	-0.0490
GATS	-0.0920	-0.0815	-0.0867	-0.2854	-0.2560	-0.2707
GCLC	-0.3281	-0.0483	-0.1882	-0.6582	-0.1971	-0.4276
GCLM	-0.0371	-0.0554	-0.0462	-0.1952	0.0220	-0.0866
GCNT3	-0.0118	0.0677	0.0279	-0.2275	0.0178	-0.1048
GDF15	0.0703	-0.0840	-0.0068	0.0427	0.0404	0.0416
GFPT1	0.0106	-0.1589	-0.0742	-0.3799	-0.0442	-0.2120
GFPT2	0.0925	0.0987	0.0956	-0.2269	-0.0386	-0.1328
GGT1	0.0929	0.2767	0.1848	0.0470	-0.0238	0.0116
GHSR	-0.0445	0.0119	-0.0163	-0.1001	-0.0379	-0.0690
GIPC1	-0.0051	-0.0467	-0.0259	-0.2107	-0.0460	-0.1284
GJA3	0.0173	0.0295	0.0234	-0.0139	0.0277	0.0069
GLCCI1	0.1321	0.0158	0.0740	0.0134	0.0705	0.0419
GLRX2	-0.0290	-0.0374	-0.0332	-0.5928	-0.0673	-0.3301
GLS	-0.0055	0.0016	-0.0020	-0.2243	-0.1102	-0.1673
GLS2	-0.0085	0.1737	0.0826	-0.2108	0.0130	-0.0989
GLUD1	0.0224	-0.0770	-0.0273	-0.5666	-0.4828	-0.5247
GLUD2	0.0548	0.1420	0.0984	0.0351	0.1037	0.0694
GLUL	0.0320	0.1490	0.0905	-0.0356	-0.0092	-0.0224
GLYATL1	0.0514	-0.1909	-0.0697	-0.0069	0.0175	0.0053
GMPS	0.0226	0.0822	0.0524	-0.2476	0.0324	-0.1076
GNAI2	-0.0025	0.0478	0.0227	-0.1719	-0.1188	-0.1453
GNAO1	0.1278	-0.0385	0.0447	-0.3184	-0.2615	-0.2900
GNAQ	-0.0487	-0.2677	-0.1582	-0.0968	-0.1267	-0.1118
GNAS	0.0485	0.1060	0.0773	-0.1917	-0.0242	-0.1080
GOT1	-0.0572	0.0807	0.0117	-0.1597	-0.0491	-0.1044
GOT2	0.0204	0.1451	0.0827	-0.0603	0.1085	0.0241
GPI	0.0056	-0.0009	0.0024	-0.1129	-0.0469	-0.0799
GPNMB	0.0098	-0.0164	-0.0033	-0.1072	0.0154	-0.0459
GPR179	-0.0546	-0.1802	-0.1174	-0.0781	-0.0524	-0.0653
GPX2	0.0095	-0.2135	-0.1020	-0.1629	-0.0213	-0.0921
GPX3	0.0026	-0.0188	-0.0081	-0.1014	-0.0067	-0.0540

GPX4	-0.0027	0.0313	0.0143	-0.0304	-0.0070	-0.0187
GPX5	0.0585	0.2990	0.1788	-0.0440	0.0707	0.0133
GPX7	-0.0054	0.0039	-0.0007	-0.0464	-0.0126	-0.0295
GRIA1	-0.0674	-0.1861	-0.1267	-0.6439	-0.0250	-0.3344
GRIA2	-0.0081	-0.0061	-0.0071	-0.4138	-0.0009	-0.2073
GRIA3	-0.0836	0.1569	0.0366	-0.6456	-0.2928	-0.4692
GRIA4	-0.0517	0.0264	-0.0127	-0.3010	0.1986	-0.0512
GRID1	-0.0043	-0.1363	-0.0703	-0.0276	-0.0488	-0.0382
GRID2	0.0164	0.2502	0.1333	-0.1031	0.0214	-0.0409
GRIK1	-0.1112	-0.3687	-0.2400	-0.2452	-0.0120	-0.1286
GRIK2	-0.0396	0.1251	0.0427	0.0176	0.1072	0.0624
GRIK3	-0.0806	-0.0290	-0.0548	-0.1088	0.0192	-0.0448
GRIK4	0.0111	0.0175	0.0143	0.0024	0.0102	0.0063
GRIK5	-0.0732	0.2287	0.0778	-0.2625	0.2268	-0.0178
GRIN1	-0.0657	0.0000	-0.0329	-0.0575	0.0967	0.0196
GRIN2A	-0.0045	0.1560	0.0757	-0.3669	-0.0505	-0.2087
GRIN2B	-0.1370	0.0109	-0.0631	-0.2693	-0.0584	-0.1638
GRIN2C	-0.1322	0.0799	-0.0262	-0.1690	-0.0160	-0.0925
GRIN2D	0.0169	0.0135	0.0152	0.0186	0.0448	0.0317
GRIN3A	0.0420	0.1040	0.0730	-0.2082	0.0919	-0.0582
GRIN3B	0.0122	0.3319	0.1721	-0.3374	0.0853	-0.1260
GRIP1	0.0869	0.0346	0.0608	-0.1166	-0.0220	-0.0693
GRIP2	0.0021	0.0039	0.0030	-0.0164	-0.0151	-0.0157
GRK2	-0.0837	-0.1557	-0.1197	-0.1608	0.0791	-0.0409
GRM1	-0.1509	-0.1169	-0.1339	-0.1905	0.0792	-0.0557
GRM2	-0.1457	-0.0113	-0.0785	-0.5336	-0.1908	-0.3622
GRM3	-0.1284	0.0895	-0.0194	-0.0706	0.0799	0.0046
GRM4	0.0414	0.2253	0.1334	0.0244	0.0933	0.0588
GRM5	-0.0052	-0.0141	-0.0096	-0.0278	-0.0021	-0.0149
GRM6	0.0342	-0.0248	0.0047	0.0139	0.0346	0.0242
GRM7	-0.0865	0.1943	0.0539	-0.4112	-0.1825	-0.2969
GRM8	-0.0837	-0.1200	-0.1018	-0.0351	0.0043	-0.0154
GRWD1	-0.0770	-0.1628	-0.1199	-0.2953	-0.1884	-0.2419
GSR	-0.1076	-0.0278	-0.0677	0.0459	0.0396	0.0428
GSS	-0.0190	-0.0115	-0.0152	0.0117	0.0426	0.0271
GSTM5	0.1003	-0.0990	0.0007	-0.0105	0.0342	0.0119
GSTP1	-0.0630	-0.2025	-0.1327	-0.4328	-0.0053	-0.2190
GSTZ1	-0.1214	-0.2615	-0.1915	-0.3054	-0.0116	-0.1585
GTF2I	-0.0976	0.3423	0.1223	-0.1710	-0.0593	-0.1151
HAL	-0.0322	0.0875	0.0277	-0.0210	0.1491	0.0641
HAO1	-0.0692	0.0587	-0.0053	-0.6266	-0.1901	-0.4084
HAUS3	-0.1601	-0.1356	-0.1479	-0.2373	-0.1719	-0.2046

HBB	-0.0261	0.0326	0.0033	-0.2330	0.0501	-0.0914
HBE1	0.0811	0.0677	0.0744	-0.0148	0.0616	0.0234
HDAC5	0.0419	-0.1345	-0.0463	-0.1602	0.0426	-0.0588
HDAC6	0.0588	0.0857	0.0722	-0.1276	0.0092	-0.0592
HERC1	-0.0051	-0.0601	-0.0326	-0.1266	0.0892	-0.0187
HES1	0.0294	-0.3183	-0.1444	-0.1006	-0.0473	-0.0740
HGD	0.0072	0.0790	0.0431	-0.0461	0.0067	-0.0197
HIF1A	-0.0719	-0.1341	-0.1030	0.0542	-0.0219	0.0162
HIP1	0.1320	-0.0110	0.0605	-0.0588	0.0447	-0.0071
HIST1H4H	0.2675	-0.1195	0.0740	0.1078	0.0293	0.0686
HMOX1	0.0312	0.5518	0.2915	-0.2470	0.0279	-0.1095
HMOX2	0.0232	-0.4839	-0.2303	-0.1823	-0.0751	-0.1287
HNF1A	-0.1051	0.1108	0.0029	-0.2357	-0.0246	-0.1302
HOMER1	-0.0123	-0.2182	-0.1153	-0.1436	0.0251	-0.0593
HOMER2	0.2055	0.1327	0.1691	-0.0100	-0.0315	-0.0207
HOMER3	0.1650	-0.1752	-0.0051	-0.3529	-0.1552	-0.2540
HP	-0.0864	-0.2028	-0.1446	-0.1077	-0.0531	-0.0804
HPCA	0.0680	0.1271	0.0976	-0.0807	0.0808	0.0000
HPRT1	-0.0568	0.0627	0.0029	-0.0704	-0.0224	-0.0464
HRASLS2	0.0808	-0.1290	-0.0241	0.0123	0.0939	0.0531
HRH3	-0.0629	0.0380	-0.0124	-0.3056	-0.0515	-0.1785
HSF1	0.0159	0.0064	0.0111	-0.0271	0.0223	-0.0024
HSPA1A	0.0052	0.0160	0.0106	-0.0288	0.0133	-0.0077
HSPA1B	0.0795	0.1544	0.1170	-0.2832	-0.1941	-0.2386
HSPD1	0.0182	0.0787	0.0485	-0.0130	-0.0113	-0.0121
HTATIP2	-0.0740	0.0306	-0.0217	0.0007	-0.0277	-0.0135
HTRA3	-0.0135	-0.0459	-0.0297	-0.1833	-0.0367	-0.1100
HYAL1	-0.0022	-0.2471	-0.1246	-0.0903	0.0151	-0.0376
HYAL2	0.0268	0.0999	0.0634	0.0501	0.0802	0.0651
IDH1	-0.1929	-0.3709	-0.2819	-0.2500	-0.1898	-0.2199
IFRD1	-0.0239	-0.0914	-0.0576	-0.1091	0.0590	-0.0251
IFT74	0.0881	-0.0616	0.0132	-0.2174	-0.1582	-0.1878
IGF2R	0.0463	0.2629	0.1546	0.0718	-0.0891	-0.0086
IL18	0.1339	0.0562	0.0951	-0.1570	-0.0043	-0.0806
IL18BP	0.0324	0.0135	0.0229	0.0309	0.0509	0.0409
IL1A	0.0456	-0.0706	-0.0125	-0.0954	0.0155	-0.0399
IL1B	0.0063	-0.1898	-0.0917	-0.0077	-0.0268	-0.0172
IL1R1	0.0190	-0.0483	-0.0146	-0.0773	-0.0171	-0.0472
IL4I1	-0.0032	0.0110	0.0039	-0.0878	-0.0204	-0.0541
IL6	-0.2690	-0.3025	-0.2858	-0.2478	-0.1060	-0.1769
IPCEF1	-0.0027	-0.3135	-0.1581	-0.2412	-0.2323	-0.2367
IPO7	0.0212	-0.0500	-0.0144	-0.2706	0.0178	-0.1264

IRF2	-0.0111	-0.0700	-0.0405	-0.0316	0.0154	-0.0081
IRF2BPL	0.1176	-0.0625	0.0275	-0.1959	0.1901	-0.0029
IVL	0.0833	0.0146	0.0490	0.0009	-0.0375	-0.0183
JAK2	-0.0922	0.1541	0.0310	-0.1570	0.0105	-0.0733
JUN	-0.0014	-0.1834	-0.0924	-0.3052	0.1427	-0.0813
KCNB1	-0.0901	0.0445	-0.0228	0.0330	0.0468	0.0399
KCNJ10	-0.1966	-0.0127	-0.1047	-0.3457	-0.1145	-0.2301
KCNN3	-0.0385	0.0489	0.0052	-0.0497	-0.0016	-0.0256
KEAP1	0.1121	0.0777	0.0949	-0.0050	0.0701	0.0325
KIAA1522	0.2068	-0.0959	0.0555	-0.0254	0.0594	0.0170
KIFC3	-0.0165	-0.1913	-0.1039	-0.4276	-0.1829	-0.3052
KLF2	-0.0093	0.0798	0.0352	-0.1811	0.0136	-0.0838
KLHL24	0.0707	-0.0419	0.0144	-0.1749	0.0019	-0.0865
KRBOX4	0.0288	-0.1715	-0.0714	-0.1732	0.0154	-0.0789
KRT1	-0.0619	-0.2852	-0.1736	-0.1549	-0.1991	-0.1770
KRT16	0.0108	0.0036	0.0072	-0.0503	0.0200	-0.0152
KYAT1	0.0354	0.2356	0.1355	-0.1821	-0.0034	-0.0928
LARS	-0.0461	-0.1785	-0.1123	-0.2647	-0.1741	-0.2194
LBR	-0.1718	-0.1550	-0.1634	-0.0726	0.0264	-0.0231
LCK	-0.0096	-0.1484	-0.0790	-0.3316	0.1065	-0.1126
LCN2	-0.0916	-0.0341	-0.0629	-0.3408	-0.0652	-0.2030
LEP	0.0607	-0.2458	-0.0926	-0.0372	-0.0112	-0.0242
LGSN	-0.0493	0.0795	0.0151	-0.2604	-0.0610	-0.1607
LIAS	-0.0369	-0.0202	-0.0285	-0.1494	-0.0643	-0.1068
LMNB2	0.0794	-0.0447	0.0174	0.0123	0.0656	0.0390
LONP1	-0.0524	-0.1422	-0.0973	-0.2769	0.0063	-0.1353
LPO	-0.0381	-0.3301	-0.1841	-0.0578	0.0787	0.0104
LRP12	-0.0284	0.1299	0.0508	0.0427	0.0514	0.0471
LRP8	-0.0049	-0.0656	-0.0353	-0.1099	0.0510	-0.0294
LRRRC8D	0.0897	-0.2508	-0.0805	-0.3298	-0.0689	-0.1993
LRRK2	-0.2213	0.1051	-0.0581	-0.3150	-0.0358	-0.1754
LY9	0.0241	-0.1049	-0.0404	-0.3033	-0.0146	-0.1590
MAFG	-0.0256	-0.1097	-0.0677	0.1939	0.0039	0.0989
MAP2K1	0.0402	-0.0313	0.0044	-0.3253	0.0009	-0.1622
MAP3K5	0.0728	-0.4699	-0.1986	-0.2369	-0.0333	-0.1351
MAPK7	-0.2293	0.2615	0.0161	-0.0206	-0.1280	-0.0743
MAPK8IP2	0.3017	0.2783	0.2900	-0.1179	0.0378	-0.0401
MAPRE3	0.0266	0.0445	0.0355	-0.0609	0.0099	-0.0255
MAPT	-0.0869	-0.2374	-0.1622	-0.1171	-0.0424	-0.0797
MARK2	-0.0177	0.0584	0.0203	-0.0055	-0.0083	-0.0069
MB	0.2100	-0.1431	0.0335	-0.1930	0.1825	-0.0053
MBL2	0.0482	-0.0888	-0.0203	-0.6316	0.0286	-0.3015

MDM2	-0.0767	-0.4674	-0.2721	-0.3627	-0.4704	-0.4165
ME1	-0.0722	-0.1926	-0.1324	-0.2319	-0.0681	-0.1500
MECP2	0.0127	0.1647	0.0887	-0.1479	0.1045	-0.0217
MEF2C	0.0505	0.0891	0.0698	-0.2913	0.0296	-0.1308
MFSD11	0.0539	-0.0560	-0.0011	-0.0799	-0.0417	-0.0608
MGST1	-0.0335	-0.0424	-0.0380	0.0139	0.0522	0.0330
MGST3	-0.0116	0.2069	0.0977	0.0515	0.0311	0.0413
MICB	0.0269	-0.0475	-0.0103	-0.1559	0.0493	-0.0533
MINK1	-0.0922	-0.0470	-0.0696	-0.0379	0.0058	-0.0160
MKLN1	-0.0621	-0.0410	-0.0515	-0.0637	0.0452	-0.0093
MMEL1	0.0332	0.0431	0.0381	-0.1612	-0.1348	-0.1480
MOV10L1	-0.1367	0.1231	-0.0068	-0.0216	-0.0054	-0.0135
MPO	-0.0182	-0.0403	-0.0293	-0.1986	0.0185	-0.0901
MPPE1	0.0607	-0.0760	-0.0077	0.0008	0.1586	0.0797
MPV17	0.0589	0.0407	0.0498	0.0318	0.0427	0.0373
MSL3	0.0871	-0.0360	0.0256	-0.1487	0.1548	0.0031
MSRA	-0.1365	-0.4060	-0.2712	-0.2150	-0.1777	-0.1963
MT3	0.0444	0.0989	0.0716	-0.0261	0.0000	-0.0130
MTF1	0.0598	0.0438	0.0518	-0.0131	0.0226	0.0047
MTF2	-0.0210	-0.0017	-0.0114	0.0473	0.0069	0.0271
MTHFS	-0.0607	-0.0939	-0.0773	-0.0798	-0.1065	-0.0932
MUC4	0.0745	-0.1747	-0.0501	-0.0151	0.1099	0.0474
MYC	0.0046	-0.0042	0.0002	-0.1392	-0.0463	-0.0927
MYH7	-0.0395	-0.1377	-0.0886	-0.0719	0.0666	-0.0026
NAA10	-0.0427	-0.0276	-0.0352	-0.0491	0.0393	-0.0049
NAA11	0.0032	-0.2787	-0.1377	-0.2759	-0.1355	-0.2057
NADSYN1	0.0119	-0.0999	-0.0440	-0.4614	-0.0452	-0.2533
NAGS	0.2840	-0.6405	-0.1783	-0.3234	-0.0230	-0.1732
NAPRT	0.0825	-0.1591	-0.0383	-0.3898	0.1091	-0.1404
NCF2	-0.0033	0.1965	0.0966	0.0009	0.0209	0.0109
NDUFA12	-0.0795	-0.2724	-0.1759	-0.1553	-0.2677	-0.2115
NDUFA6	-0.0450	-0.0897	-0.0673	-0.1646	-0.0003	-0.0824
NDUFB4	0.0113	-0.3028	-0.1457	-0.0339	0.0097	-0.0121
NDUFS2	-0.1124	-0.1041	-0.1082	-0.3510	-0.1132	-0.2321
NDUFS8	0.0488	-0.2142	-0.0827	-0.4421	-0.1047	-0.2734
NECAB2	0.1232	-0.1487	-0.0127	-0.4272	0.0241	-0.2015
NECTIN1	0.1201	-0.1096	0.0053	-0.5540	-0.0362	-0.2951
NEDD4	0.0108	-0.1487	-0.0689	-0.1006	0.1329	0.0161
NEIL1	0.0636	-0.1498	-0.0431	-0.2456	-0.0275	-0.1365
NELL2	-0.0911	0.2918	0.1004	0.0718	0.0074	0.0396
NES	0.0588	-0.0846	-0.0129	-0.1595	-0.0028	-0.0811
NET1	-0.1600	-0.2761	-0.2181	0.0170	0.0289	0.0229

NETO1	-0.0376	0.0594	0.0109	-0.1884	0.1427	-0.0229
NETO2	0.2049	-0.0776	0.0637	0.0797	0.0277	0.0537
NEUROD4	0.0023	-0.2156	-0.1066	-0.1749	0.0539	-0.0605
NF1	-0.0393	-0.2185	-0.1289	-0.1930	-0.1549	-0.1740
NFE2L2	-0.2120	-0.1971	-0.2045	-0.1139	-0.0362	-0.0750
NFKB1	-0.0311	-0.0436	-0.0374	-0.1250	0.0237	-0.0507
NIT2	-0.2012	-0.3880	-0.2946	-0.3259	-0.3545	-0.3402
NKAIN1	0.1773	0.0192	0.0983	0.0523	0.0355	0.0439
NLGN1	0.0641	0.0056	0.0348	0.0632	0.1263	0.0947
NLGN2	0.0223	-0.5027	-0.2402	-0.0812	0.0087	-0.0363
NLGN3	0.0116	-0.8995	-0.4440	-0.3023	-0.1240	-0.2132
NOS1	0.0471	0.0036	0.0253	-0.6413	0.1583	-0.2415
NOS2	-0.0824	-0.0686	-0.0755	-0.3657	-0.0949	-0.2303
NOS3	-0.0902	0.1027	0.0063	-0.0919	-0.0866	-0.0892
NOX4	-0.0611	0.0309	-0.0151	-0.0449	-0.1185	-0.0817
NOX5	0.0726	0.2180	0.1453	0.0086	0.0607	0.0347
NOXRED1	-0.0882	-0.3374	-0.2128	-0.5138	-0.4503	-0.4820
NPLOC4	0.1796	-0.1484	0.0156	-0.3243	-0.0694	-0.1969
NPTXR	0.0175	0.0480	0.0327	0.0210	-0.0031	0.0089
NPY5R	-0.1389	-0.0602	-0.0996	-0.1600	-0.0884	-0.1242
NQO1	0.0368	-0.0102	0.0133	0.0024	0.0249	0.0136
NQO2	-0.1114	-0.0098	-0.0606	-0.1958	-0.0660	-0.1309
NR1H4	0.0292	-0.3416	-0.1562	-0.1685	0.1094	-0.0296
NRCAM	-0.0932	-0.0562	-0.0747	-0.0812	-0.0802	-0.0807
NRDE2	0.1259	-0.1083	0.0088	-0.3713	-0.0145	-0.1929
NSF	-0.0333	-0.0070	-0.0202	-0.2058	0.0666	-0.0696
NTRK2	-0.0573	0.1119	0.0273	-0.1787	0.0658	-0.0565
NTSR1	-0.1051	-0.0795	-0.0923	-0.0915	-0.0537	-0.0726
NUDT1	-0.1039	-0.0760	-0.0899	-0.0106	-0.0414	-0.0260
NUMBL	0.1125	-0.0078	0.0523	-0.0575	-0.0001	-0.0288
NUP153	-0.0286	-0.0982	-0.0634	-0.6950	-0.4828	-0.5889
OAT	0.0544	0.1162	0.0853	-0.0900	0.0305	-0.0298
OGG1	-0.0376	0.4018	0.1821	-0.4317	0.0251	-0.2033
OLFM2	0.0172	-0.0132	0.0020	-0.0535	0.0073	-0.0231
OLFM3	0.0718	-0.0478	0.0120	-0.1106	0.0980	-0.0063
OLR1	-0.0908	0.0968	0.0030	-0.0769	0.0645	-0.0062
OPHN1	0.1400	0.1106	0.1253	-0.0482	-0.0256	-0.0369
OPRM1	-0.1085	0.0621	-0.0232	-0.7554	-0.4511	-0.6033
OR2AT4	-0.2392	-0.3136	-0.2764	-0.3522	-0.4444	-0.3983
OTC	-0.0425	-0.2249	-0.1337	-0.2108	-0.0077	-0.1093
non-targeting	-0.0694	-0.0679	-0.0687	-0.2872	0.0109	-0.1382
OTULIN	0.1602	0.1786	0.1694	-0.0608	0.0633	0.0013

OXR1	-0.0054	-0.0445	-0.0250	-0.0569	-0.0088	-0.0328
OXSRI	-0.0407	0.1527	0.0560	-0.2875	-0.0942	-0.1908
P2RX7	0.0356	-0.0061	0.0148	0.0066	0.0409	0.0237
P2RY10	-0.1470	-0.0391	-0.0931	-0.2957	-0.1933	-0.2445
PADI4	0.0158	0.0365	0.0261	-0.0466	0.0132	-0.0167
PAGR1	0.1251	0.4798	0.3025	0.0502	0.0897	0.0699
PARK7	0.0050	-0.0047	0.0002	-0.0438	0.0362	-0.0038
PARN	-0.2770	-0.1837	-0.2304	-0.5750	-0.3285	-0.4517
PAX2	-0.1326	-0.3478	-0.2402	-0.2960	-0.3183	-0.3072
PCNA	0.0169	0.1046	0.0608	-0.0155	0.0614	0.0230
PDCD1LG2	0.0732	-0.0652	0.0040	0.0008	0.0605	0.0306
PDCD6	-0.0596	-0.2070	-0.1333	-0.1469	-0.0668	-0.1068
PDCD6IP	0.0532	-0.0658	-0.0063	-0.1110	0.0772	-0.0169
PDLIM1	-0.0539	0.0119	-0.0210	-0.0953	-0.0220	-0.0586
PEBP1	0.0305	-0.1909	-0.0802	-0.0497	0.0184	-0.0156
PEF1	0.0335	0.0681	0.0508	0.0028	0.0420	0.0224
PER2	0.1318	-0.5432	-0.2057	-0.2887	-0.0054	-0.1470
PEX5L	-0.0054	0.1693	0.0819	-0.0107	-0.0301	-0.0204
PFAS	-0.1151	-0.1671	-0.1411	-0.5201	-0.1205	-0.3203
PGRMC2	-0.0162	0.1439	0.0638	-0.2902	-0.0193	-0.1548
PID1	0.0820	-0.1398	-0.0289	-0.0991	0.0469	-0.0261
PIEZO1	0.0184	0.0033	0.0108	-0.0968	0.0126	-0.0421
PILRB	0.1570	-0.1220	0.0175	-0.3385	-0.0401	-0.1893
PIP5K1C	-0.1510	-0.3211	-0.2360	-0.6848	-0.0317	-0.3582
PIR	-0.0016	-0.0604	-0.0310	-0.0799	0.0205	-0.0297
PKD2	0.0814	0.1221	0.1018	-0.0791	0.0875	0.0042
PKIA	-0.0198	0.0186	-0.0006	-0.1177	0.1133	-0.0022
PLA2G4A	-0.0406	0.0515	0.0055	-0.0307	-0.0382	-0.0344
PLA2G6	-0.0102	-0.0424	-0.0263	-0.0124	0.0907	0.0391
PLA2R1	0.0930	-0.0978	-0.0024	-0.0067	0.0136	0.0034
PLCB1	-0.1099	0.1598	0.0250	-0.2454	-0.0687	-0.1570
PLCG1	0.1510	0.0127	0.0818	-0.1681	0.0402	-0.0640
PLD2	0.0388	0.0985	0.0687	-0.3840	-0.0184	-0.2012
PLEKHA1	0.0622	0.0174	0.0398	-0.2399	0.1542	-0.0428
PLK1	0.0018	0.0102	0.0060	0.0064	0.0026	0.0045
PLK3	-0.0487	0.0962	0.0237	-0.4238	0.0514	-0.1862
PM20D2	0.0961	-0.1241	-0.0140	-0.1508	-0.2119	-0.1814
PMAIP1	0.0174	0.0549	0.0362	-0.0660	0.0178	-0.0241
PMF1	0.0723	-0.4690	-0.1983	-0.1021	-0.0284	-0.0652
PMFBP1	0.0945	-0.2820	-0.0937	0.0181	-0.0286	-0.0053
PML	0.0326	-0.3157	-0.1415	-0.2078	0.0459	-0.0809
PNKP	-0.1131	-0.0191	-0.0661	0.0060	0.0025	0.0043

PNPT1	0.0335	0.0348	0.0341	-0.1109	0.0036	-0.0536
PON2	-0.0062	0.2294	0.1116	0.0333	0.0302	0.0317
PORCN	-0.2558	-0.1659	-0.2108	-0.3527	-0.1970	-0.2749
PPARGC1A	-0.0597	-0.2424	-0.1511	-0.3349	-0.0880	-0.2114
PPARGC1B	0.0085	-0.1903	-0.0909	-0.0950	-0.0272	-0.0611
PPAT	0.0238	-0.2849	-0.1306	-0.3071	-0.1739	-0.2405
PPFIA1	-0.0470	0.1401	0.0466	-0.2508	-0.1103	-0.1806
PPFIA2	-0.0221	-0.0846	-0.0533	-0.2301	-0.1586	-0.1943
PPFIA3	-0.0727	0.0396	-0.0166	-0.0451	-0.0907	-0.0679
PPFIA4	-0.0978	-0.0329	-0.0654	-0.0333	-0.0111	-0.0222
PPIF	0.0278	0.2928	0.1603	-0.1348	-0.0723	-0.1036
PPP1R15B	0.0375	0.1088	0.0732	0.1344	0.0359	0.0852
PPP2CB	-0.0156	0.1780	0.0812	-0.1765	0.0867	-0.0449
PRAF2	0.0127	-0.1924	-0.0899	-0.2099	0.0477	-0.0811
PRDM1	-0.3373	-0.4212	-0.3792	-0.3092	-0.3206	-0.3149
PRDX1	-0.0596	-0.1827	-0.1211	0.0350	-0.0936	-0.0293
PRDX2	-0.0379	0.5444	0.2532	-0.1228	-0.1676	-0.1452
PRDX3	-0.2612	-0.0483	-0.1547	-0.5025	-0.3443	-0.4234
PRDX4	0.0088	0.1377	0.0732	-0.2051	-0.0676	-0.1363
PRDX5	-0.0190	0.1886	0.0848	-0.2877	-0.0139	-0.1508
PRDX6	0.0524	0.2261	0.1392	-0.0578	-0.0701	-0.0639
PREX1	0.1945	0.1910	0.1928	-0.1110	0.0482	-0.0314
PRKAA1	-0.1229	0.1446	0.0109	-0.3074	-0.0683	-0.1878
PRKACB	-0.1792	-0.0023	-0.0908	-0.6185	-0.0060	-0.3122
PRKCA	-0.1323	0.0242	-0.0541	-0.2313	0.1656	-0.0329
PRKCB	-0.0228	0.0363	0.0067	0.0009	0.1134	0.0572
PRKD1	-0.1981	-0.0231	-0.1106	-0.6816	-0.2406	-0.4611
PRNP	0.2081	-0.0462	0.0809	-0.0122	-0.0541	-0.0331
PRODH	-0.2494	-0.2429	-0.2461	-0.2094	-0.2185	-0.2139
PRODH2	-0.0254	0.3464	0.1605	-0.2280	-0.1784	-0.2032
PROK2	0.0825	0.1648	0.1237	-0.0101	0.0645	0.0272
PSAP	0.0237	-0.0885	-0.0324	0.0739	0.0801	0.0770
PSEN1	-0.0727	-0.1850	-0.1288	-0.1322	-0.1014	-0.1168
PSIP1	0.0426	-0.2868	-0.1221	-0.1073	-0.0202	-0.0637
PSMA3	0.0146	0.0332	0.0239	0.0017	0.0047	0.0032
PSMB5	-0.0635	0.0116	-0.0259	-0.0246	-0.0144	-0.0195
PSMB9	-0.0163	-0.0304	-0.0234	-0.3539	0.1742	-0.0899
PSMD14	0.0032	0.0286	0.0159	-0.0068	-0.0050	-0.0059
PTEN	-0.1227	-0.1662	-0.1444	-0.1859	0.1400	-0.0230
PTGES3	-0.0238	-0.2908	-0.1573	-0.0316	0.0845	0.0265
PTGS1	-0.0509	-0.1482	-0.0996	-0.4030	-0.0585	-0.2308
PTGS2	-0.2239	-0.0563	-0.1401	-0.5747	-0.0105	-0.2926

PTK2B	-0.0654	-0.2281	-0.1468	-0.0997	0.0151	-0.0423
PTPRK	-0.0380	0.0987	0.0303	-0.0870	0.0261	-0.0305
PTPRN	-0.0050	0.1754	0.0852	-0.1188	0.0603	-0.0293
PVRIG	0.1666	-0.5591	-0.1963	-0.5987	-0.2924	-0.4455
PXDN	-0.0299	-0.0725	-0.0512	-0.1225	0.0445	-0.0390
PXDNL	-0.0236	0.0717	0.0240	-0.0633	0.0002	-0.0315
PXN	0.0486	0.0594	0.0540	0.0145	0.0955	0.0550
PYCR1	-0.0362	0.1595	0.0617	-0.0477	0.0254	-0.0112
PYCR2	0.1007	-0.0390	0.0309	0.0271	0.0247	0.0259
PYCR3	-0.0636	0.0405	-0.0116	-0.0152	0.1662	0.0755
QARS	0.0522	0.0622	0.0572	-0.3274	-0.0319	-0.1797
QPCT	-0.0093	0.0877	0.0392	-0.3709	-0.1722	-0.2716
QPCTL	0.1712	-0.0587	0.0563	-0.0286	-0.0246	-0.0266
QRICH1	0.0261	0.0267	0.0264	-0.0676	0.0188	-0.0244
QRSL1	-0.0227	-0.0701	-0.0464	-0.0967	-0.0541	-0.0754
RAB10	-0.0849	-0.2398	-0.1624	-0.0711	-0.0026	-0.0369
RAB2B	-0.0577	0.0892	0.0158	-0.1173	0.0301	-0.0436
RAB35	0.0365	0.0514	0.0439	-0.2374	0.1380	-0.0497
RAB3A	-0.1420	-0.2120	-0.1770	-0.3678	-0.2516	-0.3097
RAB3GAP1	0.1634	-0.2475	-0.0421	-0.2923	-0.1085	-0.2004
RAB4A	0.0243	0.0568	0.0405	-0.2045	0.0370	-0.0838
RAD51B	-0.0486	-0.1287	-0.0886	-0.5672	-0.1131	-0.3402
RAMP3	-0.0736	-0.2592	-0.1664	-0.2079	0.0734	-0.0673
RAP2A	0.0317	-0.2086	-0.0884	-0.1305	-0.1971	-0.1638
RAPGEF1	-0.0291	-0.2457	-0.1374	-0.1905	-0.0003	-0.0954
RAPSN	0.1636	0.4130	0.2883	-0.0806	0.1662	0.0428
RASAL3	0.1056	-0.0557	0.0249	-0.6220	-0.2763	-0.4491
RASGRF1	0.0281	0.0815	0.0548	-0.0113	0.0060	-0.0026
RASSF6	0.0566	-0.2829	-0.1132	-0.1730	0.2020	0.0145
RCAN1	0.0146	0.1456	0.0801	-0.0615	-0.0508	-0.0561
RELA	0.0319	0.0663	0.0491	0.0845	-0.0220	0.0312
RELN	0.1447	-0.0113	0.0667	-0.0200	0.0524	0.0162
RERE	0.0867	0.1515	0.1191	0.0455	0.0356	0.0405
RFFL	0.0142	-0.0321	-0.0089	0.0058	-0.0009	0.0024
RGS14	0.1042	0.1355	0.1199	-0.0995	0.0220	-0.0387
RHOB	0.0276	-0.0282	-0.0003	-0.0242	0.0847	0.0302
RIMKLA	0.0665	0.1384	0.1025	-0.0463	0.1191	0.0364
RIMKLB	0.1201	-0.1375	-0.0087	-0.4815	-0.1720	-0.3268
RIMS1	0.0082	-0.0168	-0.0043	-0.1293	0.0487	-0.0403
RIPK1	-0.0139	0.1175	0.0518	-0.2607	-0.1791	-0.2199
RNF213	0.0143	-0.2741	-0.1299	-0.1063	-0.0054	-0.0558
RNF220	-0.0656	-0.1938	-0.1297	-0.3821	-0.0750	-0.2286

RNF7	0.1428	-0.0656	0.0386	-0.0379	0.0192	-0.0093
RNH1	0.0046	0.2807	0.1427	-0.1986	-0.0137	-0.1062
ROCK1	-0.1058	-0.0253	-0.0655	-0.2318	0.0102	-0.1108
ROMO1	0.1032	0.0388	0.0710	-0.1897	0.0814	-0.0542
RRM2B	-0.2152	-0.1048	-0.1600	-0.6411	0.0375	-0.3018
RSPH6A	0.2147	0.3318	0.2732	-0.0945	0.0580	-0.0183
RXRA	0.0523	-0.1280	-0.0379	-0.0977	0.0656	-0.0161
S100A6	-0.1093	-0.0906	-0.1000	-0.0520	0.0234	-0.0143
SACM1L	0.0291	0.0275	0.0283	-0.1507	-0.1037	-0.1272
SAR1B	0.0354	-0.0323	0.0015	-0.1132	0.0039	-0.0547
SAT1	0.2508	0.0459	0.1484	-0.0715	0.0062	-0.0327
SCARA3	0.0034	-0.1470	-0.0718	-0.1948	-0.0233	-0.1091
SCRIB	0.1411	-0.0081	0.0665	-0.0166	-0.0174	-0.0170
SDC1	0.0519	0.0403	0.0461	-0.0385	-0.0035	-0.0210
SEC61B	0.0350	0.0866	0.0608	0.0394	0.0955	0.0675
SELENOK	-0.0839	-0.1111	-0.0975	0.0080	-0.0417	-0.0169
SELENOP	0.0601	0.5573	0.3087	-0.1069	-0.0043	-0.0556
SELENOS	0.0207	-0.0414	-0.0104	-0.0693	0.0541	-0.0076
SEMA7A	0.0338	-0.2120	-0.0891	-0.1959	0.0334	-0.0812
SERPINE1	0.1773	-0.0771	0.0501	-0.2446	0.0050	-0.1198
SETBP1	0.2468	-0.2353	0.0058	-0.2712	-0.1915	-0.2313
SFPQ	-0.0069	-0.0098	-0.0083	0.0047	-0.0152	-0.0052
SFTPFB	0.1524	0.1313	0.1418	-0.0892	0.0479	-0.0206
SFTPC	0.0326	-0.0064	0.0131	-0.0746	0.0392	-0.0177
SFTPD	0.0444	0.0497	0.0470	0.0009	0.0786	0.0398
SGK2	-0.0419	0.1260	0.0420	-0.0971	-0.0291	-0.0631
SGTA	-0.0233	-0.2787	-0.1510	-0.1279	0.0666	-0.0307
SH2D6	0.0140	-0.0950	-0.0405	-0.0799	0.0004	-0.0398
SH3BP5	0.3098	0.0332	0.1715	-0.0045	0.0195	0.0075
SHANK1	0.0324	-0.2188	-0.0932	-0.1343	0.0534	-0.0405
SHANK2	0.0437	-0.0551	-0.0057	-0.0810	-0.0078	-0.0444
SHANK3	0.1202	0.0144	0.0673	-0.3119	0.1157	-0.0981
SHC1	0.0754	-0.1738	-0.0492	0.0976	-0.0110	0.0433
SHISA9	0.0912	0.1020	0.0966	0.0088	0.0856	0.0472
SIN3A	0.0148	0.0613	0.0381	-0.0456	0.0580	0.0062
SIRT1	-0.0331	-0.2661	-0.1496	-0.1506	-0.0511	-0.1008
SIRT2	-0.0888	0.0493	-0.0197	-0.0667	0.0217	-0.0225
SIRT4	0.1293	-0.0680	0.0306	-0.3316	-0.2406	-0.2861
SLAMF7	0.1082	-0.0579	0.0252	-0.0280	-0.0265	-0.0272
SLC11A2	0.0621	0.1561	0.1091	-0.0350	0.0302	-0.0024
SLC14A2	-0.1148	0.0759	-0.0195	-0.1547	-0.0635	-0.1091
SLC17A7	0.0479	0.0057	0.0268	-0.0001	-0.0997	-0.0499

SLC1A1	-0.0322	-0.1763	-0.1042	-0.0527	0.0701	0.0087
SLC1A2	-0.0268	-0.0891	-0.0579	-0.1781	-0.1108	-0.1444
SLC1A3	-0.0381	-0.0495	-0.0438	-0.1238	-0.0453	-0.0846
SLC1A4	0.0050	0.0535	0.0293	-0.2069	0.0063	-0.1003
SLC1A5	-0.0623	0.1148	0.0263	0.0067	0.0242	0.0154
SLC1A6	0.0362	0.0520	0.0441	0.0128	0.0735	0.0432
SLC1A7	-0.0281	0.1043	0.0381	0.0254	0.0403	0.0329
SLC23A2	-0.1189	-0.2170	-0.1680	-0.6014	-0.2838	-0.4426
SLC25A12	-0.0848	0.1605	0.0378	-0.0924	-0.0212	-0.0568
SLC25A13	0.0974	-0.1993	-0.0510	0.0542	-0.0054	0.0244
SLC25A18	-0.0374	-0.3223	-0.1798	-0.0128	-0.0200	-0.0164
SLC25A25	0.0450	-0.0830	-0.0190	-0.2459	-0.0998	-0.1729
SLC38A2	-0.0999	-0.0138	-0.0569	-0.4317	0.0344	-0.1986
SLC38A3	-0.0456	-0.0784	-0.0620	-0.1802	-0.0973	-0.1387
SLC38A7	-0.0936	-0.0701	-0.0819	-0.4285	-0.1376	-0.2830
SLC3A2	-0.3881	-0.3198	-0.3540	-0.2450	-0.1908	-0.2179
SLC48A1	0.0437	-0.0371	0.0033	-0.1013	-0.0142	-0.0577
SLC7A11	-0.1610	-0.4225	-0.2917	-0.7319	-0.3906	-0.5613
SLC7A7	0.0654	-0.1355	-0.0350	-0.0107	0.0140	0.0016
SLC7A9	0.0241	-0.0036	0.0103	-0.0423	-0.0124	-0.0274
SLC8A1	-0.0330	-0.2016	-0.1173	-0.1129	0.0684	-0.0223
SLCO5A1	-0.0174	-0.2242	-0.1208	-0.1173	-0.0941	-0.1057
SLX4IP	-0.0124	-0.2356	-0.1240	-0.2563	-0.2607	-0.2585
SMAD3	0.0294	-0.0408	-0.0057	-0.0220	0.0434	0.0107
SNAP25	-0.0394	0.0219	-0.0087	-0.0611	0.3844	0.1617
SNCA	0.1299	-0.0317	0.0491	0.0877	0.3535	0.2206
SOD1	0.0551	-0.1473	-0.0461	0.0827	0.1431	0.1129
SOD2	-0.0188	0.0464	0.0138	0.0101	0.0380	0.0241
SOD3	0.0899	-0.0376	0.0262	-0.0621	0.1170	0.0274
SORBS2	0.0628	-0.3226	-0.1299	-0.1903	0.0559	-0.0672
SPRY3	-0.0677	-0.0217	-0.0447	0.0711	0.0247	0.0479
SQSTM1	0.2202	-0.0886	0.0658	-0.0037	0.0278	0.0121
SRC	0.0546	0.0349	0.0447	-0.0621	0.0544	-0.0039
SREK1	-0.0349	-0.1429	-0.0889	-0.2990	-0.0711	-0.1851
SRSF5	0.0986	-0.1088	-0.0051	-0.0845	0.0126	-0.0360
SRXN1	-0.1521	0.1268	-0.0127	-0.1010	-0.0235	-0.0623
SSH1	0.0144	0.1676	0.0910	-0.2408	-0.1323	-0.1866
SSTR1	-0.0988	0.0571	-0.0208	-0.0482	-0.0254	-0.0368
ST6GALNAC1	0.0175	0.0584	0.0379	-0.0364	0.0270	-0.0047
STARD13	-0.1038	0.0498	-0.0270	-0.5072	-0.0434	-0.2753
STH	0.1800	0.0585	0.1193	-0.1466	0.0400	-0.0533
STK24	-0.0618	0.1279	0.0330	0.0185	0.0209	0.0197

STK25	-0.0675	0.2686	0.1006	-0.1304	0.0818	-0.0243
STX1A	0.0762	0.0433	0.0598	-0.1909	0.0818	-0.0545
STX3	-0.0195	-0.0317	-0.0256	-0.1473	-0.0253	-0.0863
STXBP1	0.0863	0.1615	0.1239	-0.0400	0.0497	0.0048
STXBP4	0.0910	-0.2008	-0.0549	-0.4653	-0.2574	-0.3613
SULT1A3	-0.0509	-0.1113	-0.0811	-0.1126	0.0848	-0.0139
SYNDIG1	-0.2053	-0.1491	-0.1772	-0.5043	-0.3825	-0.4434
SYT1	-0.0462	0.1848	0.0693	-0.3319	-0.1503	-0.2411
SYT4	0.1567	0.0599	0.1083	-0.0616	0.0908	0.0146
TACR1	-0.0725	0.1389	0.0332	-0.1487	-0.0033	-0.0760
TAF15	-0.0666	-0.0656	-0.0661	0.1188	-0.0425	0.0382
TANK	-0.0198	0.0590	0.0196	0.0054	0.0608	0.0331
TAT	-0.1122	0.2176	0.0527	-0.3858	-0.0744	-0.2301
TBL1X	0.0538	0.0473	0.0506	-0.0207	0.0759	0.0276
TBXAS1	-0.0644	-0.3588	-0.2116	-0.4142	-0.2290	-0.3216
TEC	-0.0186	0.1415	0.0615	-0.1089	0.0299	-0.0395
TFE3	0.0346	-0.0807	-0.0231	0.1097	0.0010	0.0553
TGM1	-0.0967	-0.0422	-0.0694	-0.0852	-0.0562	-0.0707
TGM2	-0.0564	0.2131	0.0784	-0.6793	-0.1303	-0.4048
TGM3	0.0139	0.0217	0.0178	0.0168	0.0251	0.0210
TGM4	0.0515	0.0250	0.0382	-0.1373	0.0088	-0.0643
TGM5	-0.0258	-0.0750	-0.0504	-0.0959	-0.0060	-0.0509
TGM6	-0.1379	0.1285	-0.0047	-0.3247	-0.1048	-0.2148
TGM7	0.0616	0.0661	0.0639	-0.0917	0.0345	-0.0286
THBS1	0.1952	-0.0413	0.0770	-0.1886	-0.0289	-0.1087
TIAM1	0.0051	0.1193	0.0622	-0.0379	0.0525	0.0073
TKT	-0.0264	0.2773	0.1255	-0.1538	0.0648	-0.0445
TLNRD1	0.0701	0.0261	0.0481	-0.0397	-0.0132	-0.0264
TLR4	0.0006	0.0750	0.0378	-0.0135	-0.0272	-0.0204
TMBIM6	-0.0118	0.0647	0.0264	-0.1646	0.0683	-0.0481
TMEM121	0.0286	-0.2135	-0.0925	-0.0035	-0.0411	-0.0223
TMEM140	0.0580	0.3951	0.2266	-0.0425	0.0321	-0.0052
TMEM161A	0.0186	-0.1659	-0.0736	-0.2645	0.0074	-0.1286
TMEM245	0.0226	0.0143	0.0185	-0.0960	-0.1175	-0.1067
TMTC3	0.0975	0.1128	0.1052	-0.2127	-0.0314	-0.1220
TNFAIP3	0.1156	-0.0672	0.0242	-0.0432	-0.0273	-0.0353
TNFRSF1A	-0.0178	-0.0081	-0.0129	-0.1616	-0.0859	-0.1237
TNFRSF8	-0.0351	-0.0787	-0.0569	-0.0096	0.0096	0.0000
TNFSF14	0.1109	-0.0736	0.0187	-0.1036	0.1168	0.0066
TP53	0.0281	-0.0405	-0.0062	-0.1021	-0.0353	-0.0687
TP53I3	-0.0222	0.0532	0.0155	-0.0373	-0.0344	-0.0359
TPM1	0.0396	-0.0455	-0.0029	-0.0723	0.0640	-0.0042

TPM4	-0.1422	-0.5025	-0.3223	-0.4220	-0.1297	-0.2759
TPO	0.0277	-0.0206	0.0035	-0.0641	-0.0517	-0.0579
TRAF2	0.0499	-0.0163	0.0168	-0.0387	-0.0108	-0.0247
TRAP1	-0.0850	-0.2171	-0.1511	-0.1644	0.0077	-0.0784
TRAPPC9	0.1116	-0.0737	0.0189	-0.0118	0.0447	0.0164
TRH	0.0095	0.0996	0.0545	-0.0639	0.0568	-0.0036
TRIM56	0.0459	-0.4040	-0.1790	-1.1485	-0.3098	-0.7292
TRMT112	0.1526	-0.0403	0.0561	-0.1946	-0.0227	-0.1086
TRPA1	-0.0121	-0.0130	-0.0126	-0.0539	0.2673	0.1067
TRPM1	-0.0522	0.0580	0.0029	-0.1402	0.1849	0.0223
TRPM2	-0.0157	0.0573	0.0208	-0.5723	-0.1280	-0.3502
TRPV1	0.0531	0.1904	0.1218	-0.2931	-0.0302	-0.1616
TSC22D1	-0.0281	-0.0095	-0.0188	-0.1811	-0.0552	-0.1181
TSPOAP1	0.1694	0.0026	0.0860	-0.0549	-0.0088	-0.0319
TTN	-0.0170	0.0380	0.0105	-0.2159	-0.0185	-0.1172
TXN	0.0297	-0.0509	-0.0106	-0.0729	0.0825	0.0048
TXN2	0.0367	0.0999	0.0683	-0.0900	0.0218	-0.0341
TXNIP	0.0969	-0.0573	0.0198	-0.0963	0.0653	-0.0155
TXNRD1	0.0297	-0.0003	0.0147	-0.1369	0.0000	-0.0685
TXNRD2	0.0459	0.1451	0.0955	-0.1478	0.1112	-0.0183
UACA	-0.0441	-0.1220	-0.0831	-0.2901	-0.1189	-0.2045
UBC	0.0074	0.0352	0.0213	-0.0161	-0.0015	-0.0088
UBE2E2	-0.0847	-0.2016	-0.1431	-0.3358	-0.0265	-0.1812
UBXN1	-0.0175	-0.0621	-0.0398	-0.1886	-0.1222	-0.1554
UCN	0.0386	-0.0213	0.0087	0.0233	-0.0121	0.0056
UCP2	-0.0082	-0.0703	-0.0393	-0.2925	-0.0743	-0.1834
UCP3	-0.0602	-0.3588	-0.2095	-0.1652	-0.1252	-0.1452
UFL1	0.0071	0.1158	0.0614	-0.1655	0.0798	-0.0429
UGT1A1	0.1347	0.0588	0.0968	0.1212	0.1116	0.1164
UGT1A6	-0.0055	-0.1533	-0.0794	-0.0539	0.0403	-0.0068
UGT2B7	-0.0267	-0.0891	-0.0579	-0.0548	0.0726	0.0089
UNC13A	0.0511	-0.0197	0.0157	-0.1072	0.1037	-0.0017
UNC13B	0.1440	-0.4618	-0.1589	-0.2386	0.0671	-0.0858
UNKL	0.0338	-0.1006	-0.0334	-0.0807	0.0599	-0.0104
UROC1	-0.0448	-0.0113	-0.0281	-0.1386	-0.0320	-0.0853
USP13	0.0811	-0.1977	-0.0583	-0.4118	0.0820	-0.1649
USP33	0.1502	-0.2489	-0.0494	0.0029	-0.0651	-0.0311
USP47	0.0778	-0.0976	-0.0099	-0.0189	0.0292	0.0052
VAMP2	-0.0902	-0.1592	-0.1247	-0.0811	-0.0075	-0.0443
VCP	-0.0202	0.0459	0.0129	-0.0237	0.0485	0.0124
VEZF1	-0.0460	-0.2331	-0.1395	-0.2266	-0.0093	-0.1179
VNN1	-0.0923	0.0106	-0.0409	-0.2157	-0.1025	-0.1591

VRK2	-0.0663	-0.1505	-0.1084	-0.3565	-0.1623	-0.2594
VWC2	-0.0253	-0.0132	-0.0193	0.0220	0.0074	0.0147
VWC2L	0.0611	-0.1409	-0.0399	-0.0654	-0.0149	-0.0401
WDR81	0.0697	-0.1943	-0.0623	-0.2301	0.0029	-0.1136
WDYHV1	0.1330	-0.3963	-0.1317	-0.2095	0.1010	-0.0543
WIPI2	0.1557	-0.0804	0.0377	-0.1150	0.0772	-0.0189
WRN	-0.0930	0.1218	0.0144	-0.0465	0.0553	0.0044
XPA	-0.0903	0.0157	-0.0373	-0.3113	-0.0424	-0.1768
YKT6	0.0346	-0.1356	-0.0505	-0.2711	0.1226	-0.0743
YWHAЕ	-0.0694	-0.0709	-0.0701	-0.2017	-0.0251	-0.1134
YWHAG	0.0701	-0.0357	0.0172	0.0038	0.0311	0.0175
ZAP70	-0.0040	-0.1954	-0.0997	-0.3771	0.0091	-0.1840
ZFAT	0.0561	0.0144	0.0353	-0.5653	-0.3320	-0.4486
ZMYND8	-0.0289	0.2599	0.1155	-0.0850	-0.0688	-0.0769
ZNF148	-0.1376	0.0189	-0.0593	-0.4559	-0.0261	-0.2410
ZNF3	0.0848	-0.0534	0.0157	-0.1344	-0.0562	-0.0953
ZNF469	-0.0574	0.0123	-0.0226	-0.1973	-0.1677	-0.1825
ZNF622	0.1238	-0.0954	0.0142	-0.5659	-0.1548	-0.3603

CHAPTER 4 DETERMINING THE MECHANISM OF ACTION OF EPRENETAPOPT

4.1 Preface

The aim of this chapter is to determine the mechanism of action (MoA) of eprenetapopt through an unbiased strategy. Following on from the literature discussed in **Chapter 1** and the data presented in **Chapter 2** and **Chapter 3**, which highlight the contentious nature of the MoA of eprenetapopt, here this is re-examined in detail. Through the application of unbiased technologies, including genome-wide CRISPR perturbation screening, proteomics and metabolomics approaches, the MoA of eprenetapopt is revealed.

This chapter will be submitted to a journal in the coming weeks, as per the following reference:

- **Fujihara KM**, Zhang BZ, Jackson TD, Ogunkola MO, Nijagal B, Sallman DA, Ang CS, Nikolic I, Kearney CJ, Hogg SJ, Cabalag CS, Sutton VR, Watt S, Fujihara AT, Trapani JA, Simpson KJ, Stojanovski D, Leimkühler S, Haupt S, Phillips WA, Clemons NJ. *Eprenetapopt (APR-246) triggers ferroptosis and inhibits iron-sulfur cluster biogenesis*.

The current version (submitted to Cancer Cell, October 5th, 2021) of this manuscript and associated supplementary information are presented in Chapter 4 of this thesis.

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Eprenetapopt (APR-246) triggers ferroptosis and inhibits iron-sulfur cluster biogenesis

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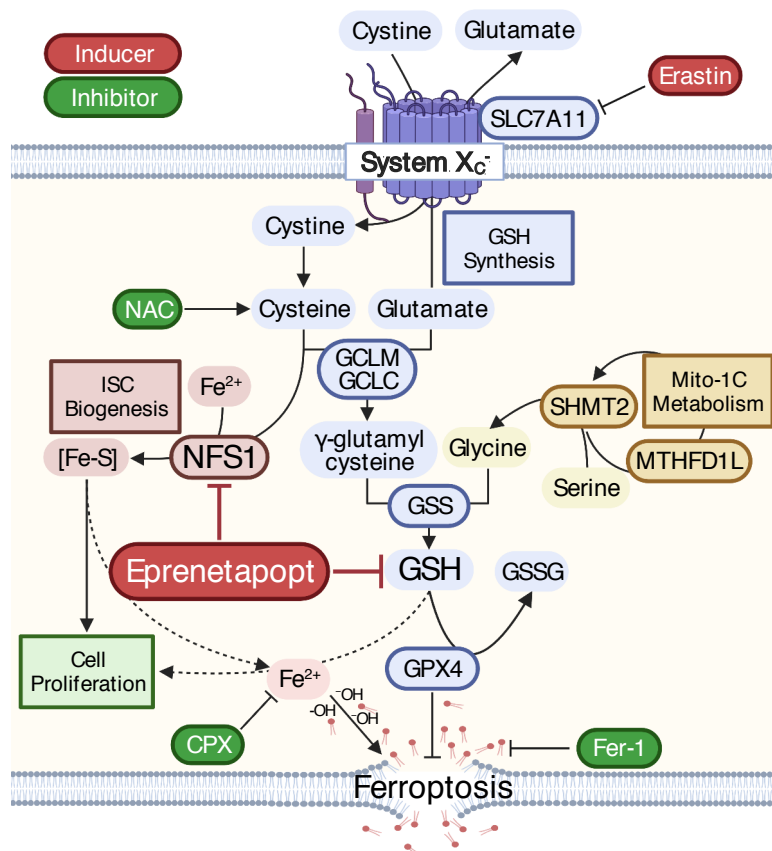
HIGHLIGHTS

- Eprenetapopt induces GSH depletion in cancer cells through an increase in the turnover of GSH, triggering ferroptosis.
- Deficiency in mito-1C enzymes and dietary SG restriction increases the potency of eprenetapopt.
- Eprenetapopt inhibits ISC biogenesis, potentiating ferroptosis and limiting proliferation.
- Clinical utility of eprenetapopt should be expanded to include MDS with ring sideroblasts.

SUMMARY

The mechanism of action (MoA) of eprenetapopt (APR-246, PRIMA-1^{MET}) as an anticancer agent remains unresolved, although the clinical development of eprenetapopt focuses on its reported MoA as a mutant-p53 (mut-p53) reactivator. Employing unbiased approaches, this study demonstrates that eprenetapopt depletes the cellular antioxidant glutathione (GSH) by increasing its turnover, triggering a non-apoptotic, iron-dependent form of cell death known as ferroptosis. Deficiency in genes responsible for supplying cancer cells with the substrates for *de novo* GSH synthesis (*SLC7A11*, *SHMT2* and *MTHFD1L*), as well as the enzymes required to synthesise GSH (*GCLC* and *GCLM*), augment the activity of eprenetapopt. Eprenetapopt also inhibits iron-sulfur cluster (ISC) biogenesis by limiting the cysteine desulfurase activity of NFS1, which potentiates ferroptosis and may restrict cellular proliferation. Evaluation of patient data from clinical investigations of eprenetapopt in *TP53*-mutated myeloid dysplastic syndromes (MDS) indicates that eprenetapopt may provide a greater therapeutic benefit to patients with MDS with ring sideroblasts (MDS-RS). These findings reframe the clinical utility of eprenetapopt from a mut-p53 reactivator to a first-in-class ferroptosis inducer.

GRAPHICAL ABSTRACT



KEYWORDS

Eprenetapopt, APR-246, PRIMA-1^{MET}, glutathione, ferroptosis, iron-sulfur cluster, mitochondrial one-carbon, CRISPR screening, mechanism of action, myeloid dysplastic syndrome, esophageal cancer, SLC7A11, p53.

SIGNIFICANCE

Currently, eprenetapopt is marketed as a mut-p53 reactivator with patient selection for clinical trials relying on *TP53* mutation status. In the context of MDS, *TP53* mutations occur in only ~7% of patients. We show that eprenetapopt is a ferroptosis inducer that also inhibits ISC biogenesis, two processes dependent on iron. Importantly, *TP53* mutations rarely co-occur with *SF3B1* mutations, which most frequently occur in MDS-RS. Therefore, restricting eprenetapopt to *TP53*-mutated MDS patients may be obscuring the therapeutic benefit eprenetapopt could provide to all MDS patients, especially MDS-RS.

INTRODUCTION

TP53 mutation occurs in ~50% of all cancers and overall is associated with poor survival (304), providing a strong impetus for the development of mut-p53 targeted therapeutics. Eprenetapopt (APR-246, PRIMA-1^{MET}) was developed as a mut-p53 targeted therapeutic and is currently under clinical investigation in *TP53*-mutated MDS and acute myeloid leukemia (AML) (174). Recent results from two phase II clinical trials in *TP53*-mutated MDS, combining eprenetapopt with standard-of-care azacitidine, reported rates of complete remission (CR) of ~50% (257,258). Furthermore, apparent improvements in median overall survival (OS) compared to previous studies of azacitidine alone were reported in *TP53*-mutated MDS (257,258,305). However, early results from the phase III randomised clinical trial in *TP53*-mutated MDS failed to meet the primary endpoint of CR rate (33.3% in eprenetapopt + azacitidine, 22.4% in azacitidine alone, $P=0.13$) (306).

The therapeutic effect of eprenetapopt has been understood to involve the reactivation of wild-type p53 activity to induce apoptosis through covalent modification of cysteine residues in the core domain of mut-p53 protein (179,239). The specific MoA of eprenetapopt, however, remains to be thoroughly defined. Recently, we demonstrated that the expression of *SLC7A11*, which encodes the functional subunit of the cystine-glutamate antiporter, system x_c^- , is the major determinant of response to eprenetapopt across cancer lineages, and neither *TP53* mutation status nor p53 protein level were associated with sensitivity (307). The import of cystine through system x_c^- provides the predominant source of intracellular cysteine, which is the rate limiting substrate required for *de novo* synthesis of the cellular antioxidant GSH (246). Eprenetapopt and its structural analogue APR-017 (also known as PRIMA-1) are prodrugs that

undergo spontaneous conversion to the active compound, 2-methylene 3-quinuclidinone (MQ), releasing formaldehyde in the process (179). MQ is a thiol-reactive Michael acceptor that forms reversible covalent bonds with thiol-containing molecules including cysteine and the GSH (143,308). As a result, the anticancer effects of eprenetapopt have been linked to GSH depletion and inhibition of the thioredoxin antioxidant system (143,214,251,308). Notably, we previously demonstrated that mut-p53 protein accumulation drives down the expression of *SLC7A11* through the inhibition of the transcription factor NRF2 (encoded by *NFE2L2*) – providing an explanation for the selectivity of eprenetapopt against mut-p53 cancer cells under some conditions (143). However, to date, limited unbiased examinations of the MoA of eprenetapopt have been attempted, and the predominant focus of prior investigations has been through the lens of eprenetapopt as a mut-p53 reactivator *a priori*.

In this study, we undertake a systematic, unbiased approach to probe the MoA of eprenetapopt, incorporating genome-wide CRISPR perturbation screening and metabolite and proteomic profiling in cancer cells with pan-cancer gene dependency and cell line sensitivity datasets. In this way, we provide a compendium of functional genomic and molecular detail into the MoA of eprenetapopt. In particular, CRISPR perturbation screens reveal that loss of mitochondrial one-carbon (mito-1C) metabolism enzymes, SHMT2 and MTHFD1L, sensitises cancer cells to eprenetapopt – an insight that can be exploited *in vivo* to sensitise tumours to eprenetapopt with serine and glycine (SG) dietary restriction. Mechanistically, we find that eprenetapopt induces GSH depletion, triggering iron-dependent, non-apoptotic ferroptosis and limits cellular proliferation, in part, by inhibiting iron-sulfur cluster (ISC) biogenesis. Clinically,

these insights provide a rational avenue for the expansion of eprenetapopt utility in MDS to include patient selection based on the presence of ring sideroblasts, rather than reliance on *TP53* mutation status.

MATERIALS AND METHODS

Compounds and reagents

Eprenetapopt was provided by Aprea Therapeutics Inc. (Boston, Massachusetts, USA). Elesclomol, erastin, staurosporine (STS) and SNS-032 were from Selleckchem (Houston, Texas, USA). 2-methylene 3-quinuclidinone (MQ), sodium formate, glycine, serine, N-acetyl-cysteine (NAC), ferrostatin-1 (Fer-1), zVAD-FMK and ciclopirox olamine (CPX) were from Sigma Aldrich (St. Louis, Missouri, USA).

Cell cultures

NCI-H1299 (H1299) (RRID:CVCL_0060), OACM5.1 (RRID:CVCL_1842), FLO-1 (RRID:CVCL_2045), and HEK-293T (293T) (RRID:CVCL_0063) were from ATCC. K562 (RRID:CVCL_0002) were provided by Prof Ricky Johnstone (Peter MacCallum Cancer Centre; PMCC). All cells were maintained at 37°C with 5% CO₂ and their identities were authenticated by short tandem repeat analysis using the PowerPlex 16 genotyping system (Promega) and confirmed mycoplasma free by PCR every two months (Cerberus Sciences). Cells were thawed from early passage stocks and passaged for no more than 4 months. Unless otherwise specified, all culture media contained 10% fetal bovine serum (FBS) supplemented with 50 U ml⁻¹ penicillin and 50 mg ml⁻¹ streptomycin (Life Technologies). MC38, FLO-1 and HEK-293T cells were grown in DMEM containing 2.5 mM L-glutamine and 4.5 g l⁻¹ D-glucose (Life Technologies). MC38 cells were maintained in 10% CO₂. H1299, OACM5.1 and K562 were grown in RPMI 1640 medium containing 2.5 mM L-glutamine. K562 were supplemented with 1× GlutaMAX (Life Technologies).

CRISPR screening

OACM5.1 cells were engineered to express the Cas9 endonuclease by transduction with the FUCas9Cherry vector (Addgene, #70182) and subsequent selection for mCherry-positive cells, or Cas9 endonuclease dead (dCas9) by transduction with the dCas9-VP64_Blast vector (Addgene, #61425) and subsequent selection for blasticidin resistance (10 µg/mL). For genome-wide CRISPR knockout (CRISPRko) and CRISPR activation (CRISPRa) screening, OACM5.1 cells expressing Cas9 or dCas9-VP64 were transduced with a genome-wide sgRNA Brunello (76,441 sgRNAs, Addgene, #73178 (309)) or Calabrese (56,762 sgRNAs, Addgene, #92379 (310)) library, respectively, at a MOI of 0.3, aiming for 500-fold starting representation of each guide. Puromycin selection (1 µg/mL) was applied for 7 days and surviving cells were subsequently split into relevant treatment conditions (CRISPRko: vehicle v 10 µM eprenetapopt; CRISPRa: vehicle v 15 µM eprenetapopt, see **Figure S4.1** for effect of eprenetapopt on cell number). Cells were passaged every four days in T175 flasks with 2×10^6 cells per flask for a total of 8 days for the CRISPRko screen and 28 days for the CRISPRa screen. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) and sequencing libraries were generated by PCR as previously detailed (311). The libraries were sequenced on a NextSeq 500 (Illumina) to generate 75 base pair single-end reads, which were demultiplexed with Bcl2fastq (v2.17.1.14). The sequencing reads were subsequently trimmed with Cutadapt (v1.7) to remove sgRNA vector derived sequences, then sgRNA reads were counted and the distribution analysed using MAGeCK (v0.5.8) using read depth (--norm-method total) normalisation. MAGeCK score was determined by $-\log_{10}(\text{neg } P\text{-value}) + \log_{10}(\text{pos } P\text{-value})$ and datasets were visualised on GraphPad Prism (v9.0). Validation experiments were performed on polyclonal OACM5.1 cells expressing individual sgRNAs (**Table S4.1**) following selection with puromycin. Formate and glycine rescue

experiments were performed under conditions matching the CRISPR screening procedure scaled down to T25 flask. SG auxotrophy experiments were performed in 96-well plates utilizing dialysed FBS and media reconstituted without SG.

DepMap Analysis

Cancer cell line gene dependency data (DepMap Public 21Q1 Release) and APR-017 cancer cell line sensitivity data (CTRPv2.0) were obtained from the DepMap web-portal (www.depmap.org) and correlation analyses were performed in RStudio (v1.1.4) using the *cor.test* function. Correlation strengths were determined by adjusting the Pearson correlations with a Fisher's z-transformation in order to account for the differences in cell lines analysed.

Metabolomics profiling

OACM5.1 cells (3.3×10^5) were plated in 6-well plates and allowed to adhere for 24 h and subsequently treated with vehicle or 50 μ M eprenetapopt for 12 h in fresh media (6 replicates per condition). Media was aspirated and cells washed with water (at 37°C) for 10 sec then snap-frozen by the addition of liquid nitrogen directly to tissue culture plates. Metabolite extraction was performed with ice-cold methanol/chloroform (9:1, v/v) solvent containing ^{13}C -sorbitol, $^{13}\text{C},^{15}\text{N}$ -valine, $^{13}\text{C},^{15}\text{N}$ -AMP, $^{13}\text{C},^{15}\text{N}$ -UMP as internal standards. Metabolite-containing supernatants were collected following centrifugation at 16,000g for 5 min to pellet insoluble material and debris.

Metabolite analysis was performed using a Agilent 6520 series quadrupole time-of-flight mass spectrometer (QTOF MS) (Agilent Technologies) with chromatographic separation on an Agilent 1200 series HPLC system (Agilent Technologies). Metabolite

separation was performed on a Merck SeQuant ZIC®-HILIC column (150 mm × 2.1 mm, 5 µm particle size) using Solvent A: 20 mM ammonium carbonate (pH 9.0; Sigma-Aldrich) and Solvent B: 100 % acetonitrile. The gradients used were as follows: negative mode – time = 0 min, 90% B; t = 0.5 min, 90% B; t = 12 min, 40 % B; t = 14 min, 40 % B; t = 15 min, 5%, t = 18 min, 5 % B; t = 19 min, 90 %. Samples were stored in the autosampler at 4°C and 7 µl injected onto the column, which was maintained at 40°C, with a solvent flow rate of 250 µl/min.

Mass spectrometry analysis was performed on an Agilent 6545 series quadrupole time-of-flight mass spectrometer (QTOF MS) (Agilent Technologies) as described previously (312). Negative mode LCMS data was collected in centroid mode with a scan range of 50-1700 m/z and an acquisition rate of 1.2 spectra/sec. Samples were analysed in the same analytical batch and randomised with a QC every 5 samples. Thirteen mixtures of authentic standards (550 metabolites) were also run to generate the library for targeted analysis. Level 1 Metabolite identification (according to the Metabolite Standard Initiative (313)) was based on matching accurate mass, retention time and MS/MS spectra to the 550 authentic standards in the MA in-house library. Metabolite abundance based on area under the curve (AUC) were obtained using Agilent Masshunter Quantitative Analysis B 0.7.00. Statistical analysis was performed applying the web-based platform MetaboAnalyst applying no missing value imputation, normalisation to median peak area and no scaling or transformation (314).

For isotopic tracing analysis, OACM5.1 cells were prepared in the same fashion as steady state experiments, however cells were cultured in cystine-free RPMI-1640 media (Sigma Aldrich) with 10% dialysed FBS supplemented with 208 µM L-[3',3'-

$^{13}\text{C}_2$]-cystine or L- ^{12}C -cystine (3 replicates per condition). For enhanced resolution, identification and detection of the isotopic species metabolite analysis was performed on a Vanquish™ Horizon UHPLC System, integrated biocompatible system coupled to Thermo Scientific Orbitrap ID-X Tribrid mass spectrometer (MS) (ThermoFisher Scientific, San Jose). The polar metabolite chromatographic separation was performed on the Metabolomics Australia Merck SeQuant ZIC®-HILIC column (150 mm $\times$ 4.6 mm, 5 μm particle size) method previously with modifications.

In short, the mobile phase of A: 20 mM ammonium carbonate (pH 9.0; Sigma-Aldrich) and B: 100 % acetonitrile was run at a flow rate of 300 $\mu\text{l}/\text{min}$ with the following gradient: time = 0.0 min, 80% B; t = 0.5 min, 80% B; t = 15.5 min, 50 % B; t = 17.5 min, 30 % B; t = 18.5 min, 5%, t = 21.0 min, 5 % B; t = 28 min, 80 %. Samples were stored in the autosampler at 4°C and 7 μl injected onto the column maintained at 30°C. Full scan ($\text{MSn} = 1$) Orbitrap ID-X data was acquired in positive and negative ionisation modes separately within the mass range of 70–1300 m/z at resolution 240,000. Ion source parameters were set as follows: Sheath gas = 40 arbitrary units, Aux gas = 10 arbitrary units, Sweep gas = 1 arbitrary unit, Spray Voltage = 3.5kV (positive ion)/ 3.2kV (negative ion), Capillary temp. = 300 °C, and Vaporizer temp = 400 °C. Metabolite and isotope abundance are based on area under the curve (AUC) deconvoluted from the labeling data using the 'detect and analyse labelled compounds' module and in-house mass list matching in Compound Discoverer 3.2. A 3 ppm mass error and a retention time range of 0.3 s in feature grouping and molecular formula and metabolite matching was applied. All molecules were annotated according to guidelines for reporting of chemical analysis results as proposed in Metabolomics Standards Initiative level 1 (313).

Quantitative proteomics

OACM5.1 cells (5.0×10^6) were plated in 15 cm plates and allowed to adhere for 24h and subsequently treated with vehicle or 50 μ M eprenetapopt for 12 h in fresh media (4 replicates per condition). Media was aspirated then cells washed with ice-cold PBS and lysed at 4°C in RIPA buffer (1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 1 mM sodium pyrophosphate in PBS) mixed with protease and phosphatase inhibitors (Roche). Debris was pelleted by centrifugation at 11,000g for 10 min. Overnight acetone precipitation of proteins was performed at -20°C to purify the samples. Proteins were resuspended in 8 M urea in 50 mM triethylammonium bicarbonate (pH 8), reduced with 10 mM Tris(2-carboxyethyl)phosphine (TCEP, Sigma Aldrich), alkylated with 55 mM iodoacetamide (IAA, Sigma Aldrich) and subjected to tryptic digest with Pierce Trypsin Protease MS-Grade (ThermoFisher Scientific) overnight at 37°C (1:50 trypsin to protein). Formic acid (1% v/v) was added to acidify the solution before loading onto Oasis cartridges (Waters). Peptides were eluted in 80% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid, freeze dried and stored at -80°C until analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

LC-MS/MS was carried out on a QExactive plus Orbitrap mass spectrometer (ThermoFisher Scientific) with a nanoESI interface in conjunction with an Ultimate 3000 RSLC nanoHPLC (Dionex Ultimate 3000). The LC system was equipped with an Acclaim Pepmap nano-trap column (Dionex-C18, 100 Å, 75 μ m x 2 cm) and an Acclaim Pepmap RSLC analytical column (Dionex-C18, 100 Å, 75 μ m x 50 cm). The tryptic peptides were injected to the enrichment column at an isocratic flow of 5 μ L/min

of 2% v/v CH₃CN containing 0.1% v/v formic acid for 5 min applied before the enrichment column was switched in-line with the analytical column. The eluents were 5% DMSO in 0.1% v/v formic acid (solvent A) and 5% DMSO in 100% v/v CH₃CN and 0.1% v/v formic acid (solvent B). The flow gradient was (i) 0-6min at 3% solvent B, (ii) 6-95 min, 3-22% solvent B (iii) 95-105 min 22-40% solvent B (iv) 105-110 min, 40-80% solvent B (v) 110-115 min, 80-80% solvent B (vi) 115-117 min, 80-3% and equilibrated at 3% solvent B for 10 min before the next sample injection. The QExactive plus mass spectrometer was operated in the data-dependent mode, whereby full MS₁ spectra were acquired in positive mode, 70 000 resolution, automatic target control target of 3×10^6 and maximum injection time of 50 ms. Fifteen of the most intense peptide ions with charge states ≥ 2 and intensity threshold of 1.7×10^4 were isolated for MSMS. The isolation window was set at 1.2 m/z and precursors fragmented using normalised collision energy of 30, 17 500 resolution, automatic target control target of 1×10^5 and maximum injection time of 100 ms. Dynamic exclusion was set to be 30 sec.

Xenograft models and treatments

All animal experiments were approved by the PMCC Animal Experimentation Ethics Committee and undertaken in accordance with the National Health and Medical Research Council Australian Code of Practise for the Care and Use of Animals for Scientific Purposes. For FLO-1 LM cell line xenografts (315), 5×10^6 cells suspended in 100 μ L of 1:1 PBS and Matrigel (BD Bioscience) were subcutaneously injected into the right flank of ~6 week-old female NOD-SCID IL-2R γ^{KO} (NSG) mice. Patient-derived xenografts (PDX) were established and implanted into a dorsal intramuscular pocket of NSG mice as previously described (316). Mice were randomised to serine/glycine (SG)-deplete or control chow *ad libitum* (AIN93G rodent diet, Specialty Feeds,

Australia) and dosed with 100 mg/kg eprenetapopt or 0.9% saline, intraperitoneally injected daily, once tumours reached 100 mm³. Tumour volume was assessed blinded to treatment group with caliper measurements every 3-4 days and calculated using the formula $(\text{length} \times \text{weight}^2)/2$. Metastatic spread was determined by bioluminescence imaging as previously described (315) involving weekly monitoring using a Xenogen IVIS 100 Imaging System (Caliper Life Science). At experimental endpoint (tumour volume > 1400 mm³), the whole mouse and its organs were imaged to determine the extent and distribution of metastases. Tumours were weighed and tumour growth inhibition was calculated with the formula $[1-(T_f-T_i)/\text{mean}(C_f-C_i)] \times 100$, where T_f , T_i and C_f , C_i represents final (f) and initial (i) tumour volume of drug treated (T) and control (C) animals respectively.

Cell death

For ferroptosis rescue experiments, cells were co-treated with rescue compounds (Fer-1, CPX, zVAD-FMK, NAC) in media containing 5 µg/mL propidium iodine (PI) in 96-well plates (FLO-1: 5,000 cells/well, H1299: 3,000 cells/well). Cell death and confluency were quantified utilizing an IncuCyte FLR (Essen BioSciences) imaging phase and red channels. Percent (%) cell death was determined by dividing the % red confluency by the % phase confluency. Note, 100% cell death was ascribed if % red confluency was greater than phase confluency.

Cell viability

For dose-response assays, 10-point log₂ serial dilutions of eprenetapopt were added to 96-well plates containing cells (OACM5.1: 10,000 cells/well, Mc38: 10,000 cells/well, K562: 5,000). After 72 h incubation, cell viability was determined using

AlamarBlue reagent (Life Technologies) and fluorescence was read at 550 nm/590 nm on a Cytation 3 Imaging Reader (BioTek).

Immunoblot

As per established protocols (143,224), cells were lysed at 4°C in RIPA buffer (1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 1 mM sodium pyrophosphate in PBS) mixed with protease and phosphatase inhibitors (Roche). Equal amounts of protein were boiled, resolved by SDS-PAGE and transferred to PVDF membranes. For total protein glutathionylation detection, lysates were not boiled and were resolved under non-reducing conditions. Membranes were incubated in blocking buffer (5% skim milk in 0.05% TBS-T) for 1 h at room temperature and probed overnight in primary antibody at 4°C. Blots were washed thrice in 0.05% TBS-T, followed by incubation with peroxidase-conjugated secondary antibody (Dako) for 1 h at room temperature. Protein levels were detected using Amersham ECL Western Blotting Detection reagents (GE Life Sciences) or ECL Plus Western blotting substrate kit (ThermoFisher Scientific). Antibodies are detailed in **Table S4.2**.

NFS1 activity assays

NFS1 cell-free and in cell assays were performed as previously described (317). Briefly, for cell-free L-Cysteine inhibition assay with MQ, a constant concentration of purified NFS1 (2 µM) was added to varying concentrations of MQ (0-2000 µM) and reaction buffer in a total volume of 1 ml. The reactions were started with varying concentrations of L-cysteine and incubated for 1 h. L-cysteine desulfurase activity was

followed by methylene blue assay as described below. Expression and purification of NFS1 are detailed in the supplemental material and methods.

Measurement of free H₂S via methylene blue

The methylene blue formation assay was performed as adapted (318). Reaction was carried out in a total volume of 400 µl containing buffer (10 mM DTT, 10 µM PLP and 2.5 mg cell lysate). The reaction was started with substrate and incubated for 1 h at 37 °C. The reaction was stopped by simultaneous addition of 20 mM N, N- Dimethyl-4-phenylendiamin-hydrochloride (DMPD) and 30 mM Iron (III)-chloride. The resulting solution was incubated further for 20 minutes for colour development and subsequently spun at 13,000 g for 5 minutes. The absorbance at the absorbance was at 670 nm using Varioskan flash (ThermoFisher Scientific, Germany).

Inhibition of persulfide formation on L-cysteine desulfurase

Purified NFS1/ISD11 was radiolabelled with ³⁵S-cysteine for persulfide formation as adapted from (319). Briefly, 2 µM of NFS1 were incubated with varying concentrations of MQ in reaction buffer containing 50 mM Tris 200 mM NaCl and 150 mM NaCl in a total volume of 35 µL. The reaction was started with 10 µCi ³⁵S-cysteine (1075 Ci/mmol) and incubated 30 °C for 1 h. Resulting solutions were mixed with SDS loading buffer without boiling and separated on a 12% SDS-PAGE. Proteins were analysed by autoradiography.

In-gel aconitase assay and aconitase activity assay

OACM5.1 cells (5.0×10^6) were plated in 15 cm plates and allowed to adhere for 24h and subsequently treated with 50 µM eprenetapopt for 0, 6, 12 and 24 h in fresh media.

Cells were collected and resuspended in lysis buffer containing 1% Triton-X 100 and protein content was determined. As previously described (320), aconitase activity gels were composed of a separating gel containing 8% acrylamide, 132 mM Tris base, 132 mM borate, 3.6 mM citrate, and a stacking gel containing 4% acrylamide, 67 mM Tris base, 67 mM borate, 3.6 mM citrate. The running buffer contained 25 mM Tris pH 8.3, 192 mM glycine, and 3.6 mM citrate. Samples contain cell lysates of equal protein content (~100 µg), 25 mM Tris-Cl, pH 8.0, 10% glycerol, and 0.025% bromophenol blue. Electrophoresis was carried out at 170 V at 4°C. Aconitase activities were assayed by incubating the gel in the dark at 37°C for 45 mins in 100 mM Tris (pH 8.0), 1 mM NADP, 2.5 mM cis-aconitic acid, 5 mM MgCl₂, 1.2 mM MTT, 0.3 mM phenazine methosulfate, and 5 U/ml isocitrate dehydrogenase (all from Sigma-Aldrich). Gels were imaged on a BioRad ChemiDoc, then stained with Coomassie to verify equal protein loading, and imaged again.

For 293T cells, the activity measurement of aconitase was adapted from (321). Three T-75 cm³ flasks per cell lines were seeded and allowed to grow to 90% confluency before collection. 50 µl cell lysates were incubated with 250 µl R1 buffer (50 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl₂, 0.5 mM NADP⁺, 0.01 U Isocitrate dehydrogenase pH 8) for 5 mins at 37 °C. 200 µl of R2 buffer (50 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl₂, 2.5 mM cis-aconitate pH 8) was added to start the reaction which was monitored for 3 minutes at 340 nm using Shimadzu UV-Vis Spectrophotometer UV-2600 manufactured in Japan.

Generating apoptosis-resistant MC38 cells

Gene disruption was carried out using CRISPR/Cas 9 technology. 150 pmol of each synthetic gRNA (2 gRNAs per gene) specifically targeting BAX, BAK1, BID, CASP3 or CASP7 (**Table S3**, Synthego) in a volume of 0.5 μ l were pooled in a final volume of 5 μ l. 200 pmol Alt-R S.p. HiFi Cas9 Nuclease in 2 μ l volume (Integrated DNA Technologies) was then added, mixed and incubated at room temperature for 10 minutes to allow formation of Cas9/gRNA complexes. Meanwhile, 3×10^5 MC38 cells for each experimental condition (harvested at 40% confluency, sub-cultured two days prior) were washed $\times 3$ in room temperature PBS, followed by re-suspension in 20 μ l of Amaxa Buffer SF. Re-suspended cells were then mixed with Cas9/gRNA complexes, transferred to a 16 well electroporation stripette (Amaxa 4D, Lonza) and electroporated using program CM-130. Immediately after electroporation, 150 μ l of pre-warmed media (DMEM, 10% FBS, Pen/Strep) was added to each well and allowed to recover in an incubator for 15 mins. Cells were then transferred to 6 well plates. After at least 5 days, the effect of gene disruption on protein levels was evaluated by western blot.

Statistical analysis and replication

Data are presented as mean $\pm$ SEM and analysed by Student's *t*-test unless otherwise indicated. GI50 dose of single agents was determined by fitting the Hill equation. Statistical analyses and data presentation were performed using Prism 9 (GraphPad). No power analyses were performed to determine sample size prior to experimentation. Blinding was undertaken during *in vivo* experiments.

RESULTS

Multomics strategy to determine the mechanism of action of eprenetapopt

To determine the MoA of eprenetapopt, we undertook a set of complementary approaches, including genome-wide CRISPR perturbation screens, quantitative proteomics and untargeted metabolomics in response to eprenetapopt (**Figure 4.1A**). We also leveraged and incorporated publicly available pan-cancer therapeutic response and cancer cell line gene dependency datasets from the Broad Institute's DepMap portal (www.depmap.org) in order to expand the validity and test the generalisability of our investigational datasets (322,323). To select an appropriate model system to systematically dissect the MoA of eprenetapopt, we first analysed cancer cell line drug sensitivity data from the Cancer Therapeutic Response Portal (CTRPv2). We found that esophageal cancer cell lines with a high rate of *TP53* mutation were the most resistant to the structural analogue of eprenetapopt, APR-017 (**Figure 4.1B**). Specifically, we selected the OACM5.1 esophageal cancer cell line for our investigation, which expresses the most common *TP53* missense mutation across all cancers, R248Q (mut-p53^{R248Q}). As a result, this model is amenable to identifying genetic determinants of sensitivity to eprenetapopt without precluding mut-p53 reactivation as a MoA.

We transduced OACM5.1 cells expressing Cas9-mCherry with the Brunello genome-wide CRISPR knockout (CRISPRko) library (309) and challenged these cells with a sublethal dose of eprenetapopt for 8 days (**Figure 4.1C**, **Figure S4.1A**). In parallel, we transduced OACM5.1 cells expressing dCas9-VP64 with the Calabrese Set A genome-wide CRISPR activation (CRISPRa) library (310) and challenged these cells with a lethal dose of eprenetapopt for 28 days. The CRISPRko screen aimed to identify

genetic deletions that increase sensitivity to eprenetapopt and thus ‘drop-out’ in the eprenetapopt treated cells compared to vehicle treated controls. Meanwhile, the CRISPRa screen aimed to identify gene overexpression that protects cells from eprenetapopt. The CRISPRa screen has the advantage of potentially identifying genetic modulators of eprenetapopt sensitivity that may not be revealed in the CRISPRko screen, where general essential genes drop-out during the puromycin selection.

Consistent with our prior observations, and those of others, that eprenetapopt triggers GSH depletion in cancer cells (143,226,249,324), the CRISPRko screen identified the *de novo* GSH synthesis genes *SLC7A11* and *GCLM*, whilst *GCLC* was the top enriched gene in the CRISPRa screen (**Figure 4.1D**). Notably, two mitochondrial one-carbon (mito-1C) metabolism genes, *SHMT2* and *MTHFD1L*, and the S-formylglutathione hydrolase gene, *ESD*, involved in GSH-mediated formaldehyde detoxification (325-327) were identified in the CRISPRko screen. We confirmed that two independent sgRNA guides targeting *GCLM*, *SLC7A11*, *SHMT2*, *MTHFD1L* and *ESD*, and one sgRNA guide for *GCLC* activation, modulated sensitivity to eprenetapopt as expected (**Figure 4.1E**). In agreement, genome-wide dependency data demonstrated that *GCLC* and *GCLM* dependency correlate strongly with pan-cancer cell line sensitivity to APR-017 (**Figure 4.1F, Figure S4.1B**). *GCLC* and *GCLM* uniquely scored strongly in both our screening approaches, indicating that the modulation of the *de novo* GSH axis is central to the MoA of eprenetapopt in cancer cells (**Figure 4.1G**).

Complementing our functional genomics approach, we performed untargeted metabolomics and label-free quantitative proteomics in OACM5.1 cells treated with eprenetapopt prior to the onset of cell death. As expected, eprenetapopt treatment decreased reduced GSH levels, without increasing oxidised GSH (GSSG) levels (**Figure 4.1H**) – indicating that eprenetapopt is triggering total GSH depletion rather than the conversion of reduced GSH to GSSG in agreement with previous studies (143,207,226,308,328). Furthermore, cystine levels were increased following eprenetapopt treatment, which could be explained by the upregulation of *SLC7A11* expression in response to eprenetapopt treatment shown in previous studies (242,308). Meanwhile, mitochondrial metabolism was altered, as demonstrated by the decrease in acetyl-CoA and NADH (**Figure 4.1H**). Notably, our proteomics study revealed that cells treated with eprenetapopt upregulated the levels of mitochondrial ferredoxin 1 (FDX1), a critical component of mitochondrial ISC biosynthesis (329), as well as two other proteins, DNAJA3 and ABCF2, which have reported roles in iron metabolism (330,331) (**Figure 4.1I**).

Altogether, these datasets provide confirmation that GSH depletion is central to the MoA of eprenetapopt and suggests as yet unappreciated roles for mito-1C metabolism and iron metabolism in the MoA of eprenetapopt.

Eprenetapopt and mitochondrial one-carbon metabolism

We next investigated the involvement of mito-1C metabolism in eprenetapopt sensitivity. Of note, mito-1C metabolism intersects with *de novo* GSH synthesis through SHMT2-mediated production of glycine from serine (332), and also supports cellular proliferation through formate production (325) (**Figure 4.2A**). First, we

attempted to rescue the increased sensitivity to eprenetapopt in mito-1C-deficient cells using exogenous formate. Pertinently, formate supplementation did not alter sensitivity to eprenetapopt (**Figure S4.2A**). Previous studies have shown that mito-1C deficient cells have altered dependency on exogenous glycine due to the loss of endogenous glycine production by SHMT2 (333). Indeed, cells expressing *SHMT2* and *MTHFD1L* sgRNAs displayed reduced growth in the absence of glycine, whilst cells transduced with control vectors did not (**Figure 4.2B, S4.2B**). Meanwhile, all cells displayed sensitivity to serine deprivation alone and combined serine and glycine (SG) deprivation. Importantly, the glycine auxotrophy in mito-1C deficient cells could be reversed with the exogenous supply of cell permeable GSH (**Figure 4.2B, S4.2B**) – in agreement with previous studies (333). This highlights that mito-1C deficient cells have a higher demand for exogenous glycine for *de novo* GSH synthesis. As a result, the increased sensitivity to eprenetapopt in mito-1C deficient cells can be reversed by supplementation of additional glycine to the cell media (**Figure 4.2C, S4.2B**). Together, these data demonstrate that mito-1C metabolism interplays with *de novo* GSH synthesis by providing endogenous glycine to regulate sensitivity to eprenetapopt in cancer cells.

Since mito-1C deficiency leads to increased demand for exogenous glycine following eprenetapopt-induced GSH depletion, we reasoned that limiting glycine availability by restricting exogenous SG would sensitise cancer cells to respond to eprenetapopt. Restriction of dietary SG has previously been shown to reduce tumour growth in a variety of xenografts and genetically engineered mouse models, and demonstrated strong efficacy in combination with biguanides, glutaminase inhibitors and phosphoglycerate dehydrogenase inhibitors (334-338). To this end, we tested the

combination of dietary SG restriction and eprenetapopt in an aggressive model of spontaneous metastatic esophageal cancer, FLO-1 LM (315). Five weeks of daily dosing of 100 mg/kg eprenetapopt monotherapy failed to inhibit primary tumour growth or limit metastatic spread (**Figure 4.2D, S4.2D,E**). Promisingly, we found that the combination of SG restriction and eprenetapopt significantly inhibited both primary tumour growth and delayed on the onset of metastatic disease, leading to prolonged overall survival (**Figure 4.2D, S4.2D**). SG in combination with eprenetapopt were well tolerated (**Figure S4.2F**) and chow consumption remained consistent across the different diet interventions (**Figure S4.2G**). We also demonstrated that the combination of eprenetapopt and SG restriction limited the growth in an esophageal adenocarcinoma patient-derived xenograft (PDX) tumour model (**Figure 4.2E**). Of note, in the metastatic and PDX models, dietary SG restriction alone inhibited tumour growth to a degree **Figure S4.2H**). Altogether, these results demonstrate that SG restriction can improve the efficacy of eprenetapopt and, for the first time, that dietary SG restriction can inhibit the growth of esophageal cancer tumours *in vivo*.

Given that *de novo* GSH synthesis pathways are central to eprenetapopt sensitivity, we hypothesised that eprenetapopt inhibits the *de novo* synthesis of GSH. First, we verified that eprenetapopt does not block cystine uptake into cells, whilst a known cystine uptake inhibitor, erastin, does (**Figure S4.2I**). Of note, we also found that mito-1C deficiency did not alter sensitivity to erastin (**Figure S4.2J**). Furthermore, in contradiction to previous investigations (251), we found that eprenetapopt treatment decreases the level of glutathionylation in cells (**Figure S4.2K**), indicating that the GSH depletion induced by eprenetapopt is not due to loss of free GSH to protein conjugation. We tracked the incorporation of isotopically labelled cystine into GSH and

other products using LC-MS. Strikingly, we observed that eprenetapopt treatment increased the proportion of cystine-derived cysteine incorporated into *de novo* synthesis products glutamylcysteine, GSH and GSSG (**Figure 4.2F**). We also detected both MQ-GSH and MQ-cysteine adducts in eprenetapopt-treated cells (**Figure 4.2F, S4.2L-N**). Meanwhile, eprenetapopt treatment also increased the proportion of incorporation into GSH breakdown intermediate, cysteinylglycine (**Figure 4.2F**). Furthermore, we found that eprenetapopt increased the proportion of unlabelled cystine in cells (**Figure 4.2F**). Given that these cells have been treated with isotopically labelled cystine, this unlabelled cystine is likely derived from the unlabelled cysteine released from degraded GSH (**Figure 4.2F**). This provides an alternative explanation for the increase in cystine we observed in our metabolomics study (**Figure 4.1H**). These data strongly indicate that eprenetapopt is not inhibiting *de novo* synthesis of GSH and instead is triggering the degradation of GSH. Together with our data that show mito-1C and *de novo* GSH synthesis deficiency drives increased sensitivity to eprenetapopt, this suggests that the capacity of cancer cells to regenerate *de novo* GSH from the available pool of GSH precursors, in particular cysteine and glycine, determines cancer cell sensitivity to eprenetapopt. This model is in keeping with our prior work, and confirmed by others (143,308,328), which demonstrated that inhibiting cystine uptake with erastin or sulfasalazine synergises with eprenetapopt both *in vitro* and *in vivo*.

Eprenetapopt triggers ferroptosis

Ferroptosis is a form of non-apoptotic cell death characterised by the induction and accumulation of toxic lipid membrane peroxidation catalysed by iron (105). Experimentally, the induction of ferroptosis is verified by the restoration of cell viability

by iron chelators and lipophilic antioxidants, and lack of cell death rescue by pan-caspase inhibitors. In our prior work, we showed that eprenetapopt induces lipid peroxidation following GSH depletion (143). However, we did not confirm that eprenetapopt induces ferroptosis as iron chelation failed to rescue cell viability following 96-hour co-treatment as determined by a resazurin-based assay, which measures the reducing capacity of cells as a surrogate for cell viability (143). Despite this, more recent investigation into the mechanisms of ferroptosis have highlighted that the use of metabolic based assays may obscure results. This is because ferroptosis induced by sustained cystine deprivation or erastin treatment perturbs cell proliferation beyond the induction lipid peroxidation (339,340).

In order to account for these factors, we performed ferroptosis rescue experiments and utilised propidium iodide (PI) uptake to directly detect dead or irreversibly damaged cells. We found that eprenetapopt-induced cell death could be reversed by ferroptosis inhibitors (ferrostatin-1 (Fer-1): lipophilic antioxidant, and ciclopirox olamine (CPX): iron chelator), but not by the poly-caspase inhibitor, zVAD-FMK in both p53-null H1299 cells and mut-p53 expressing FLO-1 cells (**Figure 4.3A,B**). In contrast, zVAD-FMK blocked cell death induced by staurosporine (STS), which kills cells through the intrinsic (mitochondrial) pathway, but ferroptosis inhibitors did not (**Figure S4.3A**), suggesting that eprenetapopt does indeed induce ferroptosis. Furthermore, cells treated with eprenetapopt underwent catastrophic destabilisation of their plasma membranes, without obvious visual evidence of nuclear fragmentation (**Movie S4.1**). Consistent with this, the activity of APR-017 correlates strongly with the efficacy of other ferroptosis inducers (erastin, 1S,3R-RSL3) included in the CTRPv2 as well as cancer cell dependency on *GPX4*, a critical gene involved in protection

against lipid peroxide accumulation and ferroptosis (341) (**Figure S4.3B**). Interestingly, ferroptosis inhibitors failed to restore cell proliferation of eprenetapopt treated cells (as assessed by confluency), which likely explains our previous results using resazurin-based assays (143) (**Figure 4.3A,B**). This suggests that eprenetapopt perturbs cell proliferation beyond the induction of ferroptosis. However, N-acetylcysteine (NAC), a thiol group donor, rescued both the cell death and the proliferation defect induced by eprenetapopt (**Figure 4.3A,B**). This is consistent with previous studies noting that exogenously supplied thiols block eprenetapopt activity in cancer cells (179,308,324,342). We also found that increasing the cell density completely blocked the induction of cell death by eprenetapopt (**Figure S4.3C**). This phenocopies previous reports of ferroptosis induced by cystine restriction and erastin being influenced by cell density (343,344).

Given that previous studies reported that eprenetapopt induces apoptosis (201,213,220), we tested the effect of eprenetapopt in a cell line model incapable of undergoing apoptosis to rule out the dependency on apoptotic machinery for eprenetapopt induced cell death. To this end, we utilised MC38 mouse colon adenocarcinoma cells in which genes encoding the key mediators of apoptosis (Bax/Bak/Bid/Casp3/Casp7, **Figure 4.3C**) were all disrupted. As expected, STS failed to induce significant cell death in the apoptosis-resistant cells, however eprenetapopt was equally potent in killing both apoptosis-proficient and -resistant cells (**Figure 4.3D,S4.3D**). Altogether, these data demonstrate that eprenetapopt induces ferroptosis and does not require apoptotic machinery in order to elicit cell death. This is in keeping with recent findings suggesting that eprenetapopt induces ferroptosis in AML models (328).

Eprenetapopt inhibits iron-sulfur cluster biogenesis

Next, we sought to explain the cell proliferation defect induced by eprenetapopt. Our screening approaches indicated a possible role of iron and mitochondrial metabolism in eprenetapopt response, including upregulation of FDX1, a mitochondrial reductase involved in ISC biosynthesis (329) (**Figure 4.1**). We hypothesised that eprenetapopt may be inhibiting mitochondrial ISC biogenesis, likely through binding to free cysteine and limiting the cysteine desulfurase activity of NFS1. NFS1 harvests sulfur from cysteine for the biogenesis of ISCs (345), which are important co-factors for at least 48 enzymes required to support several cell essential functions, including DNA synthesis and mitochondrial respiration (electron transport chain and tricarboxylic acid cycle) (346,347). Furthermore, the importance of NFS1 and ISC stability in regulation of cancer cell sensitivity to ferroptosis has also been demonstrated (348,349). Supporting our hypothesis, the top compound in the CTRPv2 dataset that correlated to *NFS1* dependency was the eprenetapopt analogue, APR-017 (**Figure 4.4A**). Moreover, we observed synergy between eprenetapopt and elesclomol (**Figure 4.4B**), where the latter has been shown to inhibit FDX1 (350). Eprenetapopt also reduced mitochondrial and cytosolic aconitase activity (**Figure 4.4C**), a surrogate marker for ISC stability (320).

Next, we utilised a cell-free NFS1 activity assay to demonstrate that the active agent of eprenetapopt, MQ, was able to inhibit the cysteine desulfurase activity of NFS1 in a dose dependent fashion (**Figure 4.4D**). MQ was found to be an uncompetitive inhibitor of NFS1 as demonstrated by the Lineweaver-Burk plot (**Figure S4.4A**). Additionally, we confirmed that MQ inhibits persulfide formation on NFS1 from

radioactive ^{35}S -cysteine – an orthogonal read-out of NFS1 cysteine desulfurase activity (**Figure S4.4B**). Together, these results suggest that MQ may inhibit NFS1 by forming inactive NFS1-cysteine-MQ complexes, and not simply by forming MQ-cysteine adducts to diminish the supply of free cysteine. Importantly, in order to confirm that these effects of MQ on NFS1 extend to intact cells, we also demonstrated that MQ inhibits NFS1 activity in HEK-293T (293T) cells (**Figure 4.4E**). This corresponded with a decrease in cell viability and aconitase activity in 293T cells (**Figure S4.4C,D**), without affecting the protein level of either mitochondrial aconitase or NFS1 (**Figure S4.4E**).

ISCs are sensitive to degradation under atmospheric oxygen conditions (standard tissue culture is 21% O_2) and thus cells require functional NFS1 to support ISC synthesis to maintain viability and proliferation (348,351). Conversely, ISCs are stabilised under low oxygen conditions (3% O_2), which allows cells to proliferate in the absence of NFS1 (348,351). Knowing this, we attempted to rescue the proliferation defect induced by eprenetapopt by culturing cells (with or without Fer-1) under low oxygen conditions, however we found that low oxygen failed to rescue the proliferation defect (**Figure S4.4F**). Interestingly, we found that low oxygen conditions alone did significantly decrease eprenetapopt induced cell death, presumably through stabilisation of ISCs (**Figure S4.4D**). Furthermore, under these conditions, the addition of a CDK2 inhibitor, which was recently demonstrated to protect against the cell proliferation defect induced by suppression of the ISC-containing protein, DNA polymerase epsilon (POLE) (351), also failed to restore proliferation (**Figure S4.4F**). Altogether, these results demonstrate that eprenetapopt inhibits ISC biogenesis through inhibition of NFS1 cysteine desulfurase activity, which potentiates ferroptosis

induced by eprenetapopt-mediated GSH depletion. It is likely that other essential cellular functions requiring GSH as a co-factor explain the continued proliferation defect induced by eprenetapopt, even in the presence of Fer-1 and under low oxygen conditions (**Figure 4.4F**).

Extending the clinical utility of eprenetapopt in myeloid dysplastic syndromes

In the context of *TP53*-mutation, eprenetapopt is currently only indicated in ~7% of all MDS cases (253). However, dysregulated iron metabolism is a hallmark of a large subset of MDS-RS, characterised by aberrant iron accumulation in erythroblast mitochondria and accounting for ~30% of all MDS cases (253,352). MDS-RS is diagnosed when at least 15% of a patient's erythroblasts are determined to be ring sideroblasts (352,353). Importantly, the most frequent genomic alteration associated with MDS-RS are *SF3B1* mutations, which are largely mutually exclusive with *TP53* mutations (354). Therefore, the selection criteria for detection of at least one *TP53* mutation for inclusion into the eprenetapopt MDS clinical trials is mostly contraindicated for patients with MDS-RS. Mechanistically, *SF3B1* mutation has been causally linked to aberrant iron accumulation in MDS patient samples through downregulation of the mitochondrial iron-sulfur export transporter, *ABCB7* (355).

To interrogate this phenomenon, we knocked down expression of *ABCB7* in human erythroleukemia K562 cells, which led to an increase in sensitivity to eprenetapopt (**Figure 4.4G**). To highlight the importance of these findings clinically, we collected and analysed data from the phase II *TP53*-mutated MDS clinical trial investigating the combination of eprenetapopt and azacitidine (258). As expected, no patients with co-occurring *TP53* and *SF3B1* mutations were detected. For patients where iron staining

were available, we found that patients with MDS-RS obtained a higher rate of CR in response to eprenetapopt and azacitidine treatment compared to patients with fewer than 15% ring sideroblasts although this was not statistically significant secondary to the small sample size (CR 75% vs 50%; $P=0.3802$, **Figure 4.4H**). Together, these data not only provide a strong premise for expansion of clinical utility of eprenetapopt beyond *TP53*-mutant MDS patients to include all patients with MDS-RS, but also highlights the potential inappropriate selecting patients based on *TP53* mutation in this indication.

DISCUSSION

Whilst pre-clinical evidence demonstrated that eprenetapopt has p53-independent anticancer activity (206,207,328), the disappointing preliminary results from the phase III clinical trial investigating eprenetapopt in combination with azacitidine highlighted the need to reexamine the patient selection strategy for eprenetapopt (306). Here, using a multiomics, unbiased strategy, we determine the MoA of eprenetapopt as a GSH-depleting agent that triggers ferroptosis and inhibits ISC biogenesis. Our work supports previous findings reporting that GSH depletion, and/or factors that regulate GSH availability, are central determinants of eprenetapopt activity in cancer cells (143,221,280,307,308). This is in keeping with previous unbiased investigation of the changes to the transcriptome in response to eprenetapopt treatment in cancer cells, which detected no changes to p53 target genes but upregulation of oxidative stress response genes, including *SLC7A11* (207,242). Further, our findings are contrary to reports that conclude eprenetapopt induces apoptosis through mut-p53 reactivation (175,187,229) and instead supports eprenetapopt as a ferroptosis inducer (328). As a result, our study reframes the potential clinical utility of eprenetapopt and provides three major insights.

First, as a ferroptosis inducer, eprenetapopt remains first-in-class. Whilst other clinical compounds including sorafenib (356), sulfasalazine (105), artemisinin (357), statins, as well as conventional chemotherapy, immune checkpoint inhibitors and radiotherapy (358) have demonstrated capacity to induce or enhance sensitivity to ferroptosis, eprenetapopt is the first compound to reach a phase III clinical trial where triggering ferroptosis is central to its anticancer efficacy. Furthermore, eprenetapopt may be unique, triggering an increase in turnover of GSH, rather than restraining *de novo* GSH

synthesis, as imposed by erastin through its inhibition of cystine uptake, or by L-buthionine sulfoximine through inhibition glutamate-cysteine ligase (359). Hematological and neurological toxicities, as well as impaired renal function have been reported in patients receiving eprenetapopt (257,258), which is in line with reports demonstrating high sensitivity to ferroptosis amongst mesenchymal-derived tissues (282,341,360).

Second, patient selection for clinical trials and usage of eprenetapopt should not rely on *TP53* mutation. The importance of this point is highlighted by the fact that mutations driving aberrant iron accumulation in MDS-RS, such as *SF3B1* mutation, rarely co-occur with *TP53* mutations (354). The aberrant iron accumulation in MDS-RS likely increases erythroblast sensitivity to ferroptosis. Therefore, restricting eprenetapopt to *TP53*-mutated MDS patients, whilst being an area of unmet therapeutic need, may be obscuring the therapeutic benefit eprenetapopt could provide to a wider range of MDS patients, especially MDS-RS. As a result, reclassifying eprenetapopt as a ferroptosis inducer and not as a mut-p53 reactivator, recontextualises the results from the phase III clinical trial to potentially be an example of poor patient selection rather than an indication of failure of eprenetapopt as an anticancer therapy. Both phase II clinical trials demonstrated that patients who responded to eprenetapopt and azacitidine had significantly improved overall survival compared to non-responders (257,258) – further highlighting the importance of patient selection.

Third, combining eprenetapopt with other agents or strategies that target cancer cell nutrient availability could provide stronger clinical efficacy. Pre-clinical evidence continues to grow, supporting the notion that dietary manipulation, including SG

restriction, could be a powerful adjunct to conventional therapeutic approaches (361). To date, no attempts to test the safety or efficacy of SG restriction in patients with cancer have been undertaken. Here, we show that priming tumours with SG restriction improves the efficacy of eprenetapopt in esophageal cancer models. A very recent study also demonstrated synergy between eprenetapopt and asparaginase (an enzyme that degrades asparagine) in acute lymphoblastic leukemia cell lines (362), which could hint that GSH depletion by eprenetapopt more generally increases cancer cell dependency on exogenous amino acid uptake. Furthermore, the combination of venetoclax (BCL-2 inhibitor) and azacitidine has been shown to target mitochondrial metabolism through inhibition of the glutathionylation of complex II of the electron transport chain (363), and also diminishes the uptake of global amino acids in primary patient AML stem cells (364). Moreover, depleting GSH through exogenous cyst(e)ine degradation with cyst(e)inase demonstrated a similar MoA in AML stem cells (365). Together with our study, these studies provide a strong rationale for the combination of eprenetapopt, venetoclax and azacitidine, which is currently under clinical investigation in a phase I/II study in myeloid malignancies (366). Preliminary results from this phase I/II clinical trial report that the study successfully reached its primary endpoint with a CR rate of 37% and found that the triple combination of eprenetapopt, venetoclax and azacitidine was well tolerated in patients (367). Further study should be undertaken to determine whether the response to the triple combination of eprenetapopt, venetoclax and azacitidine could be improved with SG dietary restriction.

In summary, our study demonstrates that eprenetapopt targets cancer cells through GSH depletion and inhibiting ISC biogenesis, leading to iron-dependent, non-apoptotic

ferroptosis. This work not only details novel determinants of eprenetapopt activity in cancer cells, including mito-1C metabolism enzymes, but also provides a clinical roadmap for targeting antioxidant pathways in tumours with eprenetapopt – beyond *TP53*-mutant tumours.

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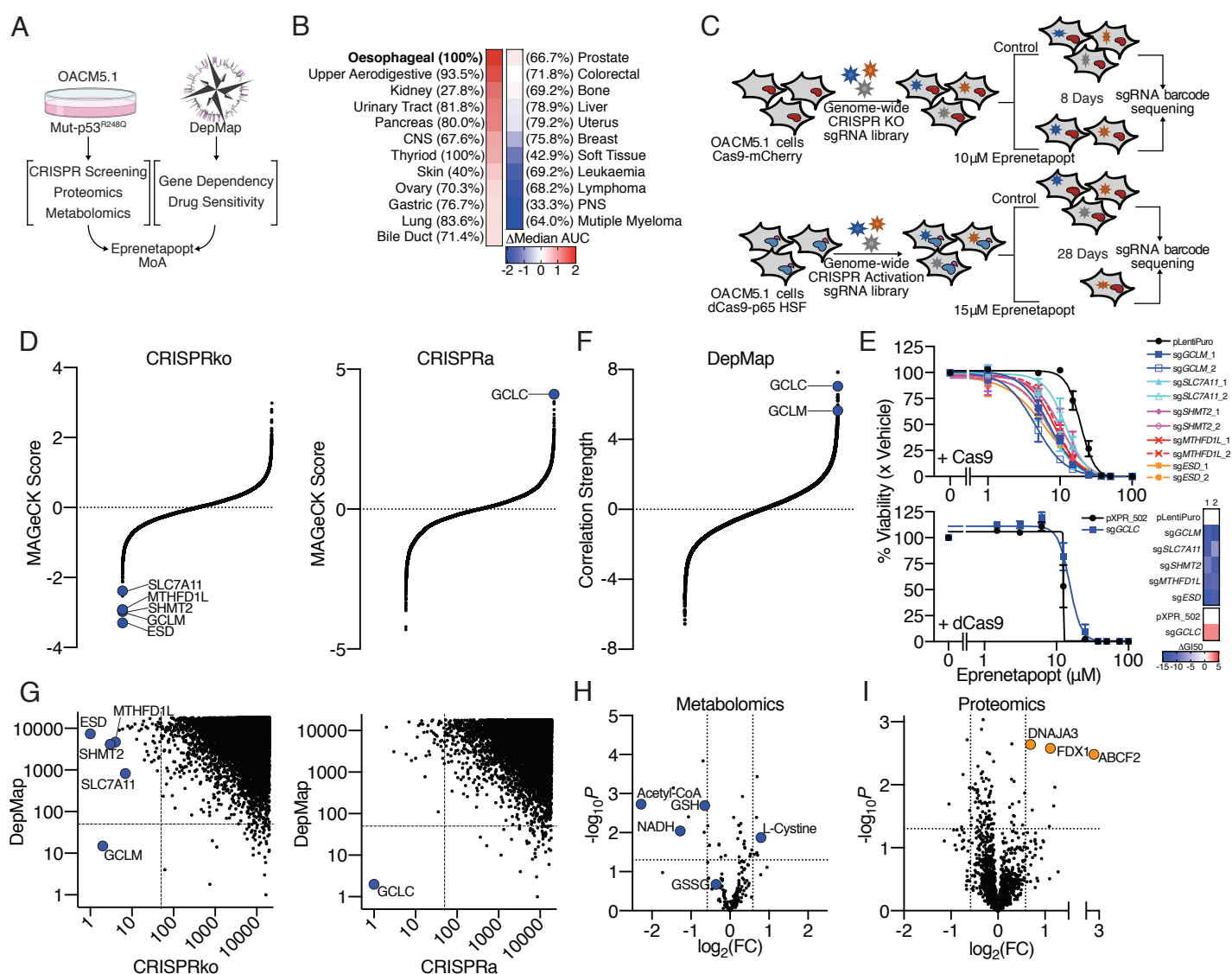


Figure 4.1 Multiomics strategy to determine the mechanism of action of eprenetapopt

(A) Schematic diagram showing a strategy to determine the MoA of eprenetapopt. **(B)** Heatmap of sensitivity to eprenetapopt analogue (APR-017). Cancer lineages are ordered by sensitivity determined by delta-median, area-under-the-curve (AUC) of compound activity. Percentages denote the frequency of *TP53* mutations in each lineage. **(C)** Schematic showing the workflow for the CRISPRko and CRISPRa screens in OACM5.1 cells. **(D)** MAGeCK scores (negative indicating 'dropout' and positive indicating 'enrichment') from CRISPR screens, plotted in order of magnitude. **(E)** Cell viability following 72 h exposure with eprenetapopt at indicated doses in cells transduced with individual sgRNA targeting identified hits. Heatmap represents the change in GI50 (dose where 50% growth inhibition is achieved) relative to control. **(F)** Fischer's transformed z-scored Pearson correlation strength of gene dependency from the DepMap database with eprenetapopt analogue (APR-017) sensitivity data from CTRPv2, plotted in order of magnitude. **(G)** Comparison of CRISPR screens and DepMap gene dependency data show overlay of glutamate-cysteine ligase units (GCLC and GCLM). Plot is representing the rank of top hits (ordered by "drop-out" in

CRISPRko, by “enrichment” in CRISPRa, by positive correlations in DepMap). Dotted lines indicate overlap of top 50 ranked genes. **(H)** Changes in polar metabolites determined by untargeted LC-MS metabolomics and **(I)** proteins determined by label-free quantitative proteomics in OACM5.1 cells following treatment with 50 μ M eprenetapopt for 12 h compared to vehicle. Dotted lines indicate significance cut-offs ($P < 0.05$, $|\log_2(\text{Fold change, FC})| > 0.5$. Two-tailed unpaired t-test **(H,I)**. Error bars = SEM. **(D)** $N=2$ for CRISPRko, $N=1$ for CRISPRa, **(E)** $N=3-4$, **(H)** $N=6$, **(I)** $N=4$. See also Fig. S4.1.

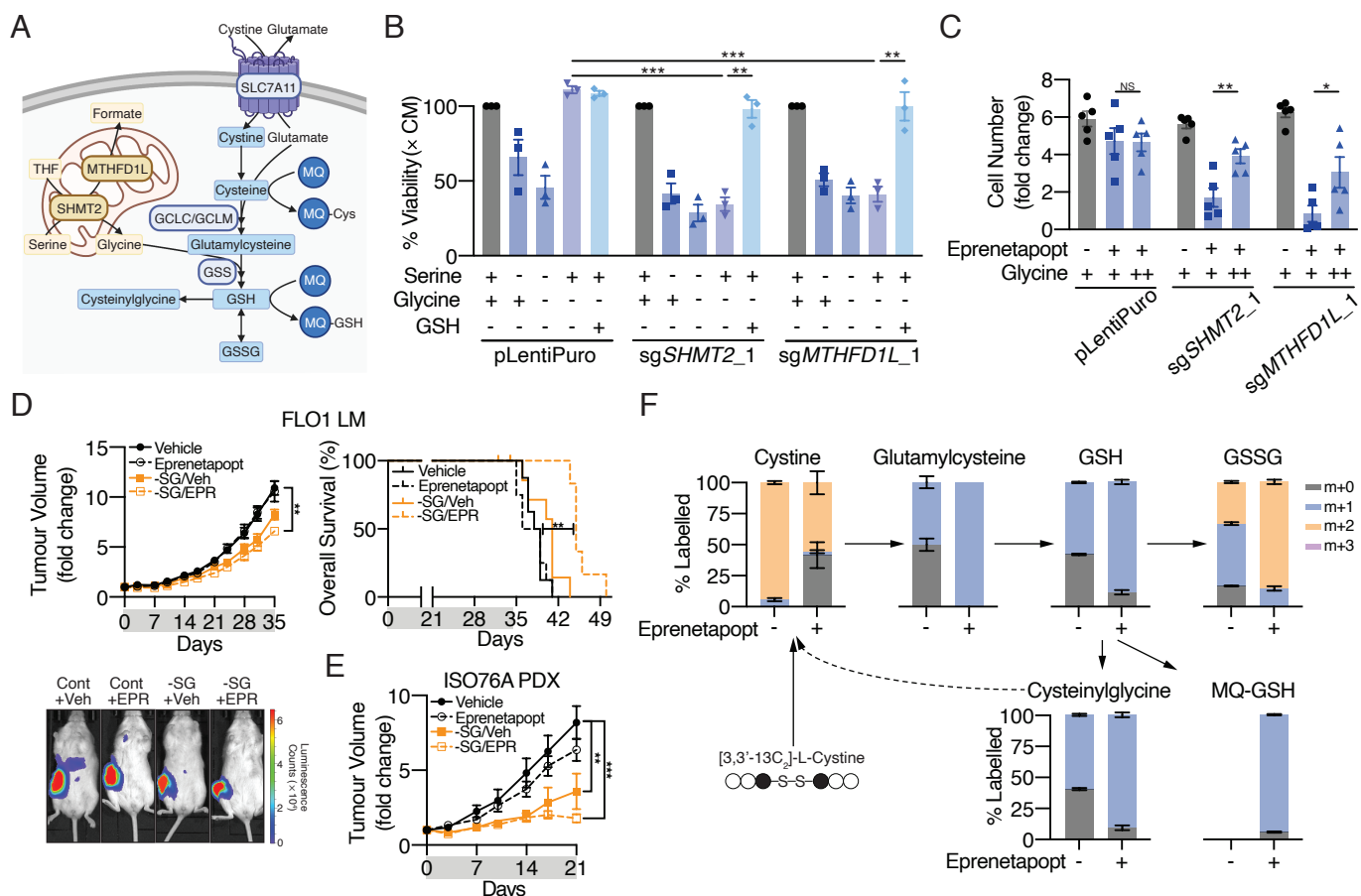


Figure 4.2 Eprenetapopt and mitochondrial one-carbon metabolism

(A) Schematic diagram illustrating the connections between *de novo* GSH synthesis and mito-1C metabolism. (THF, tetrahydrofolate; GSH, reduced GSH; GSSG, oxidised GSH; MQ-Cys, MQ-conjugated cysteine; MQ-GSH; MQ-conjugated GSH). **(B)** Cell viability compared to complete media (CM) following 72 h of serine, glycine or serine and glycine deprivation, and glycine deprivation rescued with 1 mM GSH-monoethyl ester (GSH) in OACM5.1 cells. **(C)** Relative cell number following treatment with 10 μ M eprenetapopt $\pm$ 1 mM glycine supplementation for 4 days. + indicates glycine in RPMI media, ++ indicates supplementation with additional 1 mM glycine. **(D)** Top: Growth curves and overall survival (time to reach tumour volume $\geq 1400\text{mm}^3$) of NSG mice inoculated with FLO-1 LM tumours treated with eprenetapopt (EPR, 100 mg/kg, daily) or vehicle on either normal or serine and glycine (SG)-free diets for 35 days. Bottom: Representative bioluminescence images illustrating the metastatic burden in mice after 35 days of treatment. **(E)** Growth curves of esophageal adenocarcinoma patient-derived xenografts (PDX) in NSG mice treated with eprenetapopt (EPR, 100 mg/kg, daily) or vehicle on either normal, or SG-free diet for 21 days. **(F)** Fractional labeling of *de novo* GSH synthesis intermediates from $^{13}\text{C}_2$ -cystine following 12 h exposure to 50 μ M eprenetapopt compared to vehicle in OACM5.1 cells. Two-tailed t-test (**B**, **C**), one-way ANOVA with Tukey's multiple comparisons test (**D**, **E**), log-rank (Mantel-Cox) test (**D**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bars = SEM. **(A)** $N=1$, **(B, F)** $N=3$, **(C)** $N=5$. **(D)** $N=8$, **(E)** $N=4-5$. See also Fig. S4.2.

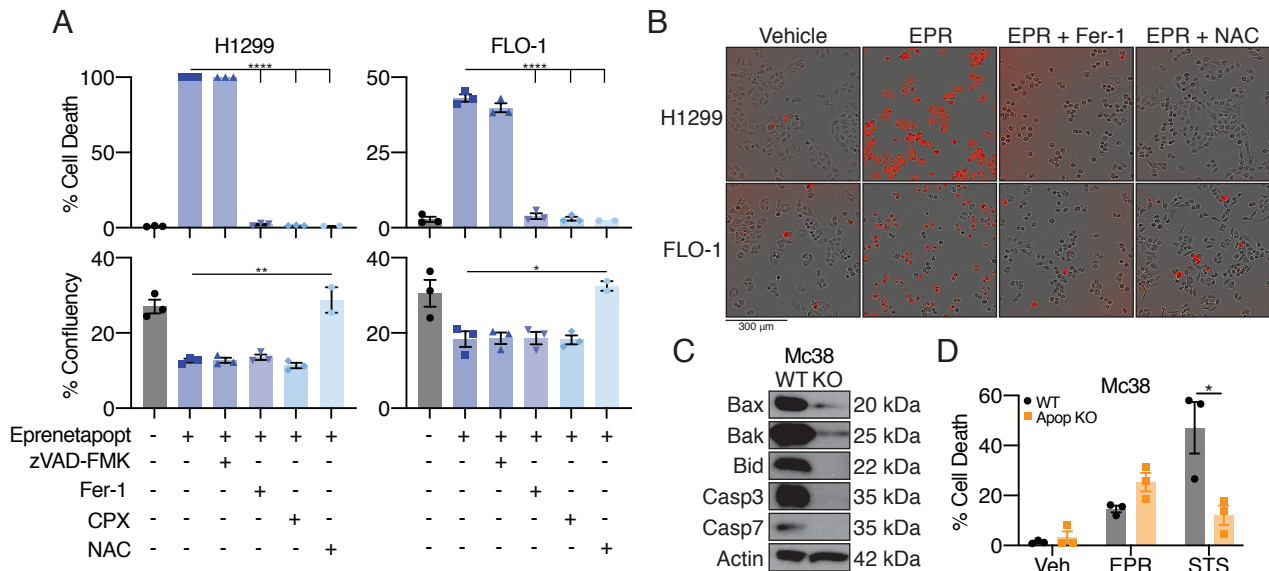


Figure 4.3 Eprenetapopt triggers ferroptosis

(A) Percentage (%) dead cells (as determined by % PI+ cells/% cell confluency) and % cell confluency following treatment for 24 h with 50 μ M eprenetapopt with or without 50 μ M zVAD-FMK (pan-caspase inhibitor), 12.5 μ M Ferrostatin-1 (Fer-1, lipophilic antioxidant), 6.25 μ M ciclopirox olamine (CPX, iron chelator) or 2.5 mM N-acetylcysteine (NAC, cysteine supplement) in H1299 (left) and FLO-1 cells (right). **(B)** Representative merged phase and red channel images of results from **(A)** acquired on Incucyte. **(C)** Immunoblot of Bax, Bak, Bid, Casp3 and Casp7 illustrating the efficiency of CRISPR editing in MC38 cells. **(D)** Cell death in MC38 wild-type (WT) and apoptosis-deficient (Apop KO) cells following 24 h treatment with 50 μ M eprenetapopt or 12.5 μ M staurosporine (STS). Two-way t-test **(A,D)**. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ **(A)** $N=2-3$, **(C)** $N=2$, representative blots shown, **(D)** $N=3$. See also Fig. S4.3 and [Movie S4.1](#).

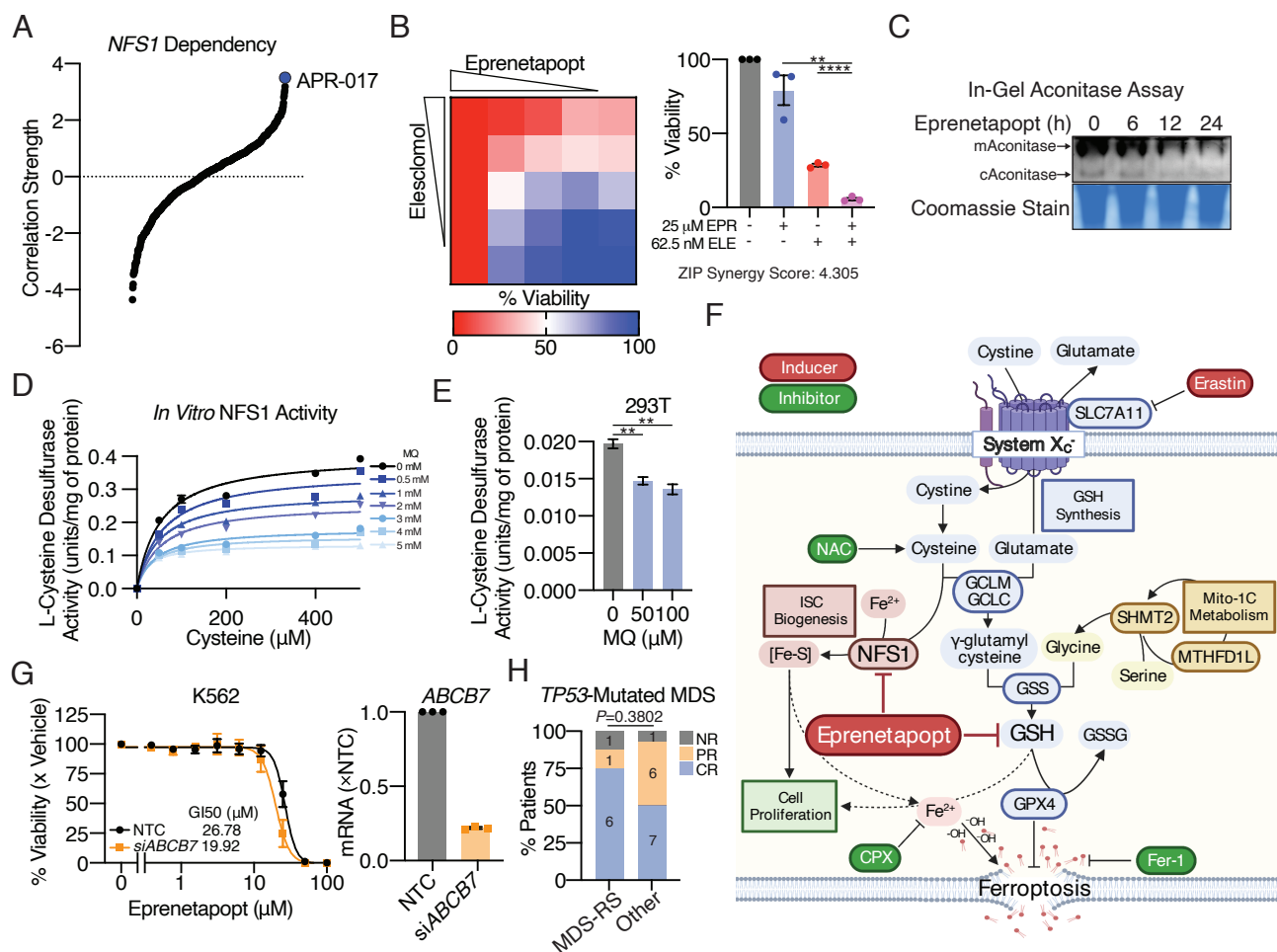


Figure 4.4 Eprenetapopt inhibits iron-sulfur cluster biogenesis

(A) Fischer's transformed z-scored Pearson correlation strength of *NFS1* gene dependency and the compound activity of the 481 Cancer Therapeutic Response Portal v2 (CTRPv2) compounds. Blue dot indicates eprenetapopt analogue (APR-017) is the top positive correlated compound to *NFS1* gene dependency. **(B)** Heatmap and bar chart demonstrating cell viability following 72 h eprenetapopt (EPR) and elesclomol (ELE) co-treatment in OACM5.1 cells. **(C)** In-gel aconitase assay and Coomassie stain (for protein loading) in OACM5.1 cells following treatment with 50 μ M eprenetapopt for indicated times (mitochondrial: mAconitase, cytosolic: cAconitase). **(D)** Cell-free and **(E)** in-cell (in HEK-293T cells) L-cysteine desulfurase assay measuring NFS1 activity at indicated doses of MQ following 1 h incubation for cell-free and 24 h for 293T cell treatment. **(F)** Schematic diagram summarizing the MoA of eprenetapopt. (GPX4, GSH peroxidase 4; Fe-S, iron-sulfur; ISC, iron-sulfur cluster) **(G)** Left: Cell viability following 72 h exposure with eprenetapopt at indicated doses in K562 cells 24 h after transfection with short interference RNA targeting *ABC7* (siABC7) or non-targeting control (NTC). Right: Relative *ABC7* mRNA levels confirming knockdown in K562 following 24 h siRNA transfection. **(H)** Response rates to eprenetapopt and azacitidine combination therapy in phase II TP53-mutated myeloid dysplastic syndromes (MDS) clinical trial stratified by the presence of ring sideroblasts in the US trial (258) (MDS-RS, MDS with ring sideroblasts; NR, non-response; PR, partial response; CR, complete remission). Two-way unpaired t-test

(B,E), Fisher's exact test **(H)**. ** $P < 0.01$, **** $P < 0.00001$. Error bars = SEM.
(B,D,E,G) $N=3$. **(C)** $N=2$, representative gel shown. **(H)** $N=8$ MDS-RS, $N=14$ for other.
See also Fig. S4.4.

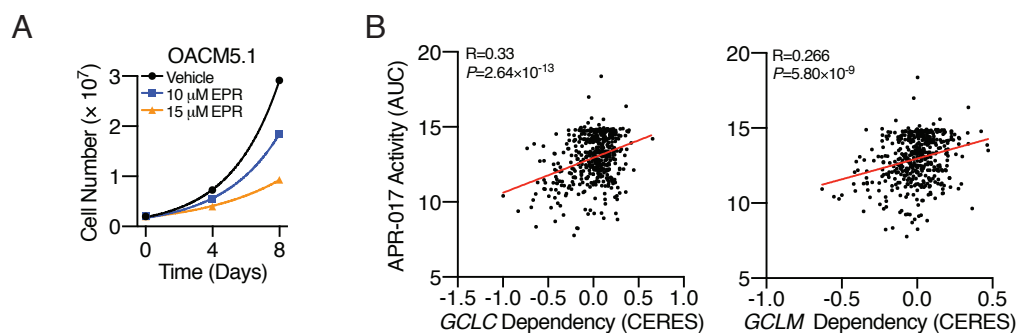


Figure S4.1 Related to Figure 4.1 Multiomics strategy to determine the mechanism of action of eprenetapopt

(A) Representative growth curve of OACM5.1 cells treated with eprenetapopt at indicated doses over 8 days. **(B)** Scatterplots correlating eprenetapopt analogue (APR-017) activity from CTRPv2 with CCL dependency on *GCLC* and *GCLM* in DepMap. (CERES, copy-number adjusted gene dependency score). Pearson's correlation **(B)**. **(A)** $N=1$. **(B)** $N=466$.

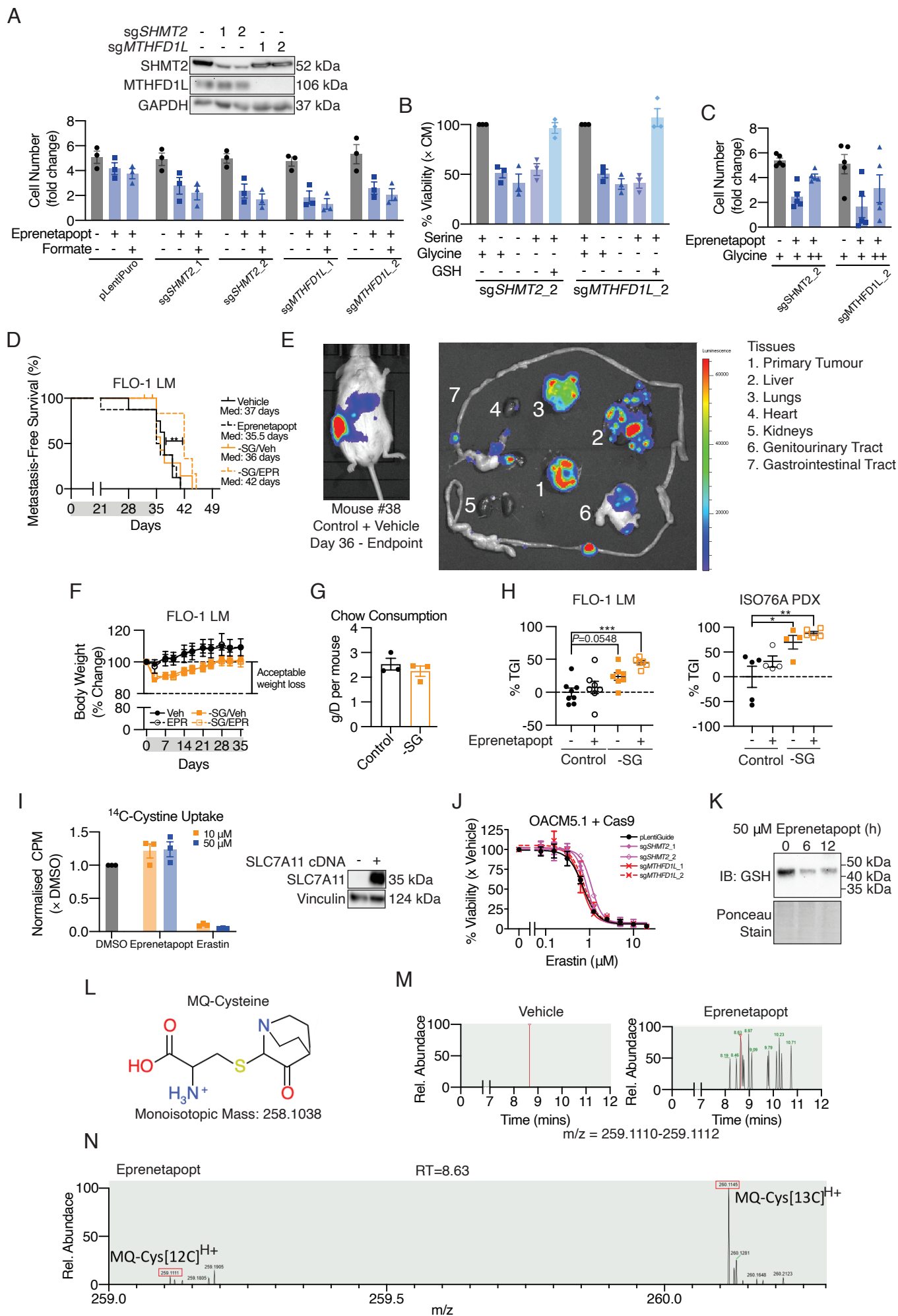


Figure S4.2 Relating to Figure 4.2 Eprenetapopt and mitochondrial one-carbon metabolism

(A) Top: Immunoblot of SHMT2 and MTHFD1L illustrating the efficiency of two independent sgRNA guides in bulk OACM5.1 cells following puromycin selection. Bottom: Relative cell number following treatment with 10 μ M eprenetapopt $\pm$ 1 mM formate supplementation for 4 days in OACM5.1 cells transduced with control (pLentiPuro) or *SHMT2* or *MTHFD1L* sgRNA. (B) Cell viability compared to complete media (CM) following 72 h of serine, glycine or serine and glycine deprivation, and glycine deprivation rescued with 1 mM GSH-monoethyl ester (GSH) in OACM5.1 cells transduced with *SHMT2* or *MTHFD1L* sgRNA_2. (C) Relative cell number following treatment with 10 μ M eprenetapopt $\pm$ 1 mM glycine supplementation for 4 days in OACM5.1 cells transduced with *SHMT2* or *MTHFD1L* sgRNA_2. (D) Metastasis-free survival of NSG mice inoculated with FLO-1 LM tumours treated with eprenetapopt (EPR, 100 mg/kg, daily) or vehicle on either normal or SG-free diets for 35 days. (E) Representative bioluminescence images of the distribution of metastatic lesions before and after dissection of a NSG mouse at ethical endpoint. (F) % change to body weight of mice over treatment period. Loss of $\leq 20\%$ body weight relative to beginning of treatment period was considered ethically acceptable. (G) Average daily chow consumption of mice on the indicated diets over 7 days. (H) % tumour growth inhibition quantified at the end of treatment (35 days for FLO-1 LM, 21 days for PDX). (I) Left: Relative radioactive cystine uptake in H1299-SLC7A11 overexpressing cells co-treated with DMSO (as control), pre-heated eprenetapopt and erastin (known cystine uptake inhibitor) at indicated doses. (CPM, counts per minute) Right: Immunoblot demonstrating overexpression of SLC7A11 in H1299 used for cystine uptake assay. (J) Cell viability following 72 h exposure with erastin at indicated doses in OACM5.1 cells transduced with indicated sgRNAs. (K) Changes to total glutathionylation level following 50 μ M eprenetapopt treatment at indicated time points in H1299 cells. (L) Chemical structure and mass of MQ-conjugated cysteine. (M) Scan to identify MQ-cysteine from LC-MS run of OACM5.1 treated with ^{13}C -cystine and 50 μ M eprenetapopt or vehicle for 12 h, over 12 mins of retention time. (N) Scan indicating relative abundance of species from retention time (RT) = 8.63, from LC-MS run of OACM5.1 treated with ^{13}C -cystine and 50 μ M eprenetapopt. MQ-Cys[^{12}C] $^{\text{H}+}$ denotes MQ-cysteine from endogenous ^{12}C -cysteine and MQ-Cys[^{13}C] $^{\text{H}+}$ denotes MQ-cysteine from exogenous ^{13}C -cysteine. Log-rank (Mantel-Cox) test (D), one-way ANOVA with Dunnett's multiple comparisons test (H). Error bars = SEM. (A,B,E,I,J) $N=3$, (C) $N=5$, (D,F) $N=8$. (K) $N=2$. Representative blot.

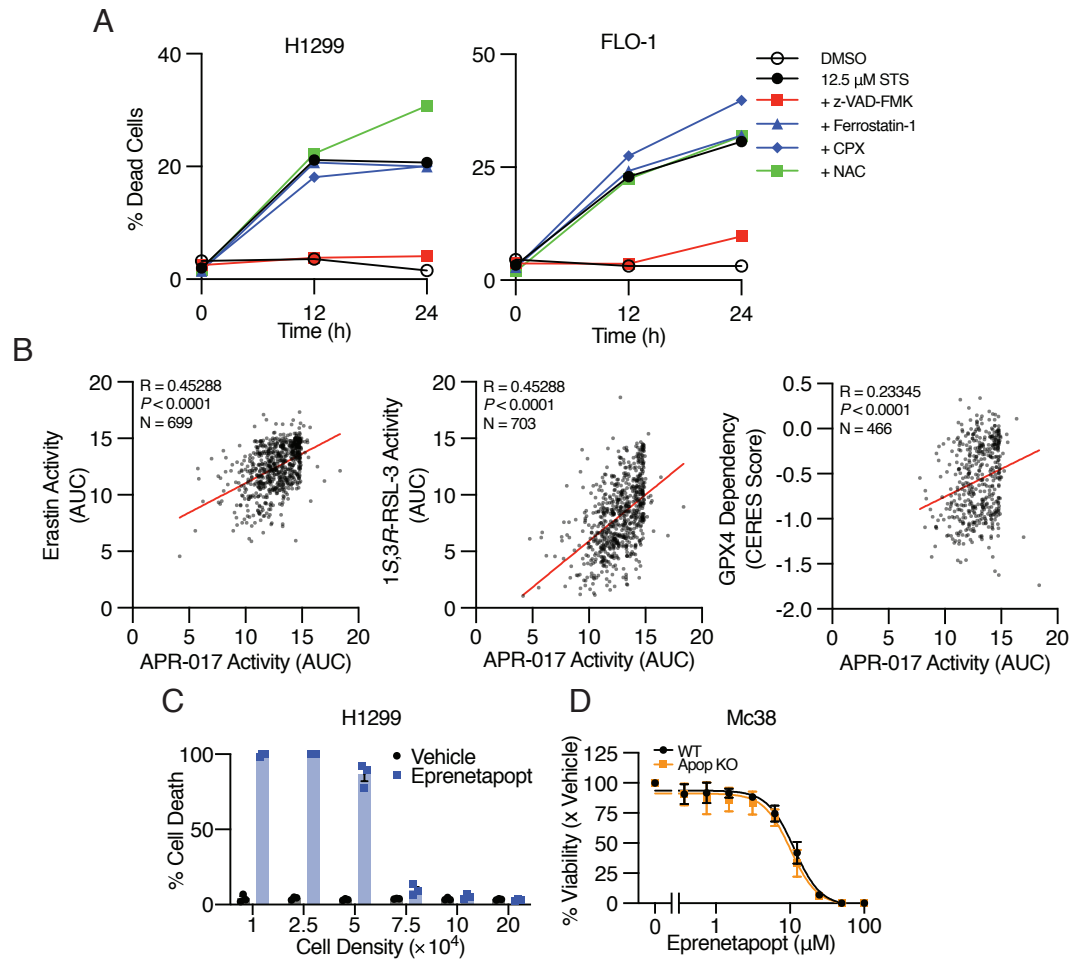


Figure S4.3 Relating to Figure 4.3 Eprenetapopt triggers ferroptosis

(A) Cell death (% PI positive) induced by staurosporine (STS, 12.5 μ M) with or without apoptosis (zVAD-FMK, 50 μ M) or ferroptosis inhibitors (Fer-1, 12.5 μ M; CPX, 6.25 μ M; NAC, 2.5 mM) in H1299 and FLO-1 cells over 24 h. **(B)** Scatterplots plots correlating eprenetapopt analogue (APR-017) activity from CTRPv2 with the activity of ferroptosis inducers, erastin (left) and 1S,3R-RSL3 (middle), and CCL dependency on *GPX4* (right) in DepMap. **(C)** % cell death determined by propidium iodide uptake following 24 h treatment with 50 μ M eprenetapopt in H1299 cells at indicated cell densities. **(D)** Cell viability following 72 h exposure with eprenetapopt at indicated doses in WT and Apop KO MC38 cells. Pearson's correlation **(B)**. Error bars = SEM. **(A)** $N=1$, **(C,D)** $N=3$.

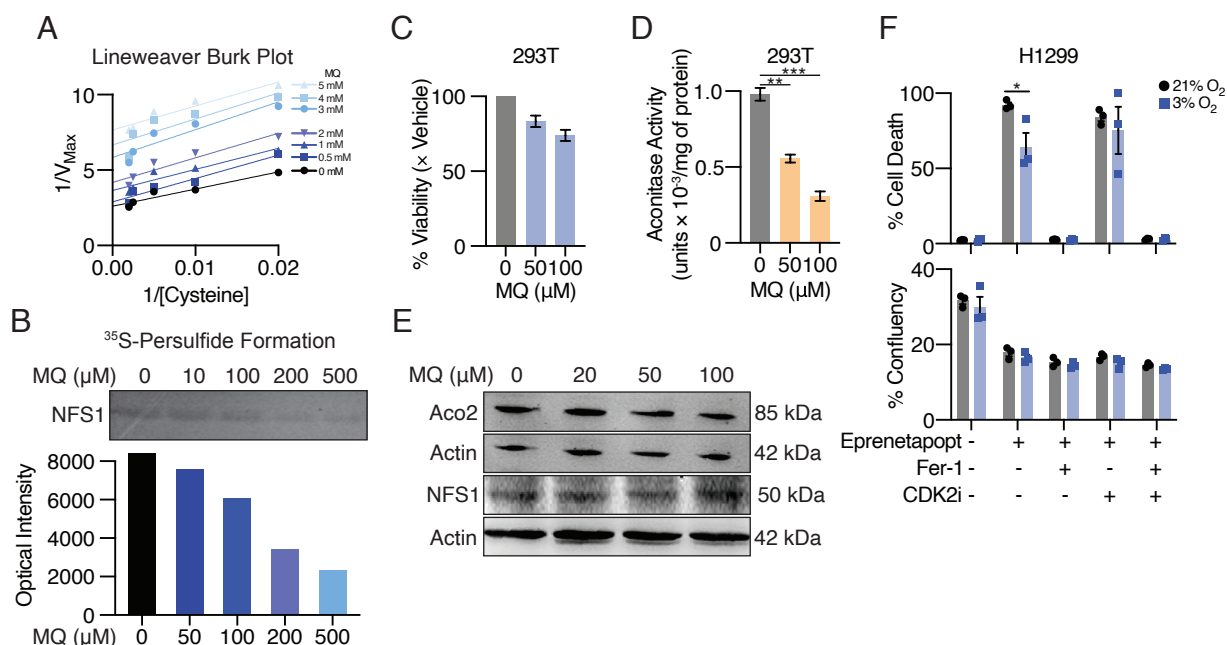


Figure S4.4 Relating to Figure 4.4 Eprenetapopt inhibits iron-sulfur cluster biogenesis

(A) Lineweaver Burk analysis of Figure 4.4D. **(B)** Top: 2 μM purified NFS1 was incubated with indicated concentrations of MQ and reaction was started with ^{35}S -cysteine for 1 h at 30 $^{\circ}\text{C}$ followed by separation on 12% non-reducing SDS-PAGE. Bottom: Image J quantification of the resulting bands. **(C)** Cell viability and **(D)** aconitase activity following 24 h exposure with MQ at indicated doses in 293T cells. **(E)** Immunoblot of Aco2 and NFS1 protein levels following 24 h exposure with MQ at indicated doses in 293T cells. **(F)** % cell death and % cell confluency following treatment for 24 h with 50 μM eprenetapopt with or without 12.5 μM Fer-1 and/or 625 nM CDK2 inhibitor (SNS-032) in 21% O_2 (black bars) or 3% O_2 (blue bars) in H1299. Two-tailed t-test **(D,F)**. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bars = SEM. **(B)** $N=1$, **(C,D,F)** $N=3$, **(E)** $N=2$, representative blots shown.

SUPPLEMENTAL MATERIALS AND METHODS

Expression and purification of Human NFS1 and ISD11

Plasmids designated pZM2 expressing an N-terminal fusion protein with His₆ tag Nfs1 Δ 1-55 and pZM4 expressing ISD11 were co-transformed into E.coli BL21 (DE3) cells. Cells were grown at 30 °C for in 1litre cultures of LB containing 150 µg/ml ampicillin and 50 µg/ml chloramphenicol. The expression was induced by 200 µM of IPTG (Isopropyl- β -D-thiogalactoside). Growth was continued for 16 h at 16 °C. Cells were clarified by centrifugation at 8000 g. Cell pellets were lysed and soluble fraction of the resulting lysed cells were obtained by centrifugation at 21000 g for 30 min. Crude extract were applied to Nickel nitrilotriacetate (Ni-NTA) resin. Nfs1 Δ 1-55/Isd11 were eluted with 50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole buffer pH 8.0. Nfs1 Δ 1-55/Isd11 were finally purified using size exclusion chromatography with Superdex 200 column (Superose12 equilibrated with 50 mM Tris, 200 mM NaCl, 10 µM PLP).

Gene expression with quantitative RT-PCR

Total RNA was extracted using NucleoSpin RNA kit (Macherey-Negal), cDNA was synthesised from 1 µg of RNA using High-capacity cDNA Reverse Transcription kit (Applied Biosystems) and gene expression was determined by SYBR-green RT-PCR on a Lightcycler 480 (Roche). Target gene expression was normalised to *ACTB* and analysed using the $\Delta\Delta$ Ct method. Primer sequences are detailed in **Table S4.4**.

Genetic knockdown using siRNA

Cells were reverse transfected in 96-well plates for dosing experiments and 6-well plate for RNA collection with 40 nM *ABCB7* or non-targeting control siRNA (Cat# D-

001810-10) SMARTpools (Dharmacon, Horizon Discovery) using Lipofectamine RNAiMAX solution (Life Technologies) according to the manufacturer's guidelines. Knockdown efficiency was determined by qPCR. siRNA IDs are detailed in **Table S4.5**.

SLC7A11 overexpression

SLC7A11 was ectopically expressed in H1299, JHesdoAD1 and FLO-1 using the GE Dharmacon Precision LentiORF pLOC downstream of the CMV promoter and contains turbo-GFP as a reporter gene. The turbo-RFP gene in place of *SLC7A11* was used as a control. Following transduction, GFP-positive cells were sorted. The expression of *SLC711* was confirmed by qPCR and western blotting.

Radioactive cystine uptake assay

H1299 *SLC7A11*-overexpressing cells were washed in pre-warmed cystine-free RPMI-1640 (uptake buffer). At this point, in each well the cystine-free-media was replaced with 300µL uptake buffer containing eprenetapopt (pre-heated at 90°C for 30 min to hydrolyse to MQ) or erastin with 0.04µCi of L-[1, 2, 1', 2'-¹⁴C]-cystine (PerkinElmer) for 10 minutes at room temperature. Cells were then washed thrice with ice-cold cystine-free-media and lysed in 400µL 0.2M NaOH with 1% SDS. This lysate was added to 4mL of scintillation fluid, and radioactive counts per minute were obtained using a scintillation counter.

SUPPLEMENTAL TABLES AND DATASETS

Table S4.1 List of oligonucleotides used for CRISPR sgRNA guides

Gene	Forward (5'-3')	Reverse (5'-3')
<i>GCLM</i> sgRNA_1	CACCGAATCAACCCAGATTTGGTCA	AAACTGACCAAATCTGGGTTGATTC
<i>GCLM</i> sgRNA_2	CACCGACTAGAAGTGCAGTTGACAT	AAACATGTCAACTGCACTTCTAGTC
<i>SLC7A11</i> sgRNA_1	CACCGAAGGGCGTGCTCCAGAACAC	AAACGTGTTCTGGAGCACGCCCTTC
<i>SLC7A11</i> sgRNA_2	CACCGTGAGCTTGATCGCAAGTTCA	AAACTGAACTTGCGATCAAGCTCAC
<i>ESD</i> sgRNA_1	CACCGAATGTACTCTTATGTCACAG	AAACCTGTGACATAAGAGTACATTC
<i>ESD</i> sgRNA_2	CACCGCCTGAGAGCCAATACAGTGC	AAACGCACTGTATTGGCTCTCAGGC
<i>SHMT2</i> sgRNA_1	CACCGCTACTCACAAGACTCTTCGA	AAACTCGAAGAGTCTTGTGAGTAGC
<i>SHMT2</i> sgRNA_2	CACCGTCATGCGGGCGTAGTCAATG	AAACCATTGACTACGCCCCGCATGAC
<i>MTHFD1L</i> sgRNA_1	CACCGAGGACAAACTCCAAAAGCTG	AAACCAGCTTTTGGAGTTTGTCTC
<i>MTHFD1L</i> sgRNA_2	CACCGTCGACCCATCTACCATCACG	AAACCGTGATGGTAGATGGGTCGAC
<i>GCLC</i> activation sgRNA	CACCGACACGCCTCCTGAGCCCCCG	AAACCGGGGGCTCAGGAGGCGTGTC

Table S4.2 List of antibodies

Antibody	Origin	Clone	Dilution	Source
Anti-SLC7A11	Rabbit	D2M7A	1:1000	Cell Signaling Technology
Anti-SHMT2	Mouse	F-11	1:1000	Santa Cruz Biotechnology
Anti-MTHFD1L	Rabbit	D8T7L	1:1000	Cell Signaling Technology
Anti-Bax	Rat	49F9	1:10000	Gift from Prof. Grant Dewson (WEHI)
Anti-Bak	Rabbit	N/A	1:10000	Sigma-Aldrich
Anti-Bid	Rat	4B5	1:1000	Gift from Prof. Ruth Kluck (WEHI)
Anti-Caspase-3	Rabbit	D3R6Y	1:1000	Cell Signaling Technology
Anti-Caspase-7	Rabbit	D2Q3L	1:1000	Cell Signaling Technology
Anti-NFS1	Rabbit	N/A	1:1000	Sigma-Aldrich
Anti-Aco2	Rabbit	N/A	1:4000	Sigma-Aldrich
Anti-Actin	Mouse	AC-74	1:8000	Sigma-Aldrich
Anti-Glutathione	Mouse	D8	1:1000	Abcam
Anti-Vinculin- HRP	Mouse	7F9	1:2000	Santa Cruz Biotechnology
Anti-GAPDH- HRP	Rabbit	14C10	1:10000	Cell Signaling Technology
Swine anti-rabbit	Swine	P0217	1:7000	Dako
Goat anti-mouse	Goat	P0447	1:7000	Dako
Goat anti-rat	Goat	3010	1:5000	Southern Biotech

Table S4.3 List of synthetic guides from Synthego for combinatorial CRISPR-editing in Mc38 cells

Gene	ID	Sequence
<i>Casp3</i>	2101	CATGCAGAAAGACCATACAT
<i>Casp3</i>	2103	AACCTCAGAGAGACATTCAT
<i>Casp7</i>	2107	GATATGCTTTAGGCATGCCG
<i>Casp7</i>	2108	TCCATGCGGTACAGATAAGT
<i>Bid</i>	1573	CCACAACATCCAGCCCACAC
<i>Bid</i>	1575	GCCAGCCGCTCCTTCAACCA
<i>Bax</i>	1429	GGACACGGACTCCCCCGAG
<i>Bax</i>	1430	GTTTCATCCAGGATCGAGCA
<i>Bak1</i>	1410	GGAACCTCTGTGTCGTAGCGC
<i>Bak1</i>	1412	GCAGGAGGCTCTTACCAGAA

Table S4.4 List of primers

Gene	Forward (5'-3')	Reverse (5'-3')
<i>ABCB7</i>	CAGGACAGTTCTTAGATGCTGC	TTTGGGCCACACATAAGAAAGC
<i>GAPDH</i>	GGTGTGAACCATGAGAAG	CCACAGTTTCCCGGAG
<i>ACTB</i>	AGAAAATCTGGCACCACACC	GGGGTGTGAAGGTCTCAA

Table S5.4 siRNA IDs for siGENOME SMARTpools

Gene	ID
<i>ABCB7</i>	M-007305-00-0005

Dataset S4.1. Source data from Figure 1D and 1F. List of genes and MAGeCK scores for CRISPRko “drop-out” screen (average of two independent screens), CRISPRa “enrichment” screen (one screen) and DepMap gene dependency Fischer’s z-transformed Pearson’s correlation strengths.

Movie S4.1. H1299 cells die from 50 μ M eprenetapopt treatment over 24 h. Here, we present a time-lapse video of cells with images taken every 5 min at 6 frames per second, therefore each second represents 1 h in real time, starting immediately after dosing with eprenetapopt. Images were acquired on IncuCyte FLR (Essen BioSciences).

CHAPTER 5 PERSPECTIVES AND CONCLUSIONS

Conclusions

As a result of its high mutation frequency and the critical nature of its role in driving cancer formation and progression, mut-p53 is a high-priority target for cancer therapy. Developing selective modalities to target mut-p53 cancers has been hailed as a “Holy Grail” for modern cancer therapeutics (114). p53 and *TP53* are perhaps the most well characterised protein and gene ever studied, returning over 100,000 articles on PubMed as of the writing of this thesis. Whilst this depth and breadth of investigation over 40 years has led to significant evolution of our understanding of tumour biology (368), this body of research has not yet yielded significant gains in the form of mut-p53 targeted therapeutics (173).

For the last 20 years, eprenetapopt has been described as the prototypical mut-p53 reactivator, transitioning through almost the entirety of the drug development pipeline: from the discovery of APR-017 in 2002 (175), to lead optimisation in 2005 with APR-246 (now called eprenetapopt) (187), to extensive investigation in pre-clinical models across almost all cancer types (369), to a first-in-human clinical trial in 2009 (254), all the way to US-FDA fast track and orphan drug designation for the treatment of *TP53*-mutated MDS and AML, following promising phase II clinical study (257,258). However, preliminary analysis, released by Aprea in late 2020, reporting on the phase III clinical trial in *TP53*-mutated MDS failed to meet the primary endpoint of improved CR rate (370). Early in the pre-clinical investigation of eprenetapopt and APR-017, data emerged demonstrating the efficacy of these compounds in wt-p53 and p53-null tumour models suggesting that mut-p53 reactivation is not sufficient to describe the

MoA of eprenetapopt and thus, *TP53* mutation may not be an appropriate marker for patient selection (181).

Together, this provided the mandate for this thesis: (1) to identify the determinants of sensitivity to eprenetapopt, and (2) to determine the MoA of eprenetapopt. In summary, through the use of unbiased strategies, this thesis demonstrates that eprenetapopt (through MQ) is a thiol-reactive GSH depleter, triggering ferroptosis and inhibiting ISC biogenesis, and its efficacy in cancer cells is determined by the expression of genes required for *de novo* GSH synthesis, in particular SLC7A11.

Based on the results presented in **Chapter 2**, it is evident that eprenetapopt and other mut-p53 target therapeutics (APR-017, MIRA-1 and Compound 7d-cis) fail to meet the first criteria for identification of a mut-p53 reactivator (discussed in **Chapter 1**) as none of these compounds selectively targets *TP53*-mutant CCLs over CCLs with no *TP53* mutation across hundreds of CCLs profiled in the CTRPv2. Furthermore, CCLs of haematological and neurological lineages are consistently more sensitive to eprenetapopt compared to other tissue lineages.

This led to the initiation of the study presented in **Chapter 3** (371) aimed at identifying a suitable biomarker for determining eprenetapopt sensitivity in cancer cells. Here, using pan-cancer cellular omics profiling of ~700 CCLs, it was revealed that expression of the cystine-glutamate transporter, SLC7A11, most significantly correlated with cancer cell sensitivity to eprenetapopt, irrespective of *TP53* mutation status. Furthermore, data reported from the APROC (APR-246 in oesophageal cancer) trial of five patients who received eprenetapopt in combination with doublet

chemotherapy (5-FU and cisplatin) showed that the only patient to maintain stable disease on trial had no detectable *TP53* mutation. Meanwhile, *KEAP1* mutation, which leads to NRF2 activation and SLC7A11 upregulation (288), correlates with resistance to eprenetapopt. A boutique siRNA synergy screen of >800 genes relating to oxidative stress, confirmed that SLC7A11 knockdown, as well as knockdown of ATF4 and MDM2, which regulate the expression of SLC7A11, most significantly sensitised both p53-null and mut-p53 expressing cancer cells to eprenetapopt.

In **Chapter 4**, the MoA of eprenetapopt was explored in detail. Leveraging complementary genome-wide CRISPRko and CRISPRa perturbation screening, proteomic and metabolomic profiling in response to eprenetapopt, combined with the pan-cancer gene dependency and compound activity datasets available on DepMap, these studies led to the conclusion that the central MoA of eprenetapopt is GSH depletion, triggering ferroptosis. Disruption of mitochondrial one-carbon enzymes, SHMT2 and MTHFD1L, sensitised cancer cells to eprenetapopt treatment by decreasing the availability of glycine for *de novo* GSH synthesis. This knowledge can be exploited *in vivo* through dietary SG restriction to overcome eprenetapopt resistance. Intriguingly, eprenetapopt continues to induce a cell proliferation defect in the presence of ferroptosis inhibitors. To this end, results from **Chapter 4** demonstrated that eprenetapopt inhibits ISC biogenesis by limiting the cysteine desulfurase activity of NFS1. This potentiates the capacity of eprenetapopt to induce ferroptosis, although ISC biogenesis inhibition is insufficient to completely explain the pleiotropic effects eprenetapopt has on cell proliferation through sustaining GSH depletion.

Critically, the results from this thesis provide a complete re-imagination of the clinical utility of eprenetapopt. As scientists and clinicians, we are all prisoners of our previous reference points. At the outset of my PhD, although evidence existed demonstrating eprenetapopt targets p53-null cancer cells with commensurate potency to mut-p53 cancer cells, the strong opinions of leaders in the field of mut-p53 anti-cancer therapeutics drove the continued narrative that eprenetapopt was a mut-p53 reactivator. This created an enormous challenge to overcome even just the nomenclature ascribed to eprenetapopt. Through the use of unbiased strategies to determine both the MoA and identify appropriate therapeutic biomarkers, is it my hope that this thesis will act as a fulcrum, providing a new reference point, for what is to come next for eprenetapopt as a novel ferroptosis inducer.

PERSPECTIVE THE CLINICAL UTILITY OF FERROPTOSIS INDUCERS IN ONCOLOGY

5.1 Preface

The aim of this perspective is to review, explore and compare the utility of ferroptosis inducers, including eprenetapopt, as anti-cancer agents. Following on from the results presented in **Chapter 4**, which demonstrated that eprenetapopt induces a form of iron-dependent, non-apoptotic cell death known as ferroptosis, here, the opportunities and challenges of developing ferroptosis inducers for clinical practice are discussed. Furthermore, this perspective deliberates on how ferroptosis induction by conventional anti-cancer agents might contribute to their efficacy in patients with cancer. Given that ferroptosis was only coined in 2012, our understanding of the challenges involved in leveraging ferroptosis as a therapeutic strategy is in its infancy and, as a result, is an area of active research.

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- **Fujihara KM**, Zhang BZ, Clemons NJ. *Opportunities for ferroptosis in cancer therapy*. *Antioxidants* 2021; 10(6), 986.

The author accepted version of this manuscript are presented within Chapter 5 of this thesis.

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Opportunities for Ferroptosis in Cancer Therapy

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Abstract

A critical hallmark of cancer cells is their ability to evade programmed apoptotic cell death. Consequently, resistance to anti-cancer therapeutics is a hurdle often observed in the clinic. Ferroptosis, a non-apoptotic form of cell death distinguished by toxic lipid peroxidation and iron accumulation, has garnered substantial attention as an alternative therapeutic strategy to selectively destroy tumours. Although there is a plethora of research outlining the molecular mechanisms of ferroptosis, these findings are yet to be translated into clinical compounds inducing ferroptosis. In this perspective, we elaborate on how ferroptosis can be leveraged in the clinic. We discuss a therapeutic window for compounds inducing ferroptosis, the subset of tumour types that are most sensitive to ferroptosis, conventional therapeutics that induce ferroptosis and potential strategies lowering the threshold for ferroptosis.

Keywords

Ferroptosis; eprenetapopt; erastin; GPX4; glutathione (GSH); SLC7A11; iron; oxidative stress; NRF2

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Introduction

A hallmark of cancer is the development of resistance to apoptosis often through genetic loss of the molecular machinery involved in programmed cell death (6). Furthermore, resistance to chemotherapeutics and molecular targeted therapies are major challenges in oncology (372). As a result, harnessing our understanding of non-apoptotic cell death pathways, such as ferroptosis, have substantial therapeutic potential for patients, especially in the metastatic setting where effective therapeutic strategies remain limited (358). Ferroptosis is an iron-dependent, non-apoptotic form of regulated cell death characterised by aberrant lipid membrane peroxidation (105). As such, the induction of ferroptosis is experimentally verified by the restoration of cell viability by iron chelators and lipophilic anti-oxidants, and lack of cell death rescue by pan-caspase inhibitors (**Figure 5.1**). Given that dysregulated iron metabolism and iron accumulation have been frequently observed across both solid tumours and haematological malignancies (373), selectively inducing ferroptosis is an attractive potential anti-cancer strategy with broad clinical implications. In this perspective, we will discuss the potential for weaponising ferroptosis in the clinic through two therapeutic avenues: (1) triggering ferroptosis in cancer cells directly with targeted agents, (2) lowering the threshold at which cancer cells undergo ferroptosis to enhance the efficacy of conventional therapies, including chemotherapy, radiotherapy and immunotherapy.

The development of ferroptosis inducers

Ferroptosis is triggered through two mechanisms, either through the depletion of the cellular anti-oxidant glutathione (GSH) or through direct inhibition of the enzyme responsible for reversing lipid oxidation, glutathione peroxidase 4 (GPX4). While more

detailed reviews of ferroptosis can be found elsewhere (358,374,375), here, we will highlight the key compounds used in the elucidation of the mechanisms of ferroptosis. The first chemical agent found to trigger ferroptosis, erastin, was originally identified in a high-throughput chemical library screen to identify compounds that were selectively lethal in oncogenic mutant HRAS^{V12} cells (376). Later, the protein target of erastin was elucidated as system x_c⁻ (encoded by *SLC7A11* and *SLC3A2*), a cell surface cystine-glutamate antiporter (105,356). Erastin was found to inhibit the activity of system x_c⁻, limiting the cellular supply of cystine, which critically leads to the depletion of intracellular GSH. Likewise, cystine deprivation *in vitro* also induces ferroptosis and phenocopies many of the cell death features induced by erastin (341). Furthermore, restricting cystine/cysteine availability to cancer cells through enzymatic degradation with cyst(e)inase triggers ferroptosis and inhibits tumour growth *in vivo* (377,378). Moreover, recent work by us and others, showed that eprenetapopt (APR-246, PRIMA-1^{MET}), previously identified as a mutant p53 reactivator, can also induce ferroptosis and has demonstrated capacity to conjugate to free cysteine and deplete GSH (300,328,379).

Following on from the discovery of erastin, 1*S*,3*R*-RSL3 (Ras synthetic lethal-3, RSL3) was identified in an analogous fashion (380). Here, RSL3, as well as erastin, were shown to induce cell death through a non-apoptotic, iron-dependent mechanism, and cells transformed with oncogenic RAS had increased levels of iron accumulation due to upregulation of transferrin receptor 1 (380). Unlike erastin however, RSL3 was found to act independently of system x_c⁻ inhibition (105), and instead covalently inhibit GPX4 (341), a unique cellular selenoenzyme that reduces phospholipid hydroperoxides to lipid alcohols using GSH as a cofactor (381,382). As a result,

inhibiting GPX4 activity either directly or indirectly through GSH depletion, triggers unrestricted lipid peroxide accumulation in the presence of iron, and subsequently results in the rupture of the plasma membrane (383). These observations are consistent with our analyses of the Cancer Dependency Map (DepMap) and Cancer Therapeutics Response Portal v2 (CTRPv2) datasets (276,277,323), which highlight that *GPX4* gene dependency correlates with cancer cell line sensitivity to GPX4 inhibitors (including RSL3), erastin and APR-017 (analogue of eprenetapopt) (**Figure 5.2A,B**). The DepMap dataset contains gene dependency data generated from pooled genome-wide CRISPR knockout screening of over 1,000 cancer cell lines, whilst the CTRPv2 dataset contains compound activity data from 481 compounds across ~700 cancer cell lines. Correlating these datasets can reveal insights into compound mechanisms of action as demonstrated.

Therapeutic index for ferroptosis

All anti-cancer therapeutics principally rely on the selective targeting and destruction of tumour cells over normal cells, known as the therapeutic index. Understanding the differences in the threshold at which cancer cells undergo ferroptosis compared to normal cells is vital for the clinical deployment of ferroptosis inducers, to both mitigate unwanted toxicities and maximise therapeutic benefit (384). Ferroptosis inducers could be used to leverage the increased levels of oxidative stress and iron in cancer cells to drive their therapeutic index (**Figure 5.3A**). Ultimately, the efficacy and safety profiles of ferroptosis inducers can only be established through clinical trials of compounds that have appropriate pharmacodynamics and pharmacokinetics. Whilst, erastin and RSL3 are not readily bioavailable, erastin analogues (eg. PRLX 93936), other system x_c^- inhibitors (eg. sorafenib, sulfasalazine) and GPX4 inhibitors (eg.

altretamine, withaferin A) are under clinical investigation across various tumours streams (358). Eprex is also being tested in a phase III clinical trial in *TP53*-mutated myeloid dysplastic syndromes (NCT03745716). Nevertheless, lessons can be gleaned from the development and usage of cytotoxic chemotherapeutics and targeted therapies in order to achieve the greatest clinical benefit for patients. These include considerations of cancer type and setting, predictive biomarkers and the use of rescue compounds to mitigate on-target toxicities.

Oxidative stress and Iron

Cancer cells experience elevated levels of oxidative stress due to increased reactive oxygen species (ROS) production arising from the augmented metabolic demands to support biomass accumulation and proliferation compared to non-transformed cells (385). In response, some cancer cells restrict ROS by elevating anti-oxidant pathways in order to avoid the deleterious effects of oxidative stress (386). For example, lung cancers frequently harbor mutations in *NFE2L2* (Nuclear factor, erythroid 2-like 2, encodes for NRF2) or *KEAP1* (encodes for KEAP1, a negative regulator of NRF2), which results in the activation of anti-oxidant pathways, including *SLC7A11* and *NQO1* upregulation (289,387). Furthermore, there is direct evidence supporting the role of NRF2 as a negative regulator of ferroptosis by promoting anti-oxidants pathways (388,389). Conversely, NRF2 acts as a guardrail to unchecked cell cycle and proliferation in haematopoietic stem cells (150), as a result NRF2 and its target genes are found at low levels in haematological malignancies compared to solid tumours (**Figure 5.2C**). Iron accumulation is found frequently in several cancer types, especially haematological malignancies (373). Iron is an important heavy metal required for a multitude of biological processes, including iron-sulfur cluster (ISC)

biogenesis to support mitochondrial metabolism and DNA synthesis, and heme synthesis to support cellular oxygen trafficking. Furthermore, iron participates in ROS generating reactions and lipid peroxidation formation via Fenton chemistry (390). As a result, the levels of iron accumulation in cancer cells compared to healthy tissues provides a therapeutic index for ferroptosis inducers.

Cancer-type

As a result of these factors, it is clear that cancer-type specific factors play a role in the sensitivity of tumour cells to ferroptosis and identifying which tumour types are most likely to benefit will be a key factor in the successful development of ferroptosis inducers as therapeutics. As such, cancer cells of mesenchymal origin were found to be selectively sensitive to ferroptosis inducers compared to epithelial-derived cancer cells (282). For example, cancers that arise from soft tissue, bone, haematological and lymphoid tissues (i.e. mesenchymal origin) display high dependency on GPX4 for survival and high sensitivity to ferroptosis activators compared to epithelial-derived cancer cell lines (eg. Oesophageal, upper aerodigestive and skin; **Figure 5.2D**). Further, cancer cells of epithelial origin that have undergone epithelial-to-mesenchymal transition (EMT) are more susceptible to ferroptosis (282). Increased polyunsaturated fatty acid (PUFA) synthesis in mesenchymal-like state cells likely drives the increased dependency on GPX4 to dissipate reactive lipid peroxides (282,391). Moreover, breast cancer cells that enter a mesenchymal-like state following lapatinib treatment become highly sensitivity to GPX4 inhibition (389). Importantly, EMT drives the metastatic potential of cancer cells (392), which suggests that metastatic cells may be more vulnerable to ferroptosis. Cell-to-cell interactions also plays a major role in ferroptosis sensitivity; cells plated at lower density display

increased sensitivity to ferroptosis compared to identical but confluent cells in culture (343). Mechanistically, cell-to-cell contacts rely on E-cadherin, which suppresses ferroptosis through activation of the NF2 and Hippo signalling pathway (343). This matches the findings relating to EMT and ferroptosis as mesenchymal-like cells lose E-cadherin expression in order to diminish cell-to-cell interactions. Collectively, these findings suggest that cancers of mesenchymal origin, especially haematological cancers, and those prone to EMT and metastasis are likely to be strong candidates for therapeutically leveraging ferroptosis inducers.

Therapeutic Biomarker

Predictive therapeutic biomarkers could also be utilised to screen and select for patients with a higher probability of response to ferroptosis inducers. A likely beneficial approach would be to screen patient tumours for low SLC7A11 expression as high expression of SLC7A11 correlates with resistance to ferroptosis inducers (**Figure 5.2E**). This further highlights the strong likelihood that patients with haematological malignancies would benefit from treatment with ferroptosis inducers as SLC7A11 expression in haematological cancers is low, correlating with their low NRF2 expression (**Figure 5.2C**).

Use of rescue agents to mitigate toxicity

Rescue interventions could be used to mitigate the on-target side effects of ferroptosis. For example, chemotherapeutic dosing with methotrexate is often followed by folinic acid supplementation in order to limit haematological and hepatic toxicities (393). To date, while it is possible to rescue the cell death induced by ferroptosis inducers, no attempts have been made to establish whether the selectivity of ferroptosis inducers

for tumour cells could be improved by selectively blocking ferroptosis in normal cells. One could hypothesise that high-dose N-acetyl-cysteine (NAC), which is routinely used to treat paracetamol poisoning (394), could be used to rescue normal cells and limit side-effects following treatments with ferroptosis inducers (**Figure 5.3B**). Furthermore, iron chelators (eg. deferoxamine) are commonly used to treat patients with iron overload and could also be utilised to limit deleterious side-effects of ferroptosis inducers.

Conventional therapeutics that induce ferroptosis

Several recent studies have identified that cancer cells undergo ferroptosis in response to conventional therapeutics, including chemotherapy, radiotherapy and immunotherapy. Cytotoxic chemotherapies typically target rapidly proliferating cells by interfering with cellular processes involved in cell division and DNA replication. Whilst the evidence indicating that ferroptosis induction directly by conventional chemotherapeutics, such as cisplatin and gemcitabine, is limited (395,396), there is pre-clinical evidence that ferroptosis inducers can synergise with traditional chemotherapeutics (397). In the case of eprenetapopt, significant pre-clinical evidence demonstrates the chemosensitisation capacity of ferroptosis activation including in oesophageal, ovarian and haematological malignancies (206,224,226,227). Meanwhile, sorafenib, a multi-tyrosine kinase inhibitor used in the treatment of advanced liver cancer, and sulfasalazine, an anti-inflammatory drug often used to treat rheumatoid arthritis, have both been shown to induce ferroptosis through inhibition of system x_c^- (356,398,399).

Radiotherapy uses high dose ionising radiation delivered locally to tumour affected tissues to kill cancer cells, predominantly by causing DNA damage. Radiotherapy is also known to induce oxidative stress in cancer cells by generating reactive oxygen species (400). Radiotherapy was recently shown to trigger ferroptosis through ATM-mediated suppression of SLC7A11 (401). Furthermore, upregulation of acyl-CoA synthetase long-chain family member 4 (ACSL4) also contributes to the promotion of ferroptosis by radiotherapy (402). ACSL4 preferentially acylates long-chain PUFAs (403), which are incorporated into plasma membranes, increasing the membrane-resident pool of oxidation-sensitive lipids (404). Interestingly, whilst ferroptosis inhibitors partially block the cell death induced by radiotherapy, they do not block the DNA damage triggered by ionising radiation (402). Importantly, strong synergy was reported between radiotherapy and ferroptosis activators, including cyst(e)inase and sulfasalazine (401,402).

Immunotherapy with immune checkpoint inhibitors (ICI) has revolutionised clinical care of cancer patients, providing an additional pillar to the suite of cancer treatment modalities. ICI predominantly elicit their anti-cancer effects by inhibiting tumour cell capacity to dampen cytotoxic T cell-mediated killing. ICI have also been shown to induce CD8⁺ T cell-mediated ferroptosis through suppression of SLC7A11 and SLC3A2 by interferon gamma (IFN γ) released by the T-cells (301). Further, PD-L1 blockage therapy synergised with ferroptosis inducers, erastin, RSL-3 and cyst(e)ine, both *in vitro* and *in vivo* (301), as well as with radiotherapy (401). More recently, high expression of the receptor tyrosine kinase, TYRO3, was shown to correlate with resistance to anti-PD-1 therapy and suppresses the induction of ferroptosis in tumour cells by activating NRF2 (405). Conversely, ferroptosis inducers have also been

shown to induce ferroptosis in CD8⁺ T cells, limiting the anti-cancer efficacy of ICI (406). Preliminary results also reported that eprenetapopt and anti-PD-1 therapy synergise in murine solid tumour models (407). This has prompted the initiation of a phase I clinical trial to test the safety of eprenetapopt and anti-PD-1 therapy, pembrolizumab, in solid tumour malignancies (NCT04383938). Given the tension between how ferroptosis inducers effect ICI and T cell killing, trials like this will provide pivotal insights into the role of ferroptosis inducers as adjuvants to ICI.

It is likely that a portion of the cell death induced by most conventional therapeutic regimens is ferroptotic. However, to date there has been no systematic attempt to quantify the contribution of cell death driven by ferroptosis in an *in vivo* or clinical setting with any therapeutic regimen. Such a study could potentially provide rationale for when and how ferroptosis inhibitors or sensitisers should be applied in combination with conventional chemotherapies to maximise therapeutic gain, particularly in the setting of tumour resistance to apoptosis.

Lowering the threshold for ferroptosis

Given that ferroptotic cell death is in part responsible for the tumour killing by chemotherapy, radiotherapy and immunotherapy, reducing cancer cell capacity to evade ferroptotic cell death would be a powerful therapeutic strategy. Following on from the study investigating ferroptosis sensitivity and mesenchymal-like cell state, statins, common anti-cholesterol drugs, were also identified as modulators of ferroptosis sensitivity in mesenchymal cells (282). Statins inhibit the rate limiting enzyme in the mevalonate synthesis pathway, HMG-CoA reductase (HMGCR), which decreases cholesterol abundance (**Figure 5.1**). Previous reports detailed that statins

inhibit isopentenylolation of the selenocysteine-charged transfer RNA, which is required for synthesis of selenoproteins like GPX4 (408). In keeping with this, statins synergise with GPX4 inhibitors through decreasing the abundance GPX4 protein and inducing lipid peroxidation (282). The mevalonate synthesis pathway also directs several other downstream pathways involved in ferroptosis, including CoQ10 and squalene synthesis – GSH-independent mechanisms of protection against ferroptosis (**Figure 5.1**) (409-411). As a result, targeting the mevalonate synthesis pathway with statins could be utilised to lower the threshold at which cancer cell undergo ferroptosis.

Limiting endogenous supply of other key nutrients may provide an alternative strategy to sensitise tumours to undergoing ferroptosis. Chronic activation of the anti-oxidant response induced by NRF2 activation increases the demand for the supply of glutamine and other non-essential amino acids due to the increased efflux of glutamate from SLC7A11 to supply cyst(e)ine for GSH synthesis (412,413). In addition, we recently demonstrated that limiting the availability of SG through dietary restriction significantly enhanced the efficacy of eprenetapopt *in vivo* by limiting the availability of glycine required for *de novo* GSH synthesis (379). However, this differs to the effects of ferroptosis induction by erastin under SG restricted conditions seen in other studies, where erastin treatment was found to reverse the sensitivity of *KEAP1* mutant tumour limits to SG restriction (412). This is likely explained by how eprenetapopt and erastin differ in their mechanisms of GSH depletion and effects on cyst(e)ine and glutamate availability (**Table 5.1**). Recently, arginine deprivation was also demonstrated protection against erastin and cystine depletion but not against GPX4 inhibition with RSL3 (414). Given that cyst(e)ine are considered non-essential

amino acids, dietary restriction of cystine could provide improved therapeutic benefit for erastin or other SLC7A11 inhibitors *in vivo*.

Table 5.1. Effect of ferroptosis inducers of GSH, cyst(e)ine uptake, glutamate release and serine/glycine (SG) restriction.

Inhibitor	GSH Depletion	Cyst(e)ine Uptake	Glutamate Release	SG Restriction
eprenetapopt	Yes	Increases	Increases	Increases activity
erastin	Yes	Decreases	Decreases	Decreases activity
1S,3R-RSL3	No	Unknown	Unknown	Unknown

Conclusion

The identification of ferroptosis as a non-redundant, regulated cell death pathway opens up opportunities for circumventing tumour cell resistance to other forms of regulated cell death, such as apoptosis. Exploiting ferroptosis in cancer therapy requires continued building of our understanding of the mechanisms underlying this cell death pathway; in particular, identification of molecular regulators of sensitivity and resistance to ferroptosis will be crucial for future clinical application. Complementary to this will be the identification of certain tumour types or cell states (such as a mesenchymal phenotype) that are particularly amenable to induction of ferroptosis. Whilst there is active interest in the development of specific ferroptosis inducers as novel therapeutics, the recognition that ferroptosis can be engaged by many current treatment modalities opens up the potential for strategies to leverage this activity indirectly by lowering the threshold for activation of ferroptotic cell death. Given the dependence on specific metabolic pathways to protect tumour cells from ferroptosis, the application of specific diets concurrent with anti-cancer treatments holds much interest in future potential.

isopentenyl pyrophosphate (IPP), which is utilised for selenoprotein synthesis (e.g., GPX4), coenzyme-Q10 (CoQ10) synthesis, which is a co-factor of ferroptosis suppressor protein 1 (FSP1, encoded by AIFM2), and squalene synthesis, which is a lipophilic anti-oxidant. Inhibiting hydroxymethylglutaryl-coenzyme A reductase (HMGCR) with statins amplifies the activity of ferroptosis inducers. Supplementation of exogenous anti-oxidants (e.g., NAC, N-acetyl-cysteine) to simulate GSH synthesis, lipophilic anti-oxidants (e.g., Ferrostatin-1, Fer-1) to detoxify lipid per-oxides, and iron chelation (e.g., DFO, deferoxamine) blocks the induction of ferroptosis. Pan-caspase inhibitors fail to rescue the cell death (e.g., zVAD-FMK) induced by ferroptosis inducers. Ferroptosis induction by traditional therapies (chemotherapy, radiotherapy, and immunotherapy) contributes to their anti-cancer activity. Figure generated on www.biorender.com (21 June 2021).

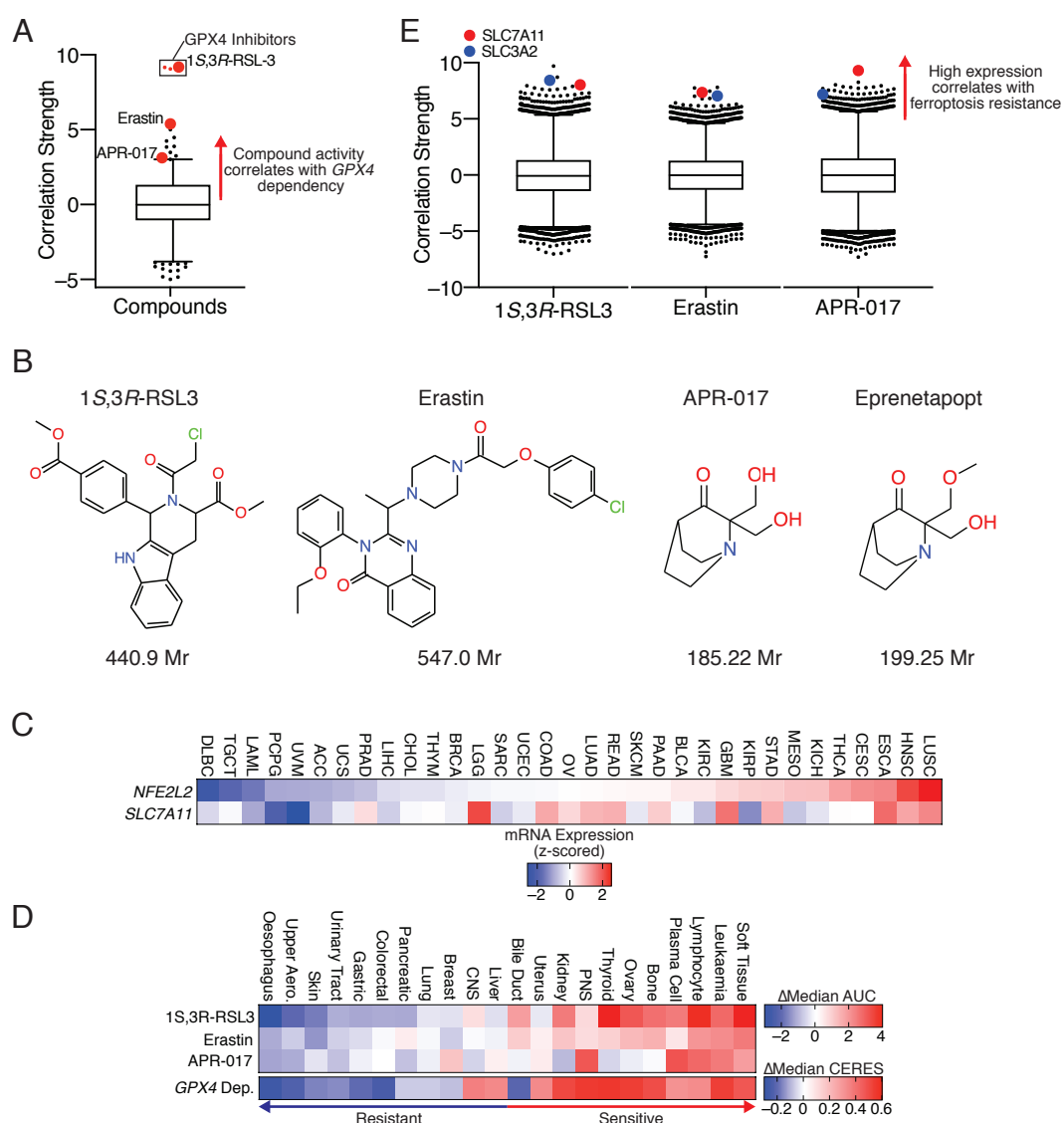


Figure 5.2 DepMap and TCGA data

(A) Box-and-whisker plot (1st–99th percentile) of Fischer's transformed z-scored Pearson correlation strength of GPX4 dependency and the 481 Cancer Therapeutic Response Portal v2 (CTRPv2) compound activity data across ~700 cancer cell lines. Red dots indicate examples of ferroptosis inducers. **(B)** Chemical structures and molecular weights (Mr) of ferroptosis inducers. **(C)** Heatmap of gene expression of NFE2L2 and SLC7A11 in patients with cancer were analysed from the TCGA, cancer type ordered by NFE2L2 expression. ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B- cell lymphoma; ESCA, oesophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukaemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma;

MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumours; THCA, thyroid carcinoma; THYM, thymoma; TPM, transcripts per million; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma. **(D)** Heatmap of sensitivity to ferroptosis inducers, RSL3, erastin, eprenetapopt analogue (APR-017), and GPX4 dependency across ~700 cancer cell lines. Cancer lineages ordered by sensitivity. AUC, area under the curve (compound activity); CERES, copy-number adjusted gene dependency score. **(E)** Box-and-whisker plot (1st-99th percentile) of Fischer's transformed z-scored Pearson correlation strength of RSL3, erastin and eprenetapopt analogue (APR-017) activity and genome-wide expression data across ~700 cancer cell lines. Red dot indicates SLC7A11 and blue dot indicates SLC3A2, which encode system x_c⁻. Data accessed from www.depmap.org (accessed 30 March 2021) and www.cbioportal.org (accessed 30 March 2021).

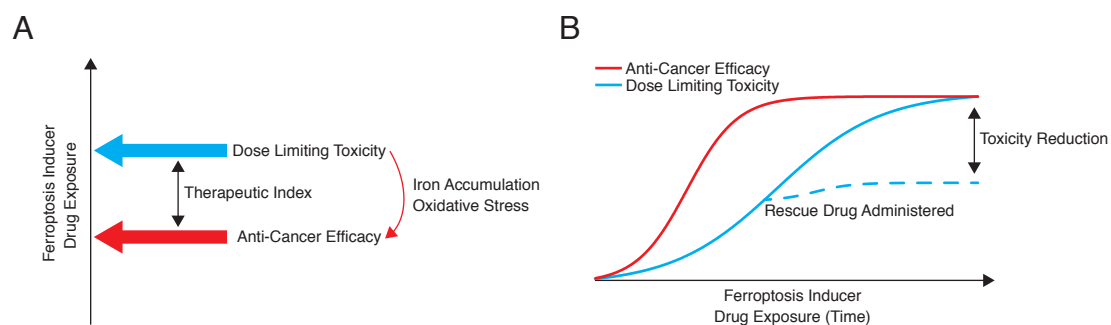


Figure 5.3 Therapeutic Index for Ferroptosis Inducers

(A) Schematic depiction of the proposed therapeutic index for ferroptosis inducers as anti-cancer agents. **(B)** The potential use of rescue drugs following ferroptosis inducer treatment. Rescue drugs could be administered once the maximal efficacy of ferroptosis inducers has been reached to reduce dose limiting toxicities without diminishing the effects on tumours.

FUTURE DIRECTIONS

How eprenetapopt will be utilised clinically in the future will ultimately be determined by clinical trials, the majority of which are funded and overseen by Aprea. How Aprea markets and discusses scientific insights into eprenetapopt, and any analogues in their development pipeline, to the clinical community as well as to their shareholders will shape this future. Pivotaly, at Aprea's most recent virtual research and development update to the public since the publication of studies demonstrating eprenetapopt is a ferroptosis inducer (328,379), Aprea has expanded its view on how eprenetapopt could benefit cancer patients, including ferroptosis induction as a MoA (415).

The intersection between cancer cell metabolism and ferroptosis

Mechanistically, several insights arising from this thesis that are relevant to the broader literature should be noted. With respect to ferroptosis induction, the intersection between this modality of cell death and cellular metabolism is obvious, but remains understudied. Experiments showing that altering mitochondrial abundance, glucose and glutamine metabolism, and mTOR/AMPK signalling augment cellular sensitivity to ferroptosis highlight the need for continued investigation of the relationship between ferroptosis and metabolism (113,339,416-418). Certainly, differences exist between the metabolic consequences of cystine deprivation-induced ferroptosis, which results in GSH depletion, and GPX4 inhibition, which does not (340), as discussed further below.

As mentioned in the Perspective in **Chapter 5** (419), similar to eprenetapopt, rescue of ferroptosis (with Fer-1) induced by erastin treatment does not restore the effects of cystine and GSH depletion on proliferation (340). In the study by Soula et al. (340),

CRISPR loss-of-function screens are utilised to probe distinct metabolic genes essential for proliferation under cystine deprivation (erastin treatment) versus GPX4 inhibition (RSL-3 treatment). *De novo* GSH synthesis genes (*GCLM* and *SLC7A11*) were determined to be essential in both erastin and RSL-3 treated cells, meanwhile genes involved in tetrahydrobiopterin biosynthesis (*GCH1*, *PTS* and *SPR*) were required for proliferation only under GPX4 inhibition. Independently, other genome-wide CRISPR screening experiments have identified GTP cyclohydroxylase-1 (encoded by *GCH1*) as essential under GPX4 inhibition (420). Conversely, loss of sideroflexin 1 (encoded by *SFXN1*), which was recently identified as the mitochondrial serine transporter required for efficient mitochondrial one-carbon metabolism (421), was found to provide a proliferative advantage under cystine starvation conditions (340). Of note, *SFXN1* was not detected in our CRISPR screens profiling eprenetapopt MoA (**Chapter 4**). Preliminary investigation in HEK-293T cells noted that *SFXN1* loss leads to sulfur-containing metabolites (taurine, hypotaurine and acetyl-CoA) accumulating in mitochondria (340). Given that *SFXN1* is required for serine transportation, one might hypothesise that *SFXN1* loss would drive increased sensitivity to ferroptosis through loss of mitochondrial glycine production through *SHMT2*. However, *SFXN1* loss, unlike *SHMT2* or *MTHFD1L* loss, does not lead to glycine auxotrophy, but instead drives serine auxotrophy that can be rescued with exogenous formate (422). *SFXN1* loss also leads to disruption to the biogenesis of Complex I and Complex III of the electron transporter chain (ETC) (422,423). This, together with other experiments indicating the ETC activity drives increased GSH utilisation and increases sensitivity to cystine deprivation (339), could provide the rationale for why *SFXN1* loss confers survival under cystine deprivation. Moreover, contrary to cystine deprivation, ETC inhibitors failed to protect cells against

eprenetapopt-induced ferroptosis (Fujihara and Clemons, *Unpublished*). This highlights the fundamental divergence of cystine deprivation, which depletes GSH through limiting *de novo* GSH synthesis and relies passively on GSH degradation, versus eprenetapopt, which depletes GSH by increasing its turnover (379). Both trigger ferroptosis, however, only cystine deprivation is amenable to modulation through limiting GSH utilisation. This may also further explain why ferroptosis induced by GPX4 inhibition is not suppressed by ETC inhibitors and SFXN1 loss (339,340). A great deal of unexplored complexity remains in this field of study, and may hold the key to how and which ferroptosis inducers are successful therapeutics and where they should be applied in the treatment of cancer.

Compared to normal cells, cancer cells have elevated levels of ROS, arising from the increased metabolic demands required to support proliferation (424-426). Pre-clinically, models of solid tumour development suggest that pre-malignant cells depend on *de novo* GSH synthesis enzymes (*GCLM* and *GCLC*) in order to support malignant transformation (427). However, GSH synthesis becomes dispensable following malignant transformation due, in part, to elevation of thioredoxin anti-oxidant pathway, which can compensate for disruption to GSH synthesis (427). Human CCL models with elevated NRF2 target gene expression (including *SLC7A11*, *GCLM* and *GCLC*) display resistance to GSH depletion and ferroptosis inducers (419). Based on these data, eprenetapopt could also be explored as a cancer preventative agent. More broadly, whether elevated blood GSH levels could be a leading indicator for malignant transformation is yet to be studied.

Broadening the clinical utility of eprenetapopt

Repurposing eprenetapopt for other disease indications beyond oncology should also be explored. A recent study identified that ISCs are important co-factors required for the replication of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) the causative agent of COVID-19 (428,429). The catalytic subunit of the SARS-CoV-2 RNA-dependent RNA polymerase (RdRp), nsp12, ligates two ISCs that are essential for structure and formation of the RdRp complex (428). As a result, destabilising ISCs was shown to block the replicative capacity of SARS-CoV-2 in cell line models of COVID-19 (428). Given that eprenetapopt inhibits ISC biogenesis **Chapter 4** (379), eprenetapopt could have selective capacity to inhibit SARS-CoV-2 replication. Similarly, apicomplexan parasites, including *Plasmodium* (the causative agent of malaria) maintain ISC biogenesis machinery in their mitochondria, like other eukaryotes, which is vital for their reproductive cycle (430,431). The hostile oxidative environment within erythrocytes in which malaria parasites reside, could potentiate sensitivity to ferroptosis induced by eprenetapopt. In support of this, artemisinin, which is an approved anti-malaria drug, induces ferroptosis (432). Moreover, blocking system x_c^- with erastin and sorafenib has been shown to reduce malaria infection and replication, especially at the liver stage of the infective lifecycle in pre-clinical models (433).

Other compounds that, similar to eprenetapopt (in the form of MQ), act as Michael acceptors with α/β unsaturated carbonyls are also under active research and have a similar broad clinical potential. In stark contradiction to eprenetapopt, which triggers ferroptosis and depletes GSH, Chalcones (1,3-diaryl-2-propen-1-one) and enoates (eg. dimethyl fumarate; DMF) are seen as anti-oxidants that, through activation of the KEAP1/NRF2 pathway, protect cells from oxidative insults and ferroptosis (434,435).

In 2013, DMF was approved by the US-FDA as an NRF2 activator for the treatment and management of multiple sclerosis as a neuroprotectant (436). As it stands, because pre-clinical and clinical deployment of eprenetapopt has focused solely on oncology, it is unclear whether or not eprenetapopt could be leveraged as an immunomodulatory therapy similar to DMF. Furthermore, this raises the question as to whether eprenetapopt is a unique Michael acceptor, or if it is in fact part of a family of soft electrophiles that mirror a similar MoA that can be exploited in oncology or other human disease.

Mutant p53: Still a feasible therapeutic target for oncology?

An unresolved question remaining from this thesis is whether mut-p53 is a good or even feasible therapeutic targeted for cancer therapy. Whilst eprenetapopt should no longer be thought of as a mut-p53 targeted therapeutic, the clinical impetus for development of mut-p53 reactivators remains strong. The most fundamental goal of a mut-p53 reactivator should be to act as the ultimate cancer cell-specific therapeutic, as mut-p53 is expressed highly in malignant tissues and not in normal cells. Two very recent results have flagged two potential compounds with mut-p53 reactivation capacity (5,437). The first is an ancient drug, mentioned at the beginning of this thesis, ATO (5), and the second is a novel compound *MANIO* (437). Worryingly, neither of these compounds successfully pass the first criteria described in **Chapter 1** for a mut-p53 reactivator - being selective for *TP53*-mutated cancers cells compared to wild-type *TP53* cells. Moreover, the clinical evaluation of ATO (in combination with decitabine) is already underway in *TP53*-mutated AML and MDS patients (5), without a study to determine appropriate therapeutic biomarker(s) in an unbiased fashion. If there is any hope of developing anti-cancer agents that truly target mut-p53 cancers

in the future, more stringent examination of compound efficacy and MoA should be undertaken prior to initiation of clinical trials in order to avoid repeating the mistakes of the past.

Improving selectivity and therapeutic activity is a major goal in the field of cancer therapeutics. However, even in this era of personalised medicine, the prospect of distinguishing treatment sensitive tumour subsets based on underlying genetics and biology, prior to the implementation of a specific drug regimen, remains in its adolescence (438). The more philosophical question remains as to whether or not a mutation-centric approach to cancer patient treatment stratification is warranted. The use of intelligent combination therapies given in schedules that aim to exploit tumour-intrinsic and tumour-extrinsic features could avoid tumour adaptation and resistance.

EPILOGUE

At times, doing research is like looking under a streetlight hoping to find your keys, only to realise you left them at home on the couch. What has become increasingly salient to me over the exercise of working and writing this thesis is that what we believe based on analytical and empirical data is always a simplification of the complexity of reality. We are constantly improving and revising, developing novel approaches and technologies, in pursuit of a deep understanding of this reality. We will, however, never complete this understanding, for the unknown unknowns are forever out there.

To paraphrase the US President George Washington's farewell address in 1796:

"Though, in reviewing the incidents of my [thesis], I am unconscious of intentional error, I am nevertheless too sensible of my defects not to think it probable that I may have committed many errors... I shall also carry with me the hope that [you will] view them with indulgence; and that, after [four] years of my life dedicated to [eprenetapopt] with an upright zeal, the faults of incompetent abilities will be consigned to oblivion, as I myself [will one day] be."

Doing research is a privilege. In the midst of a global pandemic, I have been privileged to continue working and energised by the hope that what we do makes a difference. Whilst at times the immensity of what we hope to accomplish has left me less than inspired, I remain steadfast in my belief that we are an essential part of the fabric of society.

I am left in awe of the magnitude, the depth, of the task of treating, curing and eradicating cancer for humankind. I still maintain the naïveté that we one day will.

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