

Investigating the mode of action of NMT inhibitors in MYCN-amplified neuroblastoma

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Statement of originality

I, James Zhang, confirm that the work presented in this thesis is my own. Where data has been derived from collaborators and other sources, this has been fully indicated in the thesis.

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Abstract

The *N*-myristoyltransferase (NMT) proteins catalyse the irreversible fatty acylation of >200 substrates with myristate on N-terminal glycines, modulating multiple protein properties including localisation, stability, and activity. NMTs are promising targets in cancer, however an understanding of the mechanism of action of NMT inhibitors (NMTi) is still emerging. It has been shown that deregulated MYC sensitises lymphoma cells to NMT inhibition in part through effects on respiratory complex I. However, the applicability and biological consequences of NMTi in other cancer contexts is still unclear.

In this thesis, the applicability of NMTi in neuroblastoma, the most common paediatric extracranial solid tumour, was investigated. Amplification of *MYCN*, the neural analogue of *MYC*, is both prevalent and associated with poor outcome in neuroblastoma. Using a panel of neuroblastoma cell lines, *MYCN* amplification was found to sensitise cells to NMTi. Multiple -omics based analyses of the effects of NMT inhibition in a *MYCN*-regulatable isogenic cell line revealed that NMT inhibition affects multiple pathways, with effects more rapidly induced in high *MYCN* cells. In particular, strong depletion of respiratory complex I was identified, associated with loss of the NMT substrate NDUFAF4. To validate this association in *MYCN*-amplified neuroblastoma, a NDUFAF4 G2A knock-in mutant was generated, which confirmed the importance of NDUFAF4 *N*-myristoylation alone on oxidative phosphorylation and indicated that the effects of NMTi on oxidative phosphorylation are mediated primarily through respiratory complex I. However, this mutation did not alter sensitivity to NMTi, indicating that the cytotoxicity of NMTi in *MYCN*-amplified neuroblastoma is not mediated primarily by effects on NDUFAF4, but is likely instead by simultaneous modulation of multiple biological pathways.

In total, this work suggests that NMTi would be effective in *MYCN*-amplified neuroblastomas, expanding the possible clinical applications of this compound class, and identifies pathways through which NMTi may exert its cytotoxic effects in neuroblastoma.

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- Dr. Jack Houghton optimised the chromatography and mass spectrometer settings for the timsTOF HT and data processing pipelines. He also conducted the sample preparation for the SHEP21N YnMyr optimisation experiment.
- Daria Berezina assisted in the sample preparation for the SHEP21N YnMyr experiment and with sample loading onto Evotips.
- Dr Jianan Lu ran proteomics samples on the timsTOF HT.
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Abbreviations

aa	Amino acid
ABHD	α/β hydrolase domain
ACSL	Acyl-CoA synthetase
ADC	Antibody-drug conjugate
ADRN	Adrenergic
ALK	Anaplastic lymphoma kinase
ALT	Alternative lengthening of telomere
APT	Acyl protein-thioesterase
Aq	Aqueous
AURKA	Aurora-A kinase
AUC	Area under the curve
BET	Bromodomain and extra terminal domain
BFA	Brefeldin A
bHLH-LZ	basic helix-loop-helix leucine zipper domain
CDK	Cyclin-dependent kinase
CI	Combination Index
COG	Conserved oligomeric Golgi
CRC	Core regulatory circuitry
CV	Coefficient of variation
DDA	Data-dependent acquisition
Det	Detergent
DFMO	Difluoromethylornithine
DLBCL	Double hit B-cell lymphoma
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ETC	Electron transport chain
FASN	Fatty acid synthase
FATP2	FA transport protein 2
FTase	Farnesyltransferase
gDNA	Genomic DNA
GEM	Genetically modified mouse
GGTase	Geranylgeranyltransferase
GNAT	GCN5-related N-acetyltransferase

GPCR	G protein-coupled receptor
GSEA	Gene set enrichment analysis
GTP	Guanosine triphosphate
iMET	Initiator methionine
IPA	Ingenuity Pathway Analysis
KD	Knockdown
KO	Knockout
i.p	Intraperitoneally
LFQ	Label-free quantification
LLE	Liquid-liquid extraction
LoF	Loss of function
MES	Mesenchymal
MetAP	Methionine aminopeptidase
MHC	Major histocompatibility complex
Mitoribosome	Mitochondrial ribosome
MNA	<i>MYCN</i> -amplified
MoA	Mechanism of action
MS	Mass spectrometry
Myr	Myristic acid
Myr-CoA	Myristoyl-CoA
NAC	Nascent polypeptide-associated complex
Nat	<i>N</i> - α -acetyltransferases
nGF	Nanographite fluoride
NMT	<i>N</i> -myristoyltransferase
NMTi	NMT inhibitor
N-TAILS	N-terminal amine isotopic labelling of substrates
OCR	Oxygen consumption rate
ODC1	Ornithine decarboxylase
ORA	Overrepresentation analysis
OS	Overall survival
OxPhos	Oxidative phosphorylation
Pal-CoA	Palmitoyl-CoA
PCA	Principal component analysis
PCD	Programmed Cell Death
PKA	Protein kinase A

PPI	Protein-protein interaction
PROTAC	Proteolysis-targeting chimeras
PTM	Post-translational modification
RC1	Respiratory complex I
Ribi	Ribosome biogenesis
Ribo-Seq	Ribosome sequencing
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SFK	Src family kinase
siRNA	small interfering RNA
SP4	Solvent precipitation SP3
SrtA	Sortase A
TAM	Tumour-associated macrophage
Tg	Thapsigargin
Tm	Tunicamycin
TMT	Tandem-mass Tag
UPR	Unfolded protein response
YnMyr	Alkyne myristate

Chapter 1. Introduction

1.1 The diversity of the human proteome

Proteins are responsible for the vast majority of cellular processes. However, with up to 6 million possible proteoforms but only 20,000 protein-coding genes in the human genome¹, the complexity of the proteome is not described by genetic encoding alone. Proteome complexity arises at the genetic level by mutations, the transcript level by alternative splicing and mRNA processing and the translational level by alternative translational start sites². However, the greatest contribution to proteome expansion arises from co- and post-translational modifications (PTMs) of protein primary sequences, which increases proteome diversity by orders of magnitude³.

Over 600 PTMs are currently described and multiple PTMs can be simultaneously present on a single protein⁴. PTMs can alter the activity, localisation, stability and interactions of a protein and the specific effects of the modification will vary by both type and site of the modification and its co-occurrence with other PTMs. As almost every eukaryotic protein is expected to be post-translationally modified, PTMs therefore play a central role in diverse biological processes and cellular phenotypes. Certain PTMs are irreversible and therefore influence the baseline function of the protein, however most are enzymatically reversible and therefore dynamically control protein function. These modifications occur on a timescale shorter than protein turnover and therefore allow cells to rapidly adapt to changing conditions and stimuli⁴.

The importance of PTMs is highlighted by the numerous pathologies associated with abnormal protein modifications. For example, hyperphosphorylation of tau proteins can lead to neurodegenerative disorders including Alzheimer's disease⁵ and continuous activation of multiple pro-oncogenic signalling pathways is mediated by altered phosphorylation in cancer⁴. As such, PTMs represent a rich area for therapeutic intervention, as the activity of potentially undruggable proteins can be modulated⁶. As examples, an estimated third of all drug development endeavours are targeted towards protein kinases⁷ and therapeutic interest in developing proteolysis-targeting chimeras (PROTACs), which induce protein ubiquitination and degradation, has recently intensified⁸.

1.2 A general overview of protein lipidation

Uniquely among the known protein PTMs, a key aspect of protein lipidation is to directly target proteins to cellular membranes through the hydrophobicity of the lipid moiety³. Such protein-membrane interactions are vital to many biological processes including vesicle-mediated protein transport and protein signalling, although the lipid group can also directly affect protein function. As a result, protein lipidation is a generally biologically essential process, with global ablation of key lipidation pathways poorly tolerated⁶. In disease contexts, roles of protein lipidation have been identified for infection, cancer, and cardiovascular and neurological diseases, among others⁹. As many key oncogenic proteins are lipidated and the lipid group is often essential for their function, the clinical development of lipid transferase inhibitors has been pursued, with recent successes⁶.

Many different protein lipidation events have been identified, however protein S-prenylation, S-acylation and N-myristoylation are generally the most studied. The first two are briefly described here, whereas N-myristoylation, the focus of this thesis, is described in more detail in section 1.3.

S-prenylation is the irreversible post-translational attachment of either a farnesyl (C15) or geranylgeranyl (C20) isoprenoid group onto cysteines located near the protein C-terminus³ (**Figure 1.1A**). The modification is catalysed by four heterodimeric enzymes, farnesyltransferase (FTase) and three geranylgeranyltransferases (GGTase 1-3). Proteins modified by FTase and GGTase I are mono-prenylated on C-terminal CaaX boxes, where “a” represents aliphatic residues and “X” can be any amino acid; the type of prenylation is generally determined by the identity of the X residue. GGTase II recognises C-terminal CC or CXC motifs and modifies Rab proteins, usually as a double geranylgeranylation event, while the full substrate scope of the recently identified GGTase III is yet to be determined. Due to the clinical importance of known S-prenylated proteins including the Ras oncogenes, and the requirement of the prenyl group for their oncogenic activity, multiple prenyltransferase inhibitors have been developed¹⁰. These have generally been limited in clinical trials by low efficacy or high toxicity^{11,12}, although the farnesyltransferase inhibitor lonafarnib has recently obtained FDA approval for use in progeria¹³. This is the first clinically approved lipid transferase inhibitor, providing proof that targeting such enzymes provides therapeutic value in the correct contexts.

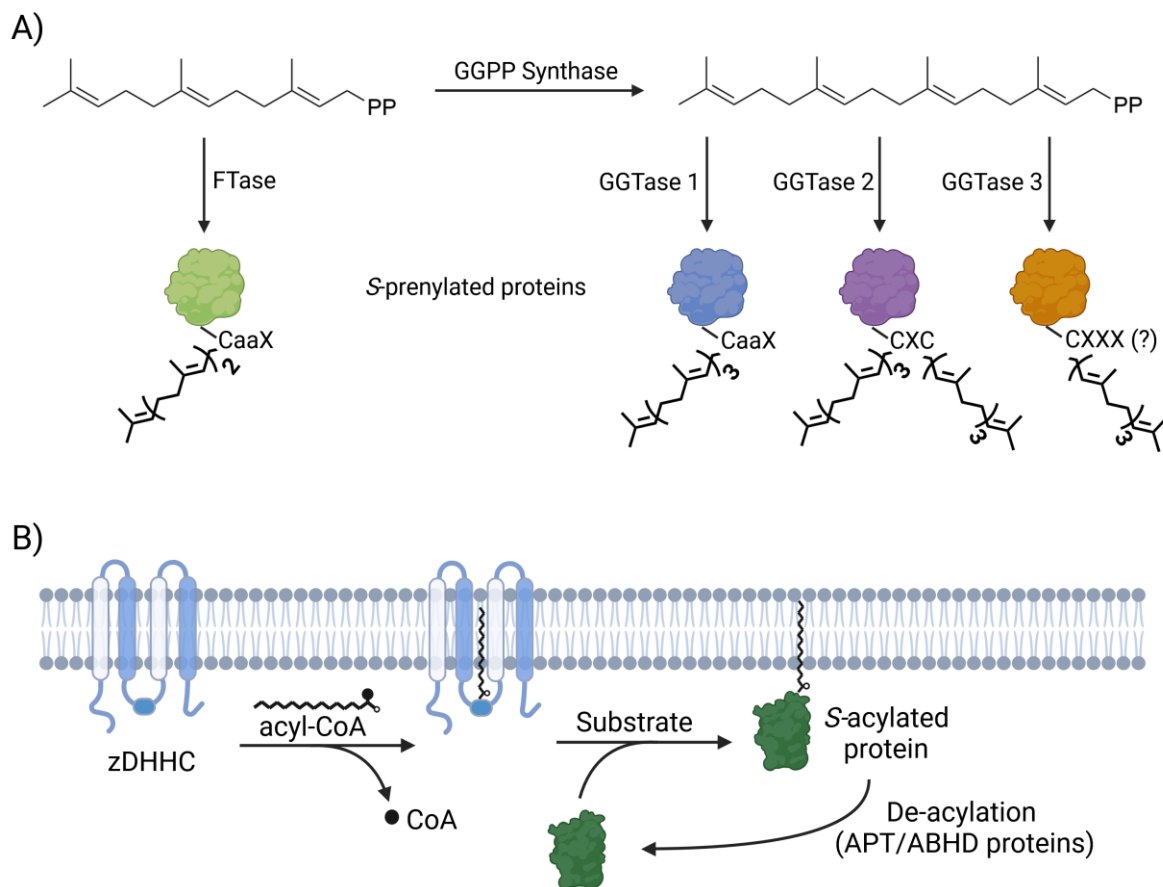


Figure 1.1 Protein S-prenylation and S-acylation. A) S-Prenylated proteins are defined by recognition motifs (CaaX, CXC and CXXX) and are modified by FTase or GGTase I-III. The full substrate scope of GGTase III is still unknown. **B)** S-Acylated proteins are modified by zDHHC proteins. The zDHHC is first auto-acylated, followed by lipid transfer onto a substrate protein. The acyl group may be removed by APT/ABHD proteins. X: any residue, a: aliphatic residue.

S-acylation is the only major reversible lipidation event and is the post-translational attachment of long chain fatty acids, generally 14 to 20-carbon long, to the thiol of protein cysteines³ (**Figure 1.1B**). Although a heterogeneous lipidation, attachment of palmitate (C16:0) is most common, and the modification is often referred to as S-palmitoylation as a result. This modification is catalysed by the 23-member zDHHC family of protein acyltransferases. S-acylation is widely prevalent in cells, with over 5000 putative substrates identified in human palmitoyl-proteomes across a range of cell types (SwissPalm)¹⁴, although a consensus sequence for the modification has not been identified¹⁵. On the other hand, various acyl protein-thioesterase (APT) or α/β hydrolase domain (ABHD) proteins can remove the acyl chain from modified cysteines, thereby

subjecting *S*-acylated proteins to an additional level of dynamic regulation³. This PTM has been implicated in a variety of disease states, including neurodegenerative diseases¹⁶ and cancer⁶, however the field of *S*-acylation is less developed in terms of therapeutic intervention relative to the other types of major lipidation (*S*-prenylation, *N*-myristoylation). This can be attributed to two main factors. Firstly, specific and selective zDHHC inhibitors are lacking and their development is challenging¹⁷. The widely used 2-bromopalmitate also inhibits general lipid metabolism¹⁸, while inhibition of specific zDHHCs is likely to be difficult given the conserved nature of the zDHHC active site¹⁹. Secondly, conventional techniques are poorly optimised to characterise *S*-acylation, with the integral membrane nature of zDHHCs and the labile and complex nature of the *S*-acylated group complicating structural and proteomic studies respectively^{3,20}. Indeed, the first structures for zDHHCs^{21,22}, as well as methods to identify specific substrates for individual zDHHCs, have only recently been reported²³. It is likely, however, that advances in cryo-EM and chemical genetic techniques will help overcome these obstacles.

1.3 Protein *N*-myristoylation: Biology

1.3.1 *N*-myristoylation overview

N-myristoylation is the irreversible attachment of myristate (C14:0) onto the free amine of the N-terminal glycine of over 200 proteins³ and is catalysed by *N*-myristoyltransferase (NMT) enzymes (**Figure 1.2**). The modification usually occurs co-translationally at the ribosome following excision of the initiator methionine (iMet) by methionine aminopeptidases (MetAPs) early in translation (<100 residues polymerised)²⁴. However, the modification is also known to occur post-translationally after protease cleavage to reveal an internal glycine at neo-N-termini^{25,26}. The modification has been shown to influence a range of protein properties, discussed further in section 1.3.6²⁷.

1.3.2 *N*-myristoyltransferases: redundant or unique?

NMT enzymes are ubiquitous in eukaryotes²⁸, and have been characterised in all such species investigated, including pathogenic fungi²⁹⁻³¹ and protozoa^{32,33}, yeast³⁴ and mammals²⁷. Lower eukaryotes express only one NMT, whereas mammals express two NMT isozymes (NMT1 and NMT2)³⁵. Furthermore, two different isoforms of NMT1 can be expressed as a result of alternative splicing or translation initiation at a later initiation site³⁶.

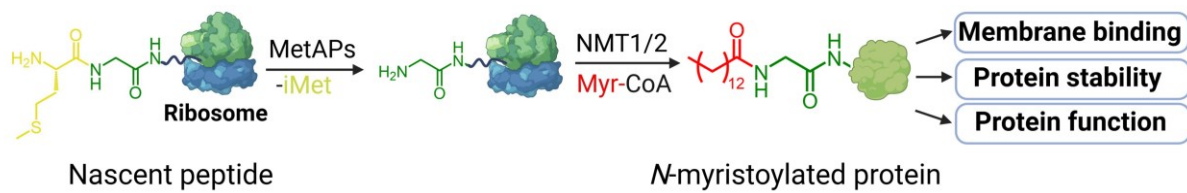


Figure 1.2 Co-translational protein *N*-myristoylation. The initiator methionine is first removed from nascent peptides by MetAPs to reveal a N-terminal glycine. Subsequently, NMT1 or NMT2 transfers a myristoyl group from the Myr-CoA co-substrate onto the N-terminal amino group of selected proteins. The myristoyl group regulates multiple protein properties including localisation, stability, and function.

N-myristoylation is highly important in eukaryotes; NMTs are essential for survival in yeast³⁷, fungi³⁸, and parasitic protozoa^{39,40}, and is required for normal development in *D. melanogaster*⁴¹. In mammals, whether NMT1 and NMT2 are redundant or have specialised functions has been a matter of some investigation. Genetically, NMT1 and NMT2 share 77% sequence identity, however the homology between mouse and human NMTs is 96-97%, suggesting evolutionarily conserved, distinct roles for each isozyme³⁵. The two isozymes differ mainly in the N-terminal region, which is dispensable for catalysis but is thought to mediate ribosomal targeting⁴². Indeed, *in vitro* peptide assays have identified almost complete overlap in the substrate specificity and catalytic efficiency of the two isozymes^{35,43}, although there appears to be minimal differences in their localisation. Both isoforms are present in the cytosol and plasma membrane, whereas NMT2 is reportedly also present in the Golgi⁴⁴. However, cellular *N*-myristoylation in mammals appears to be catalysed primarily by NMT1 and homozygous *Nmt1* knockout (KO) is embryonic lethal in mice despite *Nmt2* expression⁴⁵⁻⁴⁷. In line with this hypothesis, CRISPR essentiality screens have identified that NMT1 is essential in most (cancer) cell lines, whereas no lines appear to require NMT2⁴⁸⁻⁵⁰. Indeed, proteomic studies in NMT1 and NMT2 knockout Panc-1 cells identified significant changes only in NMT1 KO cells, in both total protein levels and *N*-myristoylation state⁵¹. Therefore, NMT2 appears to be dispensable for global *N*-myristoylation under normal conditions. However, differential roles of the two isozymes may arise during apoptosis, as caspase cleavage leads to predominantly cytosolic localisation of NMT1 but membrane localisation of NMT2⁵².

1.3.3 The structure and mechanism of action of NMTs

NMT enzymes are structurally well characterised, with 147 crystal structures of NMTs across all species listed on the Protein Data Bank, including 43 structures of human NMT1

(HsNMT1)⁵³. HsNMTs belong to the GCN5-related *N*-acetyltransferase (GNAT) family and possess two GNAT domains joined by a loop region⁵⁴. Myristoyl-CoA (Myr-CoA) binds to the N-terminal GNAT domain, whereas both domains contribute to peptide substrate binding⁵⁵. The catalytic cycle of NMTs has also been investigated and was found to be a sequential, ordered Bi:Bi mechanism based off of initial data from *S. cerevisiae* NMT⁵⁶. More recently, the catalytic mechanism of HsNMT1 was elucidated by a series of crystal structures of the protein in complex with reactive substrates (**Figure 1.3**)^{43,57}. First, the Myr-CoA co-substrate binds to NMT, which induces a conformational change to form the peptide acceptor site. A peptide can then bind, with the protonated Gly2 positioned by multiple contacts with the Thr282 residue and connected to the Gln496 catalytic base by a water molecule from a solvent channel. Peptide binding induces a conformational change which distorts the Myr-CoA thioester and triggers the nucleophilic substitution reaction by concerted deprotonation of the Gly2 and nucleophilic attack by the Gly2 amine onto the thioester of Myr-CoA. After product formation, the CoA product is released first, followed by the Myr-peptide species; product release is likely the rate determining step^{57,58}.

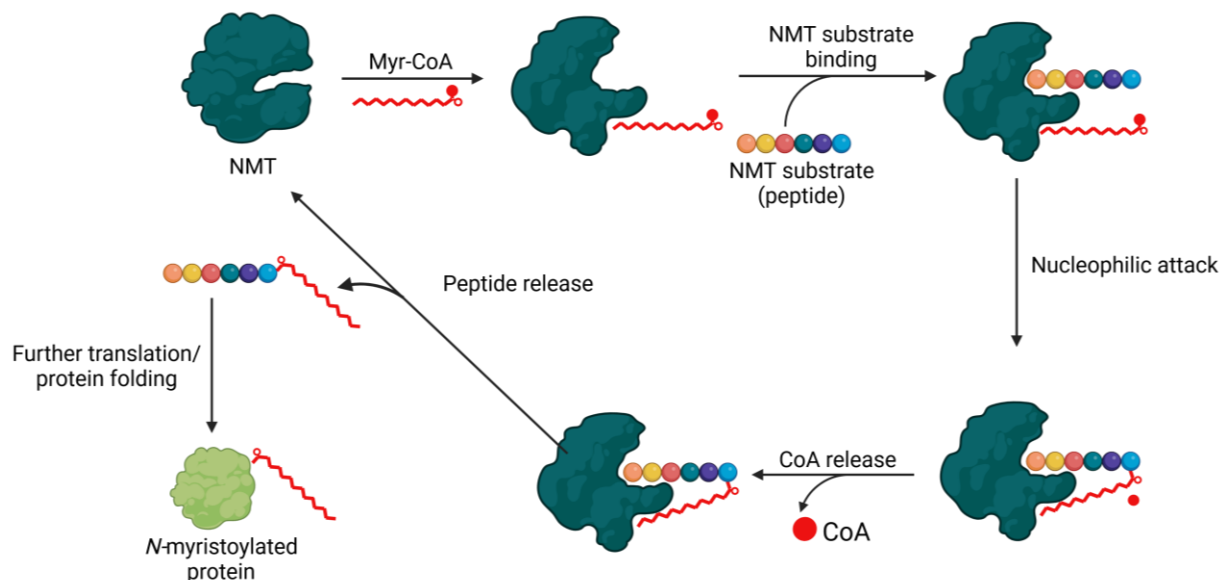


Figure 1.3 NMT catalytic cycle. Binding of Myr-CoA to NMT induces a conformational change forming the peptide binding pocket. The nascently translated NMT substrate then binds. NMT facilitates a nucleophilic attack of the N-terminal amine onto the thioester of Myr-CoA. CoA is released first, followed by the *N*-myristoylated peptide. After further translation and folding, the mature *N*-myristoylated protein is formed.

1.3.4 NMT substrate specificity

The myristoyl-CoA binding site has been extensively investigated, predominantly in *S. cerevisiae* NMT (ScNMT), although the pocket is highly conserved across species²⁷. The chain length of the acyl-CoA species appears to be the main determinant of whether it is accepted by NMT⁵⁹; the acyl-CoA binds in a bent conformation, such that the length of the acyl chain is measured^{60,61}. Therefore, apart from Myr-CoA, C13:0 and C15:0 acyl-CoAs, which are physiologically rare, are well tolerated, but other chain lengths are not, ensuring selectivity *in vivo*⁶². Interestingly, cases of heterogeneous acylation on N-terminal glycine have been reported^{63,64}, although the consequences of this have not yet been thoroughly investigated.

Multiple studies investigating peptide substrate specificity have also been carried out (**Figure 1.4**). A comprehensive *N*-myristoylation motif has proved elusive, although amino acid preferences for residues 2-7, which reside inside or at the peptide binding pocket, have been identified through crystal structures⁴³. A N-terminal glycine is required; for co-translational *N*-myristoylation, this is usually at position 2 (except in the case of MRPS36, which has a N-terminus with two methionines and is presumably processed twice by MetAPs to reveal a N-terminal Gly3)^{64,65}.

The small and acidic nature of the binding pocket for the next amino acid (aa3), preventing the inclusion of large (Arg, Phe, Tyr, Trp) and negatively charged (Glu, Asp) residues⁴³. Proline is also prohibited, due to steric restraints^{34,66}. Minimal restrictions on amino acid identity are placed by the aa4 pocket, whereas the hydrophobic nature of the aa5 binding pocket excludes charged (Asp, Glu, Lys, Arg) amino acids. Finally, aa6 again favours small residues, although Ser6 is most common (~60% of known NMT substrates)⁵⁴, and aa7 favours positively charged residues (Lys, Arg) due to formation of a salt bridge with three conserved aspartic acid residues at the mouth of the binding pocket. However, selectivity for peptide substrates may rely also on residues outside of the binding pocket, up to aa18; these residues either interface with the surface of NMT (aas8-11) or act as a hydrophilic linker without secondary structure (aas12-18)⁶⁷. It is important to note that the above preferences are not absolute. As examples, ARF1 lacks a conventional recognition motif but is *N*-myristoylated⁶⁸, while ARL2 is not, although other ARFs and ARLs are NMT substrates⁶⁹. Therefore, experimental evidence for *N*-myristoylation is still required.

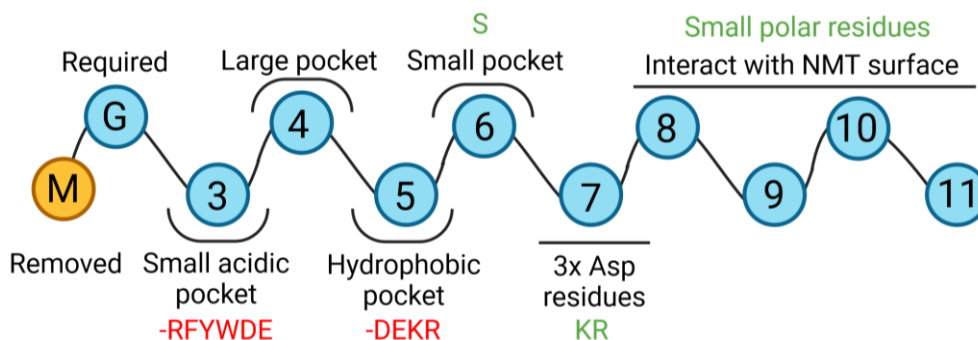


Figure 1.4 The conventional *N*-myristoylation motif. A N-terminal glycine is the only requirement. The nature of the binding pockets for positions 3, 5 and 6 restrict the accepted amino acids at these positions, while the presence of three adjacent Asp residues favours positively charged amino acids at position 7. Positions 8-11 interact with the surface of NMT and are less important for substrate selectivity but tend to be small and polar. Red: disfavoured amino acids, Green: favoured amino acids.

Classically, *N*-myristoylation occurs only on glycines, however recent reports have suggested that NMTs may catalyse post-translational *N*-myristoylation of lysine ϵ -amino groups^{57,70,71}, and that such a modification is reversible due to the ability of sirtuins or histone deacetylases to remove the myristate group⁷²⁻⁷⁵. It is speculated that such a modification may regulate signal transduction or act as a protein quality control mechanism⁵⁷. Indeed, one report suggests that ARF6 is dynamically *N*-myristoylated on lysines *in vivo*, modulating its GTPase cycle⁷⁰. However, to date, lysine *N*-myristoylation has only been unambiguously detected in cells using overexpression or knockout systems with high (50 μ M) levels of exogenous myristic acid added. Whether this modification occurs under physiological conditions therefore remains unproven.

1.3.5 Regulation of *N*-myristoylation

The mechanisms by which NMT activity is regulated have not been well defined, although various methods have been suggested (**Figure 1.5**). The most obvious regulatory mechanism is the availability of the co-substrate Myr-CoA. Myr-CoA is generated from myristic acid by acyl-CoA synthetases (ACSLs). However, myristic acid is rare in cells (<1% of total fatty acid content in cells)⁷⁶ and its availability may therefore limit NMT activity^{61,77}. Indeed, knockdown (KD) of ACSL4 may limit global *N*-myristoylation in prostate cancer models⁷⁸. A major source of myristic acid is from the diet, however it can be synthesised *de novo* from acetyl-CoA by fatty acid synthase (FASN)⁷⁹. Other sources

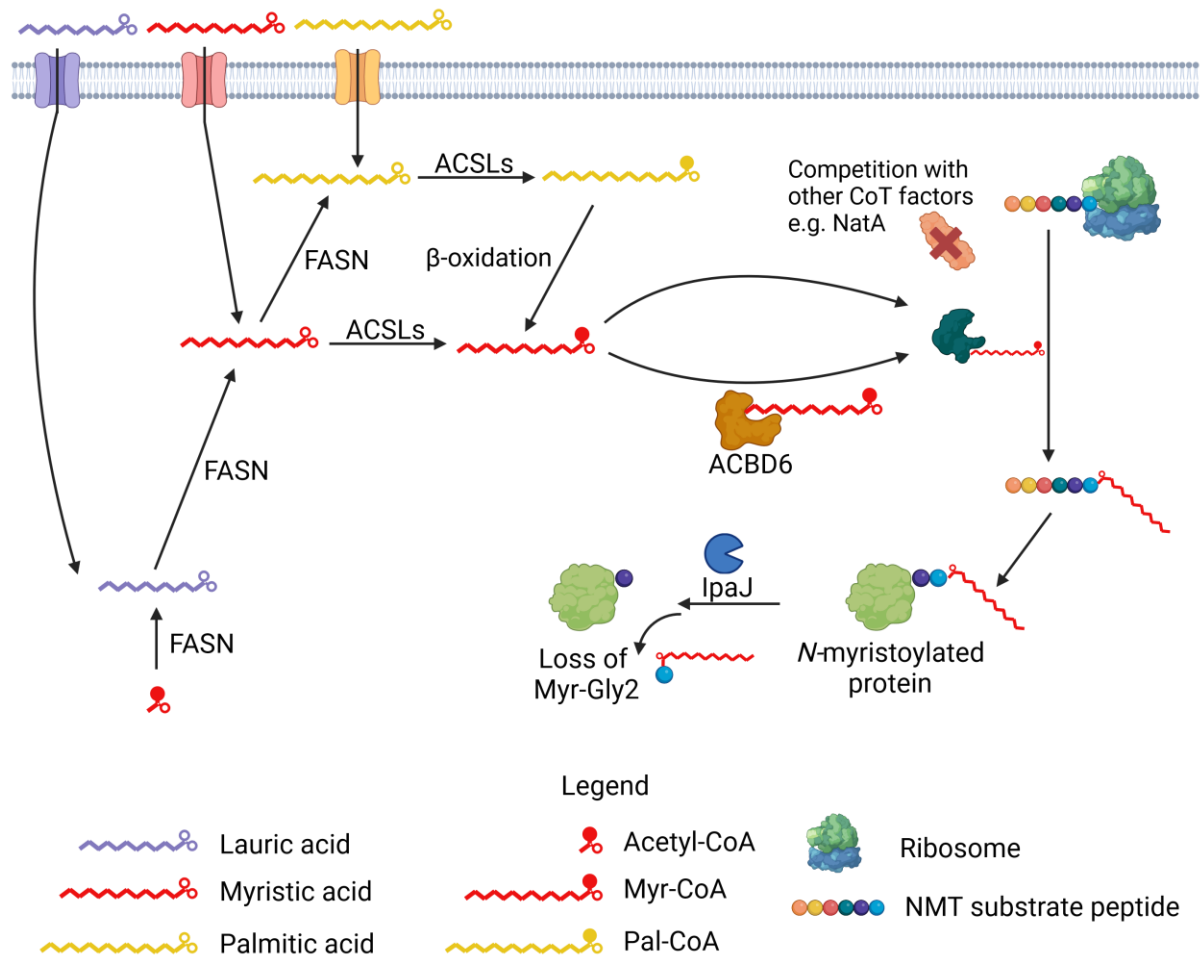


Figure 1.5 Regulation of N-myristoylation. Protein N-myristoylation is likely regulated on multiple levels, including by regulation of Myr-CoA availability and delivery to NMT and spatiotemporal regulation at the ribosome, for example potential competition with NatA. In the context of *S. flexneri* infection, ARF proteins are demyristoylated by the bacterial protease IpaJ.

of myristic acid include β -oxidation of the highly abundant palmitic acid or chain elongation of lauric acid (C12:0).

In contrast with to Myr-CoA, palmitoyl-CoA (Pal-CoA) is not accepted as a lipid donor by NMTs, although it can still enter the Myr-CoA binding pocket⁶¹. As the physiological concentration of Pal-CoA is much higher than that of Myr-CoA⁸⁰, Pal-CoA may therefore act as a competitive inhibitor of NMT, leading to the hypothesis that NMT requires protection from endogenous Pal-CoA. The acyl-CoA binding protein ACBD6 has been suggested to promote selective Myr-CoA binding through its interaction with NMT⁸¹⁻⁸³. ACBD6 may bind to Myr-CoA to for delivery to NMTs, whereas its binding of Pal-CoA may prevent its access to NMT. Indeed, bi-allelic loss-of-function (LoF) mutations in ACBD6 or

ACBD6 KO affects the incorporation of exogenous myristate probes into NMT substrates without affecting overall protein abundance, suggesting that ACBD6 regulates *N*-myristoylation through direct or indirect mechanisms⁸⁴.

As the majority of *N*-myristoylation occurs co-translationally, it is expected that an additional layer of regulation arises at the ribosome. NMTs need to act in a coordinated fashion with other co-translational and nascent chain factors, such as MetAPs, *N*-α-acetyltransferases (Nats) and the nascent polypeptide-associated complex (NAC)⁸⁵. Although no studies examining the structure of NMT bound to ribosomes have been reported, other co-translational factors are known to bind to the ribosome as well as the nascent peptide chain. These include MetAPs⁸⁶ and NatA⁸⁷, which catalyses N-terminal glycine acetylation and shares a GNAT fold with NMT⁴³. Recent studies have also shown that the NAC spatiotemporally recruits and controls MetAP/NatA activity to regulate co-translational activity^{88,89}. Therefore, it is possible that NMT binds to the ribosome in a similar position to NatA, and indeed may compete with NatA. The substrate preferences of NMT and NatA are different; NatA prefers acidic N-termini, which is disfavoured for NMT⁴³. However, the degree of *N*-myristoylation for a given substrate in cells is not necessarily stoichiometric and some NMT substrates also exist in a N-terminally acetylated or unmodified state in cells^{43,90}. Competition of NMTs and NatA for ribosome binding, in addition to their inherent affinities for the polypeptide chain, may therefore determine the modification state of these proteins.

Finally, although *N*-myristoylation is conventionally irreversible, the *S. flexneri* enzyme IpaJ can catalyse demyristoylation through the recognition and cleavage of the *N*-myristoylated glycine⁹¹. Although promiscuous *in vitro*, IpaJ is remarkably selective for ARF proteins in the context of *Shigella* infection⁹², and is the only known demyristoylase to date.

1.3.6 The impact of *N*-myristoylation on proteins

As already mentioned, a key function of the myristate group is to increase the local hydrophobicity of the modified protein to target otherwise cytosolic proteins to membranes. Although an irreversible modification, *N*-myristoylation alone does not necessarily mediate stable membrane localisation. The modification is often essential but not sufficient for protein membrane localisation as the Gibbs free binding energy of myristate to membranes

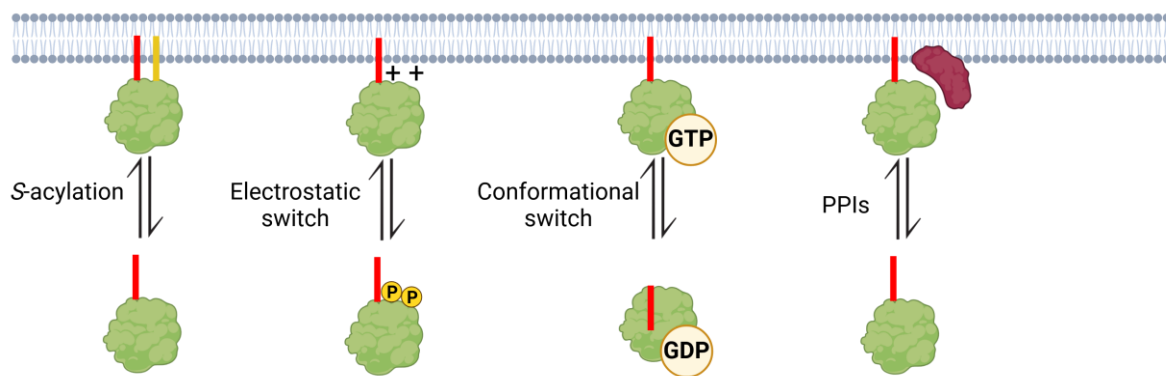


Figure 1.6 Two-signal membrane binding model. In addition to *N*-myristoylation, membrane association can be induced through additional *S*-acylation of the NMT substrate, polybasic domains which interact with the negatively charged phospholipid head groups, ligand binding or protein-protein interactions. These secondary signals are usually reversible, allowing dynamic control of protein localisation.

(8 kcal/mol) is insufficient for stable membrane insertion⁹³. Therefore, a second signal is often required, producing a two-signal membrane binding model (**Figure 1.6**). These secondary signals can be modulated, giving rise to “myristoyl switches” that dynamically regulate protein localisation²⁷. In this way, the activity of signal transduction pathways can be controlled. Multiple potential secondary signals are possible and are described below.

Protein *S*-acylation is a common mechanism by which *N*-myristoylated proteins are anchored into the membrane, with examples including most Src family kinases (SFKs)⁹⁴ and GNAI (Gai) proteins⁹⁵. As zDHHCs are integral membrane proteins⁹⁶, *S*-acylation occurs after initial *N*-myristoylation, which likely directs the protein to the membrane for zDHHC processing. It has been shown that *N*-myristoylation of proteins such as CCNY⁹⁷ and the SFK Fyn⁹⁸, among others, is required for *S*-acylation, supporting this model. Removal of the *S*-acyl group by APTs or ABHDs can cause membrane disassociation⁹⁵. Stable membrane association can also be achieved by the presence of polybasic domains in the modified protein, which electrostatically interact with the negatively charged head groups of membrane phospholipids; examples of proteins exploiting this mechanism include c-Src⁹⁹ and MARCKS¹⁰⁰. Phosphorylation of residues in the polybasic site ablates their associated positive charge and leads to loss of membrane localisation. Ligand-induced myristoyl switches have also been described. In these cases, the myristoyl group is sequestered in an internal hydrophobic pocket. Binding of the protein ligand induces a conformational change, extruding the myristoyl group and allowing it to insert

into the membrane. This mechanism has been demonstrated for various proteins including recoverin¹⁰¹, which binds Ca^{2+} , and ARF1¹⁰², which exchanges GDP with GTP to expose the myristate group and activate signalling.

In addition to localisation, the myristate group often controls protein stability. In the case of cAMP-dependent protein kinase (PKA), this is due to direct stabilisation by the myristate group¹⁰³. More generally, *N*-myristoylation protects proteins from the recently described N-degron pathway in which two Cullin-RING E3 ubiquitin complexes, Cul2^{ZYG11B} and Cul2^{ZER1}, recognise and direct proteins with free N-terminal glycines to the proteasome¹⁰⁴. This pathway may play a role in quality control of *N*-myristoylation, as mutation of ZYG11B and ZER1 partially rescues destabilisation of NMT substrates in NMT1/2 KO contexts.

N-myristoylation also directly regulates protein catalytic activity, most notably for the tyrosine protein kinase c-Abl. Crystal structures have revealed that myristate binds at the base of the kinase domain, docking the SH2 domain in an auto-inhibitory conformation¹⁰⁵. Indeed, loss of *N*-myristoylation in the BCR-ABL fusion protein is responsible for its constitutive and oncogenic activity. On the other hand, c-Src *N*-myristoylation directly enhances its kinase activity, showcasing the diverse effects of the modification¹⁰⁶.

Finally, the myristate group may be directly involved in protein-protein interactions (PPIs). Crystal structures of calmodulin and UNC119A in complex with *N*-myristoylated, N-terminal substrate peptides have identified hydrophobic channels in each protein which bind the myristoyl group^{107,108}. Furthermore, photo-crosslinking experiments have also suggested that the myristate of CHP1 directly interacts with and activates GPAT4¹⁰⁹.

1.3.7 Probing *N*-myristoylation

Early methods to assess analyse *N*-myristoylation were limited to metabolic labelling with radiolabelled myristic acid and immunoprecipitation of targeted proteins, which was lowly sensitive and low throughput, given the low immunogenicity and reactivity of the lipid group³. The development of biorthogonal “click” reactions and chemical proteomics therefore represented a significant advance into the study of lipidation as a whole, including for *N*-myristoylation. Specifically, incorporation of alkyne or azide groups into myristic acid analogues, followed by metabolic incorporation into cells, allows the global labelling of *N*-myristoylated proteins at endogenous levels (**Figure 1.7A**). Such tools, in particular the alkyne analogue of myristic acid (YnMyr), have been applied to investigate

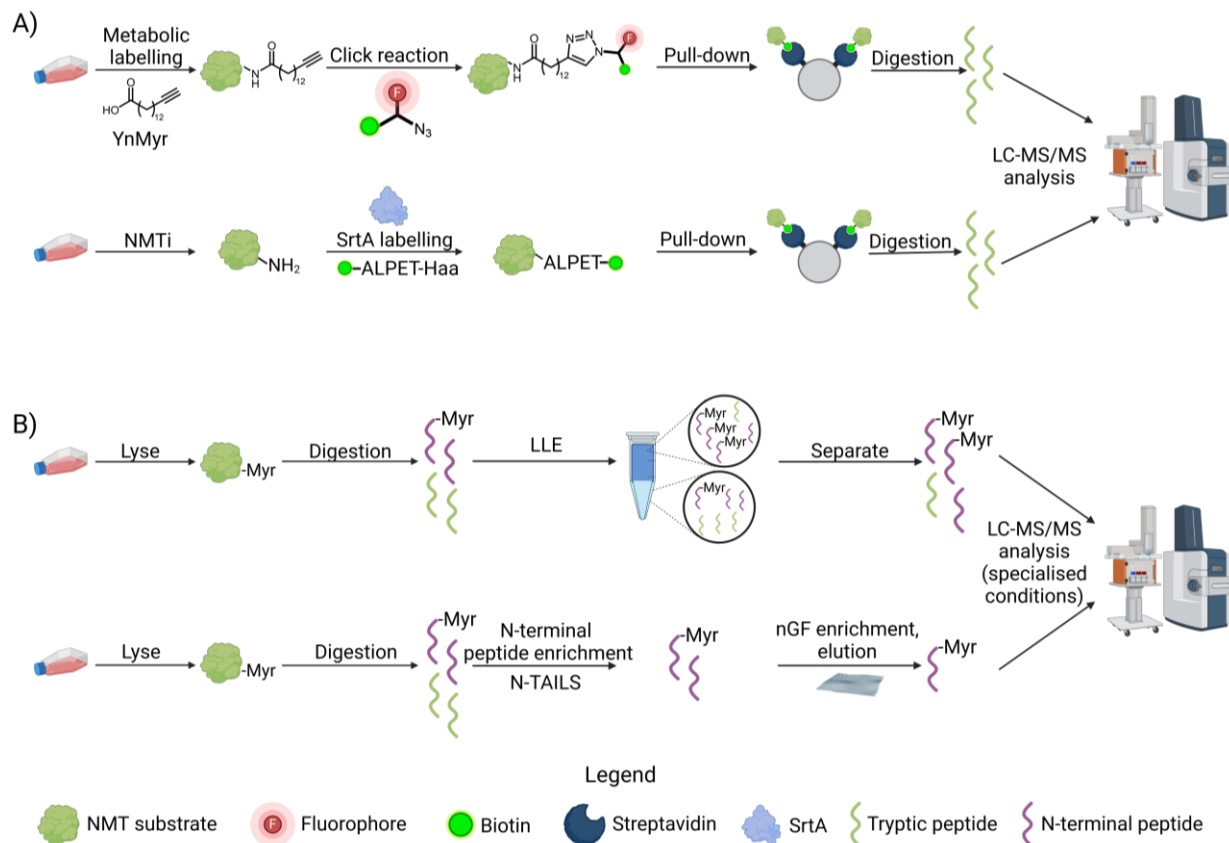


Figure 1.7 Proteomic methods to assay N-myristoylation. **A)** Chemical proteomic methods to identify NMT substrates. Top, metabolic labelling of cells with e.g. YnMyr labels newly translated NMT substrates with an alkyne functional group. After cell lysis, these proteins are ligated via a "click" reaction to a capture (biotin) or visualisation (TAMRA) group (AzTB, with both groups, is shown here). Tagged proteins are enriched by pull-down on Neutravidin beads, digested and analysed by LC-MS/MS. Bottom, cells treated with NMTi generates NMT substrates with unmodified N-termini. These can be tagged with biotin using SrtA enzyme and enriched by Neutravidin pull-down. **B)** Proteomic methods to identify endogenous N-myristoylated N-terminal peptides. Top, cells are lysed, proteins digested and N-myristoylated peptides enriched by LLE. Bottom, cells are lysed, proteins digested and N-terminal peptides enriched using N-TAILS. N-myristoylated peptides are then enriched using nGF. For both methods, peptides are analysed using optimised LC-MS/MS conditions to improve detection of lipidated peptides.

the N-myristoylated proteome in multiple experimental models, including human cell lines, parasites, zebrafish, and frogs^{26,40,84,110}. In such experiments, cells are treated with YnMyr. Cellular uptake of YnMyr and conversion to the acyl-CoA species allows it to act as a co-substrate to NMT and label native NMT substrates. Following YnMyr incorporation, cells are lysed and proteins subjected to a biorthogonal click reaction; the incorporated alkyne tag may be selectively ligated to a variety of azide-conjugated reporters, such as fluorophores (e.g. TAMRA) or capture tags (e.g. biotin). Total cellular N-myristoylation can

therefore be visualised through in-gel fluorescence or biotin blots, whereas enrichment of tagged proteins (e.g. by pull-down of biotinylated proteins onto Neutravidin beads) allows the visualisation of specific NMT substrates by Western blotting. Alternatively, enriched proteins can be digested by trypsin and the peptides submitted for LC-MS/MS analysis for global identification of *N*-myristoylated proteins.

However, it must be noted that such experiments will always be non-physiological, as supra-physiological concentrations of myristate analogue are used. This may induce physiologically non-significant *N*-myristoylation through increased cellular (alkyne) myristoyl-CoA concentrations or alterations in fatty acid metabolism pathways. Furthermore, incorporation of myristate analogues into proteins is not unequivocal evidence for *N*-myristoylation, unless site-level information is obtained. Such analogues may be further processed in the cell, for example by incorporation into GPI anchors or conversion into other lipid species and then ligated to proteins e.g. by zDHHCs²⁶. Unfortunately, detection of the lipid-modified peptide is challenging owing to the low abundance of such peptides, retention of the modified peptide on avidin beads and the detrimental effects of the lipid on peptide ionisation¹¹¹. In this regard, two enhancements to this protocol aid identification of true NMT substrates. First, trypsin-cleavable capture reagents, such as AzRB, allow the elution of probe-tagged peptides from streptavidin beads and improves peptide ionisation through the incorporation of an extra arginine residue, giving access to site-level information¹¹⁰. Second, the development of potent and selective NMT inhibitors have greatly aided identification of the *N*-myristoylated proteome, as pre-incubation of cells with NMT inhibitors prior to YnMyr treatment prevents protein incorporation of YnMyr only for native NMT substrates²⁶.

Although the above approach is very powerful, it requires metabolically active samples, preventing its use in *ex vivo* and tissue samples. Furthermore, the amount of YnMyr required quickly precludes its use in many *in vivo* models. As a result, a complementary method to profile *N*-myristoylation was also developed by the Tate lab, which exploits the ability of the Sortase A (SrtA) enzyme to selectively ligate a peptide substrate onto free N-terminal glycines¹¹². Fluorescence or capture tags can be incorporated into the peptide substrate, and NMTi-treated samples will be enriched in NMT substrates possessing free N-terminal glycines, allowing identification of NMT substrates through mass-shift assays, or by mass spectrometry (MS) following enrichment.

Recently, two publications have reported methods to enrich for endogenous *N*-myristoylated peptides without the requirement for protein labelling, allowing the unequivocal identification of endogenous *N*-myristoylation and classification of NMT substrates in any sample^{64,113} (**Figure 1.7B**). Both these methods depend on hydrophobic enrichment of the myristoyl group, as well as a high MeCN chromatographic gradient during LC-MS/MS analysis, to overcome the challenges of low peptide abundance and solubility preventing detection by mass spectrometry. The first method implements parallel processing of the same sample by three different digestion enzymes (trypsin/LysC, GluC and chymotrypsin), followed by liquid-liquid extraction (LLE) to enrich *N*-myristoylated peptides and maximise identifications, whereas the second method first enriches for N-termini using N-terminal amine isotopic labelling of substrates (N-TAILS), followed by enrichment of *N*-myristoylated peptides using nanographite fluoride (nGF). Indeed, these studies each identified over 50 *N*-myristoylated N-termini, comparable to the number of NMT substrates inferred by the metabolic labelling and SrtA methods and provided direct evidence for *N*-myristoylation of 8 and 9 previously unknown or low confidence substrates, respectively.

The above methods have been leveraged to identify multiple NMT substrates, both co-translational and post-translational. Since *N*-myristoylation is predominantly co-translational, as indicated by the incorporation of radiolabelled myristate in nascent polypeptide chains¹¹⁴ and the interaction of NMT with the ribosome, it is useful to clarify the classification criteria for co- or post-translational substrates. Generally, *N*-myristoylated proteins which possess a N-terminal glycine are considered co-translational substrates, whereas proteins for which *N*-myristoylation is detected at internal glycines (usually by metabolic labelling) are considered post-translational substrates^{25,26,115}.

Finally, it should be mentioned that while selective NMTi are available, a recent study has shown that commonly used tool NMTi are not *bona fide* NMT inhibitors (**Figure 1.8**), including the commonly used 2-hydroxymyristic acid and D-MAPPD (B13)¹¹⁶. While the inactivity of B13 as a NMT inhibitor in this study has been attributed to the commercial compound tested being an enantiomer of the originally synthesized compound^{117,118}, comprehensive validation of the correct enantiomer has not been carried out. Furthermore, as other publications have used the commercially available compound, interpretation of such studies should still be approached with caution.

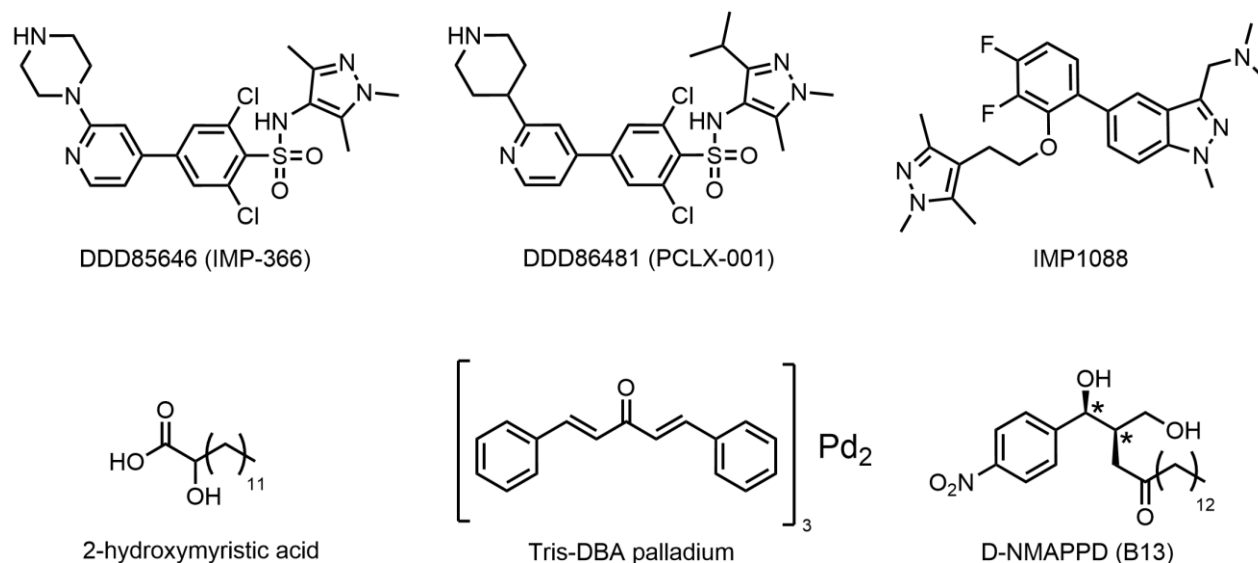


Figure 1.8 Commonly used NMT inhibitors. Validated (top row) and invalidated (bottom row) NMT inhibitors. DDD86481 is currently being evaluated in clinical trials under the tradename zelenirstat¹¹⁹. IMP1088 is highly used as a tool compound in the Tate group.

1.4 Targeting *N*-myristoylation in disease

1.4.1 Targeting NMT in infectious diseases

Pharmacological inhibition of NMTs has been suggested as a therapeutic strategy for decades. Initial efforts at developing clinical NMTi were focused on their anti-fungal potential, as NMT is essential for clinically relevant species and current anti-fungal treatments are limited by toxicity and resistance¹²⁰. A range of NMTi with high potencies against purified fungal NMTs were developed based on multiple scaffolds, including peptidomimetics, benzofurans and benzothiazoles. Unfortunately, none of these proved successful, with low anti-fungal efficacy or narrow spectrum as major reasons¹²¹. More recent efforts to develop anti-fungal NMTi have therefore focused on repurposing more recently developed NMTi or *in silico* studies to identify novel scaffolds¹²¹⁻¹²³.

Similarly, NMT has been investigated as a drug target against parasitic protozoa, including those from the genus *Leishmania*³⁹, *Trypanosoma*³⁹, *Plasmodium*^{40,124-126} and *Cryptosporidium*¹²⁷, which are responsible for multiple tropical diseases, namely leishmaniasis, Chagas disease, sleeping sickness and malaria. Many high-throughput screens and structure-activity relationship studies have been published, giving rise to many different scaffolds^{124,125,128-131}. Notably, multiple potent parasitic NMTi have been developed, including the pyrazole sulfonamides DDD85646¹²⁹ and DDD100097¹³² and the

aminomethylindole IMP1002¹³³. However, these compounds have low selectivity for the parasite NMT over *HsNMT*¹³⁴, which currently limits the translational potential of such inhibitors.

Although viruses do not possess NMTs, multiple viral proteins are *N*-myristoylated by host NMTs which is essential for their function²⁸. For example, the HIV-1 protein Gag requires this modification for membrane localisation and processing, with NMT inhibition preventing the formation of infectious HIV-1 particles¹³⁵. Furthermore, picornaviruses, which include the common cold, require *N*-myristoylation of the capsid protein VP4 to complete capsid assembly¹³⁶. NMT inhibition therefore prevents the production of infectious particles and protects against virus-induced cytotoxicity. This was shown using the highly potent and selective NMTi IMP1088, which possesses sub-nanomolar affinities to *HsNMTs*¹³⁷. Most recently, IMP1088 was demonstrated also to be effective against the vaccinia virus, further expanding the anti-viral potential of NMTi to poxviruses¹³⁸. Due to the requirement of targeting host NMTs, the therapeutic window is a concern. However, the anti-viral effects of IMP1088 occurred before cytotoxicity in host cells, in which washout of NMTi rapidly restored NMT activity, suggesting NMTi are viable anti-viral agents^{137,139}.

1.4.2 *N*-myristoylation as a liability in cancer

Currently, the most active translational area of research for NMTi is in cancer. NMT was originally suggested as a cancer target given that the major proto-oncogene c-Src is regulated by *N*-myristoylation, and subsequent studies linked increased NMT1 expression to increased cancer grade in a range of cancers such as gallbladder and breast^{140,141}. Indeed, genetic ablation of Src *N*-myristoylation inhibits Src-mediated tumorigenesis in a mouse model of prostate cancer⁷⁷ and loss of phosphorylation downstream of Src has been observed upon NMT inhibition¹⁴², indicating that NMTi may mediate some of their effects through Src. However, it is now known that many more pro-oncogenic pathways involve proteins regulated by *N*-myristoylation (**Figure 1.9**) and effects on these proteins will also affect cell viability. To take some specific examples, the anti-ferroptotic role of FSP1 (formerly known as apoptosis-inducing factor 2, AIF2) is dependent on its myristate-induced membrane localisation^{143,144} and this has been associated with resistance to platinum chemotherapies in ovarian cancer¹⁴⁵. Furthermore, the mTORC1 complex is anchored to the lysosome through interactions with the Ragulator complex

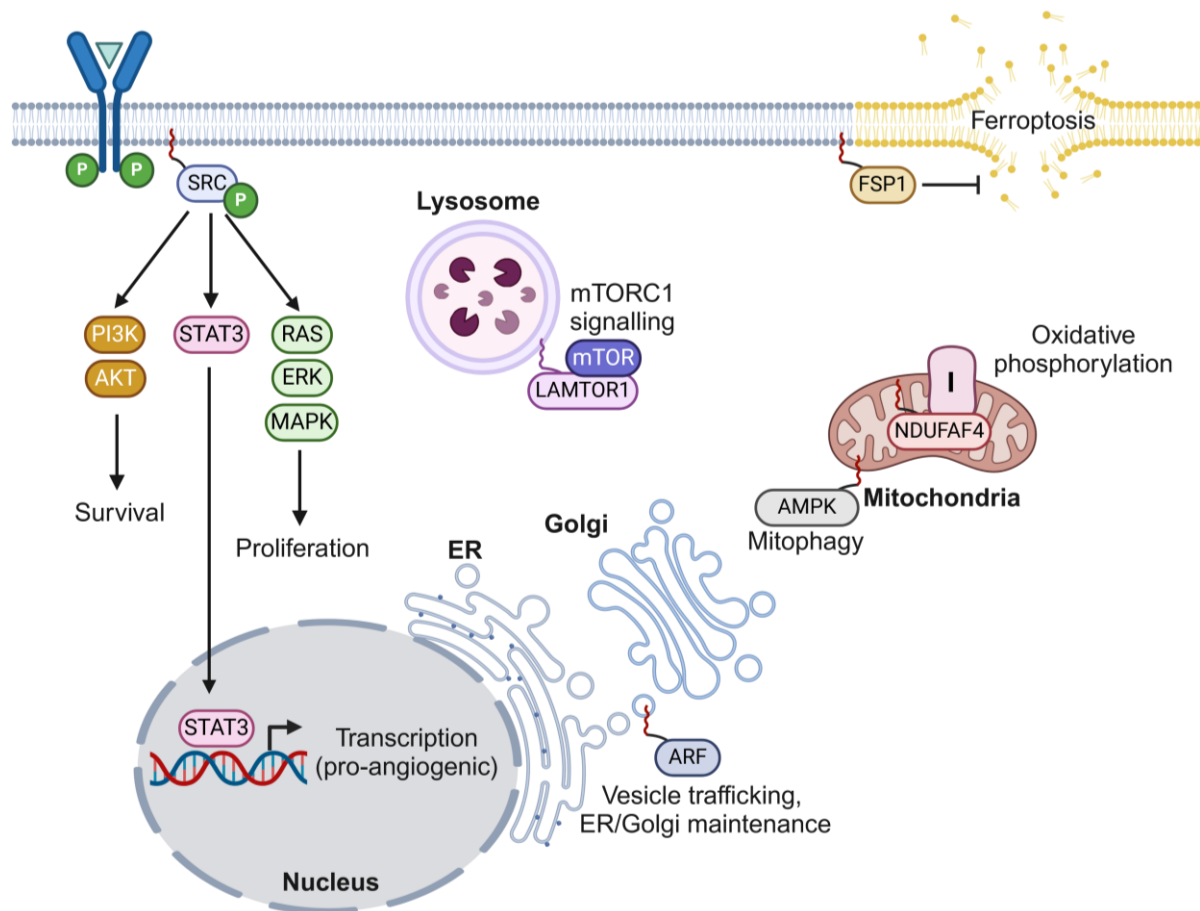


Figure 1.9 NMT substrate functions in pro-oncogenic pathways. Pathways through which NMT substrates have been identified to support oncogenic growth are shown here. Enhanced Src signalling leads to pro-survival, pro-proliferative and pro-angiogenic responses via the PI3K/AKT, STAT3 and RAS/ERK/MAPK pathways. LAMTOR1 anchors mTOR to the lysosome and promotes signalling of the mTORC1 complex. ARF and ARL proteins mediate ER-Golgi vesicle trafficking and maintain the integrity of both compartments. AMPK is recruited to mitochondria upon damage and induces mitophagy. NDUFAF4 promotes respiratory complex I assembly to support oxidative phosphorylation. FSP1 inhibits cell death through ferroptosis, which may otherwise be induced by increased ROS generation.

through dually *N*-myristoylated and *S*-acylated LAMTOR1. Mislocalisation and degradation of LAMTOR1 due to NMT inhibition therefore inhibits mTORC1 signalling and reduces tumour growth in bladder and lung cancer models^{146,147}. *N*-Myristoylation of numerous Golgi proteins, including ARF and ARL proteins, supports protein sorting and trafficking through the ER-Golgi pathway¹⁴⁸. There, NMTi treatment inhibits protein secretion and thereby provokes both ER stress and the unfolded protein response (UPR) in cancer and senescent cells^{149,150}. In the latter context, cellular IpaJ expression was shown to be sufficient to induce defects and cell death, indicating a particular dependence

of senescent cells on ARF1 *N*-myristoylation. Recent reports have also linked dysregulation of lipid metabolism, mediated by upregulation of ACSLs, with a general increase in cellular *N*-myristoylation capacity, increased metastatic potential and treatment resistance^{78,151}, further indicating a role for *N*-myristoylation in cancer.

It is therefore not surprising that genetic and pharmacological targeting of NMT is efficacious in multiple cancer models both *in vitro* and *in vivo*^{66,117,118,142}. However, a major current obstacle in the use of NMTi for cancer treatment is selectivity. Specifically, it is anticipated that the pleiotropic effects of NMT inhibition will limit the therapeutic window of systemic NMTi⁶. To address this issue, the dosing schedule and intensity will need to be carefully optimised and those cancer subtypes most sensitive (or resistant) to NMTi identified to increase the therapeutic window. Furthermore, the key underlying biological pathways driving responses to NMTi should be identified; previous mechanistic studies have largely focused (somewhat arbitrarily) on individual preselected NMT substrates, with only a few exceptions^{66,149,152}. To identify NMTi sensitive cancers, large-scale cell line screens against various NMTi have been carried out. These have identified a particular sensitivity of haematological cancers to NMTi^{142,153}, although expression of NMT1 and NMT2 do not generally correlate with NMTi IC₅₀ values^{66,152}, in contrast with previous claims that NMT2 deficiency correlates with NMTi sensitivity. Furthermore, analyses conducted by a previous Tate group member Dr. Gregor Lueg identified a subset of genes which were enriched in NMTi sensitive cell lines, called the NMTi sensitivity signature⁶⁶. Further analysis using this signature revealed a key finding, namely that NMT inhibitors were highly active in MYC-deregulated cells such as Burkitt's lymphoma, which is driven by chromosomal translocations involving *MYC*¹⁵⁴. *MYC* is a ubiquitously expressed transcription factor which has been extensively linked to cancer and drives protein translation¹⁵⁵. Additionally, another NMTi sensitivity signature, termed the MISS-54, has been recently described¹⁵². Interestingly, *MYC* target proteins are overrepresented in the MISS-54 and *MYC* itself is present in this signature.

Mechanistically, NMT inhibition induces apoptosis in highly MYC expressing lymphoma cells in part due to loss of NDUFAF4 *N*-myristoylation, which is a MYC-regulated mitochondrial respiratory complex I (RC1) assembly factor, leading to its proteasomal degradation and subsequent mitochondrial dysfunction not seen in cells with low MYC expression (low MYC) (**Figure 1.10**)⁶⁶. This result was identified in the MYC-modulatable P493-6 cell line, a model for Burkitt's lymphoma in which MYC expression is turned off in

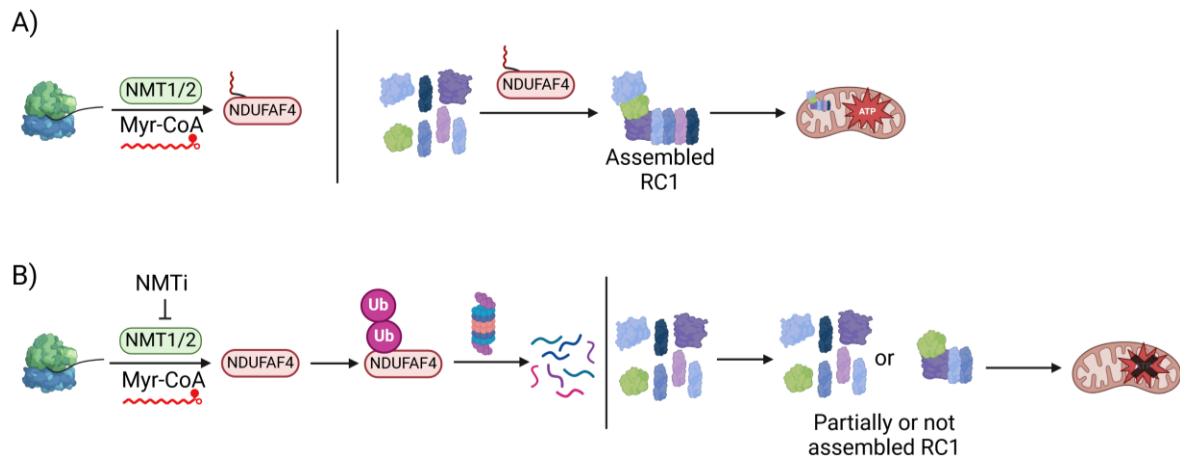


Figure 1.10 Loss of mitochondrial function in high MYC P493-6 cells upon NMTi is due to effects on NDUFAF4. A) Under normal conditions, NDUFAF4 is *N*-myristoylated and assists in assembly of respiratory complex I, the first complex of the electron transport chain, enabling oxidative phosphorylation. **B)** Upon NMT inhibition in high but not low MYC conditions, newly synthesized NDUFAF4 is not *N*-myristoylated leading to its proteasomal degradation. Respiratory complex I is partially/not assembled causing loss of oxidative phosphorylation.

the presence of doxycycline. Similar results have been reported very recently in HAP1 (chronic myelogenous leukaemia) and MV-4-11 and U937 (acute myeloid leukaemia) cells, confirming that this occurs in a range of haematopoietic cancers^{152,156}. These observations are in line with the known function of MYC to upregulate mitochondrial biogenesis¹⁵⁷ and with the recently observed efficacy of RC1 inhibitors in MYC-driven lymphomas¹⁵⁸.

The work described above have marked a shift in our understanding of NMT biology, however questions still remain. For example, how else do NMT substrates interact with MYC-driven biology to induce NMTi sensitivity and what other mechanisms underlie the cytotoxic effects of NMTi in MYC-deregulated cancers? Is the increase in sensitivity in MYC driven cancers due to a general increase in protein turnover or reliance on general *N*-myristoylation, or an increased reliance on specific pathways, as appears to be the case in senescent cells¹⁵⁰? Furthermore, do currently identified pathways mediating the effects of NMTi represent general MYC vulnerabilities, or cancer-type specific effects, given that a majority of mechanistic studies have focused on haematological cancers? And most importantly, is it possible to expand the use of NMTi beyond haematological cancers with MYC deregulation? To investigate these questions, this thesis assesses the use of NMTi in *MYCN*-amplified (MNA) neuroblastomas, a solid tumour cancer with unmet need which NMTi has the potential to address.

1.4.3 Current outlook of NMTi for the clinic

The first clinically approved drug targeting a *N*-myristoylation event, asciminib, was not an NMTi, but instead a selective inhibitor of the leukaemia driver BCR-ABL¹⁵⁹. Asciminib binds the myristate pocket to induce allosteric inhibition of the kinase, mimicking the function of the endogenous myristate group. Importantly, this inhibitor is efficacious in patients resistant to previous BCR-ABL inhibitors, which have all targeted the kinase domain.

To bring NMTi to the clinic, however, two main approaches are being advanced. First, zelenistat (also known as PCLX-001 or DDD86481) has recently completed Phase I tolerability studies (NCT04836195) and is now being assessed in Phase II trials as a systemic single agent for refractory and relapsed B-cell non-Hodgkin's lymphoma and in advanced refractory colorectal cancer patients¹¹⁹. The maximum tolerated dose was determined to be 210 mg when given once daily, a dose expected to provide efficacious concentrations. Although no objective responses were observed, stable disease was seen over a minimum of 16 weeks in 8/29 patients enrolled, albeit none of the four relapsed/refractory B cell lymphoma patients were among this cohort.

Secondly, a spin-out from the Tate group and the Francis Crick Institute, Myricx Bio, is leveraging NMTi as novel payloads for antibody-drug conjugates (ADCs), with preclinical proof-of-concept studies showing that NMTi-ADCs are able to eliminate tumours in three different solid cancer models (breast, gastric and prostate) *in vivo* without toxicity¹⁶⁰. These cancers are generally considered insensitive to systemic NMTi, but efficacy likely arises from prolonged inhibition of an essential lipid modification, with tolerability driven by the targeted delivery of NMTi to the tumour. These results indicate that this modality will improve the scope and therapeutic index of NMTi.

Finally, it is important to note that all current NMTi are dual NMT1/2 inhibitors with equal potency. Development of selective NMT1 inhibitors may be a strategy to improve the therapeutic index of NMTi⁶. While the structural similarities of NMT active sites makes development of selective inhibitors difficult, this may be alleviated through the ability of PROTACs to turn promiscuous binders into selective degraders¹⁶¹.

1.5 MYCN and neuroblastoma

1.5.1 Neuroblastoma background

Neuroblastoma is the most common extracranial paediatric solid tumour¹⁶² and accounts for ~15% of all deaths caused by childhood cancers¹⁶³. Neuroblastomas originate from neural crest cells in the developing sympathetic nervous system and are most commonly found in the abdomen, in either the adrenal glands or sympathetic nerve ganglia¹⁶⁴. Neuroblastoma is a clinically heterogeneous disease and patients are classified as low, medium and high-risk upon diagnosis. While low and intermediate-risk neuroblastomas have excellent prognosis (>95% 5-year overall survival), with some cases of such neuroblastomas spontaneously regressing, patients with high-risk disease have a 5-year overall survival rate of only 50-60%¹⁶⁵. As approximately half of neuroblastomas at diagnosis are high-risk, improved treatment strategies for this cohort of patients is currently a highly active area of research in neuroblastoma.

Currently, the treatment of high-risk neuroblastomas is multi-modal and involves intensive chemotherapy, tumour resection, myeloablative surgery followed by autologous stem cell transplantation, radiotherapy and finally maintenance therapy using isotretinoin and anti-GD2 antibodies. The recent development and approval of anti-GD2 antibodies (dintuximab) for high-risk neuroblastoma in particular represents a significant milestone and is predicted to reduce the 70-month relapse rate by 32% (COG ANBL0931).

However, there is still an urgent need for improved treatments for high-risk patients. While outcomes have improved for this cohort of patients in the past two decades (5-year event free survival (EFS) of 36.1% to 57.2%^{165,166}) due to the introduction of autologous stem cell transplantation and anti-GD2 therapies, these treatments are associated with high acute toxicities. For example, the full therapeutic benefit of anti-GD2 therapies is limited by on-target, off-tumour neuropathic pain¹⁶⁷. In addition, 40% of high-risk patients develop relapsed disease, for which the survival rate is <10%¹⁶⁸. Furthermore, current multimodal therapies are associated with adverse long-term effects. Severe therapy-related late onset adverse effects such as hearing loss and learning difficulties are observed in >50% of survivors¹⁶². A recent study of neuroblastoma survivors (>5 years) also found that grade 3-5 chronic health conditions were enriched in high-risk patients (hazard ratio of 16.1) compared to low risk patients (hazard ratio of 1.8)¹⁶⁹. Importantly, the 25-year cumulative incidence of mortality in survivors of high-risk disease was 22.6%, compared to ~3% for

survivors of low and intermediate disease; the majority of these (50/69) were due to late recurrence of disease. Therefore, novel treatments which minimise toxicities and improve both initial and long-term outcomes are still needed.

To further improve high-risk neuroblastoma treatment, multiple other modalities are being developed. These include immunotherapies, such as bispecific antibodies¹⁷⁰ and CAR-T cells¹⁶⁷, antibody-drug conjugates^{171,172}, targeted radiotherapies (through neuroblastoma-selective uptake of ¹³¹I-metaiodobenzylguanidine)¹⁷³, anti-angiogenic therapies¹⁷⁴ and in particular precision therapies against validated targets in neuroblastoma.

1.5.2 The molecular background of neuroblastoma

From a molecular standpoint, neuroblastoma, like all paediatric cancers, is characterised by a low mutational burden (0.6 mutations per megabase)¹⁶². Instead, chromosomal alterations are common, occurring in 90% of tumours, with segmental chromosomal alterations (especially 1p loss, 11q loss and 17q gain) associated with a worse prognosis. However, significant genetic alterations are known in neuroblastoma (**Figure 1.11**). For example, activating mutations or amplification of anaplastic lymphoma kinase (ALK) occurs in 15% of newly diagnosed neuroblastomas, 80% of familial neuroblastomas, and are enriched in relapsed disease¹⁶³. ALK activation primarily increases signalling through the RAS-MAPK and PI3K-AKT pathways resulting in increased cellular proliferation and survival. Advanced ALK inhibitors such as lorlatinib, originally developed for adult cancers, are currently being trialled, with promising results so far¹⁷⁵, and will be given as a first-line treatment in a phase III trial (TITAN) in 2025.

Telomere maintenance is also upregulated in high-risk neuroblastoma. Chromosomal rearrangements can bring TERT under the control of super-enhancers causing its overexpression in a quarter of high-risk neuroblastomas, and alternative lengthening of telomere (ALT) mechanisms have been identified a further 25%¹⁶². The most common genetic event associated with ALT (~60% of cases) is mutation of ATRX, a transcriptional regulator, generally causing loss of function¹⁷⁶. Targeting such mechanisms in neuroblastoma is currently being investigated at the pre-clinical stage.

Although not a genetic event, it is worth mentioning that neuroblastoma cells exist in two major cellular states, namely adrenergic or mesenchymal, between which cells can interconvert¹⁷⁷⁻¹⁷⁹. These are defined by separate feedforward networks of master

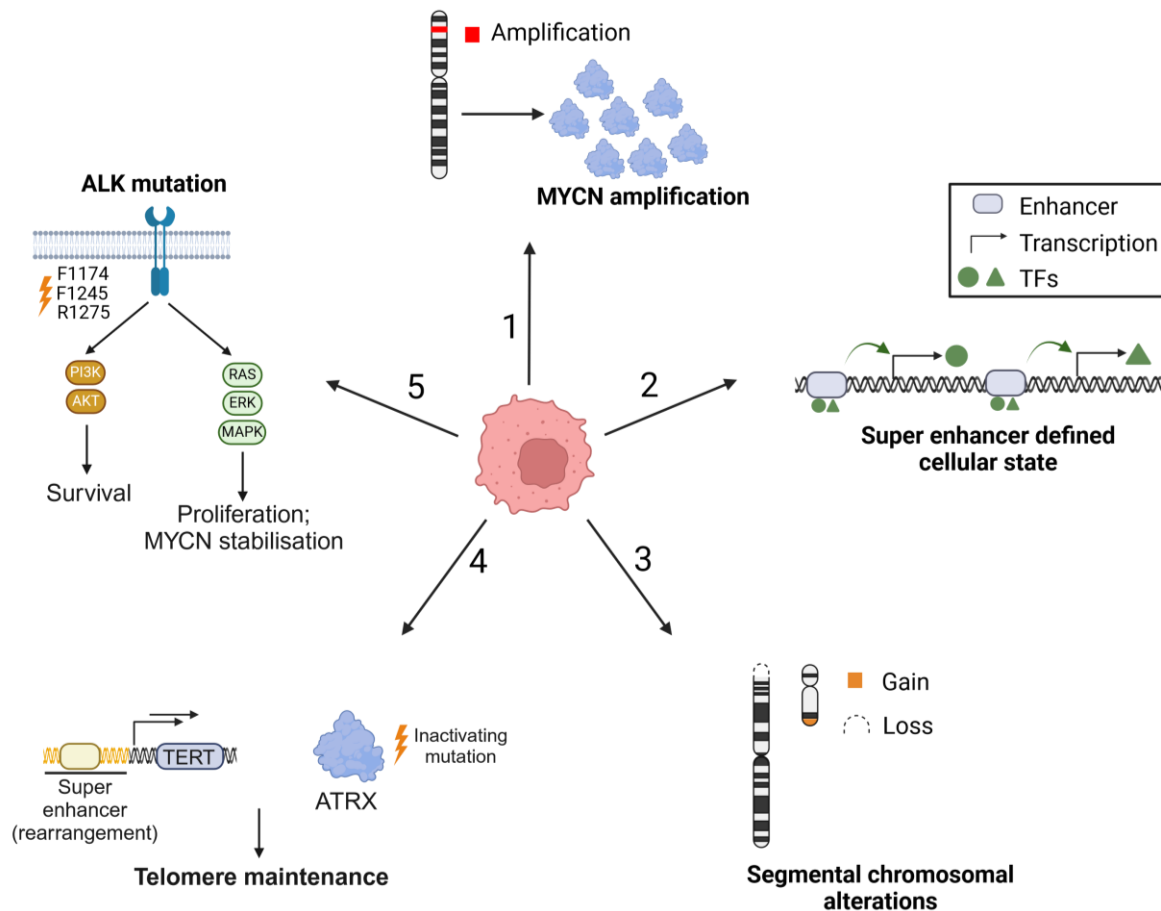


Figure 1.11 Genetic and epigenetic background of neuroblastoma. Selected highly prevalent aberrations identified in neuroblastoma. 1: MYCN amplification increases MYCN protein concentration and activity, leading to multiple pro-oncogenic effects. 2: Neuroblastoma cells exist in two cellular states (mesenchymal and adrenergic) maintained by feed-forward super enhancer networks. 3: Segmental chromosomal alterations are common in neuroblastoma; 1p loss, 11q loss and 17q gain in particular are associated with high-risk disease. 4: Known mechanisms of telomere maintenance in neuroblastoma include TERT rearrangements which increase TERT expression and inactivating mutants in ATRX to promote alternative lengthening of telomeres. 5: Activating mutations in ALK support tumour growth via increased PI3K/AKT and RAS/ERK/MAPK signalling. Common mutation sites are indicated. TF: transcription factor.

transcription factors, called the core regulatory circuitry (CRC), which include for example PHOX2B, GATA2 and HAND2 for the adrenergic state and for the mesenchymal state, PPRX1 and AP-1. Importantly, the cellular state influences drug sensitivities. Mesenchymal cells are more resistant to standard chemotherapies and are enriched in relapsed tumours, but may be more responsive to immunotherapies^{178,180}.

However, by far the most important alteration in neuroblastoma is *MYCN* amplification. *MYCN* is the neural analogue of *MYC* and *MYCN* amplification is a driver of

neuroblastoma; ectopic *MYCN* expression is sufficient to generate neuroblastomas in mouse and zebrafish models^{181,182}. *MYCN* amplification almost always classifies the tumour as high-risk and is highly prevalent in patients (~25% of all tumours, and 40% of high-risk patients)¹⁸³. Patients with *MYCN* amplification often relapse after initial treatment and this is almost always fatal¹⁸⁴. In addition, a further 10% of high-risk neuroblastomas are associated with MYC overexpression, which appears to cause similar effects as *MYCN* amplification¹⁸⁵. The effects of *MYCN* amplification are extensive and discussed further in section **1.5.4**, after a brief introduction to the MYC protein family.

1.5.3 MYC family proteins

The proto-oncogenic MYC family is comprised of three paralogues: MYC, MYCN and MYCL. These are general transcription factors – up to 15% of all genes may be direct MYC targets – which drive pro-survival and pro-growth cellular programs and have been implicated in many of the hallmarks of cancer¹⁵⁵. MYC deregulation is widespread in cancer, occurring in up to 70% of cancers, whereas MYCN deregulation is more restricted, generally occurring in tumours of neuronal and neuroendocrinal origin, and MYCL is implicated mainly in small cell lung cancer¹⁸⁶. Given the ubiquitous expression of MYC and observed similarities between MYC and MYCN (described below), it is the most studied paralogue. Therefore, while the focus of this section will be on MYCN, insights from MYC biology will be mentioned where relevant.

Structurally, MYC paralogues possess high conservation in only specific regions (**Figure 1.12**), namely their basic region, helix-loop-helix and leucine zipper domain (bHLH-LZ), as well as six regions termed “MYC boxes,” which are the main mediators of MYC PPIs¹⁸⁷. Both the N- and C-termini are intrinsically disordered and require interacting proteins for stable folding¹⁵⁵. To mediate their function, MYC proteins interact with related families of bHLH-LZ proteins, termed the “proximal MYC network”¹⁸⁸. The most important interaction of MYC proteins is that with MAX to form a heterodimer which then binds to DNA and modulates gene expression. MAX is crucial for MYC protein function and MYC-mediated transformation¹⁸⁹. The sequence 5'-CACGTG-3' defines canonical, high affinity MYC binding sites (E-boxes), however MYC proteins do not bind exclusively to these sites and this is mediated by other factors such as PPIs; for example, WDR5, which binds to methylated H3K4, is a MYC and MYCN interactor and therefore localises them generally to active promoters¹⁹⁰.

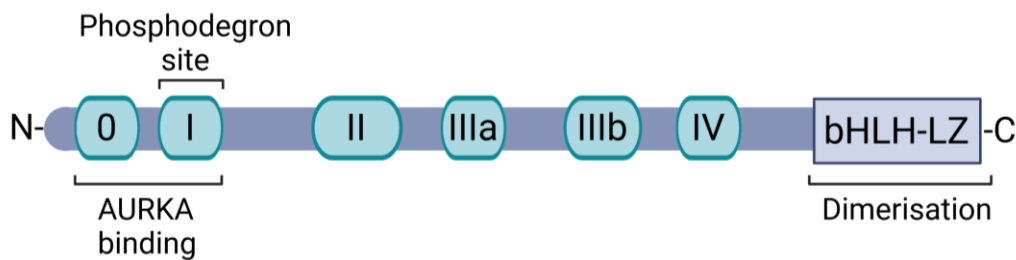


Figure 1.12 MYCN protein structure. MYC proteins dimerise with members of the proximal MYC network and are recruited to DNA through their bHLH-LZ domain. MYC boxes 0-IV primarily mediate protein-protein interactions. MYC box I additionally controls protein stability through the presence of a phosphodegron at T58; the MYCN-AURKA interaction prevents ubiquitination of MYCN by FBXW7 leading to its stabilisation.

The biochemical and phenotypic effects of MYC and MYCN are similar. Homozygous loss of either paralogue is embryonic lethal in mice and both induce cell cycle progression and transformation^{191,192}. MYCN can also compensate for MYC and vice versa; the normal morphology and proliferation rates of mouse embryonic stem cells homozygous for *MYC* or *MYCN* loss is attributed to this effect. Additionally, *MYCN* expression at the *MYC* locus (*MYC^{N-N}*) rescues mice from the embryonic lethality of *MYC* deletion¹⁹³. This has led to the suggestion that the main difference between MYC paralogues is their spatiotemporal expression¹⁸³. MYC is generally ubiquitously expressed across tissues and developmental stage whereas MYCN expression is more prevalent generally in tissues of neuronal origin and is largely not expressed in adult mice. However, there do appear to be intrinsic biochemical differences between MYC and MYCN which leads to distinct phenotypes depending on the paralogue expressed. *MYC^{N-N}* mice, although viable, suffer from skeletal muscle dystrophy¹⁹³. MYC and MYCN amplifications are also associated with different molecular subgroups of medulloblastoma and this has been associated with the differential affinities of MYC and MYCN with MIZ-1 to mediate gene repression^{194,195}. Furthermore, MYCN recruits BRCA1 to chromatin to clear stalled RNA Pol II to a greater extent than MYC, suggesting differential responses to transcriptional stress¹⁹⁶.

While it is clear that MYC proteins are central for tumorigenesis and tumour maintenance in many cancers, the molecular mechanisms through which these proteins are oncogenic is still not known. The prevailing assumption over the past few decades involves activation of a transcriptional program downstream of oncogenic levels of MYC proteins, although

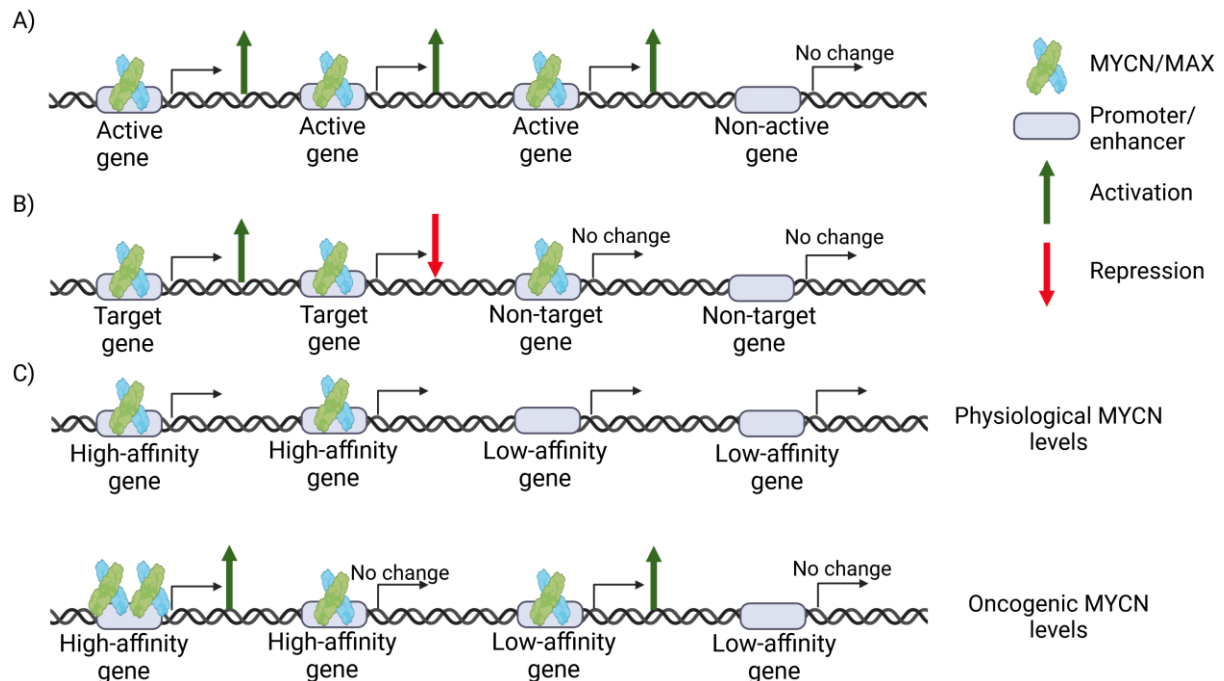


Figure 1.13 Current models for MYCN oncogenic signalling through transcriptional upregulation. **A)** Global amplifier model. Oncogenic levels of MYCN invade all active promoters, globally increasing transcriptional output. **B)** Specific gene regulation model. MYCN binding at promoters does not necessarily modulate transcription in this model. Instead, only specific gene sets are modulated by MYCN. **C)** Affinity-dependent model. At physiological MYCN levels, only promoters (or enhancers) with high MYCN affinity are bound. As MYCN levels increase, lower affinity promoters and enhancers are also bound by MYCN. Genes whose promoters are saturated at physiological MYCN levels will not be further regulated by oncogenic MYCN levels.

three separate models have been proposed to describe the effects of MYC (**Figure 1.13**). First, MYC proteins accumulate at all active promoters, and some enhancers, in the cell, therefore acting as a global amplifier of the existing transcriptional program in the cell and increasing overall cellular mRNA concentrations^{197,198}. Alternatively, it has been observed in some models that only a subset of MYC-bound genes are regulated by changing MYC levels^{199,200}, and therefore MYC selectively regulates only a subset of transcribed genes. The third model suggests that a promoter or enhancer's affinity to MYC is the key determinant for MYC activity^{201,202}. Therefore, at physiological MYC levels, only the high affinity promoters will be bound, possibly at saturating levels. However, at oncogenic levels, MYC invades lower affinity promoters and enhancers, and it is these genes, defined by lower MYC affinity, which are responsible for the oncogenic effects of MYC proteins. To date, there is still a lack of consensus on which model best describes MYC protein activity. This picture is further complicated by difficulties in defining oncogenic MYC target genes.

While consensus sets of MYC target genes have been defined^{203,204} and appear to be related primary to translation and purine biosynthesis, direct MYC targets are largely tissue and cell-type specific; this has been associated with the expression of tissue-specific transcription factors which co-localise with MYC or MYCN at enhancers²⁰². The mechanisms by which MYCN mediates gene repression are also poorly characterised but may involve increased transcription of microRNAs²⁰⁵. Recently, there is rising interest in the pro-oncogenic functions of MYC proteins which are unrelated to increased target gene transcription but instead to genome maintenance and resolution of transcriptional stress. MYCN-mediated resolution of torsional stress²⁰⁶, R-loop formation¹⁹⁶ and transcription/replication conflicts²⁰⁷ have all now been proposed to maintain viability of *MYCN*-amplified neuroblastoma, without effects on the transcriptome. Such effects are consistent with the global binding of MYCN to all active promoters and may be mediated by phase separation of MYCN, although the relevance of this process is still unclear^{208,209}.

1.5.4 MYCN in neuroblastoma

Consistent with MYCN's status as a master transcription factor, its deregulation in neuroblastoma is associated with modulation of many biological pathways. As these have been comprehensively described in the literature, only selected pathways are briefly discussed below (**Figure 1.14**).

MYC and MYCN are best described to drive enforced flux through the cell cycle and MNA neuroblastomas fail to arrest in the G1 phase upon irradiation²¹⁰. Indeed, direct targets of overexpressed MYCN which also correlated with poor overall survival (OS) were enriched in cell cycle proteins²¹¹ and MYCN knockdown reduces expression of cell cycle related genes²¹². MYCN therefore mediates its effects on cell cycle through many effectors, such as the E2F transcription factors²¹³, which also directly mediate the MYC-induced G1-S transition²¹⁴.

An increased rate of cell proliferation requires concomitant increases in macromolecular biosynthesis. MYC proteins increase the transcription of ribosomal and transfer RNAs by RNA Pol I and III, therefore directly contributing to global increases in protein synthesis, in addition to their effects on mRNA transcription¹⁵⁵. Various mechanisms for how MYCN promotes the latter have been identified, and the most prominent appears to be via promoter-proximal pause release of RNA Pol II²¹⁵. Additionally, MYCN increases the

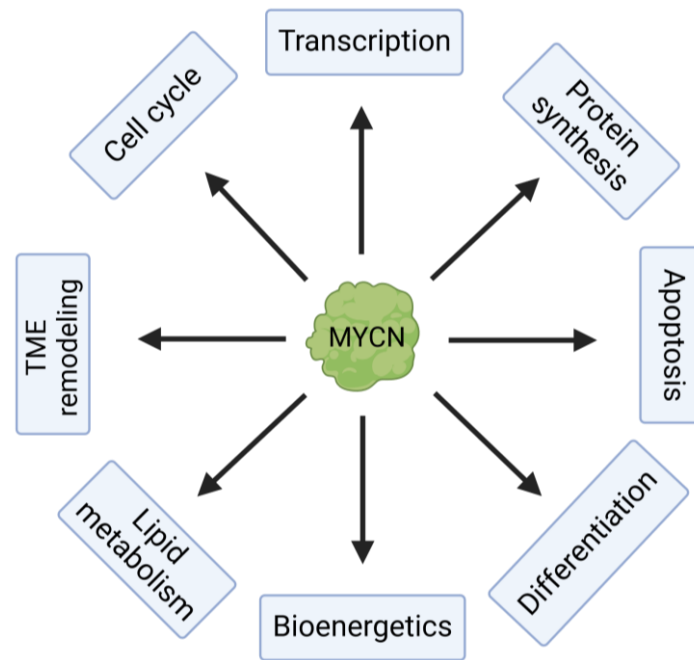


Figure 1.14 MYCN regulates many processes in neuroblastoma. The transcriptional program induced by MYCN regulates many cellular processes to support tumour growth and maintenance.

uptake of exogenous fatty acids through upregulation of FA transport protein 2 (FATP2), likely to support both membrane lipid synthesis and energy generation²¹⁶. In this respect, it has been shown that MYCN significantly alters the metabolic profile of neuroblastoma, for example inducing glutamine addiction²¹⁷ and altering amino acid metabolism²¹⁸. Notably, MYCN significantly increases energy generation by upregulating both glycolysis and oxidative phosphorylation (OxPhos)²¹⁹. Multiple studies have confirmed the increased glycolytic nature of MNA neuroblastomas, consistent with the Warburg effect, through ¹⁸F-fluorodeoxyglucose imaging or -omic studies of cell lines and primary tumours²²⁰⁻²²². This is at least in part due to the upregulation of multiple glycolytic enzymes such as HK2 and LDHA by MYCN²²³. The role of OxPhos in MNA neuroblastoma is less well characterised, although some MNA cell lines appear to have a greater dependence on OxPhos²²⁴. Interestingly, fatty acids were identified as the predominant mitochondrial fuel in at least one MYCN-modulatable neuroblastoma model, and treatment with etomoxir, a fatty acid oxidation inhibitor, controlled tumours in mouse xenograft models of MNA neuroblastoma, albeit modestly²¹⁹.

Additionally, MYCN expression in neuroblastomas is inversely related to their differentiation state¹⁸³, consistent with its ability to promote transformation of fibroblasts into induced pluripotent stem cells²²⁵. MYCN promotes a stem-like state in neuroblastoma

both through the upregulation of pluripotency genes, such as *KLF2* and *KLF4*, and suppression of differentiation pathways¹⁸³. Accordingly, single cell transcriptomic analyses have shown that MNA neuroblastoma cells most closely resemble early but not late normal neuroblasts²²⁶.

Paradoxically, a pro-apoptotic effect has been ascribed to both MYC and MYCN. TP53 is a direct MYC and MYCN target²²⁷ and mediates MYC-induced apoptosis²²⁸; furthermore, *MYCN* amplification sensitizes neuroblastoma cells to apoptosis in part through upregulation of NOXA²²⁹. Therefore, cancers driven by MYC and MYCN must acquire apoptosis avoidance mechanisms and multiple have been proposed. While TP53 mutation is rare upon diagnosis in neuroblastoma¹⁸³, alterations in its regulatory pathway have been observed. For example, MDM2, an E3 ligase which marks TP53 for degradation, is directly transcriptionally activated by MYCN²³⁰. Additionally, caspase 8, which cleaves Bid to induce apoptosis upon Fas stimulation, is often silenced in MNA neuroblastoma, contributing to their survival²³¹.

Finally, *MYCN* amplification has also been associated with non-cell-autonomous effects. MYCN appears to promote angiogenesis through both the secretion of pro-angiogenic factors, such as VEGF²³² and repression of angiogenesis inhibitors, as conditioned media from a MYCN normal neuroblastoma cell line contained inhibitors of endothelial cell proliferation absent in media from a MYCN overexpressing cell line²³³. Furthermore, MYCN contributes to immune evasion in neuroblastoma through the downregulation of multiple factors including major histocompatibility complex (MHC) Class I proteins²³⁴ and the cytokines CCL2²³⁵ and CXCL10²³⁶, leading to reduced (cytotoxic) lymphocyte infiltration in MNA neuroblastomas compared to non-MNA neuroblastomas.

1.5.5 Targeting MYCN

Given the importance of MYCN in MNA neuroblastomas, MYCN inhibitors would have a strong therapeutic impact in neuroblastoma, a conclusion supported by studies targeting *MYCN* using antisense oligonucleotides²³⁷ or small interfering RNA (siRNA)²³⁸. Furthermore, its limited expression in regular tissues suggests that such drugs would possess a wide therapeutic window. To date, three approaches have largely been explored to target MYCN: direct targeting, indirect targeting, and inhibition of MYCN-induced dependencies (**Figure 1.15**).

Direct targeting of MYC proteins has proven difficult as they are considered “undruggable” due to the unstructured nature of their N- and C-termini, the lack of conventional small molecule binding pockets and their lack of enzymatic activity²³⁹. Currently, the most advanced approach to directly inhibiting MYC is through the use of a negative dominant mini-protein termed “Omomyc.” This is a synthetic MYC mutant, comprising of the bHLH-LZ domain, which binds to all MYC proteins as well as MAX, forming inactive dimers^{240,241}. An analogue of Omomyc, OMO-103, has advanced to phase I trials for MYC-driven solid tumours (NCT04808362, NCT06059001) and was well tolerated, with indications of on-target efficacy, albeit no objective responses were recorded²⁴². Small molecules targeting the MYCN/MAX interaction have also been developed, such as 10058-F4 and

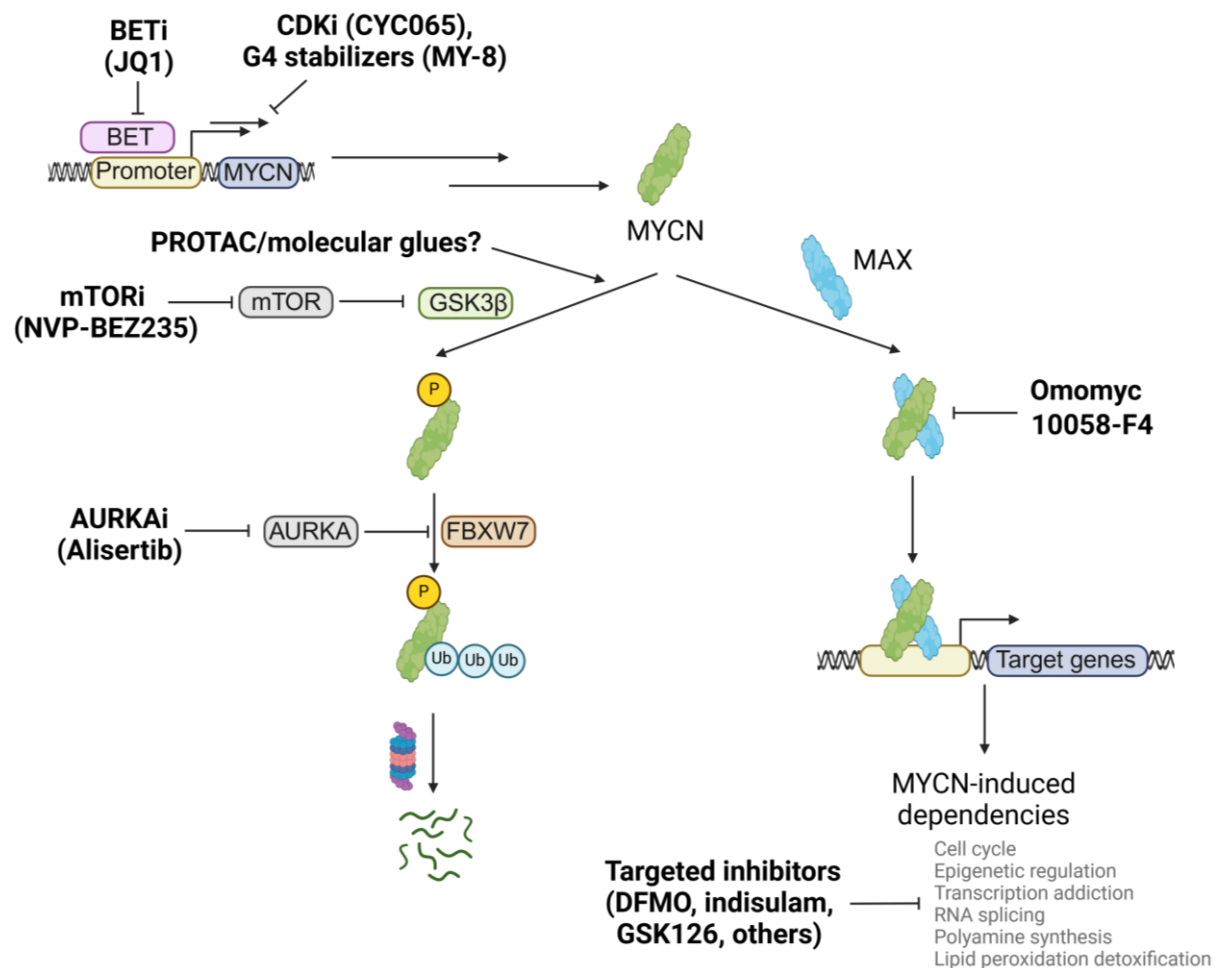


Figure 1.15 Strategies to target MYCN. Multiple strategies to inhibit MYCN have been developed, including inhibition of *MYCN* transcription, MYCN/MAX dimerization, destabilization of the MYCN protein and inhibition of MYCN-induced dependencies. Improvements in degrader technologies may represent a new avenue to target MYCN. Bold: possible therapeutic interventions.

MYCi975, although these are limited by the high concentrations required for activity, up to 100 μM ²⁴³. Finally, recent advances in protein degrader technology, namely molecular glues and PROTACs, have generated optimism that MYCN may be targetable through such an approach, although no such degraders have yet been reported²³⁹.

Indirect targeting of MYCN takes the form of inhibiting MYCN transcription and promoting its degradation. Abrogation of MYC transcription by bromodomain and extra terminal domain (BET) protein inhibition has been extensively investigated and this approach has been extended to MYCN^{244,245}. However, BET proteins also generally regulate transcription and indeed MYC status does not always correlate with BET inhibitor sensitivity²⁴⁶. BET inhibitors have been limited by toxicity or low efficacy in clinical trials thus far. Other approaches targeting MYCN transcription include the stabilisation of a G-quadruplex in the first intron of MYCN²⁴⁷ and inhibition of cyclin-dependent kinases (CDKs)²⁴⁸. Of note, the dual CDK2/9 inhibitor CYC065 (fadraciclib) is now available to neuroblastoma patients under the ESMART clinical trial (NCT02813135). CDK9 inhibition by CYC065 inhibits MYCN transcription, although notably CDK2 inhibition is required for apoptosis and more generally the adrenergic cellular state is also affected independent of MYCN expression²⁴⁸.

MYCN expression is also highly regulated on a post-translational level, with a half-life of only 30 minutes²⁴⁹. This is primarily due to the presence of a phospho-degron in MYC Box I. Phosphorylation of Thr58 by GSK3 β occurs subsequent to Ser62 phosphorylation by CDK1. After PP2A-mediated pSer62 dephosphorylation, the E3 ligase FBXW7 binds to pThr58 and mediates the proteasomal degradation of MYCN¹⁸⁶. Aurora-A kinase (AURKA) antagonises the FBXW7-MYCN interaction through its own interaction with MYCN and AURKA inhibitors can disrupt this PPI, inducing MYCN degradation. As such, AURKA inhibitors such as alisertib have been extensively investigated for efficacy in neuroblastoma, including in a Phase II clinical trial²⁵⁰, although dose-limiting toxicities were identified, suggesting combination treatments would be required. Furthermore, mTOR inhibitors promote MYCN turnover through the stimulation of GSK3 β and inhibition of PP2A and selectively induces apoptosis in MNA neuroblastoma cells²⁵¹, suggesting this may be a viable approach.

Many approaches have been proposed to target MYCN-induced dependencies. These include inhibition of epigenetic regulators²⁵², global transcription²⁵³, RNA splicing²⁵⁴, cell cycle²⁵⁵ and fatty acid uptake²¹⁶, or induction of apoptosis²²⁹ and ferroptosis²⁵⁶⁻²⁵⁸. The

most successful of these approaches thus far is the inhibition of polyamine synthesis by difluoromethylornithine (DFMO), which has recently been approved by the FDA as a maintenance therapy²⁵⁹. DFMO inhibits ornithine decarboxylase (ODC1), the rate-limiting enzyme for polyamine synthesis, and was prioritised as many MNA neuroblastomas upregulate this pathway to support proliferation²⁶⁰.

1.6 NMT inhibition in MNA neuroblastoma: a new strategy?

It is evident from the above that many therapies to target MYCN are in preclinical and clinical development. However, development of novel therapies is still desirable, given the high attrition rates in drug development and the fact that preclinical efficacy does not necessarily translate into clinical success. For example, despite the initial promise of BET and AURKA inhibitors, these have not yet translated into approved therapies^{246,250}. In addition, the recently approved DFMO is again associated with high toxicity and is currently only indicated as a maintenance therapy for patients who responded to initial therapy²⁶¹. Notably, this drug is cytostatic and therefore may not reduce the rate of late disease recurrence. Therefore, there is still a need for improved induction therapies, especially for primary refractory disease, and for improved clearance of residual cancer cells. NMTi may be able to address this need. Firstly, NMT inhibition is expected to act pleiotropically through multiple pathways, which may allow NMTi to control currently refractory disease⁶. Secondly, a common mechanism of therapy avoidance in cancer cells is induction of senescence. This may not be an issue for NMTi as senescent cells are highly sensitive to NMT inhibition¹⁵⁰. While a possibly low therapeutic index is a general concern for systemic NMTi, the tolerance of NMTi in a Phase I trial¹¹⁹ and the identification that MYC-deregulated cancers are especially sensitive to NMTi⁶⁶ suggests that an actionable therapeutic window may be present in MNA neuroblastoma.

The notion that NMTi may be especially efficacious in MNA neuroblastoma leads to the question of how selectivity over non-MNA neuroblastomas may arise, and which NMT substrates would mediate the cytotoxicity of NMTi. The simplest hypothesis would be that MNA neuroblastomas have an increased dependence on the *N*-myristoylation of specific NMT substrates. To investigate this, publicly available CRISPR essentiality screening data (Dependency Map, DepMap) in 37 neuroblastoma cell lines (26 MNA, 11 non-MNA) was examined^{48,50}. Gene effect scores, a measure of the essentiality of a gene in a given cell line, were compared between the MNA and non-MNA samples. However, neither NMT1

nor NMT2 were identified as selectively dependent in MNA cell lines. This was due to the general importance of NMT1 (average gene effect score of -0.74 and -0.71 in MNA and non-MNA cell lines respectively; the mean gene effect score of all common essential genes is -1) especially at the long timepoint used (21 days), and a general lack of essentiality of NMT2 (average gene effect score of 0.18 and 0.15 in MNA and non-MNA cell lines). Additionally, no selective dependence on any NMT substrate based on MYCN status was seen. However, several NMT substrates were important in all screened neuroblastoma lines, suggesting that these may be relevant for general NMTi cytotoxicity in neuroblastoma cells (**Table 1.1**). Similar results were also seen from a siRNA essentiality screen²⁵⁶, which aimed to identify MYCN-specific protein dependencies in an isogenic cell line. One NMT substrate was identified as a MYCN-specific dependency in this screen: FMNL2, also a direct MYCN transcriptional target, perhaps due to a specific dependency in the cell line used. Overall, these results indicate that MNA neuroblastomas do not have an increased dependence on NMTs or any one NMT substrate over a long-term period. It also implies that extended, constitutive loss of *N*-myristoylation will be toxic in all cancer cells and further suggests that careful optimisation of clinical NMTi dosing schedules will be necessary. However, such inferences from CRISPR screen analyses only consider a

NMT/NMT substrate	<i>N</i> -myristoylation evidence	References	Average Gene Effect Score		
			MNA lines (n=26)	non-MNA lines (n=11)	All (n=37)
CHMP6	1,2,4	26,262	-1.66	-1.58	-1.62
TOMM40	1,2,4	26,263	-1.42	-1.33	-1.38
PSMC1	1,2,3,4	26,43,264	-1.21	-1.12	-1.17
NMT1	N/A	N/A	-0.74	-0.70	-0.72
DNM2	3	113	-0.71	-0.06	-0.63
NDUFB7	1,2,3,4	26,43,265	-0.56	-0.44	-0.50
ARF4	1,2,3	26,43	-0.50	-0.58	-0.54

Table 1.1 Neuroblastoma cells are dependent on a subset of NMT substrates. Average gene effect scores for the NMTs/NMT substrates most essential in neuroblastoma cell lines in CRISPR essentially screens (DepMap) by MYCN status. Only NMT substrates with a gene effect score <-0.5 are included. Evidence for *N*-myristoylation is as follows: 1; protein is enriched upon YnMyr treatment by mass spectrometry, 2; protein enrichment by YnMyr is abolished upon NMTi treatment by mass spectrometry, 3; direct identification of the *N*-myristoylated peptide (native or YnMyr labelled) by mass spectrometry, 4; incorporation of myristic acid analogues (³[H]-myristate, YnMyr or AzMyr) is abolished by the G2A mutant.

very simplified scenario. For one, all NMT substrates will be affected simultaneously, and it is likely that a combination of substrates would mediate increased sensitivity in MNA contexts. For another, NMT substrate essentiality does not imply importance of protein *N*-myristoylation. In addition, it is also plausible that toxic gain-of-function effects of non-*N*-myristoylated substrates could contribute to increases in NMTi sensitivity of MNA neuroblastomas, perhaps because of their upregulation by MYCN. 63 NMT substrates are annotated as direct MYCN targets in aggregated ChIP-X datasets²⁶⁶, including the protein phosphatase subunits PPM1A, PPM1B and PPM1G. Excessive deregulated protein phosphatase activity could therefore contribute to increases in NMTi sensitivity.

1.7 Project aims

While there is an initial rationale for using NMTi in MNA neuroblastoma based off the findings in MYC-deregulated lymphoma cells, prior to the initiation of the work described in this thesis, no mechanistic studies of NMTi in MNA neuroblastoma had been carried out, and the sensitivity of only one neuroblastoma cell line to NMTi was investigated⁶⁶. Therefore, it remained necessary to first confirm that NMTi would be efficacious in MNA neuroblastoma, and also to identify the effects downstream of NMT inhibition that contribute to cell death in a MYCN-dependent manner. An additional important question to answer is whether the effects identified in MNA neuroblastoma correlate with those in MYC-deregulated lymphoma cells, given the role of tissue-specific enhancers in defining MYC/MYCN target proteins²⁰². Therefore, this work attempted to address these gaps using the following approaches:

- **Chapter 2:** Verification of the broad NMTi-sensitising effects of MYCN using a panel of neuroblastoma cell lines. Furthermore, the contributions of several cell-death inducing mechanisms to NMTi MoA were investigated based off their proven relevance to NMT inhibition. Finally, the NMT substrate landscape in neuroblastoma cells were profiled to provide an initial understanding of the effects of NMTi in these cells.
- **Chapter 3:** Global transcriptomic and proteomic profiling in a MYCN-modulatable cell line was carried out to identify NMTi-mediated effects in a MYCN dependent manner.

- **Chapter 4:** Application of detergent-based cell fractionation to investigate the effects of NMTi with a focus on the membrane proteome. A follow up functional study on OxPhos, which was highly affected upon NMT inhibition was also conducted.

Chapter 2. MYCN sensitises neuroblastomas to NMT inhibition

2.1 Validation of MYCN as a sensitising factor to NMTi in neuroblastoma

2.1.1 Choice of cytotoxicity assay

The cellular response to NMT inhibition was studied using a cytotoxicity assay developed by a previous Tate lab member, Dr Andrea Goya Grocin, which uses the Incucyte S3 live cell imaging platform to monitor cells in real time using both phase and fluorescent imaging⁵¹ (**Figure 2.1**). Importantly, the proportion of dead cells in a population can be quantified by the use of SYTOX Green, a nucleic acid stain which strongly fluoresces only upon DNA binding. This reagent is cell impermeable, meaning that it will only bind to DNA when the membrane is compromised, a characteristic of dead cells. Normalised cell death is then calculated as the green area over the phase area, a measure of cell number. Both time and concentration-dependent effects of NMTi can therefore be assessed using this assay and IC₅₀ values obtained from dose-response curves calculated from the area under the curve (AUC) for each concentration.

2.1.2 NMTi efficacy correlates with MYCN status

Using the above assay, the effects of IMP1088¹¹⁶, a highly potent and selective NMTi and a commonly used tool compound in the Tate group, was assessed over 5 days of treatment in a panel of four MNA and three non-MNA neuroblastoma cell lines (**Appendix Figure 1**). The engineered SHEP21N cell line²⁶⁷ (parental line SHEP), was also included in the panel. MYCN is exogenously expressed in these cells under the control of a tetracycline-off promoter, allowing the assessment of MYCN effects in an isogenic cell model (**Figure 2.2A**). Overall, absolute IC₅₀s (henceforth IC₅₀) for IMP1088 strongly correlated with MYCN status compared with other common genomic alterations in neuroblastoma (**Figure 2.2B, Appendix Table 1**). Less than 50% total cell death was observed for the 3 non-MNA cell lines even at 1 μ M IMP1088, the highest concentration used in this assay, whereas greater amounts of cell death were observed for MNA cell lines. Furthermore, the IC₅₀ of IMP1088 increased 6.2-fold in SHEP21N cells upon repression of MYCN expression (434

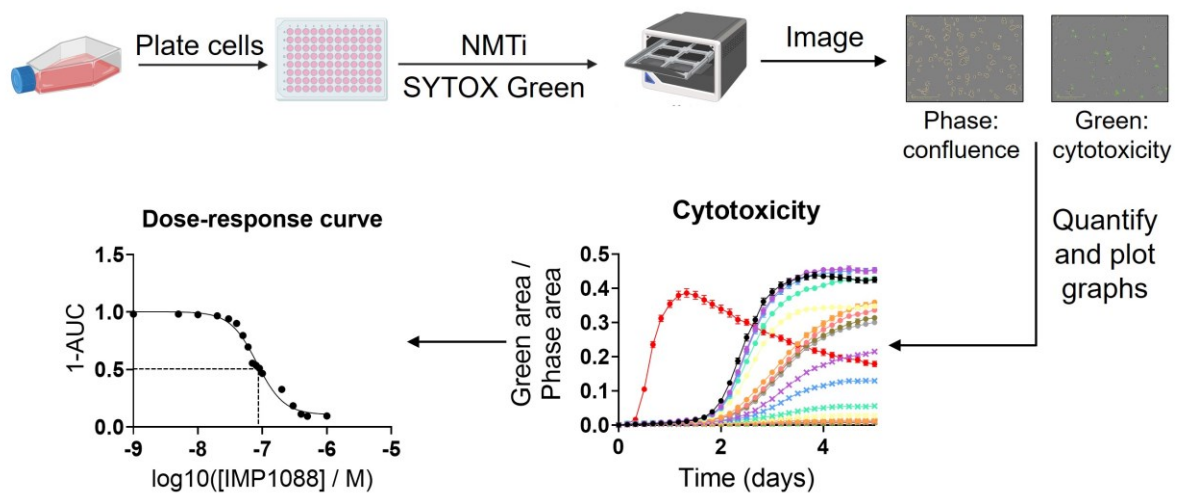


Figure 2.1 The Incucyte platform allows assessment of time and concentration-dependent effects of NMTi. Schematic of the cytotoxicity assay used to assess NMTi efficacy. Cells were plated, allowed to adhere overnight, and treated with 2X concentrations of NMTi and SYTOX Green. Phase and green fluorescent images were taken every 4 h. Normalised cell death was plotted as the green area over phase area. To obtain absolute IC₅₀s, the AUC of each curve was calculated, normalised to the puromycin AUC and 1-AUC was plotted against concentration. Curves were fitted in Prism using the absolute IC₅₀ method.

vs 70 nM, **Figure 2.2C**). The CHP-212 cell line was the only slight exception, with maximal cell death of only 40-60% observed despite being MNA. This may be due to their cellular state, as they are the only MNA cell line which exist the mesenchymal state, associated with drug resistance¹⁸⁰. All non-MNA cells assayed were also mesenchymal. However, this may also be due to other genetic alterations in CHP-212 cells as SHEP21N cells are also mesenchymal but were highly sensitive to IMP1088 when expressing MYCN. Therefore, an association between the cellular state and NMTi sensitivity is not clear cut and further studies to investigate this possible association, for example by expanding the panel to include mesenchymal MNA and adrenergic non-MNA cell lines, would be necessary. However, CHP-212 cells were still more sensitive to high concentrations of IMP1088 compared to non-MNA cells, confirming the overall trend that MYCN amplification sensitises to IMP1088.

Inspection of the cytotoxicity plots generated by this assay further revealed that a reduced time required for the onset of cell death upon NMTi treatment was another indicator of increased sensitivity to NMTi, as exemplified by the SHEP21N model (**Figure 2.3A**). Cell death was induced in MNA cells after only one day of IMP1088 treatment, whereas two or

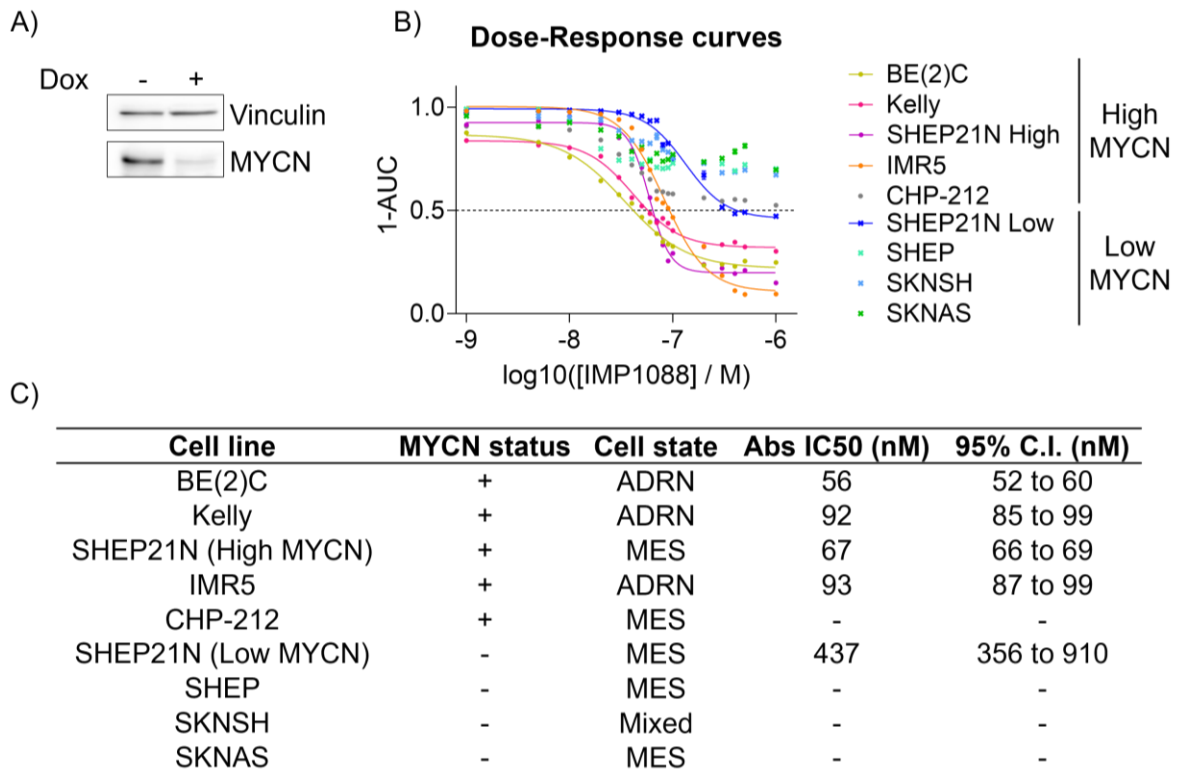


Figure 2.2 MYCN correlates with NMTi sensitivity in a panel of neuroblastoma cell lines.

A) Western blot for MYCN in SHEP21N cells treated with 0.5 µg/mL doxycycline (24 h). **B)** Dose-response curves for IMP1088 in a panel of neuroblastoma cell lines. Curves have been fitted to cell lines for which IC₅₀ values were calculatable using the absolute IC₅₀ method in Prism. One representative biological repeat, carried out in technical triplicate, is shown as mean ± s.e.m.; only error bars larger than the symbol are plotted. **C)** MYCN status, cell state and absolute IC₅₀ values for each cell line in the panel. C.I.: confidence interval. ADRN: Adrenergic, MES: Mesenchymal

three days of NMTi treatment was required for low MYCN cells. Additionally, while the degree of cell death was low at 5 days, it had not plateaued at this point for the non-MNA SKNAS (**Figure 2.3B**) and SKNSH (**Appendix Figure 1**) cell lines in part due to the delayed onset of cell death in these cells. Further monitoring of SKNAS cells over a 10-day period revealed significant amounts of cell death at higher concentrations of IMP1088 which was not attributable to overconfluency or lack of nutrients (**Figure 2.3C** and **D**). These results indicate that sustained NMT inhibition can eventually lead to robust cell death even in non-MNA neuroblastoma contexts and that both time and concentration factors are reflective of NMTi sensitivity. On the other hand, the extent of cell death in SHEP cells had plateaued by 5 days of NMTi treatment and did not increase even with extended (10 day) treatments with IMP1088 (**Appendix Figure 2**). A possible explanation

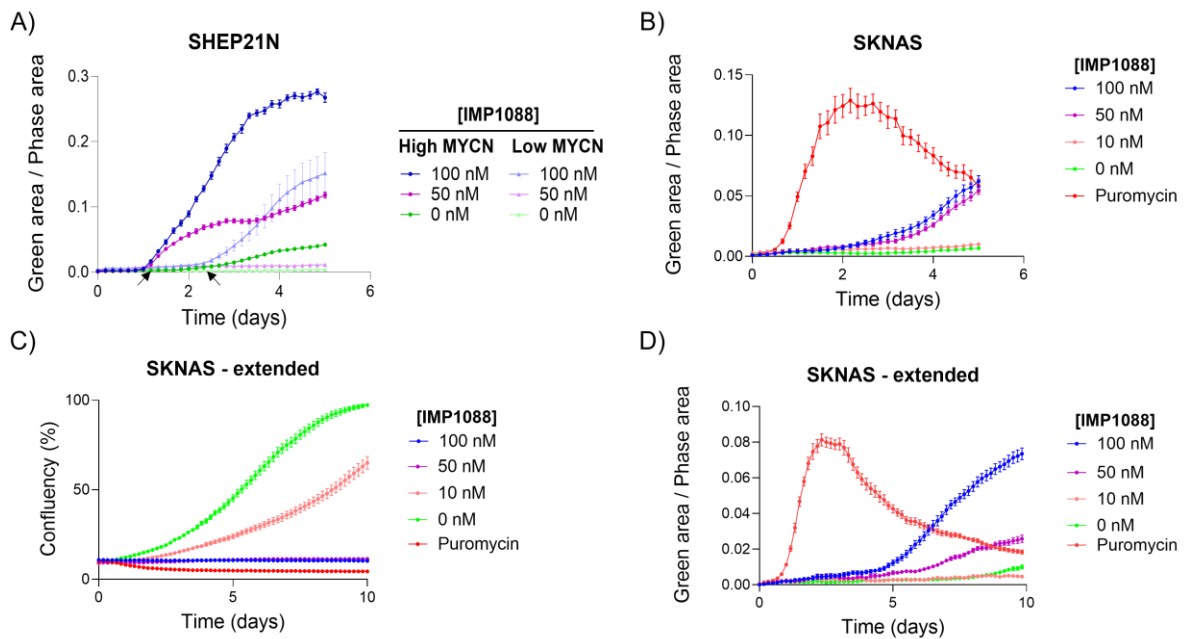


Figure 2.3 The time required for NMTi-induced cell death is a measure of NMTi sensitivity. (A) Cytotoxicity of IMP1088 in SHEP21N cells. High MYCN cells begin to die after 24 h treatment whereas low MYCN cells begin to die after 48 h treatment (arrows). (B) Cytotoxicity of IMP1088 in SKNAS cells. Cell death had not plateaued after 5 days of NMTi treatment. (C) Confluency of SKNAS cells treated with IMP1088 over 10 days. (D) Cytotoxicity of IMP1088 in SKNAS cells over 10 days. Data are represented as mean $\pm$ s.e.m.

for these differences could be the overall MYC family protein expression in these cells; SKNAS and SKNSH cells express MYC, whereas SHEP cells do not express MYC or MYCN¹⁸⁵. Since *N*-myristoylation is predominantly co-translational, cells with higher protein synthesis and turnover rates should be more sensitive to NMT inhibition; MYC family proteins promote protein synthesis and drive cell cycle progression¹⁵⁵. Possibly, SHEP cells transition into a protective quiescent state upon NMTi challenge, in which protein synthesis is blunted, due to the lack of MYC or MYCN-enforced cell cycle progression. This may be prevented in MYC or MYCN-expressing cells, but in the context of neuroblastoma MYCN appears to induce a greater sensitivity to NMTi than MYC. This could be due to differences between MYC and MYCN-mediated transcriptional programs, such as their differential binding to MIZ-1¹⁹⁵. Therefore, it may be that in the context of neuroblastoma, the cellular state induced by MYCN expression is particularly sensitive to NMTi, whereas MYC-expressing cells eventually also succumb to NMT inhibition. Lack of any MYC or MYCN expression may allow a cellular response blunting the effects of NMTi.

Cell line	MYCN status	Abs IC50 (nM)	95% C.I. (nM)
SHEP21N (High MYCN)	+	257	250 to 264
IMR5	+	308	300 to 317
SHEP21N (Low MYCN)	-	1006*	751 to 1461
SKNAS	-	-	-

Table 2.1 Sensitivity to DDD86481 also correlates with MYCN status. Abs IC50 values for DDD86481 in the indicated cell lines. * Extrapolated IC50s

Finally, to confirm that these results are due to on-target NMT inhibition, the effects of the chemically distinct NMTi DDD86481/PCLX-001¹⁴² was investigated. As expected, DDD86481 was efficacious in high but not low MYCN cells in a dose-dependent manner, with a 3.9-fold increase in IC50 upon MYCN ablation in SHEP21N cells (**Table 2.1**, **Appendix Figure 3**). The IC50 values obtained for DDD86481 were also higher than those for IMP1088, consistent with IMP1088 being more potent than DDD86481⁶⁶ (**Figure 2.4**).

2.1.3 NMTi-induced cell death is not due to MYCN downregulation

As discussed in the introduction, induction of MYCN degradation is considered a therapeutically viable strategy in neuroblastoma, given the oncogenicity associated with MYCN in this disease. As mTOR inhibitors can stimulate MYCN degradation²⁵¹, and as NMT inhibition has been shown to induce mTORC1 inhibition through degradation of the NMT substrate LAMTOR1^{146,147}, the potential contribution of MYCN depletion to NMTi-mediated cell death was investigated in a reduced panel of neuroblastoma cell lines

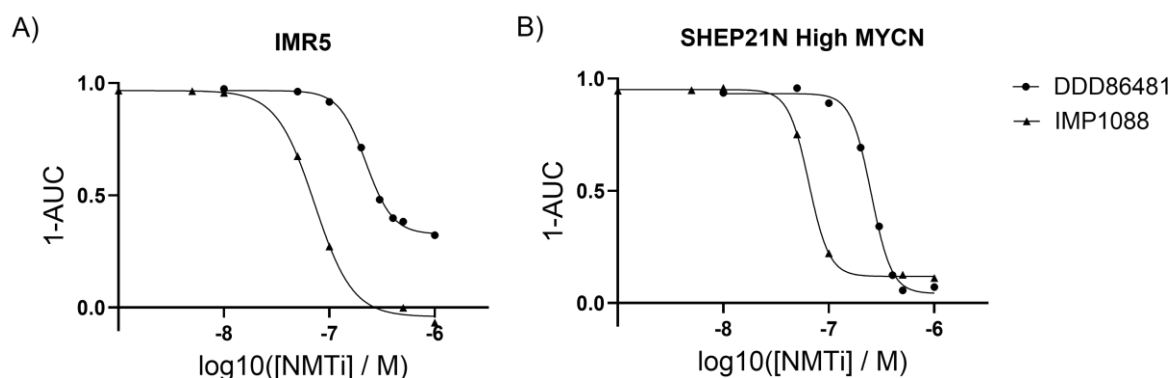


Figure 2.4 IMP1088 is more potent than DDD86481. Dose-response curves for IMP1088 and DDD86481 in **A)** IMR5 and **B)** high MYCN SHEP21N cell lines. One representative biological replicate, carried out in technical triplicate, is shown. Curves were fitted in Prism using the absolute IC50 method.

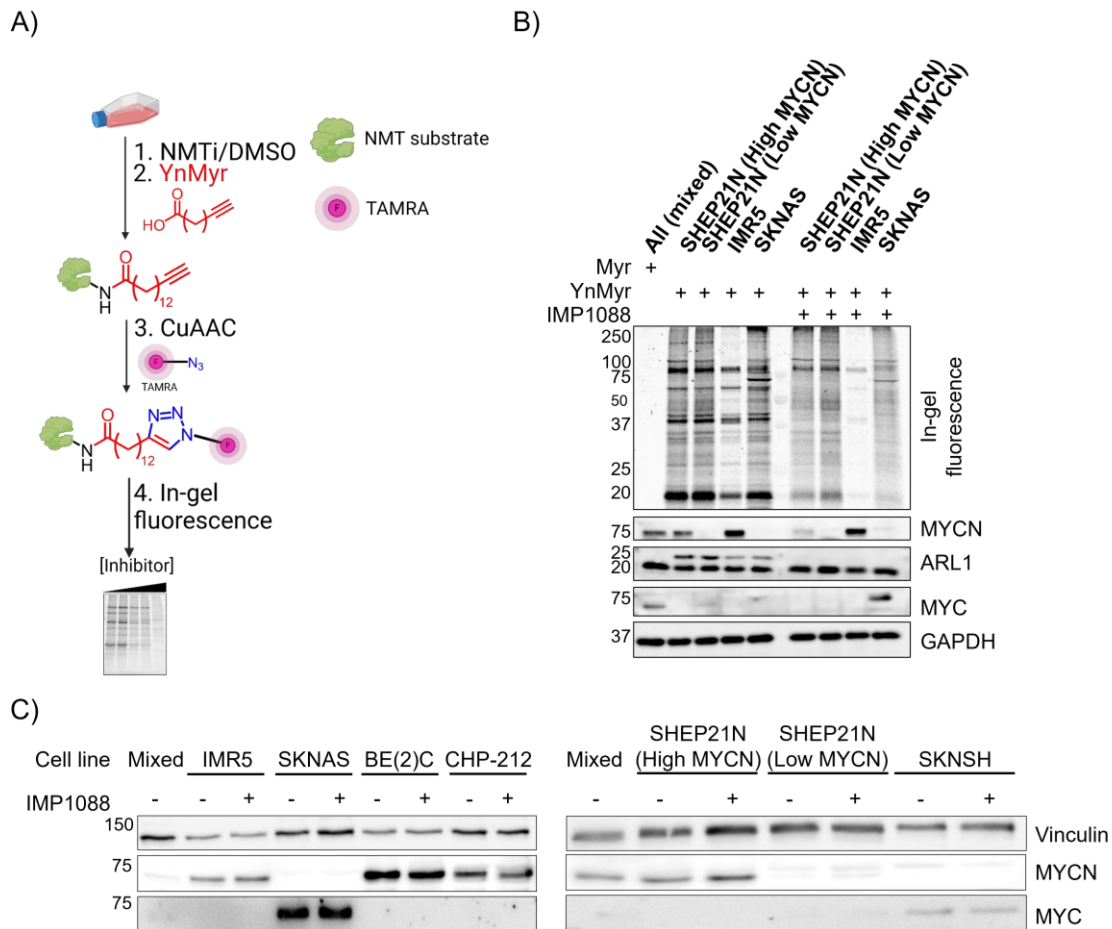


Figure 2.5 The cytotoxicity of IMP1088 is not due to downregulation of MYC family proteins. A) Schematic of the target engagement workflow. Cells were pre-treated with 100 nM IMP1088 or DMSO for 30 min, treated with YnMyr for 18 h and lysed. YnMyr-tagged proteins were ligated to a TAMRA azide fluorophore and metabolic incorporation assessed by in-gel fluorescence. NMT inhibition results in reduced fluorescence. **B)** Initial target engagement assessment and western blots for MYC family proteins. The presence of a higher MW band in ARL1 blots corresponds to YnMyr-tagged and clicked ARL1. **C)** Western blots for MYC family proteins across a panel of neuroblastoma cell lines.

(IMR5, SKNAS and SHEP21N). Cells were treated with DMSO or 100 nM IMP1088, sufficient to induce robust cell death, and labelled with YnMyr for 18 h to measure target engagement (**Figure 2.5A**). Under these conditions, robust inhibition of cellular *N*-myristoylation was seen in all cell lines, as identified by in-gel fluorescence and the loss of a higher molecular weight band for ARL1; this corresponds to YnMyr-labelled and TAMRA-modified ARL1 (**Figure 2.5B**)¹¹⁶. Initial results were mixed on the effect of MYCN, with a decrease in SHEP21N but not IMR5 MYCN levels upon NMTi treatment observed and an absent MYC signal in the DMSO treated SKNAS samples, indicating technical issues with the western blot workflow. Subsequent repeats of western blots for MYC and

MYCN across a range of neuroblastoma cell lines revealed however that MYC proteins were not depleted by NMTi treatment (**Figure 2.5C**). Therefore, MYC protein depletion does not contribute to NMTi cytotoxicity, consistent with a mechanism of action downstream of MYCN expression.

2.2 Investigating the contribution of ferroptosis to NMTi-induced cell death

2.2.1 Introduction to ferroptosis

While apoptosis is the best characterised form of programmed cell death (PCD), multiple other mechanisms of PCD have been characterised, including necroptosis, pyroptosis and ferroptosis²⁶⁸. In particular, interest in ferroptosis induction as an anti-cancer strategy has risen significantly in recent years due to the finding that both conventionally drug-resistant and metastatic cells are sensitised to ferroptosis^{269,270}. Mechanistically, ferroptosis is a form of iron-dependent cell death, in which intracellular iron promotes the generation of reactive oxygen species (ROS, **Figure 2.6A**). These then oxidise membrane phospholipids containing polyunsaturated fatty acids to generate lipid peroxides and thence lipid peroxyl radicals, which induces membrane damage and cell death. Ferroptosis can be prevented on the one hand by the system xc⁻/GSH/GPX4 axis, in which GPX4 catalyses the reduction of lipid peroxides using glutathione (GSH)²⁷¹, or alternatively by trapping of peroxyl lipid radicals by ubiquinone, which is regenerated by FSP1^{143,144}. Notably, FSP1 is a NMT substrate and *N*-myristoylation is required for its activity.

2.2.2 Ferroptosis does not contribute to NMTi-induced cell death

As MNA neuroblastomas have recently been reported to be highly sensitive to ferroptosis induction²⁵⁶⁻²⁵⁸, whether NMTi induced ferroptosis in the MNA IMR5 cell line was studied using the ferroptosis inhibitor UAMC-3203, which inhibits lipid peroxidation²⁷². UAMC-3203 alone (1 nM to 1 µM) did not affect the viability or proliferation of IMR5 cells, however UAMC-3203 did inhibit RSL3-induced ferroptotic cell death in a dose-dependent manner at low nanomolar concentrations (**Appendix Figure 4**), verifying its suitability as a tool compound. However, UAMC-3203 was not able to inhibit IMP1088-mediated cell death even at 1 µM, indicating that cell death due to NMT inhibition is not ferroptotic, although notably the combination of IMP1088 and RSL3 displayed synergistic effects (**Figure 2.6B**).

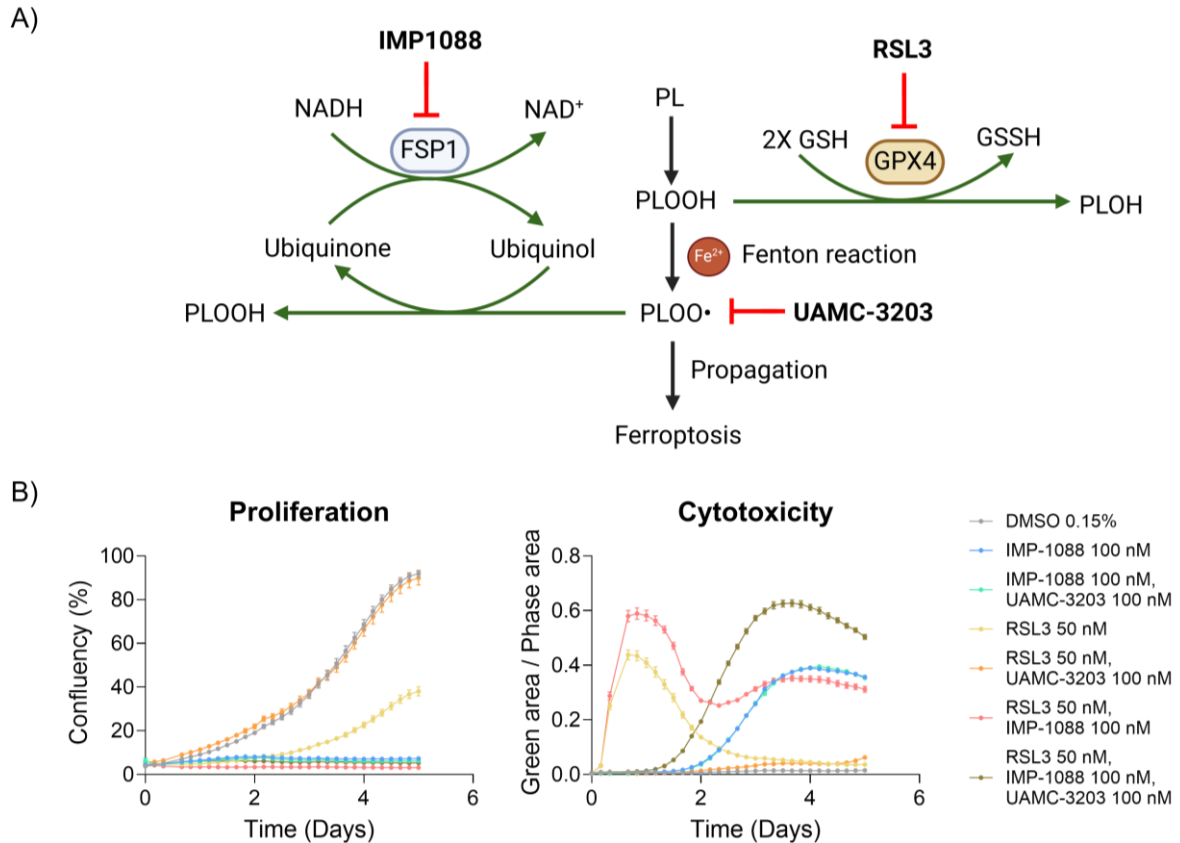


Figure 2.6 IMP1088 does not induce ferroptosis in IMR5 cells but synergises with RSL3.

A) The ferroptosis pathway and its major regulatory pathways. Intracellular iron catalyses the formation of phospholipid radicals, which can propagate, resulting in membrane damage and cell death. GPX4 is the main antioxidant system regulating ferroptosis and reduces lipid peroxides using glutathione to prevent radical formation. Alternatively, ubiquinol traps phospholipid radicals and is regenerated by the NMT substrate FSP1. RSL3 induces ferroptosis by direct inhibition of GPX4, while the myristate group is critical for FSP1 activity. **(B)** Effects of the indicated compounds on IMR5 proliferation (left) and survival (right). Data are plotted as mean $\pm$ s.e.m.

Synergy was quantified using the Bliss independence model, which assumes that the two drugs are independent and do not mechanistically overlap²⁷³. As RSL3 induces ferroptosis through direct inhibition of GPX4²⁷⁴, which is not a NMT substrate, this was considered a reasonable assumption. The combination index (CI) of 50 nM RSL3 with 100 nM IMP1088 was 0.55, whereas a $CI < 1$ indicates synergy. Inspection of the cytotoxicity curves (**Figure 2.6B**) showed that the main contribution to this effect was an increase in the early peak of cell death corresponding to RSL3-induced ferroptosis, indicating that IMP1088 treatment induces lipid peroxidation in a relatively short timeframe. However, it is likely that such levels of lipid peroxidation are rescued by GPX4 upon IMP1088 treatment alone, precluding ferroptosis. Furthermore, triple treatment of IMR5 cells with RSL3, IMP1088

and UAMC-3203 showed a distinct profile of cell death, with an earlier and greater induction of cell death than IMP1088 treatment alone. This is notable given that a significant indicator of sensitivity to NMTi is the time required to induce cell death. Therefore, GPX4 inhibition potentiates NMTi-induced cell death even in the absence of lipid peroxidation, possibly by accelerating general oxidative damage. Such an effect was probably not seen in the RSL3/IMP1088 double treatment due to induction of ferroptosis.

Overall, these results indicate that ferroptosis does not contribute to NMTi-induced cell death, which is likely apoptotic as has been previously observed in multiple models^{142,149}, although it has recently been reported that NMTi can induce parthanatos in lung cancer models²⁷⁵. Regardless, the results also indicate that NMTi induces oxidative stress and synergise with ferroptosis inducers to elicit a greater degree of cell death compared to single agent treatments in MNA neuroblastomas cells.

2.3 Investigating the contribution of ER stress and the UPR to NMTi-induced cell death

2.3.1 Introduction to the UPR

Previous mechanistic studies have linked induction of endoplasmic reticulum (ER) stress and subsequent activation of the unfolded protein response (UPR) to NMT inhibition^{142,149}. Notably, NMTi are potent senolytics and induce ER accumulation of cytokines in senescent (cancer) cells, leading to UPR induction and cell death¹⁵⁰. Therefore, the induction of the UPR in neuroblastoma was also investigated.

Mechanistically, ER stress, arising from the accumulation of misfolded or unfolded proteins in the ER lumen, is detected by the three sensor proteins IRE1 α , PERK and ATF6 via the chaperone protein BiP²⁷⁶. While BiP usually associates with these sensors, it preferentially binds to misfolded proteins. BiP disassociation therefore allows PERK and IRE1 α to self-dimerise and auto-phosphorylate, and for ATF6 to translocate to the Golgi under conditions of ER stress. These changes then induce UPR activation to promote clearance of misfolded proteins, or apoptosis if ER stress is unresolvable.

2.3.2 The UPR is not activated by NMTi in MNA neuroblastomas to induce cell death

In order to assay UPR activation, western blotting against canonical UPR markers was carried out. Additionally, three ER stress inducers were used as positive controls: tunicamycin (Tm), thapsigargin (Tg) and Brefeldin A (BFA), as it has been reported that the UPR may be differentially activated depending on the mechanism of ER stress induction²⁷⁷. The treatment conditions for the positive controls were first optimised and 18 h treatment with each control most robustly induced the UPR in SHEP21N cells (**Appendix Figure 5**). It was also noted that the signal for phosphorylated eIF2 α , which is downstream of PERK activation, was very low when 20 μ g of total protein input was used, and therefore 40 μ g was subsequently used when blotting for phosphorylated proteins.

Next, the effect of 100 nM IMP1088 (18, 36 and 60 h treatment) was investigated in high MYCN SHEP21N cells. Overall, the UPR did not appear to be activated, except only slightly after 60 h of IMP1088 treatment, well after initial induction of cell death (**Figure 2.7**). This was clearly demonstrated by the lack of upregulation of BiP, CHOP and ATF4, contrary to the results in the positive controls. Both ATF4 and CHOP are upregulated by PERK signalling; ATF4 mediates transcription of genes involved in protein folding and amino acid metabolism whereas CHOP mediates apoptosis²⁷⁶. CHOP is also upregulated downstream of IRE1 α activation. Similar results were also observed for eIF2 α phosphorylation. While these showed a clear lack of UPR signalling, the interpretation of the IRE1 α and PERK blots was less clear. Total IRE1 α was not affected by IMP1088 but was greatly increased in the Tg and BFA treated positive controls, indicating a lack of UPR induction by IMP1088 in a similar fashion to these positive controls. The phospho-IRE1 α blot also showed accumulation of a higher molecular weight band ~250 kDa in size upon IMP1088 treatment, which was apparent even after 18 h treatment and was also present upon Tm but not Tg or BFA treatment, suggesting that effects on IRE1 α activation by IMP1088 are more similar to the effects of Tm. This molecular weight corresponds to dimerised IRE1 α , potentially due to incomplete denaturing prior to SDS-PAGE. As dimerised, phosphorylated IRE1 α is the active form, this suggests IRE1 α activation by IMP1088, although the results should be interpreted with caution given that this higher molecular weight band is not seen in the total IRE1 α blots. This could be because the antibody against total IRE1 α is unable to bind to the dimeric form; the antibody binds residues around His963, which is relatively close to the dimer interface. As normalisation

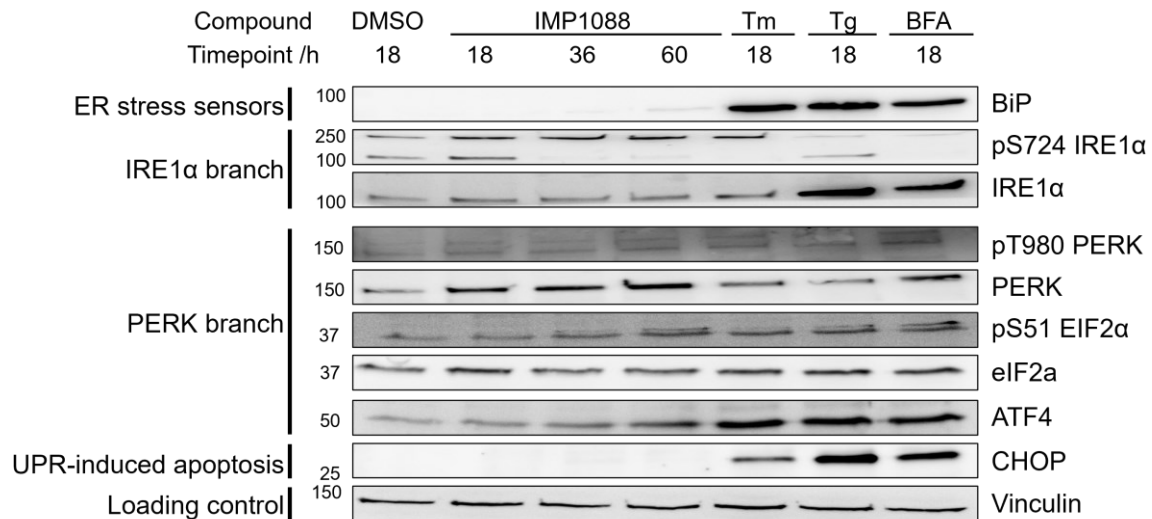


Figure 2.7 ER stress and the UPR do not initiate cell death in high MYCN SHEP21N cells upon NMT inhibition. Western blots for proteins involved in the UPR upon treatment with 100 nM IMP1088 for 18-60 h. Tunicamycin (5 mg/mL), thapsigargin (100 nM) and brefeldin A (2 µg/mL) were used as positive controls.

of phospho-blot against total protein amounts is necessary to quantify phosphorylation activation, robust interpretation of the current results is not possible. This likely contributes to the differences seen in the positive controls, although the cells may also be at different stages of the UPR depending on the positive control used. For example, Tg or BFA treated cells may be in a more apoptosis committed state than Tm treated cells, as suggested by the higher levels of CHOP in these samples. Such a state may also correspond to attenuated IRE1α phosphorylation but increased total protein accumulation. When considering the PERK results, it was not possible to draw a definitive conclusion about its phosphorylation due to the low signal. This is a known difficulty for direct measurement of PERK phosphorylation by western blot²⁷⁸ due to the low total abundance of the proteins and variable phospho-antibody performance. Additionally, total PERK levels increased in NMTi treated samples which has been seen to correlate with UPR induction²⁷⁹, although this was only the case with the BFA positive control in this study. Finally, western blots in IMP1088-treated IMR5 cells showed similar effects, with slight induction of UPR only after 60 h treatment with 100 nM IMP1088, although 1 µM IMP1088 may induce UPR in this cell line at earlier timepoints (**Appendix Figure 6**).

Taken together, these results indicate that the UPR does not contribute significantly to initiating IMP1088-induced cell death in high MYCN neuroblastoma contexts. Some indications of possible IRE1α or PERK activation was seen, although these do not

translate into activation of downstream signalling and indeed further validation of their activation is required. As western blotting for phosphorylation status has proved difficult, alternative approaches such as the use of Phos-tag gels or measurement of XBP1 splicing directly downstream of IRE1 α should constitute better approaches²⁷⁸. A likely explanation for the lack of observed UPR activation in the neuroblastoma context comes from the duration of NMTi treatment. UPR activation was observed after three (HeLa)¹⁴⁹ or five (senescent cells)¹⁵⁰ days of NMTi treatment whereas the latest timepoint measured in this study was 60 h (2.5 days), when UPR induction was just beginning to occur, consistent with the time-dependent induction of UPR in HeLa cells. While NMTi may therefore eventually induce the UPR in MNA neuroblastoma cells, it appears that other effects of NMTi induce cell death first.

2.4 YnMyr profiling of NMT substrates in IMR5 cells

2.4.1 Introduction

As mentioned in the introduction, metabolic tagging of NMT substrates using YnMyr and subsequent LC-MS/MS based proteomic analysis is a powerful technique to interrogate the *N*-myristoylated proteome. As *N*-myristoylation has not been profiled in neuroblastoma to date, this method was applied to IMR5 cells as a representative MNA cell line to identify NMT substrates possibly unique to neuroblastoma and investigate the NMT substrate profile in neuroblastoma. Following previously established protocols¹¹¹, three biological conditions were prepared in triplicate for LFQ proteomic analysis: myristic acid (Myr) treated (negative control), YnMyr treated, and both YnMyr and IMP1088 treated. Cells were pre-treated with DMSO or 100 nM IMP1088 for 30 min followed by an 18 h incubation with YnMyr before cell lysis. Proteins were then ligated to AzRB, biotin-labelled proteins enriched on neutravidin beads, digested, and submitted for analysis. 337 proteins were identified in at least one replicate, and principal component analysis (PCA) plots showed that the samples segregated well based on experimental condition (**Appendix Figure 7**).

2.4.2 Direct detection of NMT substrates

The most unambiguous method to identify NMT substrates is direct detection of the modified peptide; in this case, the AzRB-YnMyr adduct. Direct detection of 14 such peptides, corresponding to 15 proteins, were identified in YnMyr labelled samples

(**Appendix Table 2**). These all corresponded to NMT substrates for which direct detection of the N-terminally modified peptide had already been identified, further indicating that the experiment worked well. All but three peptides were previously identified in analogous YnMyr proteomic experiments^{26,110}, whereas the native *N*-myristoylated peptide for the other three (PRKAB2, FMNL2, PRKACB) had been identified in human brain extracts⁴³.

2.4.3 Initial profiling of NMT substrates: protein enrichment in YnMyr-treated samples relative to Myr-treated controls

Due to the limitations on detecting modified peptides, measuring protein enrichment upon YnMyr labelling remains the predominant way to identify NMT substrates. As an initial approach, CoT NMT substrates were assayed by comparing the Myr-treated samples with the YnMyr-treated samples. A stringent filtering strategy was initially tested (requiring 2 valid values in each condition to exclude low confidence protein identifications), but this only yielded 70 proteins, including just one canonical NMT substrate, MARCKS, which was highly enriched in the YnMyr-treated condition ($\log_2FC=10.26$; FC=fold change) (**Figure 2.8A**). The next most enriched proteins did not possess a Gly2. However, many of the proteins were filtered out due to missing values in the Myr-treated group, indicative of selective pull-down only upon metabolic labelling. As a result, this filtering strategy was considered to be too stringent and a more permissive filter (3 valid values in at least one condition, which was always at least the YnMyr treated sample) was used. 198 proteins passed this filter including 62 canonical NMT substrates, confirming that selective enrichment of YnMyr-labelled NMT substrates was a major cause of the missing values. Missing values were imputed from a downshifted normal distribution as default in Perseus, the processing software used, and the results visualised in a volcano plot (**Figure 2.8B**).

In order to avoid over-interpretation of the results due to the use of imputation, significantly enriched proteins were classified as those proteins with a $p\text{-value}<0.05$ ($-\log_{10}(p\text{-value})>1.3$), a standard significance cut-off used in such experiments, and a $\log_2FC>3.18$. This FC cut off corresponded to that of YWHAЕ, which was the most enriched non-NMT substrate using the stringent filtering threshold and 80% of identified canonical NMT substrates passed this threshold. This resulted in the identification of 55 canonical CoT

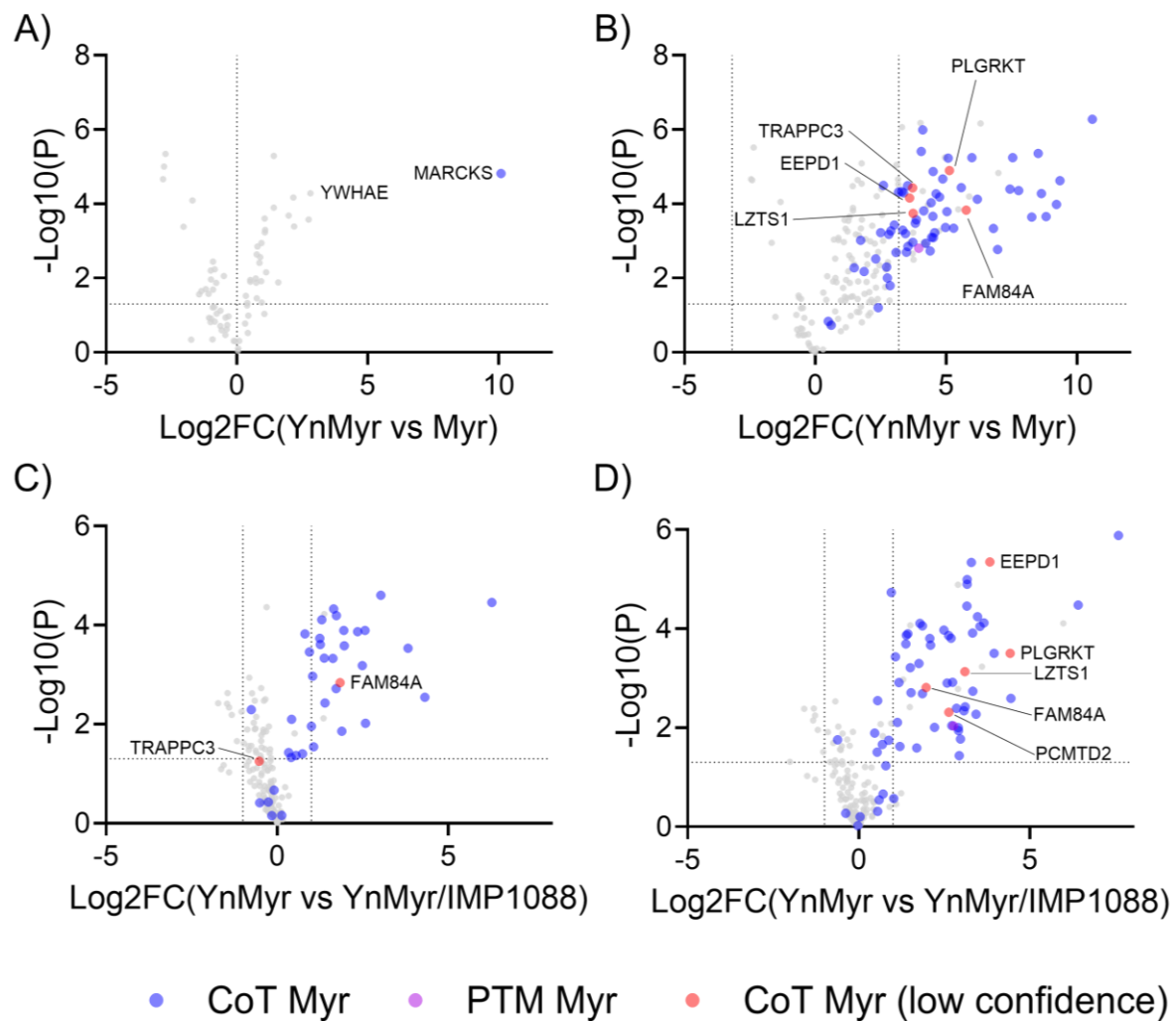


Figure 2.8 YnMyr proteomics in IMR5 cells confirms five proteins as endogenous CoT NMT substrates. Volcano plots showing proteins enriched upon YnMyr treatment compared to Myr control using a stringent (A) or a relaxed (B) filter or compared to samples pretreated with 100 nM IMP1088 for 30 minutes using a stringent (C) or a relaxed (D) filter threshold. Stringent filter thresholds required two valid values in both conditions. Relaxed filter thresholds required three valid values in at least one condition with missing values imputed. Proteins in the top right sector were considered significantly enriched in YnMyr samples. Volcano plots were constructed in Perseus using a two-tailed student's t-test. CoT Myr; proteins with a N-terminal glycine which incorporate YnMyr in a NMTi-dependent manner or for which the N-myristoylated N-terminal peptide has been identified, PTM Myr; proteins lacking a N-terminal glycine which incorporate YnMyr in a NMTi-dependent manner during e.g. apoptosis, CoT Myr (low confidence); proteins with a N-terminal glycine which incorporate YnMyr in an NMTi-independent manner. All assignments are based primarily from in-house and previously published proteomic datasets (references 26,43,110,112,116).

NMT substrates as well as 5 glycine starting proteins (Gly2) not previously verified in our in-house high confidence NMT substrate list: FAM84A (also known as LRATD1), TRAPPC3, LZTS1, EEPD1 and PLGRKT. It is relevant to re-iterate here that enrichment

upon YnMyr treatment by itself is not enough to qualify a Gly2 starting protein as a native high confidence CoT NMT substrate, due to possible incorporation of YnMyr through alternative lipid processing methods and co-enrichment of NMT substrate interactors²⁶. However, four of the five non-high confidence Gly2 proteins identified had all been annotated as potential NMT substrates: previous YnMyr proteomic datasets had identified both PLGRKT and EEPD1 as enriched upon YnMyr incorporation^{26,110}. Wild type, but not G2A EEPD1 additionally incorporated radiolabelled myristate ($[^3\text{H}]\text{Myr}$) when transfected in HEK293 cells²⁸⁰. Similarly, $[^3\text{H}]\text{Myr}$ labelled wild type but not G2A rat LZTS1 expressed in COS7 cells²⁸¹; rat and human LZTS1 only differ at one amino acid in the first 20 amino acids. Finally, LRATD1 was previously suggested to be a NMT substrate based on peptide studies⁴³, but evidence for its *N*-myristoylation was only shown in cells for the first time shortly before this study. Transiently expressed LRATD1 was labelled by YnMyr, which was abolished by treatment with the NMTi DDD85646 or the G2A mutation²⁸². These evidence show that the aforementioned proteins are NMT substrates under the experimental conditions tested, although the use of overexpression systems can induce non-physiological lipidation. Therefore, confirmation that these proteins are endogenous NMT substrates is still desirable.

2.4.4 Validation of identified CoT NMT substrates: identification of NMTi-responsive proteins

To verify the results of the above analysis, the YnMyr treated condition was then compared with the YnMyr + IMP1088 treated condition. Stringent filtering of proteins as above yielded 163 proteins and showed robust enrichment of known CoT NMT substrates in YnMyr-treated samples over IMP1088-treated samples ($p < 0.05$, $\log_2\text{FC} > 1$, 22/38 identified substrates) including FAM84A, confirming it as a CoT NMT substrate at physiological concentrations. This fold-change cut off, although arbitrary, corresponds to a 2-fold difference in abundance and again is a commonly used threshold for differential proteomics to represent a meaningful abundance change. However, TRAPPC3 intensities were not affected by NMT inhibition, disqualifying it as a NMT substrate (**Figure 2.8C**). Two other proteins were also significantly enriched in this analysis, PSMD2 and PSMC2, although they were likely enriched through their interactions with PSMC1, a known NMT substrate²⁸³.

When the more relaxed filter was used (3 valid values in at least one condition), a further 38 proteins were identified, with a majority (27) of these again annotated as canonical NMT substrates. Using this dataset, 47 canonical NMT substrates were significantly NMTi responsive (**Figure 2.8D**). Furthermore, *EEPD1*, *LZTS1* and *PLGRKT* were also identified. These were significantly NMTi responsive and more robustly so than multiple canonical NMT substrates, confirming them as endogenous NMT substrates. In addition, one further low-confidence NMT substrate was identified as NMTi sensitive: *PCMTD2*, which was previously only identified as enriched upon YnMyr treatment. This protein did not meet the required FC threshold in the previous YnMyr vs Myr analysis to be considered enriched, however this is likely due to stochasticity introduced by imputation, as no *PCMTD2* was detected in the Myr treated samples.

2.4.5 Comparison with the HeLa *N*-myristoylated proteome

To investigate whether an altered expression profile of NMT substrates could affect NMTi sensitivity, the NMTi sensitive NMT substrates identified in IMR5 cells were compared with those previously identified in HeLa cells, which are less phenotypically sensitive to NMTi^{116,149}. A majority of such NMT substrates were identified in both cell lines, with only 5 substrates only identified in IMR5 cells:

CHCHD6: This protein is part of the core of the MICOS complex, which maintains the cristae in the mitochondrial inner membrane and maintains ATP production through oxidative phosphorylation²⁸⁴.

EEPD1: *EEPD1* has been suggested to be important to maintain genomic stability through the repair of oxidative damage at replication forks²⁸⁵ and appears to promote homologous recombination over non-homologous end-joining. Furthermore, *EEPD1* induces cholesterol efflux in macrophages to inhibit inflammatory signalling²⁸⁶.

FAM84A/LRATD1: This is a poorly characterised protein, although interestingly expression of this protein is undetectable in normal tissues but upregulated in certain cancer contexts²⁸⁷. Assays have demonstrated that *LRATD1* expression upregulates the migratory, but not proliferative, capacity of cells, suggesting a role in metastasis.

LZTS1: The canonical role of this protein is to inhibit cell cycle progression and mitosis. Under normal conditions, LZTS1 interacts with the CDK1-Cyclin B complex and assembled microtubules in late G2-M phase, however LZTS1 destabilisation induces exit from mitosis and increased proliferation and hypertetraploidy²⁸⁸. Accordingly, LZTS1 is an established tumour suppressor; an estimated 60% of tumours downregulate LZTS1 and LZTS1^{-/-} mice spontaneously develop neoplasms²⁸⁹.

PCMTD2: This protein appears to be part of an Elongin B and C–Cul2 or Cul5–SOCS box protein (ECS) E3 ubiquitin ligase complex and therefore mediates protein quality control²⁹⁰. In CD8⁺ T cells, PCMTD2 appears to solely mediate aspects of CUL5 function and promote protein ubiquitination leading to T cell activation.

However, as only a few differences were observed in HeLa and IMR5 cells, it was considered that the NMT substrate scope in IMR5 cells did not significantly differ from those in HeLa cells and that the effects of NMTi are mediated through generally similar pools of NMT substrates. While LZTS1 is not expressed in HeLa cells²⁹¹, which would explain its selective detection in IMR5 cells, the other proteins are expressed in HeLa cells. Selective detection in these cases most probably arises from technical factors, including differences in the sample preparation workflow, LC-MS/MS conditions such as run time and in particular the use of data-dependent acquisition (DDA)²⁹². DDA selects a given number of precursor (peptides) for ionisation for MS/MS analysis, generally the top N most abundant peptides in a given retention time and m/z window. Alongside inherently biasing quantification towards more abundant proteins, this method introduces stochasticity between separate LC-MS/MS runs and therefore missing values.

2.5 YnMyr profiling of NMT substrates in SHEP21N cells

2.5.1 Re-optimisation of the proteomic workflow

To further assess NMTi mechanism of action (MoA) and differences between high and low MYCN conditions, the SHEP21N cell line was chosen as the main cell model due to its isogenic nature. Therefore, the NMT substrate profile and their response to NMTi was also

Input protein (μg)	#CoT NMT substrates	
	Ctrl	YnMyr
100	1	91
250	11	91
500	6	97
750	8	96
1000	7	86

Table 2.2 Protein input for YnMyr proteomics can be scaled down without loss of depth using a timsTOF HT mass spectrometer. The number of CoT NMT substrates identified in the optimisation experiment (n=1) in Myr and YnMyr treated samples respectively.

assessed in this cell line. Due to the acquisition of a new timsTOF HT mass spectrometer, which has increased sensitivity compared to the QExactive used in the IMR5 study, the experimental protocol was first reassessed. The goal was to minimise the protein input amount without loss of NMT substrate detection while also reducing avidin contamination, which was prevalent in the previous experiments and contributes to reduced protein identifications and lowered LCMS/MS performance. If a reduced protein input is tolerated, then automation of this workflow, of growing interest in the Tate group, would also be enabled. Cells were treated with Myr or YnMyr for 18 h and different protein input amounts were tested (100, 250, 500, 750 and 1000 μg), whereas the bead volume ratio was kept as 1:10 bead volume:input protein. For input amounts requiring <30 μL beads,

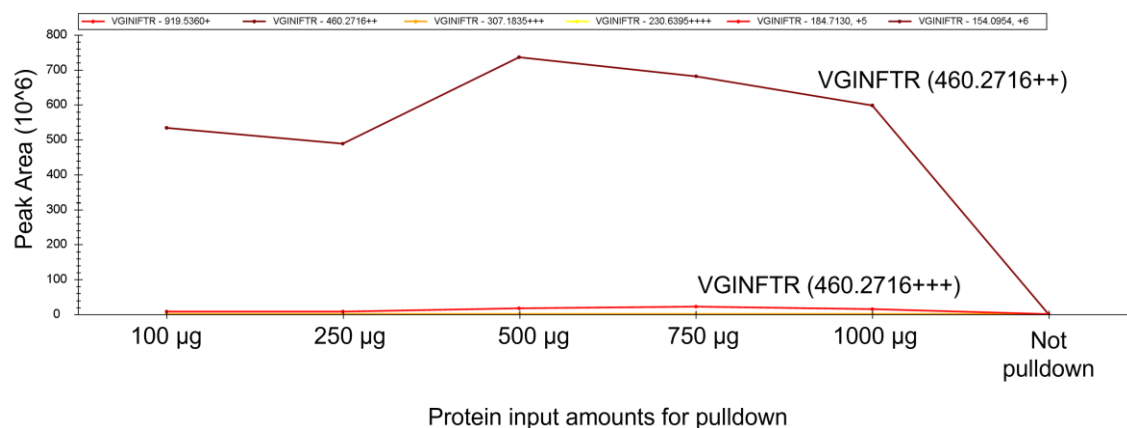


Figure 2.9 Streptavidin contamination in YnMyr pull-down samples. Peak area of peptides corresponding to streptavidin contaminant peptides identified in the YnMyr pull-down optimisation samples. A global proteomics sample prepared without streptavidin beads is used as a negative control (rightmost sample, not pulldown). The peptide species (mass/charge) are shown.

blank agarose beads up to 30 μ L were added. Samples were then processed using an updated protocol described in the Methods section, although the general steps were the same; Dr Jack Houghton assisted with the sample preparation. The results of this optimisation experiment showed that the number of identified NMT substrates (~90) was similar across all input amounts in YnMyr, and highly increased over Myr treated control samples (generally <10), confirming that protein identification and quantification using a timsTOF HT enables scaling down of input amounts without loss of depth (**Table 2.2**). Although only one replicate was prepared per condition, comparable results were observed for other pull-down protocols in the Tate group. Additionally, the amount of avidin contamination was relatively similar for all samples, but lowest for the 100 and 250 μ g protein conditions, which use the lowest number of beads (**Figure 2.9**). Therefore 100 μ g protein input was taken forward for pull-down proteomics.

2.5.2 NMT substrate profiles in SHEP21N cells

To assess NMT substrates in SHEP21N cells, only the more robust comparison between the YnMyr treated and the YnMyr/IMP1088 treated samples was considered. Cells were treated as for IMR5 cells, except quadruplicates were prepared for each condition, and samples prepared using the optimised protocol. Sample preparation was conducted with Daria Berezina. Generally, the PCA plots for both MYCN conditions showed reasonable separation of the samples by treatment condition especially by PC2, although some intra-group variation was seen (**Appendix Figure 8**). Seven modified N-terminal peptides corresponding to canonical NMT substrates were detected and these again corresponded to those previously identified (**Appendix Table 3**). For differential enrichment analysis, proteins were filtered against the requirement for three valid values in at least one condition and the missing values imputed as for the IMR5 experiment. Significantly NMTi responsive proteins, according to the same criteria used in the IMR5 experiment ($\log_2FC > 1$, $p < 0.05$, 88% confidence interval), in high MYCN cells that were not known NMT substrates did not possess N-terminal glycines apart from SCFD1 (**Figure 2.10A**), where a Gly2 is present in a secondary isoform due to the absence of amino acids 1-67 of the canonical sequence. However, the peptides responsible for this enrichment were residues 2-16 of the canonical sequence, disqualifying it as a CoT NMT substrate. Similarly, the only non-canonical NMT substrate with a N-terminal glycine in the low MYCN condition was YARS1 (**Figure 2.10B**), which was previously non-NMTi responsive in HeLa

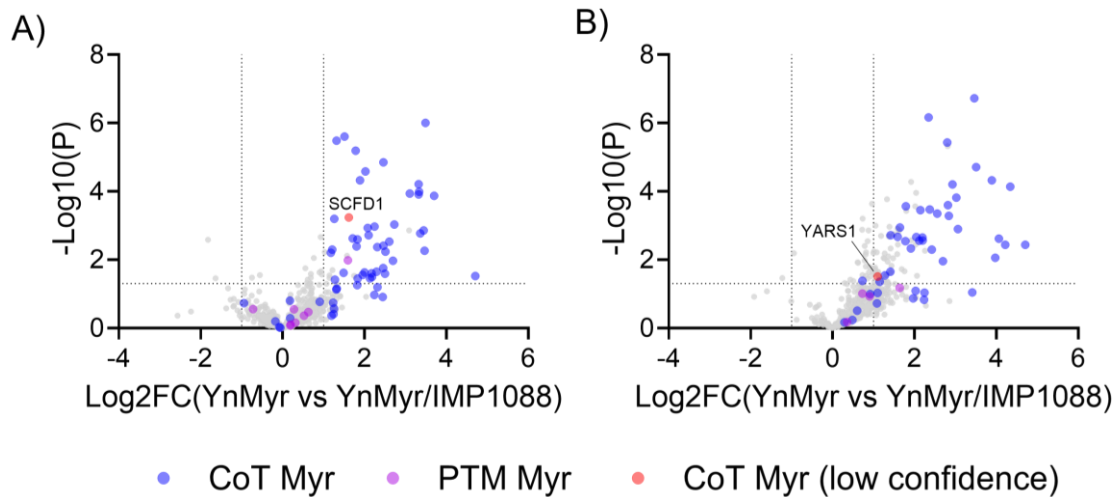


Figure 2.10 YnMyr proteomics in SHEP21N cells identifies no novel NMT substrates. Volcano plots showing proteins enriched upon YnMyr treatment compared to samples pretreated with 100 nM IMP1088 for 30 minutes in high (A) or low (B) MYCN SHEP21N cells. Protein filter thresholds required three valid values in at least one condition and missing values imputed. Proteins in the top right sector were considered significantly enriched. Volcano plots were constructed in Perseus using a two-tailed student's t-test.

cells and thus classified as not a NMT substrate²⁶. However, this is most likely a false positive resulting from imputation, as the p-values and fold changes were close to the thresholds used ($\text{log}_2\text{FC}=1.10$, $p=0.03$) and the protein was lowly abundant in the YnMyr samples to begin with. Furthermore, YARS1 is not NMTi-responsive in the high MYCN state, where it was detected in at least two replicates for each condition and most importantly the N-terminal peptide of YARS1 has previously been detected as *N*-acetylated but not *N*-myristoylated⁴³. Therefore, no new NMT substrates were detected in SHEP21N cells.

2.5.3 Comparison of NMT substrate profiles in high and low MYCN contexts

Finally, because abundance depletion upon NMTi treatment reflects how sensitive a given protein's *N*-myristoylation state is to NMT inhibition, which may provide a rationale for observed phenotypic differences, the fold changes for known substrates in the high and low MYCN cells were compared. Indeed, multiple NMT substrates were differentially NMTi sensitive depending on MYCN status (Figure 2.11). In this figure, the effect of IMP1088 on the *N*-myristoylated proteoform of NMT substrates is compared in the high and low MYCN states. For the proteins on the left-hand side of the graph, loss of the *N*-myristoylated proteoform (as indicated by the enriched YnMyr-labelled subpopulation) is

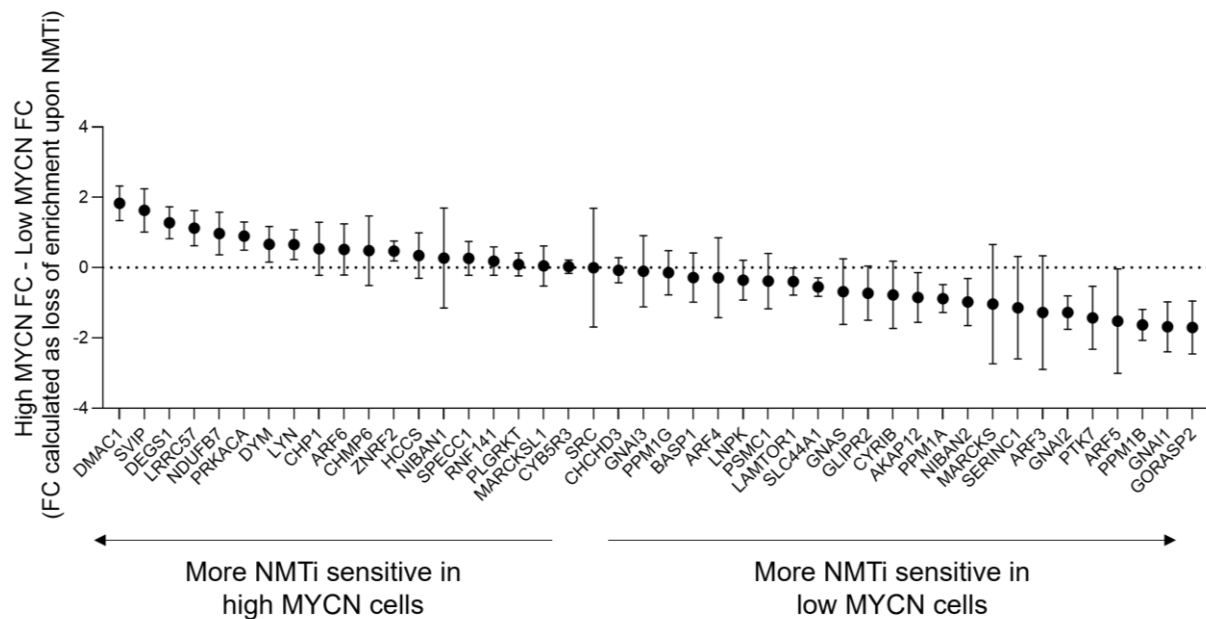


Figure 2.11 Multiple NMT substrates are differentially sensitive to NMTi depending on cellular MYCN status. Ranked responses of NMT substrates to IMP1088 as measured by loss of YnMyr enrichment. Positive values indicate greater depletion upon IMP1088 treatment in high MYCN states. Data are shown as mean + s.e.m. of $n = 4$ biological replicates.

greater in the high MYCN state than the low MYCN state upon NMTi treatment. The pathways regulated by these proteoforms may therefore be disproportionally affected in the high MYCN state and could explain the greater sensitivity of the high MYCN state to NMT inhibition. The functions for the proteins most differentially affected in the high and low MYCN states are highlighted below:

More affected in high MYCN:	
DMAC1	This is an essential assembly factor for RC1 of the electron transport chain (ETC) ²⁹³ . Notably, loss of <i>N</i> -myristoylation in the G2A mutant is associated with significant loss of protein expression, although it does not affect its localisation ²⁶⁵ .
SVIP	SVIP is an interactor of p97/VCP, a widely expressed protein with key roles for protein maintenance, being known to regulate protein ubiquitination and autophagy. In particular, SVIP has been shown to

	regulate VCP localisation, promoting lysosomal recruitment and inhibiting ER-associated degradation (ERAD) ^{294,295} . <i>N</i> -myristoylation of SVIP is necessary for its <i>S</i> -acylation and subsequent localisation and may also modulate protein interactions with VCP ²⁹⁶ . Therefore, SVIP appears to regulate VCP in a <i>N</i> -myristoylation dependent manner.
DEGS1	This fatty acid desaturase participates in sphingolipid metabolism and catalyses the last step of ceramide biosynthesis ²⁹⁷ . DEGS1 is localised in the ER and mitochondria and therefore DEGS1 deficiency is associated with mitochondrial dysfunction and defective lipid metabolism ²⁹⁸ . The myristoyl group of DEGS1 promotes mitochondrial localisation of the protein and also increases the enzyme's activity.
LRRC57	This is a broadly uncharacterised protein with unknown function. Structurally, the protein contains leucine rich repeats, which primarily facilitate protein-protein interactions (PPIs) ²⁹⁹ .
NDUFB7	This is a functionally relevant accessory component of RC1; an intronic mutation in this gene which depletes NDUFB7 also reduces activity of RC1 and is associated with severe congenital lactic acidosis in humans ³⁰⁰ . <i>N</i> -myristoylation of NDUFB7 is required for its mitochondrial localisation and acts as an anchor to the intermembrane space leaflet ³⁰¹ . The myristoyl group also participates in PPIs, binding in a groove in MT-ND5, although the functional importance of this interaction has not been investigated.
More affected in low MYCN:	
GORASP2	This is a protein which regulates multiple aspects of Golgi structure. Most prevalently, the Golgi exists as stacks of cisternae, and GORASP2 appears to link cisternae together through protein-protein interactions with GORASP domains on adjacent cisternae, although some debate over the exact function of GORASP proteins exists ^{302,303} . GORASP2 has also been linked to multiple other functions including formation of Golgi ribbons, disassembly and reassembly of the Golgi after mitosis and unconventional secretion of proteins ³⁰⁴ . Studies on the

	function of GORASP2 <i>N</i> -myristoylation have been limited to <i>in vitro</i> , non-cellular contexts, but indicate its importance in membrane localisation and dimerisation ³⁰⁵ .
GNAI1	Binding of G protein-coupled receptors (GPCRs) by a ligand trigger the activation of GTP binding small heterotrimeric G-proteins, consisting of α , β and γ subunits ³⁰⁶ . This is an inhibitory α subunit (Gai), meaning that upon activation, it inhibits cAMP production by adenylate cyclase and therefore downregulates protein kinase A (PKA) signalling, leading to a variety of effects. The myristoyl group is required for membrane association.
PPM1B	This is a protein phosphatase 2C (PP2C) serine/threonine protein phosphatase; these phosphatases negatively regulate cell stress pathways. PPM1B has multiple targets, including CDKs, AMPK and IKK β , through which it regulates cell cycle, metabolism and NF- κ B signalling, among others ³⁰⁷ . The myristoyl group appears to be involved in PPM1B substrate recognition, decoupled from its effects on localisation ³⁰⁸ .
ARF5	A member of the GTP binding ADP-ribosylation factor (ARF) family which are primarily active at the Golgi. Active ARFs recruit a variety of effectors in their active state, in particular vesicle coat proteins and lipid modifying proteins, to regulate vesicular transport and membrane composition ³⁰⁹ . <i>N</i> -myristoylation is important for ARF proteins to maintain membrane localisation and therefore activity.
PTK7	A receptor tyrosine kinase (RTK), PTK7 notably does not possess intrinsic catalytic ability, but acts as a dimerisation scaffold to potentiate active RTKs ³¹⁰ . The best characterised role of PTK7 is its participation in the Wnt signalling pathway through which it has been implicated in cytoskeletal reorganisation and cell proliferation. PTK7 is overexpressed in multiple cancers and has been linked to metastatic disease and worse prognosis, including in breast, lung, and cervical cancers.

It is notable that of the proteins most selectively affected in high MYCN cells upon NMT inhibition, two were directly related to RC1, and a third related to mitochondrial organisation, suggesting that the mitochondria may be disproportionately affected in high MYCN states. Conversely, Golgi-associated functions or general signalling may be more perturbed in low MYCN states, although whether selective dysregulation of specific pathways contributes to differences in NMTi sensitivity requires further validation.

2.6 Chapter conclusions and future work

A range of phenotypic assays investigating the impact of NMTi, as well as chemical proteomic profiling of NMT substrates in neuroblastoma cell models was described in this chapter. Cytotoxicity assays in a panel of neuroblastoma cell lines, including the isogenic SHEP21N cell line, confirmed that MYCN expression sensitises to NMTi in neuroblastoma cells (**Figure 2.2**). One consideration, however, is that the assay protocol used involved treatment of cells with NMTi for at least 5 days without media replacement. Therefore, it is possible that depletion of some media nutrients, especially in the longer assays, would alter the metabolic profile of the cells and contribute to the cellular responses observed. Therefore, repetition of these experiments in the future with media replenishment would be beneficial. Additionally, when considering the results in the SHEP21N cell line, the effect of doxycycline on the cells, independent of its effect on MYCN expression, was not considered. This could be addressed in future experiments by treatment of the parental SHEP cell line with doxycycline and NMTi simultaneously. However, generally, the results are consistent with the sensitivity previously observed in MYC-deregulated cells⁶⁶ and more generally for haematopoietic cancers such as Burkitt's lymphoma, in which MYC-associated chromosomal translocations are known drivers¹⁵⁴. Therefore, the mechanisms mediating increased sensitivity to NMTi are likely to be similar between MYC paralogues and are downstream of MYC/MYCN themselves in tumours, as MYCN levels were not affected by NMTi (**Figure 2.5**). This latter result was also demonstrated for MYC in the P493-6 cell line (manuscript under review).

As recent reports have demonstrated significant sensitivity of MNA neuroblastomas to ferroptosis induction, the contribution of this PCD mechanism in the context of NMTi was assessed but found to be negligible (**Figure 2.6**). This is probably because any loss of anti-ferroptotic mechanisms by loss of FSP1 *N*-myristoylation is compensated by a still functional GSH/GPX4 antioxidant pathway; indeed, similar results were recently reported

in a lung cancer cell model²⁷⁵. However, it is possible that ferroptosis will contribute to NMTi-mediated cell death in GPX4-deficient contexts. In particular, IMP1088 synergised with RSL3, a GPX4 inhibitor, to increase the amount of ferroptosis-associated cell death, indicating that NMT inhibition does induce oxidative stress. This could be further verified by the measurement of ROS or lipid peroxidation levels using BODIPY 581/591 C11. Combination treatments with ferroptosis inhibitors would therefore be an interesting avenue to further investigate, and synergy could extend to a range of interventions affecting cellular anti-oxidation, such as cystine deprivation or transsulfuration inhibition (both of which support glutathione synthesis)²⁵⁶. To confirm the exact mechanism through which NMTi-treated neuroblastoma cells die, analogous experiments using apoptosis, necroptosis and pyroptosis inhibitors should be carried out, or activation of these PCD mechanisms measured. It is expected that these cells will die by apoptosis, although NMTi-induced parthanatos has been reported²⁷⁵. Notably, parthanatos shares similarities with apoptosis, meaning that standard methods to measure apoptosis, such as Annexin V staining, do not distinguish between the two mechanisms, requiring further assays to measure the contribution of apoptosis and parthanatos in different contexts.

The contribution of the UPR to NMTi cytotoxicity in this study was also determined to be negligible, with no UPR activation observed prior to the onset of cell death (**Figure 2.7**). This is contrary to previous reports^{149,150} but is probably due to the shorter duration of NMTi treatment used and different cell-type dependencies on this pathway; senescent cells are particularly sensitive to ER stress due to their high dependence on protein secretion, termed the senescent-associated secretory phenotype¹⁵⁰. Hence, NMTi cytotoxicity in MNA neuroblastoma is primarily due to its other effects and their intersection with the MYCN-defined cell state.

Finally, the NMT substrate profile was profiled in neuroblastoma for the first time in this chapter: in IMR5 cells (**Figure 2.8**) and high and low MYCN SHEP21N cells respectively (**Figure 2.10**). These profiles were highly similar to those previously generated, although five proteins, namely FAM84A, EEPD1, PLGRKT, LZTS1 and PCMTD2 were confirmed as endogenous NMT substrates (that is, without protein overexpression, which may lead to non-physiological *N*-myristoylation) for the first time. Indeed, the native *N*-myristoylated N-terminal peptide for PCMTD2 was recently identified¹¹³, confirming this result. Furthermore, an altered NMT substrate expression profile does not underlie the

heightened sensitivity of IMR5 cells to NMTi, as the expression profile was highly similar to HeLa cells.

Differences in the apparent sensitivity of individual NMT substrates was identified between high and low MYCN SHEP21N cells (**Figure 2.11**). Assuming these substrates are *N*-myristoylated at similar stoichiometries, these differences could arise as a result of differential synthesis or degradation rates. Alternatively, cellular *N*-myristoylation could also be controlled by MYCN-dependent cofactors. In terms of selectively affected biological processes, mitochondrial substrates appeared to be particularly NMTi sensitive in high MYCN cells, whereas Golgi and general signalling proteins were more affected in the low MYCN cells. These experiments therefore provide initial insights into which NMT substrates are most affected by NMT inhibition in the given cell lines, although it should be noted that complete coverage of NMT substrates was not obtained and that the use of imputation introduces a degree of uncertainty on the accuracy of these fold changes. Whether the differences in NMTi sensitivities translates to selective regulation of biological processes depending on MYCN state and phenotypes therefore requires further validation by e.g. microscopy or global transcriptomics or proteomics. The latter two methods are used in the next chapter to investigate global effects of NMTi.

Chapter 3. Global -omic approaches to study NMTi-induced cellular changes in high and low MYCN cells

3.1 Introduction

Previous studies into the downstream effects of NMTi have included a range of global analyses, including transcriptomics, both mRNA-sequencing and total RNA sequencing¹⁵³, global proteomics¹⁴⁹ and phosphoproteomics¹⁵³. These global analyses have primarily been carried out in two main cell lines, BL-41 and HeLa, and have identified important effects of NMTi. However, cell type specific effects have been observed. For example, a main finding in BL-41 cells, defective splicing, was not observed in similar studies in HeLa cells, whereas the UPR was not transcriptionally activated in BL-41 cells. This is congruent with previously published studies identifying cell type specific effects of drugs^{311,312}. As NMTi may have different impacts depending on the pathways present in diverse cell lineages and disease states, it was therefore considered necessary to conduct global profiling analyses in a neuroblastoma background to account for such effects. Furthermore, previous studies have focused on identifying pathways which contribute to cell death in sensitive contexts but have not examined the effect of NMTi in less sensitive contexts, which would help define a rationale for differential sensitivity to NMTi. Therefore, the effects of NMTi in high and low MYCN SHEP21N cells have been assessed in this chapter by mRNA sequencing and global proteomics. These are complementary techniques widely used to characterise drug mode of action, with separate advantages. mRNA sequencing is highly sensitive, being able to profile transcripts corresponding to all protein-coding genes³¹³. However, it is proteins which directly regulate the majority of cellular processes and only a moderate correlation between protein and transcript levels exists, especially when the system is not at steady state^{314,315}. On the other hand, mass spectrometers have been generally limited by both their cost and their sensitivity, with only ~3000-5000 proteins (out of ~20,000 expected) detected per sample run by commonly used protocols and spectrometers^{149,316}. Improvements in instrumentation³¹⁷⁻³²⁰ and data analysis pipelines³²¹ have however recently significantly addressed this limitation, with ~10,000 proteins now identifiable using standard 24-minute methods (reference³¹⁹ and in-house data). Additionally, the effects of NMTi in an *in vivo*, immunocompetent mouse model of MNA neuroblastoma was assessed by global proteomics in this chapter.

3.2 mRNA sequencing analysis of IMP1088 treated SHEP21N cells

3.2.1 Experimental design and workflow

To assay the transcriptomic effects of NMTi in neuroblastoma, SHEP21N cells (high and low MYCN) were treated in quadruplicate with 50 nM IMP1088 for 2, 6 and 18 h (**Figure 3.1**). This concentration of IMP1088 was chosen as it induces cell death selectively in high but not low MYCN SHEP21N cells (**Figure 2.3A**), whereas the timepoints were chosen to give insight into early, intermediate and late effects of NMTi before the onset of cell death, as cellular changes induced by apoptotic programs were otherwise expected to interfere with downstream analyses. RNA was extracted and purified from cells using the Qiagen RNeasy kit and submitted to the Advanced Sequencing Facility (ASF) for library preparation and sequencing. cDNA libraries were prepared by Ashley Fowler and Gavin Kelly from the Bioinformatics and Biostatistics (BABS) team processed the raw reads to provide a raw gene count matrix. This count matrix was processed using DESeq2³²² for differential expression analysis of the conditions of interest, namely with or without NMTi at each timepoint in each MYCN condition (**Appendix Figure 9**). An initial examination of the data using PCA revealed that samples segregated primarily by MYCN status, as expected from a master transcription factor (**Figure 3.2**). The effects of IMP1088 were much smaller in comparison and only clearly separated samples after 18 h of treatment, although a small degree of separation was observed at 6 h.

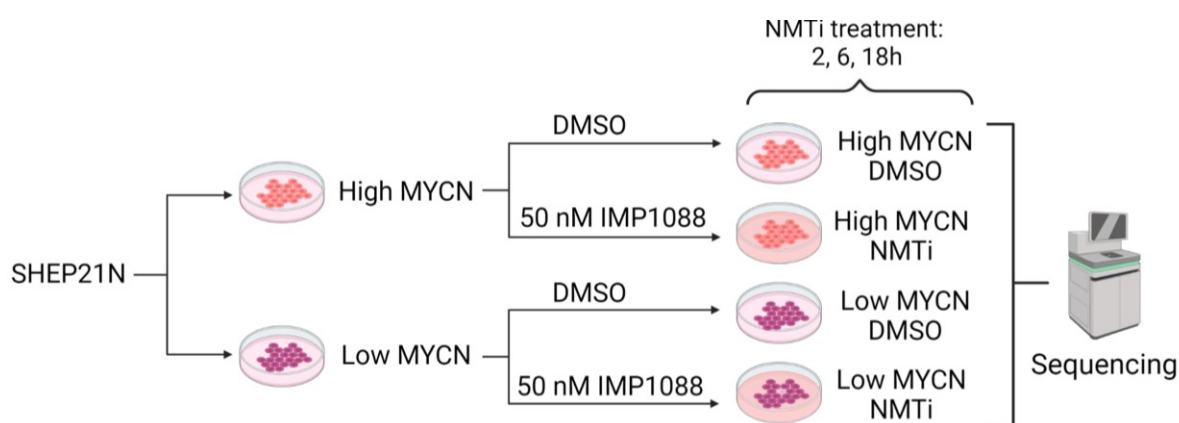


Figure 3.1 Schematic of the sample preparation workflow for mRNA sequencing. SHEP21N cells were first induced into high or low MYCN states, then treated with DMSO or 50 nM IMP1088 for 2, 6, or 18 h. Cellular RNA was extracted, purified, and submitted for library preparation and sequencing by the ASF at the Francis Crick Institute.

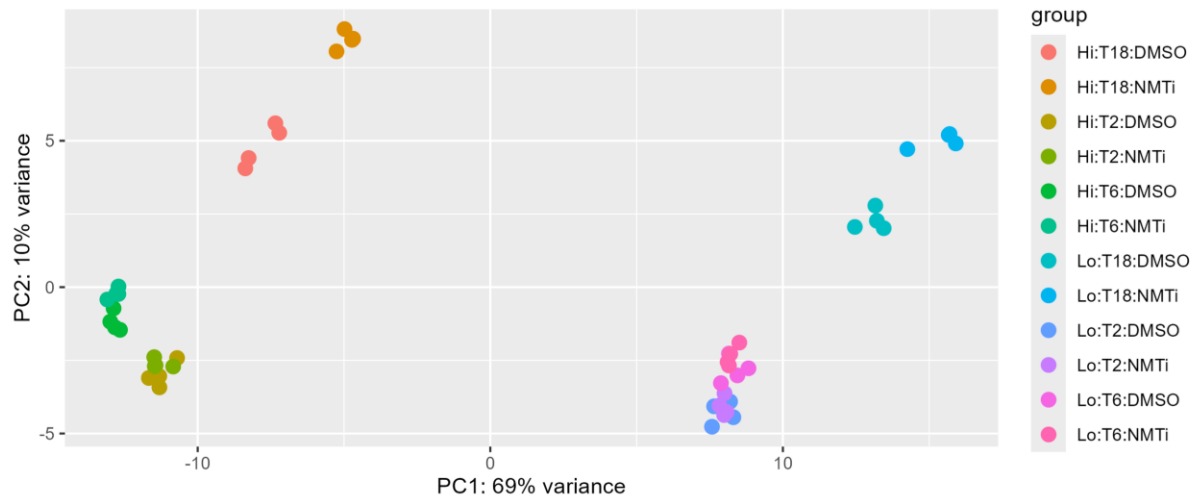


Figure 3.2 Time-dependent separation of SHEP21N cells treated with IMP1088. PCA plot for the samples submitted for mRNA sequencing. Samples predominantly separate by MYCN state. Sample separation due to IMP1088 increases with incubation time. Hi: High MYCN, Lo: Low MYCN, T: Time

3.2.2 Effects of MYCN on the transcriptome

The effects of MYCN depletion by doxycycline was first examined by comparing the DMSO-treated samples at each timepoint. As similar results were observed for each timepoint (**Appendix Figure 10**), only the results of the 2 h timepoint will be presented here. As expected, MYCN transcripts were highly (almost 5-fold) depleted in doxycycline-treated samples, and more generally many genes were significantly and highly differentially expressed (adj. p-value<0.05, $|\log_2(\text{FC})|>1$). The biological relevance of these changes was assessed using gene set enrichment analysis (GSEA)³²³ against the Hallmark and KEGG_Legacy canonical pathway gene sets²⁰³ (**Figure 3.3**). As expected, pathways most significantly enriched in high MYCN cells were those already associated with MYC or MYCN expression, including in a previous analysis of SHEP21N cells^{155,276,324}. These included upregulation of the cell cycle, MYC targets, transcription, OxPhos, the UPR and mTORC1 signalling.

3.2.3 Effects of NMTi on the transcriptome

3.2.3.1 NMTi cause minimal transcriptomic effects after 2 h of treatment

The PCA indicated that the effects of NMTi on the transcriptome were relatively modest (in comparison to the effect of MYCN) and time-dependent (**Figure 3.2**). Accordingly, the

Global -omic approaches to study NMTi-induced cellular changes in high and low MYCN cells

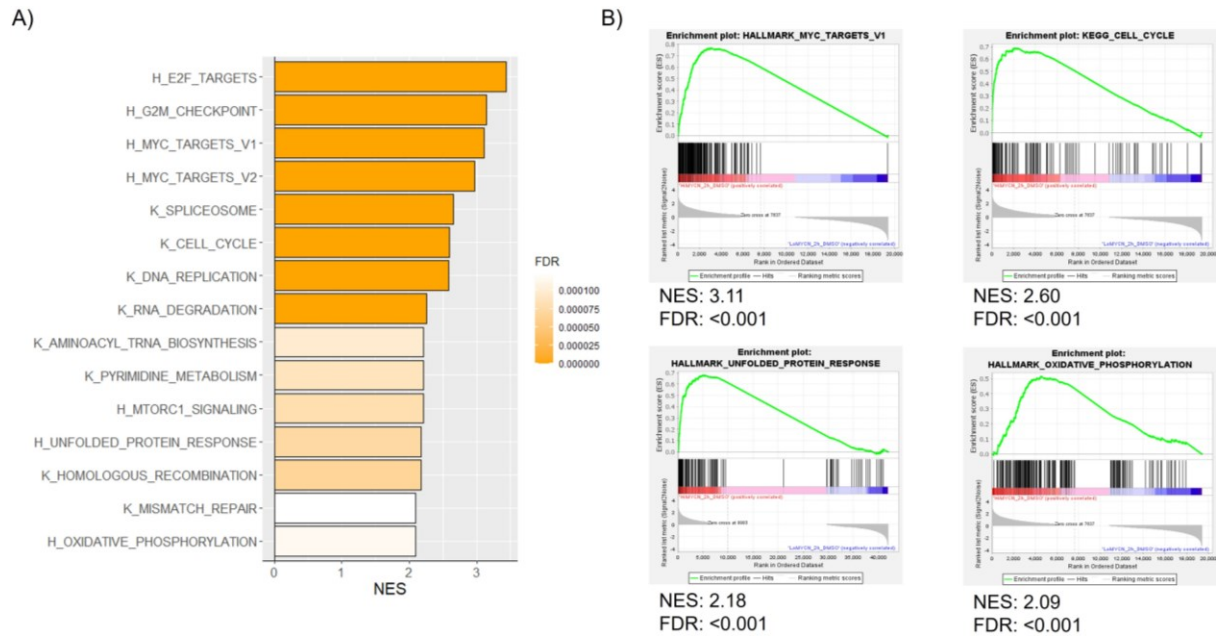


Figure 3.3 Impact of MYCN on the transcriptome. (A) The top 15 pathways enriched in high MYCN over low MYCN SHEP21N cells by GSEA (2 h timepoint results). (B) GSEA enrichment profiles for selected gene sets enriched in (A). NES: Normalised Enrichment Score, FDR: false detection rate, K: KEGG, H: Hallmark.

number of significantly affected transcripts and the magnitude of the fold changes increased over time (**Table 3.1**). There was a bias towards downregulation of transcripts upon NMTi, consistent with previous studies¹⁵³, and the effects of NMTi were generally slightly larger in high MYCN cells. Of note, 2 h treatment with IMP1088 caused extremely few significant transcriptomic effects. This was particularly the case in low MYCN cells, where apart from one protein (KLF10, $\log_2FC = 0.31$), the $|\log_2FC|$ values were all less than 0.01 (**Figure 3.4A**, **Appendix Figure 9B**); in high MYCN cells, only two proteins (FOS/EGR1, $\log_2FC$ s of -0.91 and -0.68) had a $|\log_2FC|$ greater than 0.11 (**Figure 3.4B**).

	High MYCN			Low MYCN		
	2h	6h	18h	2h	6h	18h
#Significant genes	108	165	4926	12	97	4073
#Genes with $FC < -1.5$	2	9	143	0	4	125
#Genes with $FC > 1.5$	0	1	44	0	0	74
FC range	-0.91-0.09	-1.59-0.62	-5.85-1.16	-0.00-0.31	-1.25-0.56	-1.53-1.66

Table 3.1 Time-dependent effects of IMP1088. Summary statistics for transcripts affected by IMP1088 treatment in SHEP21N cells.

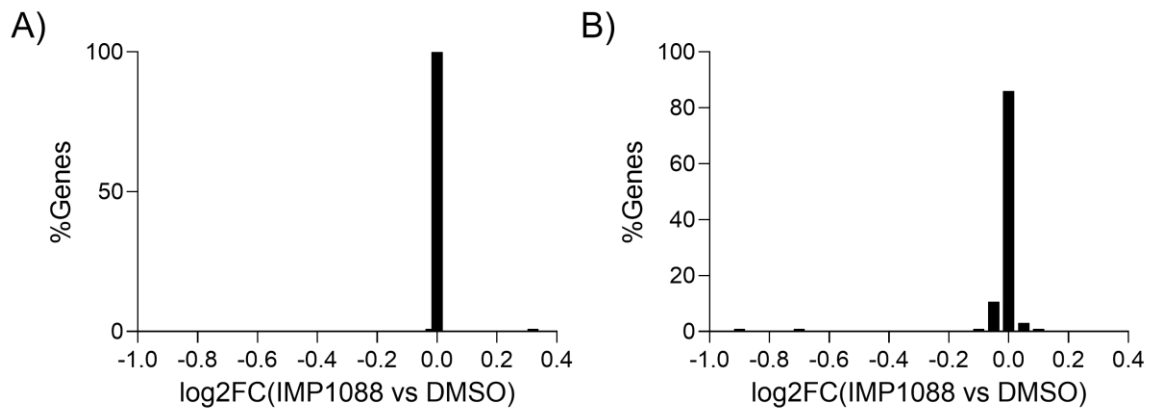


Figure 3.4 IMP1088 does not cause major transcriptomic effects at the 2 h timepoint. Histograms of transcript fold changes in low **(A)** and high **(B)** MYCN SHEP21N cells at the 2 h timepoint.

Therefore, it was concluded that 2 h treatment with NMTi was too short to cause any major, concerted transcriptomic responses, likely because the primary effects of NMTi are on nascently translated proteins, requiring time to filter down to the transcriptome.

3.2.3.2 Assessment of impacted biological processes by GSEA

To obtain further biological insight, GSEA was performed on the 6 and 18 h datasets against the Hallmark and the KEGG gene sets. A particular advantage of GSEA in this case is that this method better considers incremental contributions from multiple genes acting in concert³²³, and therefore is able to identify affected pathways without the need for genes to be highly affected, as is implicit for overrepresentation analysis (ORA) approaches such as GO enrichment.

In high MYCN cells, 6 h of NMTi treatment only significantly (FDR q-value<0.05) affected two gene sets, namely translation initiation (upregulated) and TNF α signalling by NF- κ B (downregulated). Many more gene sets however were significantly affected at 18 h, although again only gene sets relating to translation initiation were upregulated. Downregulated gene sets were related to multiple biological processes, and EnrichmentMap clustering³²⁵ and analysis of these gene sets revealed three main themes **(Figure 3.5A)**:

- Cell cycle and mitosis, in agreement with the observation that NMTi induces cell cycle arrest¹⁴⁹ and prevents proliferation even at lowly cytotoxic doses **(Appendix Figure 1)**

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- DNA replication and repair; downregulation of DNA replication is consistent with cell cycle arrest and therefore reduced flux through S-phase. This may also contribute to the downregulation of DNA repair, as these processes act coordinately, and genes associated with these processes often overlap.
- Microtubule dynamics: This would again be consistent with cell cycle arrest, as microtubules are essential for cytokinesis, being involved in mitotic spindle assembly and cellular contraction³⁰⁶. Microtubules additionally regulate other processes including apoptosis³²⁶.

In addition to these themes, it is also interesting to note that MYC Targets (both V1 and V2) are downregulated upon NMTi, suggesting that NMTi may induce the shutdown of various MYCN-driven pathways, placing the effects of NMTi in direct opposition to the cellular state induced by MYCN and ultimately inducing apoptosis. Also in this context, the robust downregulation of E2F targets is interesting (**Figure 3.5B**), as E2F transcription factors mediate MYC protein-induced cell cycle progression^{212,214} and inhibition of E2F activity has been shown to be lethal in a MYC-overexpressing osteosarcoma model³²⁷.

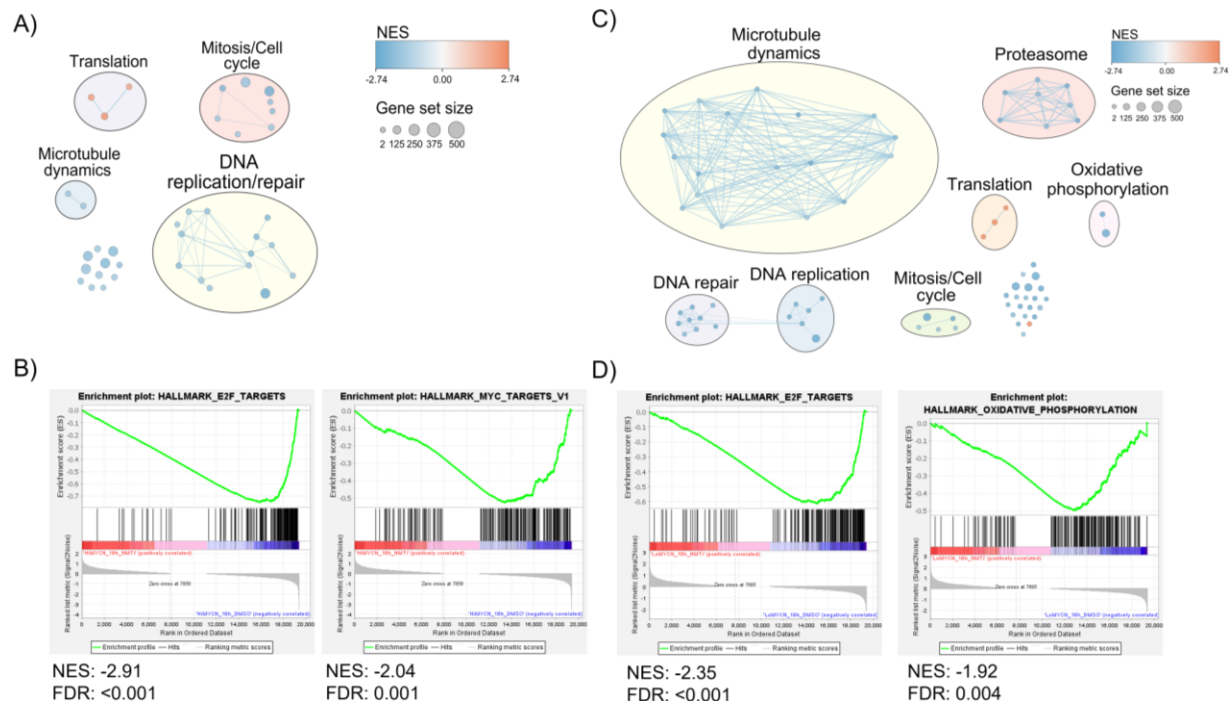


Figure 3.5 Effects of IMP1088 on the transcriptome generally relate to cell cycle arrest. EnrichmentMap analysis of significantly enriched gene sets (FDR<0.05) in high (**A**) and low (**C**) MYCN cells at the 18 h timepoint. Example GSEA enrichment profiles are shown for high (**B**) and low (**D**) MYCN comparisons.

Similar analysis in the low MYCN samples identified no significantly affected pathways at the 6 h timepoint, but at 18 h gene sets related to translation were again upregulated, while cell cycle, DNA replication and repair, and microtubule gene sets were downregulated (**Figure 3.5C**). However, the number of downregulated gene sets relating to microtubule dynamics was much higher in low MYCN cells. This may be a result of the high MYCN but not low MYCN cells undergoing apoptosis-related cytoskeletal rearrangements at this timepoint. Proteasomal and OxPhos gene sets were downregulated selectively in low MYCN cells (**Figure 3.5D**), which could also contribute to the differential phenotypes seen in these cells.

Overall, these results show that major transcriptomic effects only occur at 18 h but not 6 h of NMTi treatment. The transcriptomic response of cells irrespective of MYCN status is predominantly cell cycle arrest, whereas gene sets corresponding to translation (generally ribosomal proteins) are enriched, suggesting additional translational responses to NMTi. The effects of NMTi were highly similar in both cell states, suggesting increased sensitivity in high MYCN cells may primarily be due to their increased reliance on the affected pathways such as E2F or MYCN targets, or perhaps due to their more inherently stressed basal state which predisposes them to apoptosis^{229,276}. However, oxidative phosphorylation and the proteasome were downregulated selectively in low MYCN cells, which could represent pro-survival adaptive responses to NMTi challenge. High MYCN cells may not have the capacity to respond in the same way if these pathways are critical in this context and indeed both were upregulated by MYCN in this cell model (**Figure 3.3**). For example, MNA neuroblastomas predominantly rely on OxPhos over glycolysis in both basal and stressed conditions^{219,224}. In particular, previous analyses of MYCN induction have shown that RC1 is the most broadly upregulated component of the respiratory ETC by MYCN, which was similarly seen here³²⁴. RC1 is also a major site of ROS generation, which when uncontrolled leads to oxidative damage³²⁸. Multiple NMT substrates are mitochondrially located and contribute to OxPhos such as the importers SAMM50 and TOMM40²⁶³, but it is notable that three NMT substrates are directly connected to RC1: the assembly factors NDUFAF4²⁶ and DMAC1²⁹³ and accessory component NDUFB7²⁶⁵. NDUFAF4 is generally required for assembly of functional RC1²⁹³ and notably is the most upregulated RC1-associated transcript upon MYCN induction in this study, while NDUFB7 and DMAC1 proteins were highly affected by NMTi in high MYCN cells as shown in the previous chapter (**Figure 2.11**). These factors combine to give a possible explanation of

how OxPhos downregulation could act as a pro-survival response in low MYCN cells: NMTi may generate dysfunctional or misassembled RC1 due to its effects on *N*-myristoylated RC1-associated proteins, leading to an increase in ROS generation (as has been shown in high-MYC P493-6 cells⁶⁶), oxidative damage and apoptosis. Downregulation of OxPhos would therefore limit the amount of oxidative damage induced. A possible protective role of proteasome downregulation is also possible but considered less likely. This complex is responsible for the majority of cellular protein degradation and is essential in protein homeostasis and quality control³²⁹. As such, proteasomal upregulation generally promotes cancer cell viability and indeed proteasomal inhibitors are clinically approved for the treatment of myeloma and lymphoma³³⁰. However, it is possible that proteasomal downregulation could result in stabilisation of certain pro-survival proteins in the context of NMTi, although no evidence for such a causal relationship exists as present.

3.2.4 Applicability of mRNA sequencing to measure NMTi-induced cellular changes

While the mRNA-Seq data showed some interesting results, its applicability to describe the NMTi-induced changes in cell state was uncertain. As previously mentioned, generally the correlation between mRNA and protein is only moderate, due to the contribution of many regulatory factors, such as translational control (e.g. through miRNA interactions or charged ribosomal availability), post-translational regulation of protein stability or protein buffering³³¹. Yet, proteins are the primary functional molecules in a cell and are therefore closest to the phenotype. Furthermore, primary effects of NMTi are mainly on a co-translational level given the nature of the *N*-myristoylation modification, and this may lower the correlation between protein and transcript levels even further, as has been suggested for other drugs operating on the post-translational level³¹². This is supported by the observation that transcripts of most NMT substrates (87%) were lowly affected (less than 20% change, $|\log_2FC| < 0.26$) by 18 h treatment of NMTi in high MYCN cells, although multiple are expected to be depleted upon NMT inhibition e.g. LAMTOR1¹⁴⁶. An increase in ribosomal protein transcripts upon NMTi also suggested a translational response to NMTi. As a result, global proteomics was also employed to assess the effect of NMTi.

3.3 Global proteomic analysis of IMP1088 treated SHEP21N cells

3.3.1 Effects of NMTi on the global proteome

Samples were prepared in triplicate using the same conditions as in the mRNA sequencing experiment, except only the 18 h timepoint was used, identifying fold changes for ~3600 protein groups after filtering (2 valid values in each group) in each MYCN condition (**Figure 3.6A**) First, the transcript and protein fold changes were compared. This revealed that the transcriptome changes do not accurately reflect protein-level changes, with a Pearson correlation coefficient of only ~0.1 in both MYCN states, shown for the high MYCN state in **Figure 3.6B**. Additionally, NMT substrates known to be destabilised by loss of *N*-myristoylation, such as GNAI2, PRKAB1 and LAMTOR1^{104,146,332} were depleted by NMTi by at least 20% ($|\log_2FC| > 0.26$) on the protein but not the transcript level. Therefore, while the transcriptomics may reflect subsequent adaptations that the cell makes, it does not describe the current cell state. The results of the global proteome experiment were then analysed by ORA using Ingenuity Pathway Analysis (IPA). Distinct from most ORA software, IPA integrates information from both up and downregulated proteins to predict whether a statistically enriched pathway is activated or repressed. This is quantified by a z-score, in which negative values represent repression and positive values, activation. Additionally, IPA enriches against a manually curated database, which increases statistical

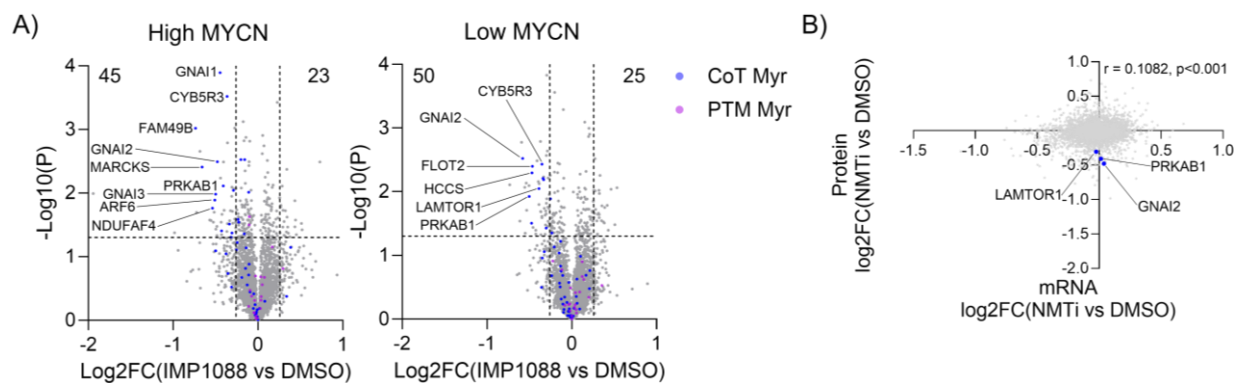


Figure 3.6 Transcript and protein fold changes upon IMP1088 treatment do not correlate. (A) Volcano plots for total protein abundance in IMP1088 treated cells (50 nM, 18 h) compared to DMSO treated cells in high and low MYCN states. The number of significantly differentially expressed proteins ($p\text{-value} < 0.05$, $|\log_2FC| > 0.26$) is indicated. (B) Correlation of protein and transcript fold changes upon IMP1088 treatment in high MYCN SHEP21N cells. The Pearson correlation coefficient is shown.

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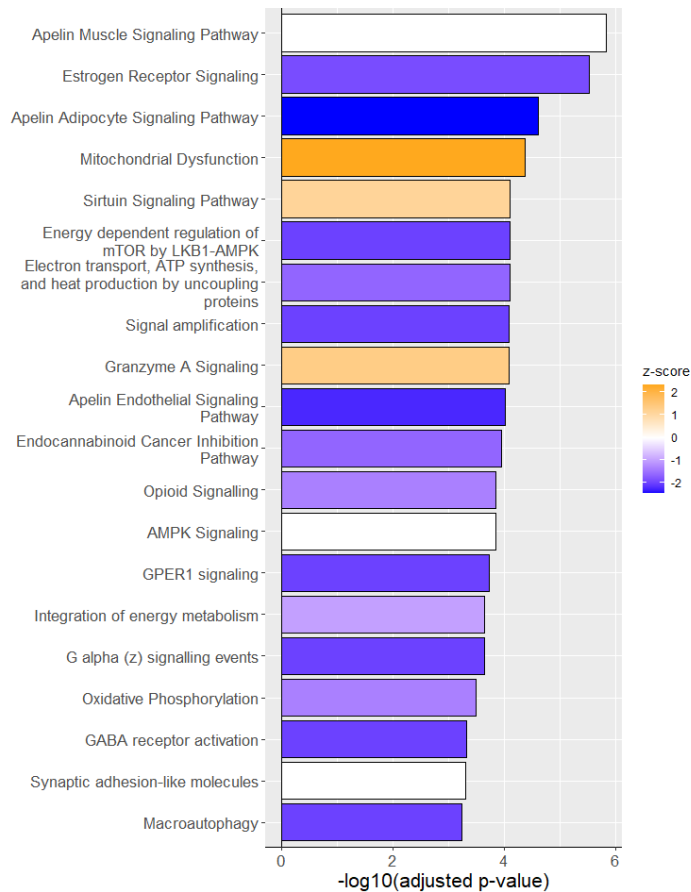


Figure 3.7 Affected Canonical Pathways upon IMP1088 treatment in high MYCN SHEP21N cells include induction of mitochondrial dysfunction. The top 20 significantly (adj. p-value<0.01) affected Canonical Pathways in high MYCN SHEP21N cells. A positive z-score indicates pathway activation upon IMP1088 treatment.

power by reducing the number of redundant gene sets. Proteins with a p-value<0.05 and $|\log_2\text{FC}|>0.26$ were submitted for analysis, yielding 68 proteins (45 downregulated, 23 upregulated) and 75 proteins (50 downregulated, 25 upregulated) for the high and low MYCN states respectively. This fold change threshold was chosen as it was considered that even small but concerted abundance changes in interacting proteins may lead to significant biological effects. Of these, 14 were commonly downregulated in both MYCN conditions, including 10 NMT substrates, reflecting the role of the myristoyl group on their stability, and only 1 protein was commonly upregulated.

The results of the IPA analysis in high MYCN cells (**Figure 3.7**) revealed that a range of pathways were affected. In particular, mitochondrial dysfunction was induced upon NMT inhibition. This may be a result of many factors. In this analysis, loss of multiple RC1 proteins was identified (**Appendix Figure 11A**), suggesting loss of ATP generation

through RC1 function, consistent with predicted downregulation of two further OxPhos-related pathways. Also contributing to the mitochondrial dysfunction phenotype were downregulation of MGST3 and CYB5R3, a NMT substrate. Both have antioxidant activities^{333,334}, suggesting oxidative damage. In the context of mitochondrial dysfunction, it is interesting that three major metabolic sensing pathways were identified as significantly affected, suggesting that NMTi induces energetic dysfunction:

Sirtuin signalling: Sirtuins have been shown to regulate ETC function through their NAD⁺ dependent deacetylase activity. For example, SIRT3 deacylation has been reported to increase the activity of respiratory complexes I, II and IV³³⁵. Sirtuin activation may therefore be a compensatory mechanism, although RC1 dysfunction would lead to a decrease in NAD⁺ and may therefore eventually attenuate such a response.

AMPK: AMPK is a major ATP sensor composed of a catalytic α subunit and regulatory β and γ subunits³³⁶. AMP binding activates AMPK and induces its relocalisation from the cytosol to organellar membranes to promote ATP generation and catabolism. AMPK is a master regulator of mitochondria, mediating both mitochondrial biogenesis and mitophagy. Notably, AMPK is *N*-myristoylated on its β -subunit^{26,337,338}. The role of *N*-myristoylation of AMPK is of some debate, however it represses AMPK activity *in vivo* and modulates AMPK localisation³³². Indeed, AMPK-mediated mitophagy has been described to be reliant on recruitment of the protein to mitochondrial membranes by the myristoyl group³³⁹.

mTOR: A master regulator of cell growth, this protein is activated in high ATP conditions and activates an anabolic program, promoting translation, macromolecule synthesis and mitochondrial biogenesis³⁴⁰. The effects of mTOR are often opposite of those of AMPK; for example, ULK1 phosphorylation by mTORC1 prevents its activation by AMPK³⁴¹. Therefore, AMPK and mTOR together regulate cellular metabolism. As mentioned in the introduction, mTORC1 activity is also dependent on its lysosomal localisation, mediated by the NMT substrate LAMTOR1¹⁴⁶.

In addition to the above pathways, other signalling pathways were also downregulated, including the apelin pathway. This pathway is involved in angiogenesis³⁴², which would be consistent with reported anti-angiogenic effects of NMTi³⁴³. However, these results should not be over-interpreted. Only a small number of proteins were submitted for ORA analysis

and close inspection of the proteins responsible for the pathways enriched showed that a small core of proteins with broad function were responsible for most of these results:

- GNAI1-3 (Gai proteins) and their interactor GNB1
- The AMPK subunits PRKAA1 and PRKAB1
- Respiratory complex I associated proteins (NDUFA12, NDUFA9, NDUFAF4, NDUF51 and NDUF57)
- LAMTOR1

These proteins all NMT substrates or close interactors of NMT substrates, which suggests enriched pathways are primary effects of NMTi. However, some pathways were enriched exclusively due to these proteins. For example, GNAI1-3 and GNB1 were the only proteins contributing to the enrichment of the pathways “Signal Amplification”, “GPER1 signalling”, “G alpha (z) signalling events” and “GABA receptor activation”. Many other pathways signal through these proteins and these are likely not statistically enriched in this study due to a technical limitation, namely that smaller gene sets are more likely to be statistically significant. This would increase the number of false negatives identified in the analysis.

Overall, the enrichment analysis on this dataset suggested disruption of Gai signalling and energetic dysfunction. Both effects may contribute to NMTi-mediated cell death in these cells. For example, GPCRs are upregulated in a range of cancers and are validated therapeutic targets, with 35% of all FDA-approved drugs targeting GPCRs³⁴⁴. Interestingly, while therapeutic control of cancer by targeting a single GPCR is challenging, pertussis toxin reduces tumour growth in breast cancer cell lines presumably through the global ablation of Gai/o signalling and simultaneous inhibition of multiple GPCRs³⁴⁵; similar effects may occur upon loss of Gai protein *N*-myristoylation. Additionally, mitochondrial dysfunction may contribute to NMTi-induced cell death in MNA neuroblastoma cells in a similar way as in MYC-deregulated lymphoma cells⁶⁶.

When the same analysis was applied to the low MYCN results, the enriched pathways identified were similar to those in the high MYCN state, including induction of mitochondrial dysfunction and downregulation of mTOR regulation by AMPK, further indicating that NMTi causes generally similar global effects regardless of MYCN status (**Figure 3.8, Appendix Figure 11B**). The extent of regulation of these pathways was generally similar, although consistently more significant in the high MYCN state (**Appendix Figure 12**). One pathway which was more downregulated in low MYCN cells was the Rho GTPase cycle (z-score of

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cellular changes in high and low MYCN cells

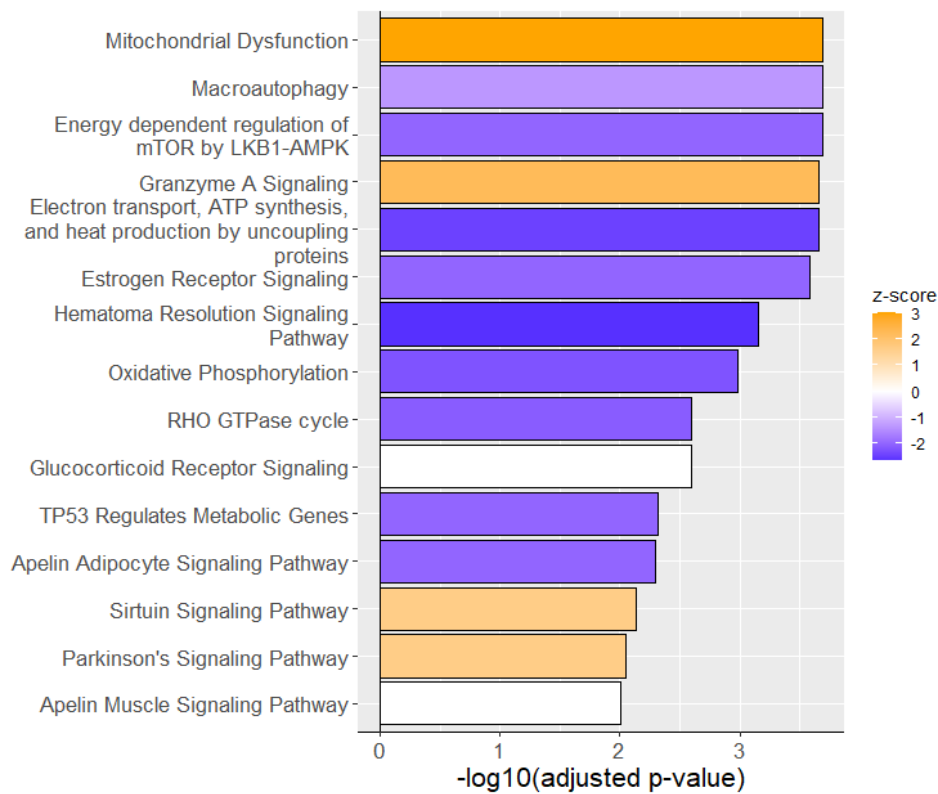


Figure 3.8 Affected Canonical Pathways upon IMP1088 treatment in low MYCN SHEP21N cells. Significantly (adj. p-value<0.01) affected Canonical Pathways in low SHEP21N cells. A positive z-score indicates pathway activation upon IMP1088 treatment.

(-2.1 vs -1.1 in high MYCN cells). These are master regulators of the cytoskeleton and therefore regulate multiple functions including apoptosis³⁴⁶. Notably, a previous study showed that the cytotoxicity of metformin in neuroblastoma cells was mediated in part through activation of the CDC42 and RAC1 GTPases but repression of RHOA GTPase activity³⁴⁷. Therefore, low MYCN cells could protect against apoptosis through the downregulation of CDC42 and RAC1 activity. This would also be consistent with the mRNA sequencing results in which cytoskeletal effects were more depleted in low MYCN cells. GNAI-regulated pathways were also downregulated much more in high MYCN cells. However, the low number of proteins submitted for ORA means that caution should be used to interpret these results, as differences of only one or two proteins in the lists used for ORA can lead to these observed differences.

3.4 Investigating the effects of NMTi in an *in vivo* model of MNA neuroblastoma

3.4.1 NMTi is efficacious in a mouse model of MNA neuroblastoma

Concurrent with the work above, Myricx Bio, a spin out from Imperial College London and the Francis Crick Institute developing NMTi for use in the clinic, were separately collaborating with the Chesler group to test NMTi *in vivo* in a mouse model of MNA neuroblastoma. Initial experiments tested the efficacy of IMP1320, a NMTi from the same chemical class as IMP1088 with similar potency (IMP1320 K_D : 0.09 nM; IMP1088 K_D : 0.10 nM) but improved pharmacokinetic properties⁶⁶, in the immunocompetent Th-MYCN syngeneic mouse model. Drs Evon Poon and Barbara Costa carried out this experiment. The Th-MYCN genetically engineered mouse (GEM) model overexpresses MYCN in the neural crest to spontaneously induce tumours phenotypically similar to clinical human neuroblastoma and is the most widely used transgenic mouse model of neuroblastoma¹⁸¹. Tumour cells from this GEM model are extracted, expanded *in vitro*, and engrafted into

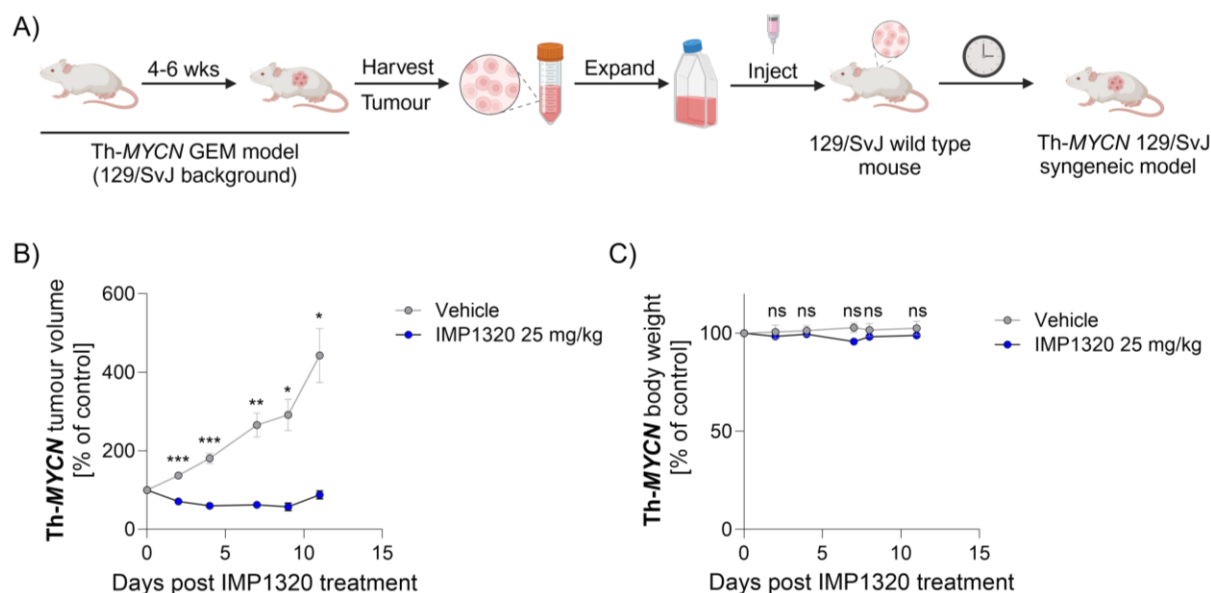


Figure 3.9 The NMTi IMP1320 is efficacious in the Th-MYCN syngeneic mouse model. (A) Generation of the syngeneic Th-MYCN mouse model. (B) Efficacy of intraperitoneally administered IMP1320 in the Th-MYCN syngeneic mouse model. Only timepoints where >50% of vehicle-treated mice are alive are plotted. (C) Body weight of vehicle and IMP1320 treated mice. Significance was determined by mixed-effects analysis. ns: not significant; * $p<0.05$, ** $p<0.01$; *** $p<0.001$

129SvJ mice to develop the syngeneic model (**Figure 3.9A**). Mice were dosed intraperitoneally (i.p.) with IMP1320 (25 mg/kg QD) on a three day on, four day off cycle. NMTi treatment led to robust tumour reduction compared to vehicle treated mice (**Figure 3.9B**), which all reached the humane endpoint and were sacrificed after two weeks of treatment. Further, no significant effect on body weight was observed (**Figure 3.9C**), indicating IMP1320 was tolerable at efficacious doses *in vivo*.

3.4.2 Proteomic profiling of IMP1320-treated TH-MYCN mice

3.4.2.1 Confirmation of IMP1320 target engagement by Sortase A labelling

NMTi are clearly directly cytotoxic to cancer cells, as demonstrated by the *in vitro* cytotoxicity assays from the last chapter and the robust tumour elimination seen in xenograft models of double hit B-cell lymphomas (DLBCL)^{66,142}. However, NMTi can also repolarise macrophages from a M2-like phenotype, which promotes tumour proliferation, to a M1-like phenotype possessing anti-tumour activity³⁴⁸. Macrophage repolarisation, and more generally immune activation, may therefore support NMTi efficacy *in vivo*. To investigate the mechanism of action of NMT inhibition *in vivo*, global proteomics of tumours isolated from Th-MYCN syngeneic mice treated with IMP1320 or vehicle (n=4 per group) was carried out. To maximise the amount of tumour material obtained in IMP1320-treated mice, tumours were harvested after three days of IMP1320 treatment (25 mg/kg, QD, i.p.), at which point tumours had shrunk by 25-50% (**Appendix Figure 13**).

Target engagement was first assayed using the SrtA assay¹¹²; tumours were lysed and proteins ligated to biotin-ALPET-HAA. Western blotting indicated target engagement through the appearance of a higher molecular weight form of ARL1 in IMP1320-treated mice, corresponding to biotin-labelled ARL1, although the bands were low intensity in multiple samples (**Figure 3.10A**). Therefore, target engagement was further assessed by proteomics. Proteins were enriched on dimethylated Neutravidin-agarose beads and digested. Peptides were then labelled with tandem-mass tag (TMT) reagents, fractionated, and submitted for LC-MS/MS analysis. TMT-based proteomics was used for these *in vivo* samples due to the higher sensitivity, precision and coverage (i.e. fewer missing values) obtained by this quantification method compared to the standard label-free quantification (LFQ) method used in the previous *in vitro* analyses²⁹². This is because the TMT tags are isobaric at the MS1 level but generate unique reporter ions depending on the TMT tag

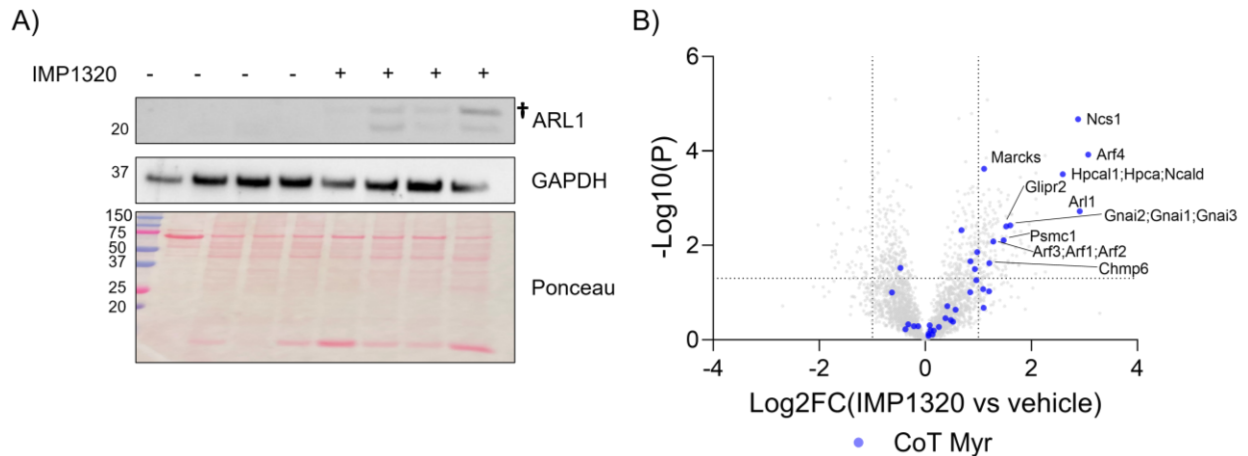


Figure 3.10 SrtA profiling of IMP1320 treated mice confirms target engagement in tumour samples. (A) Western blot for ARL1 in tumour samples from Th-MYCN syngeneic mice. The higher MW form of ARL1 (dagger) corresponds to biotin-labelled ARL1 indicative of NMT target engagement. **(B)** Volcano plot of proteins identified after SrtA labelling and enrichment in tumours from IMP1320 vs vehicle treated mice. CoT *N*-myristoylated proteins are defined as human NMT substrates which also have a N-terminal glycine in the mouse homolog.

used upon peptide fragmentation at the MS2 level, allowing sample multiplexing and simultaneous analysis in a single LC-MS/MS run. PCA analysis indicated that the samples generally clustered well by treatment condition except one outlier in the control sample (**Appendix Figure 14**). However, there was a clear trend of CoT NMT substrates being enriched in the NMTi-treated samples, indicating on-target NMT inhibition (**Figure 3.10B**). For these protein groups, the intensities for all IMP1320 treated mice clustered well, confirming target engagement in all treated mice. While only 10 protein groups corresponding to CoT NMT substrates were significantly enriched by SrtA labelling ($p\text{-value} < 0.05$, $\log_2\text{FC} > 1$, the same thresholds as YnMyr proteomic experiments in the previous chapter which also measures NMT target engagement in a similar but complementary manner), the reported fold changes are underestimates of the true fold change due to protein quantification by TMT isobaric labelling at the MS2 level which introduces ratio compression²⁹². This arises as a result of the low dynamic range of MS2 intensities and co-elution of near isobaric peptides at the MS1 level, increasing the noise-to-signal ratio. Annotation of the identified proteins revealed that highly affected NMT substrates included Arf and Arl proteins as well as neuronal calcium sensors, pointing towards potential disruption of calcium signalling and Golgi vesicular transport. These may not necessarily be the most functionally disrupted NMT substrates, however, as the detection power of SrtA for NMT substrates by this method is relatively low and crucially

requires a free N-terminal glycine. Therefore, NMT substrates which are degraded or alternatively N-terminally processed upon NMT inhibition would not be identified as differentially affected in this analysis¹¹².

3.4.2.2 NMTi induces immune infiltration of tumours in vivo

Having confirmed target engagement, global proteomics was next carried out on the tumour lysates, again using TMT quantification, and significantly differentially expressed

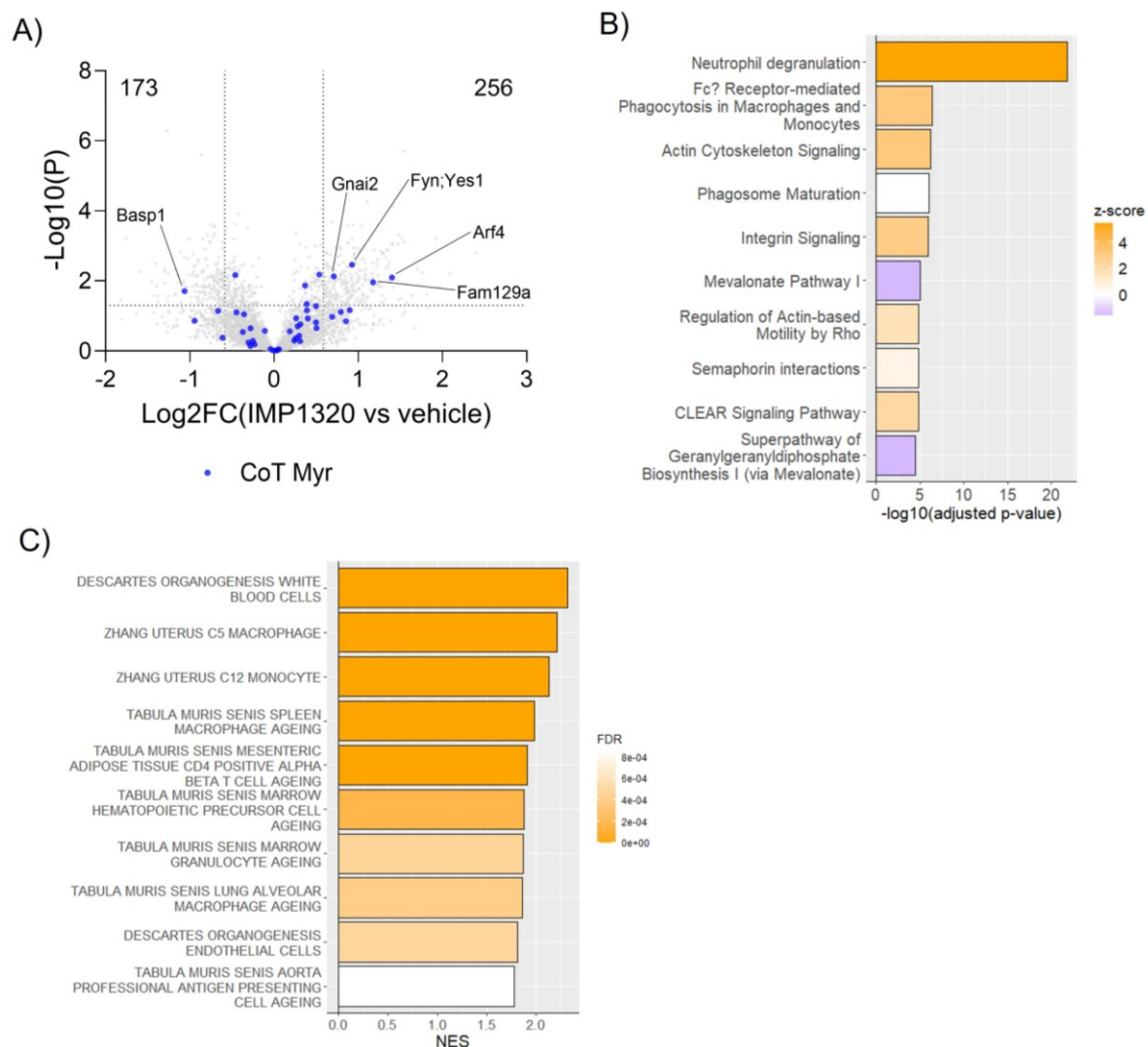


Figure 3.11 Robust immune activation is observed upon IMP1320 treatment. (A) Volcano plot for total protein abundance in tumours from IMP1320 vs vehicle treated mice. The number of proteins with $p < 0.05$ and $|\text{log}_2\text{FC}| < 0.585$ are indicated. **(B)** Significantly (adj. p-value < 0.01) affected Canonical Pathways in Th-MYCN mice. A positive z-score indicates pathway activation in tumours from IMP1320 treated mice. **(C)** Cell types enriched in IMP1320-treated tumours as revealed by GSEA.

proteins ($p\text{-value} < 0.05$, $|\log_2\text{FC}| < 0.585$) were submitted to IPA for analysis. A more stringent fold change cut-off was used here to try and reduce noise in the subsequent analysis due to the more pronounced effects of IMP1320 compared to vehicle, with now hundreds of proteins significantly up- and down-regulated (**Figure 3.11A**). Notably, the strongest effect in the proteomics directly implicated immune infiltration and activation (**Figure 3.11B**). Neutrophil degranulation was by far the most significantly enriched pathway (adj. $p\text{-value} < 10^{-20}$), followed by phagocytosis by macrophages. Additionally, multiple actin-based pathways were activated as well as the CLEAR signalling pathway which promotes lysosomal biogenesis, both of which also support phagocytosis^{306,349}. To further investigate this finding, GSEA was carried out against the proteomics results using mouse cell type signature gene sets curated from single cell studies²⁰³ (**Figure 3.11C**). This analysis also indicated a strong enrichment of immune cells in tumour tissue upon NMT inhibition, including of monocytes,

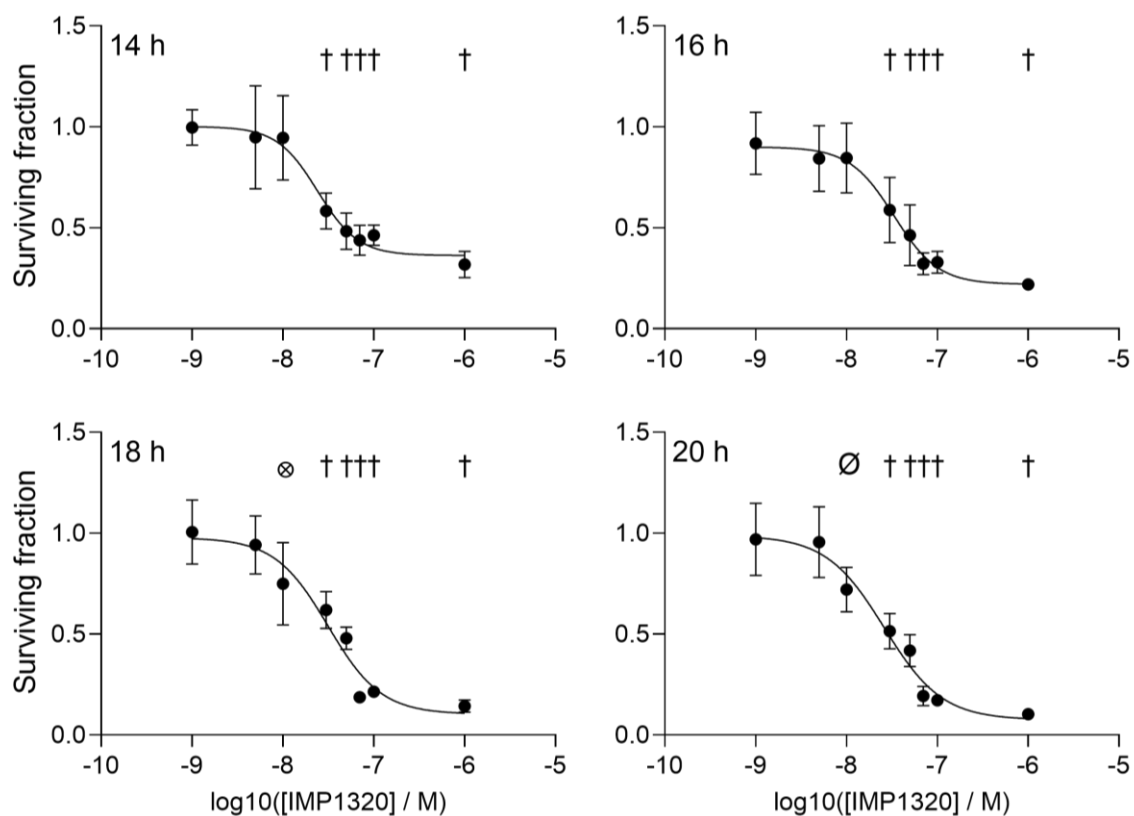


Figure 3.12 IMP1320 is highly effective in Th-MYCN tumour cells *in vitro*. (A) Dose-response curves for IMP1320 in Th-MYCN tumour cells at the indicated timepoints. Concentrations of IMP1320 that induce significant viability loss relative to DMSO control are indicated. ⊗ $p < 0.01$, ∅ $p < 0.001$, † $p < 0.0001$

macrophages, and granulocytes. NMTi may induce immune activation in several ways. First, repolarisation of M2-like tumour associated macrophages (TAMs) to a more M1-like phenotype may induce direct phagocytosis of tumour cells and recruitment other immune cells with an anti-tumour phenotype. Alternatively, NMTi may first directly induce immunogenic cell death in tumour cells. This is a form of PCD in which damage-associated molecular patterns are released, recruiting immune cells to the tumour and activating an adaptive immune response³⁵⁰.

When considering possible tumour-intrinsic effects of IMP1320, it was notable that none of the pathways identified in the SHEP21N *in vitro* study were re-identified. To examine the possibility that this was due to intrinsic differences in response between Th-MYCN and SHEP21N cells, the effects of IMP1320 on Th-MYCN tumour cells *in vitro* was investigated. Cell viability upon IMP1320 treatment was first assessed by the CellTiter-Glo assay (**Figure 3.12**). These cells were highly sensitive to NMTi, with <20% viable cells present after only 18 h treatment with 100 nM IMP1320 and statistically significant cell death observed after only 14 h treatment with higher concentrations of IMP1320. Next, Th-MYCN tumour cells were treated with 10 nM IMP1320 for 14 h *in vitro* and changes in the global proteomic profile measured (**Figure 3.13A**). These conditions were used as 10 nM is the minimum IMP1320 concentration to induce significant cell death at 18 h treatment, but not at 14 h. IPA analysis of the significantly differentially expressed proteins

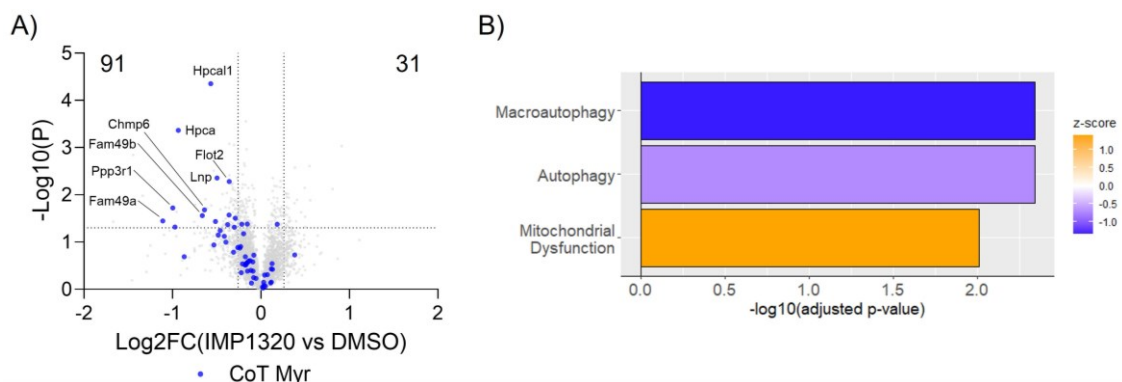


Figure 3.13 IMP1320 induces mitochondrial dysfunction in Th-MYCN tumour cells *in vitro*. (A) Volcano plot for total protein abundance in IMP1320 vs vehicle treated Th-MYCN tumour cells. The number of proteins with $p < 0.05$ and $|FC| > 1.2$ are indicated. (B) Significantly (adj. p-value < 0.01) affected Canonical Pathways in Th-MYCN tumour cells as assessed by IPA analysis. A positive z-score indicates pathway activation in IMP1320 treated mice.

($p < 0.05$, $|\log_2FC| < 0.26$], in line with the previous *in vitro* experiments) identified only three significantly (adj. p -value < 0.01) affected pathways, perhaps due to species-specific differences in protein association for a given pathway. However, those identified represented inhibition of autophagy and induction of mitochondrial dysfunction (**Figure 3.13B**) in line with the SHEP21N study (**Figure 3.7**). Therefore, it is likely that such cell-intrinsic effects were masked by the prominent immune-related signals in the *in vivo* study.

3.5 Chapter conclusions and further work

This chapter reports the results of global -omics analyses carried out on neuroblastoma cells treated with NMTi *in vitro* as well as *in vivo* to elucidate the global biological consequences of NMT inhibition. Initially, mRNA sequencing was carried out in high and low MYCN SHEP21N cells to identify the effects of NMT inhibition. IMP1088 treatment caused generally similar transcriptomic effects in both MYCN states after 18 h treatment, predominantly a cell cycle arrest phenotype (**Figure 3.5**). This suggested that high and low MYCN cells responded similarly to NMTi and that high MYCN cells may therefore be more sensitive as a result of increased reliance on the affected pathways, such as enforced cell cycle progression through E2F transcription factor activity or transcription of canonical MYC targets. Bulk mRNA sequencing only identified limited effects of NMTi after 6 h of treatment, which may be in part due to biological variance within the bulk sample, for example the cells initially being in different cell cycle stages. Single-cell transcriptomics, or synchronisation of cells prior to NMTi treatment, may therefore provide more insights into the effect of NMTi at earlier stages. However, the transcriptomic changes were not representative of the proteomic changes at the protein or the pathway level (**Figure 3.6B**). As translation is not instantaneous, any change on the transcript level will not immediately be reflected on the protein level, giving rise to lower mRNA-protein correlations at any given timepoint³³¹. However, the direct effects of NMT inhibition on the proteome are mediated at the co-translational level. Therefore, it was still considered that effects on the transcriptomic level would not be the most informative proxy to assess the direct phenotypic consequences of NMT inhibition. The effects of NMT inhibition on the global proteome *in vitro* under the conditions of this study are modest, exemplified by the low number of proteins passing the significance and fold change cut-offs implemented for downstream analysis (**Figure 3.6A**), but again indicate that high and low MYCN cells

respond similarly to NMTi, pointing towards mitochondrial dysfunction and loss of energy homeostasis as a general effect in particular. Notably, as impacts on RC1 upon perturbation of cellular *N*-myristoylation by NMTi or NMT1 KO have been noted in prior^{66,149} and recently reported^{152,156} analyses in different cell models, this appears to be a conserved and cell-agnostic effect of NMTi. Furthermore, this would be consistent with the reported high turnover of RC1 proteins³⁵¹, which should selectively sensitise this complex to effects induced by NMT inhibition. The effects of NMT inhibition on OxPhos in a MNA neuroblastoma model are investigated further in the next chapter.

One factor limiting the global proteomics analysis was the confident identification of only a few differentially expressed proteins, and only ~3500 proteins overall. This meant that differences in only one or two proteins in the protein lists submitted for ORA significantly influences the pathways identified and that those identified require targeted validation. As an example, Granzyme A activation was identified, however this is a serine protease released from cytotoxic lymphocytes³⁵². This mechanism would not function in this cell culture model, however it is enriched in this analysis as RC1 proteins are part of this gene set. Future efforts to identify the cellular reprogramming induced by NMT inhibition by global proteomics in neuroblastoma may benefit from multiple different strategies. First, processing of these samples by data-independent analysis on the timsTOF HT instrument would increase the proteome depth to ~10,000 proteins and facilitate the identification of significantly differentially regulated but less abundant proteins which may have biological relevance. Furthermore, the low effect size (protein log₂FC) of NMT inhibition under the conditions used could be addressed by assaying its effects in a cell line panel followed by aggregation analysis, which emphasises proteins which are consistently regulated in the same way in each cell line. This approach has been reported to increase the signal-to-noise ratio of affected proteins and identify known effects of inhibitors which were not detectable in single cell line experiments³¹². To further help MoA deconvolution, the effects of NMTi on the proteome (a proteome fingerprint) could also be compared to those of drugs with known function, with the rationale that drugs that induce similar cellular changes will have similar proteomic fingerprints. Such approaches have been successfully applied on the transcript³⁵³ and protein^{311,312,354,355} level. An initial comparison of the IMP1088-induced proteomic changes in high MYCN SHEP21N cells with an online database of proteomic fingerprints (<http://wren.hms.harvard.edu/DeepCoverMOA/>)

yielded only poor correlations, although this is likely due to differences in study design and cell line models.

Additionally, protein abundance alone is only one part of the picture. Protein localisation and PTMs such as phosphorylation add an extra layer of biological regulation, and it has recently been shown that profiling of PTM changes in a dose-dependent manner gives insight into drug MoA³⁵⁶. Given the relevance of key pathways such as Gai and Src family protein signalling to NMT biology, investigating changes in the phosphoproteome would likely also be illuminative. An initial investigation on the effect of NMTi on protein localisation is conducted in the next chapter.

Finally, this chapter also reports investigations into the effects of NMTi *in vivo* in MNA neuroblastoma models, in which NMTi displays significant efficacy. In particular, global proteomics indicated immune activation upon NMTi treatment, although further experiments to confirm these findings are necessary. These would include confirmation of immune cell infiltration and type by immunohistochemistry or cytometry, comparative assessment of NMTi efficacy in (partially) immunodeficient contexts generated through macrophage or neutrophil depletion or in immunodeficient allograft mouse models, and *in vivo* vaccination assays. While the pathways found in this analysis did not overlap with the *in vitro* results in SHEP21N cells, this is most likely due to the prominence of the immune signal in this study, although the drastic differences in cell growth conditions (cells grown in nutrient-rich media compared to the 3D architecture of a tumour *in vivo*) will undoubtedly also contribute.

Chapter 4. Application of Triton X-114 phase separation to investigate the effects of NMTi at membranes

4.1 Introduction

While global proteomic and transcriptomic profiles have been successfully leveraged to elucidate drug MoA, as mentioned in the previous chapter, protein activity is also controlled on the post-translational level by multiple methods including by PTMs or control of subcellular localisation³⁵⁷. For example, NF- κ B is usually held inactive in the cytosol by interaction with I κ B proteins but translocates to the nucleus upon I κ B phosphorylation, inducing transcription of its target genes³⁵⁸. Such mechanisms are particularly relevant to *N*-myristoylation, given the importance of this modification in dynamic membrane localisation to regulate protein activity, for example in the case of ARF1¹⁰². However, systematic investigation of such effects are not widely applied to drug discovery. The global proteomic analysis in the previous chapter, while identifying some affected pathways, was characterised by a small global impact, limiting the power of subsequent analyses and suggesting that effects on overall protein abundance may not reveal the major effects of NMT inhibition under the conditions used. Further, the baseline expression of a subset of membrane proteins was able to predict sensitivity to NMTi with 90% accuracy in a panel of 245 cancer cell lines (unpublished data). We therefore hypothesized that a key downstream effect of NMT inhibition may be to prevent the localisation of NMT substrates and their interactors at membranes, without necessarily affecting protein abundance, to inhibit their function and induce cellular dysfunction. In this chapter, the effects of NMTi on membrane localised proteins was studied in SHEP21N cells. This revealed loss of RC1 associated with depletion of NDUFAF4. The causal relationship between *N*-myristoylation of NDUFAF4, oxidative phosphorylation and NMTi cytotoxicity in SHEP21N cells was further investigated.

4.2 Proteomic experimental design and initial validation

In order to effectively probe membrane-resident proteins, two key challenges must be addressed. First, their hydrophobic nature promotes protein aggregation, complicating extraction and solubilisation³⁵⁹. Secondly, these proteins are lowly abundant, contributing to their underrepresentation in proteomic datasets. A protocol to selectively enrich for the

Application of Triton X-114 phase separation to investigate the effects of NMTi at membranes

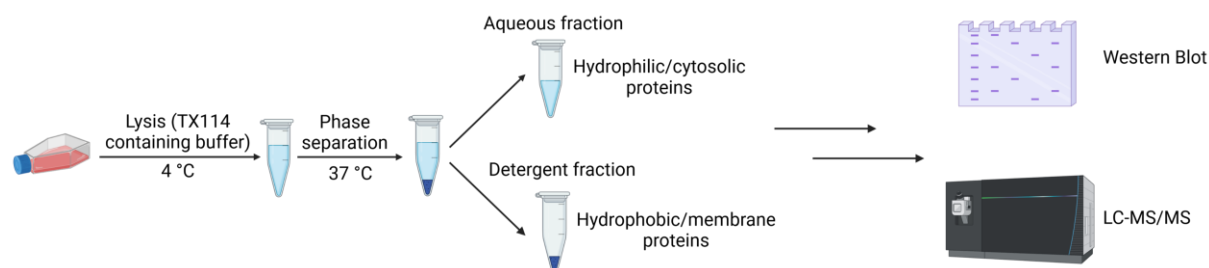


Figure 4.1 Schematic of the TX-114 fractionation protocol. After treatment, cells are first lysed in a homogenous buffer containing 2% pre-condensed TX-114 at 4 °C. Lysates are then phase separated by incubation at 37 °C for 10 minutes to obtain detergent-rich (detergent) and detergent-poor (aqueous) phases respectively, which are consolidated by centrifugation. Hydrophilic (cytosolic) proteins mainly partition to the aqueous fraction and hydrophobic (membrane) proteins to the detergent fraction. Proteins in these fractions can be further analysed by standard techniques.

membrane proteome was therefore required, and enrichment by Triton X-114 (TX-114) phase partitioning was chosen due to its accessibility and proven efficacy in membrane protein enrichment³⁶⁰⁻³⁶⁴ (**Figure 4.1**). TX-114 is a non-ionic, non-denaturing detergent which disrupts lipid-lipid and lipid-protein interactions, but less so protein-protein interactions, by forming mixed lipid/detergent micelles containing detergent-protein interactions. At temperatures below 22 °C, the cloud point of TX-114, these micelles are homogeneously distributed within the solution, whereas above this temperature the solution separates into two distinct phases, a detergent-rich and a detergent-poor phase, due to the formation of micellar aggregates. Partitioning of hydrophobic (generally membrane) proteins in the detergent-rich phase and hydrophilic (generally cytosolic) proteins in the detergent-poor phase therefore allows analysis of these subcellular fractions upon phase isolation and further processing, in this case by LC-MS/MS based proteomics. These phases will be referred to as the aqueous fraction, or the detergent or detergent-enriched membrane fraction, respectively. Given the mildness of the detergent, as well as the low temperatures at which phase partitioning occurs (the cloud point for a similar detergent, Triton X-100, is 65 °C), it was thought that protein-protein interactions could be kept intact using this detergent, which would be an advantage for these experiments.

The fractionation ability of this protocol was first confirmed by western blot for the integral membrane protein Na⁺/K⁺ ATPase, the peripheral membrane protein Rab27a and GAPDH, a cytosolic protein (**Figure 4.2**). The membrane proteins were strongly enriched in the detergent fraction but depleted in the aqueous fraction as compared to lysates

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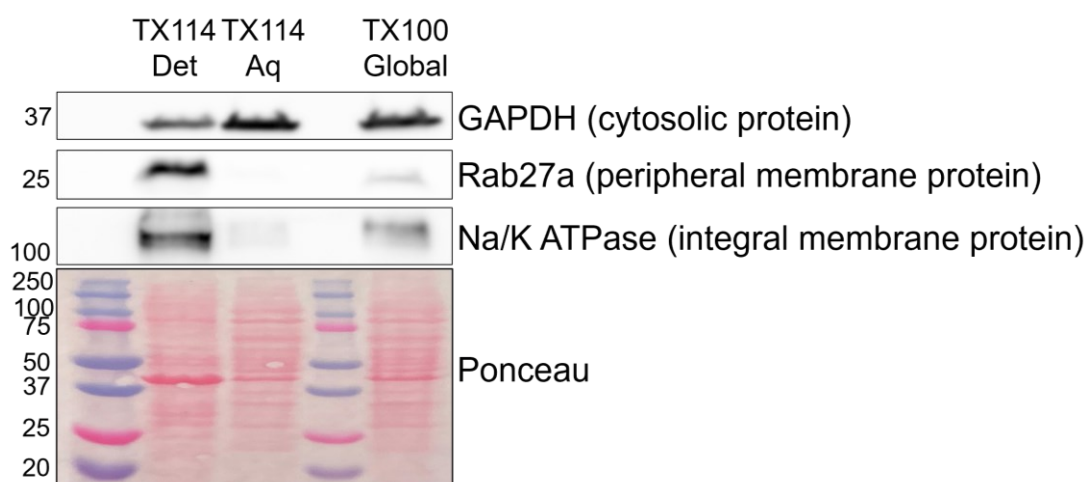


Figure 4.2 TX-114 fractionation successfully enriches for membrane proteins. Western blot for GAPDH, Rab27a and Na⁺/K⁺ ATPase in P493-6 cells in detergent (Det) and aqueous (Aq) fractions or global proteome lysates (global).

obtained using the 0.1% (w/v) SDS and 1% Triton X-100 lysis buffer used to solubilise the global proteome in this thesis (global samples), confirming successful fractionation. GAPDH was depleted in but not excluded from the detergent fraction, although this was not considered a major issue as many proteins, including GAPDH, are transiently associated with the membrane as a result of activated signalling⁴⁴.

4.3 Pilot proteomics experiment

As the fractionation worked well, a pilot proteomics experiment was prepared, in which SHEP21N cells were induced into different MYCN states and treated with 50 nM IMP1088 or DMSO for 18 h, analogous to the global proteomic experiment (**Figure 4.3A**). Aqueous and detergent fractions were extracted and prepared for LC-MS/MS analysis; these were further compared with the unfractionated global proteomic samples presented in the previous chapter. The primary goal of this experiment was to assess the technical suitability of the protocol. The secondary goal was to obtain initial insights into the biological effect of NMTi under these conditions.

4.3.1 The sample preparation workflow is applicable for proteomics

Three assessments were carried out to assess the suitability of this workflow for LC-MS/MS analysis: PCA, coefficient of variation (CV) and contaminant analysis. PCA

Application of Triton X-114 phase separation to investigate the effects of NMTi at membranes

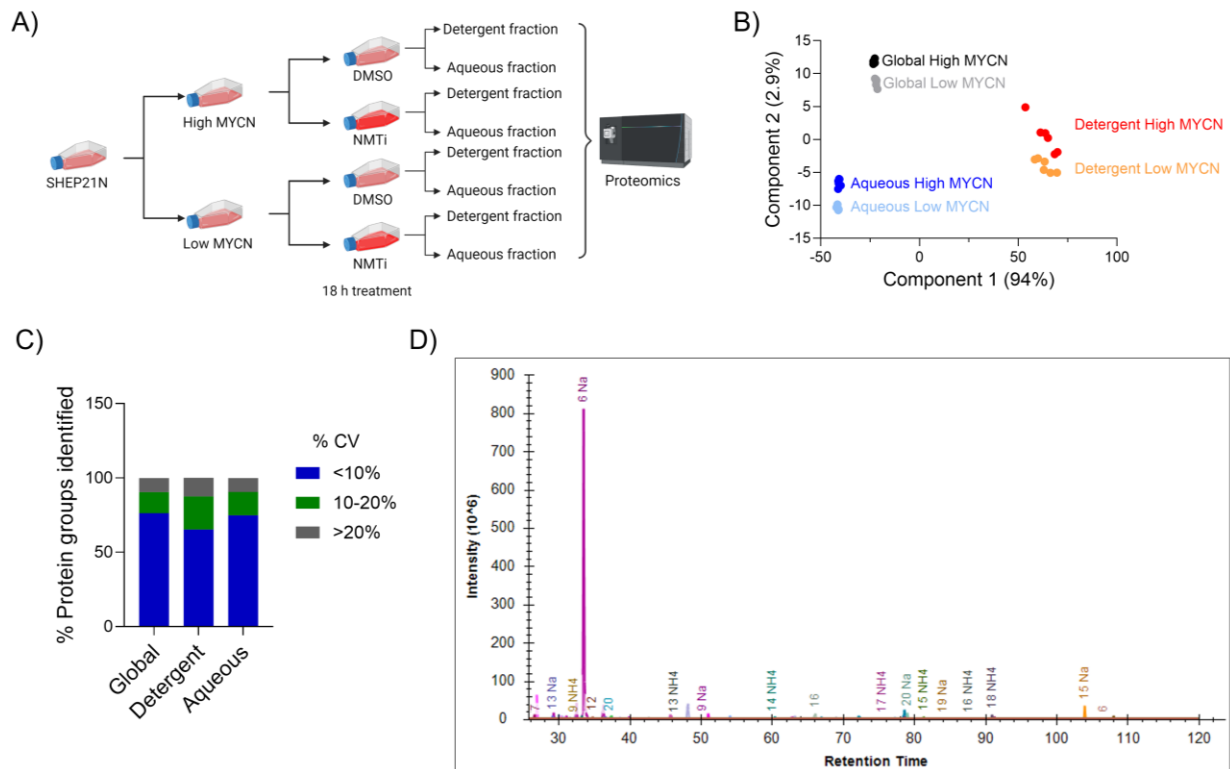


Figure 4.3 A pilot proteomic experiment confirms the suitability of TX-114 fractionation for proteomics. **A)** Schematic of the sample preparation workflow for the pilot proteomic experiment. SHEP21N cells were induced into the respective MYCN states, then treated with 50 nM IMP1088 or DMSO for 18 h. Cells were then lysed in 2% TX114 in PBS and subjected to phase separation. Proteins in the detergent and aqueous phases were then processed for LC-MS/MS analysis. **B)** PCA plot for the pilot proteomic fractions, additionally compared to the global proteomics samples from the previous chapter. Samples cluster primarily by fraction type. **C)** CV analysis of proteins identified in each fraction. **D)** Skyline analysis for Triton contamination in a representative detergent fraction sample. All MS1 signals assigned to Triton contamination are shown.

confirmed high reproducibility and separation of these samples, with sample fraction followed by MYCN status accounting for the majority of separation as expected (**Figure 4.3B**). CV values are also a measure of protocol reproducibility. Although CV values for the log₂ LFQ intensities tended to be higher in the detergent phase, the proportion of proteins with CV<20%, a common threshold for proteomic reproducibility³⁶⁵, is similar across all fraction types indicating general reproducibility (**Figure 4.3C**). Furthermore, the median CVs for all fractions was <10% (**Appendix Figure 15**). Finally, although samples were cleaned up by solvent precipitation SP3 (SP4)³⁶⁶, which efficiently removes detergents, residual TX-114 contamination was a possibility due to the high concentrations

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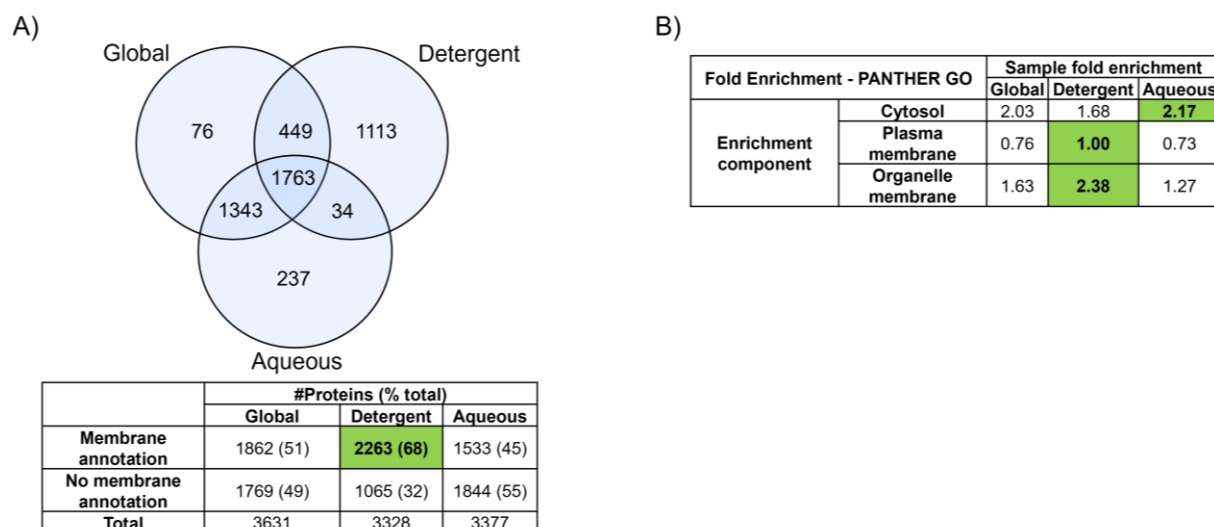


Figure 4.4 TX-114 fractionation globally enriches for membrane associated proteins, particularly those associated with organelles. A) Venn diagram of identified proteins in each fraction. The number and percentage of membrane-associated proteins in each fraction is indicated in the table below. **B)** Pathway enrichment results for identified proteins against the cytosol, plasma membrane and organelle membrane gene sets from the GO Cellular Compartment database. The fractions with the highest enrichment for a given compartment are shaded in green.

used (~12% in the detergent fraction). However, contaminant analysis indicated that no residual TX-114 was present. One high intensity MS1 peak was assigned to TX-114, but this is likely to be a misassigned peak due to its early retention time and lack of a repeating peak envelope characteristic of progressive loss of Triton C₂H₄O subunits³⁶⁷ (**Figure 4.3D**). Therefore, this protocol was considered compatible with LC-MS/MS analysis.

Next, differences in the identified proteins in each fraction were compared. The global samples and aqueous fractions shared the biggest overlap, with >3000 shared protein groups, whereas 1113 protein groups were only found in the detergent fraction (**Figure 4.4A**). Localisation annotation was retrieved for 981 of these 1113 proteins and revealed that 856 were membrane associated, confirming identification of an alternate, membrane-associated proteome. Correspondingly, the total percentage of proteins with membrane annotation was highest in the detergent fractions (68%), compared to 51% in the global sample and 45% in the aqueous sample. GO enrichment (PantherDB)³⁶⁸ further showed that proteins associated with organelle membranes were more enriched by this protocol compared to plasma membrane-associated proteins; this may be due to differences in membrane composition or protein hydrophobicity affecting extraction

(Figure 4.4B). Consistent with *N*-myristoylation promoting membrane association, identification of canonical CoT NMT substrates almost doubled in the detergent fraction compared to the global samples (81 vs 45) but were depleted in the aqueous fraction, where only 34 were identified. Overall, these results show the suitability of this protocol for LC-MS/MS based assessment of membrane-associated proteins.

4.3.2 Initial insights into the effects of NMT inhibition at membranes

The differences upon NMT inhibition in the detergent fractions was next investigated. It is important to note, however, that this enrichment method does not isolate pure intact membranes and therefore is most accurately an assessment of the detergent-enriched proteome. However, these results are still a useful surrogate for a membrane-associated

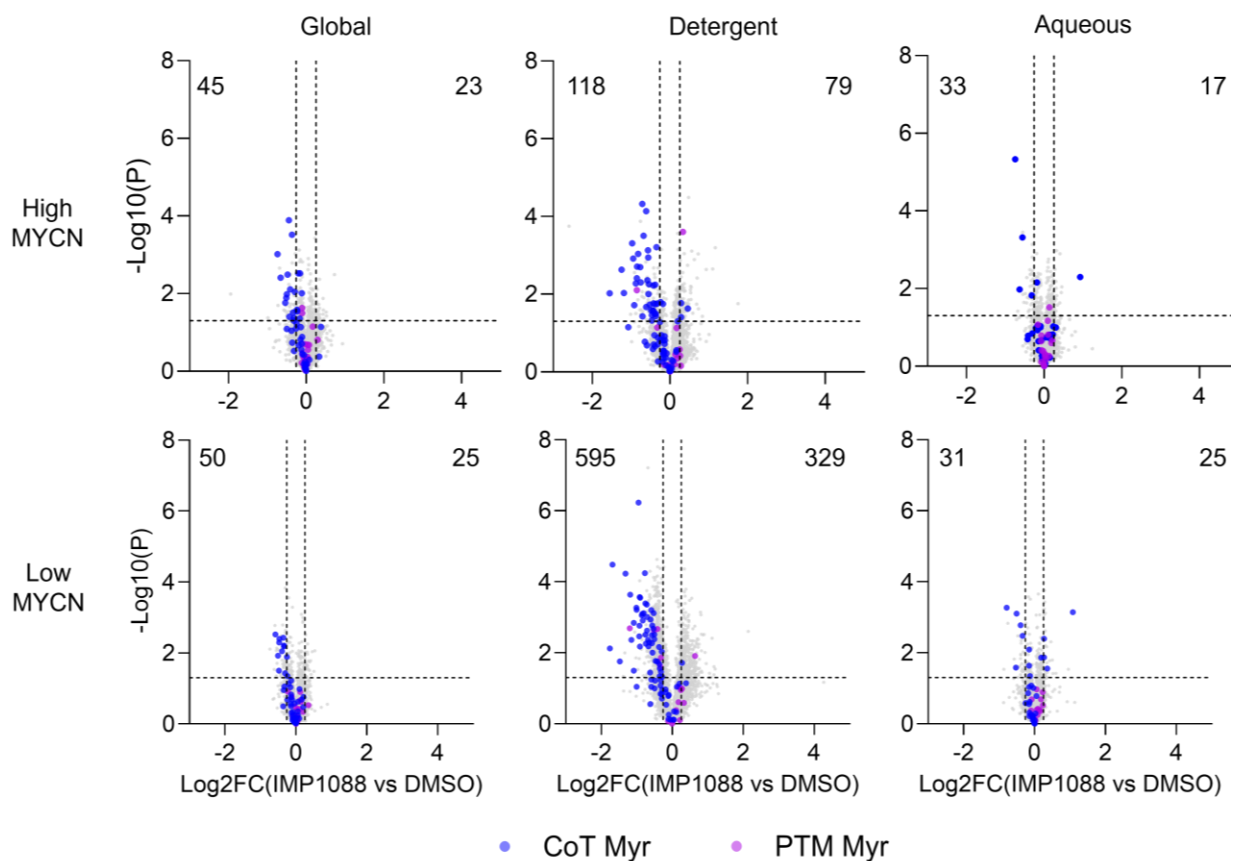


Figure 4.5 Summary volcano plots for the pilot TX-114 fractionation proteomic experiment compared to the global experiment. Volcano plots for NMTi-induced effects in the global samples, detergent fractions, and aqueous fractions. The number of significantly differentially expressed proteins ($p < 0.05$, $|\log_2FC| > 0.26$) is indicated.

proteome, as such proteins are inherently more likely to be membrane localised and as TX-114 enriches for proteins predominantly by disrupting lipid-protein interactions³⁶⁹. Therefore, differential abundance in these analyses may arise from changes in enrichment efficiency, total protein abundance and protein localisation upon NMTi treatment. The predominant effect of NMTi, regardless of fraction, is to deplete CoT NMT substrates. This is most prevalent in the detergent fraction but less so in the aqueous fraction compared to global samples (**Figure 4.5**). On the other hand, post-translational NMT substrates did not show a strong trend of upregulation or downregulation in any fraction, suggesting that the effects of NMT inhibition are mediated primarily through CoT NMT substrates. Proteins were classified as significantly differentially affected using the same thresholds as the global analysis to enable comparison between these two datasets ($p\text{-value} < 0.05$, $|\log_2\text{FC}| > 0.26$), although the minimum biologically significant fold change likely differs between global and subcellular contexts. The number of significantly differentially affected proteins was much higher in the detergent fractions of low MYCN samples than high MYCN samples due to better sample separation by treatment in the former as shown by PCA (**Appendix Figure 16**). While it is possible that this is due to true biological differences in response depending on MYCN condition, variability introduced by sample preparation or instrumentation drift likely also contributes.

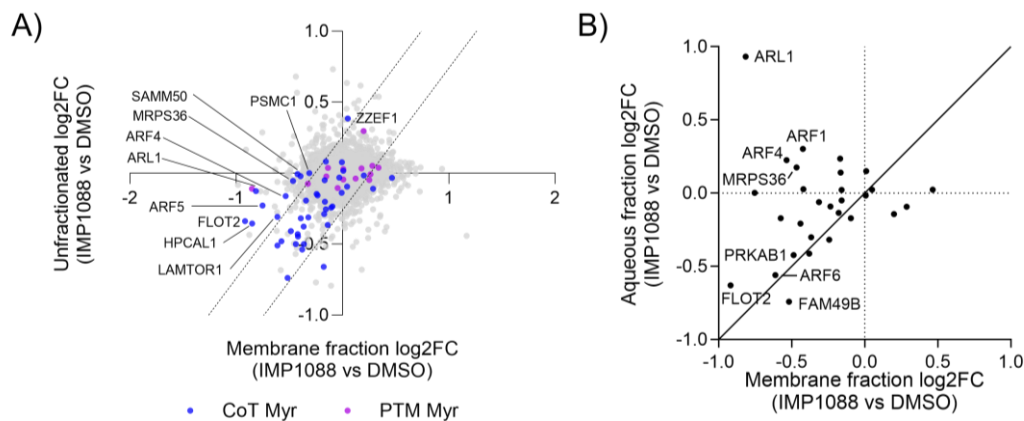


Figure 4.6 Protein abundance changes in detergent fractions reflect both abundance and localisation effects. **A)** Comparison of protein fold changes in global proteome samples (total abundance) against detergent fractions (membrane-associated) in high MYCN cells. Proteins outside the thresholds shown have a $\log_2\text{FC}$ difference greater than 0.26, the threshold for differential expression in single experiments. **B)** Comparison of protein fold changes for NMT substrates in aqueous and detergent fractions in high MYCN cells. Proteins in the bottom left quadrant are depleted in both fractions and proteins in the top left are depleted in the detergent fraction but enriched in the aqueous fraction upon NMT inhibition. A line of origin is shown.

To investigate the extent to which changes in protein localisation contribute to the effects seen in detergent fractions, fold changes upon IMP1088 treatment in the detergent fraction and global samples were compared in the high MYCN samples. Greater fold changes were generally identified in the detergent fraction compared to the global samples (**Appendix Figure 17**). Additionally, the fold changes of 24% of proteins identified in both fractions differed by over 20% ($|\Delta\log_2FC| > 1.2$), the threshold taken as a significant differential fold change (**Figure 4.6A**). This may be due to localisation changes also contributing to the fold changes seen in the detergent fraction for these proteins. This was the case for 13 NMT substrates, the majority of which were more depleted in the detergent fraction than in the global proteome samples. These included PSMC1 and SAMM50, for which it has been demonstrated that loss of *N*-myristoylation primarily affects their localisation but not total abundance^{104,264}.

To further characterise the effects of NMT inhibition on NMT substrate localisation, the fold changes for NMT substrates in the detergent and aqueous fractions were compared (**Figure 4.6B**). Many substrates were depleted in both fractions to some degree indicating overall protein degradation, likely by the glycine N-degron pathway. A smaller subset was depleted in detergent fractions but enriched in aqueous fractions upon NMTi treatment, indicating mislocalisation of non-*N*-myristoylated proteoforms into the cytosol without efficient degradation. This was particularly obvious for ARL1, although ARF1/4 and MRPS36 also behave similarly; these four proteins were also not highly downregulated in the previous global proteomic analysis. Similar results were also found in the low MYCN condition (**Appendix Figure 18**). Therefore, for these proteins, the myristate group appears to be important for protein localisation but not stability.

4.3.3 NMTi affects multiple protein complexes at the membrane

Loss of cellular *N*-myristoylation may inhibit the recruitment of protein complexes to membranes to cause biologically relevant perturbations. In particular, protein clusters which are depleted in membranes and interact with NMT substrates likely mediate primary effects of NMT inhibition. In order to identify these complexes, significantly differently affected proteins (depleted and enriched) in the detergent fraction were separately analysed by STRING PPI analysis, considering only high confidence (STRING score > 0.7) physical interactors³⁷⁰. If the resulting network did not result in defined clusters, data were clustered using the in-built MCL algorithm. Clusters were manually inspected and functions

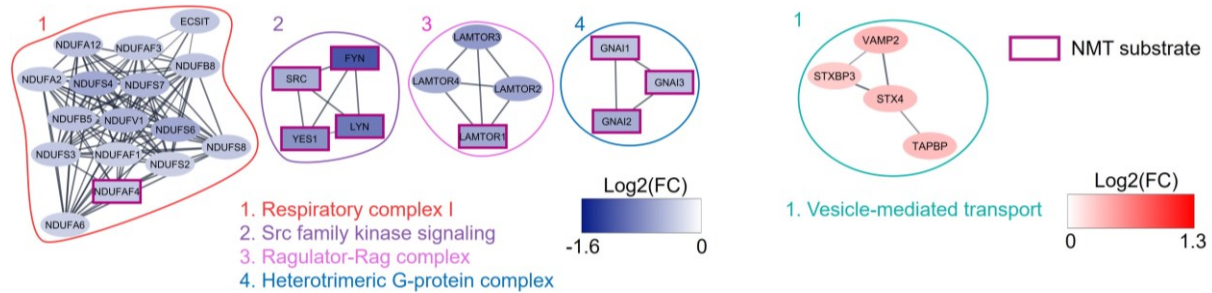


Figure 4.7 The effects of NMTi in detergent fractions in high MYCN cells in the pilot study. Results of STRING analysis for significantly affected ($p < 0.05$, $|\log_2FC| > 0.26$) proteins with negative (left) and positive (right) fold changes in high MYCN detergent-enriched membrane fractions after IMP1088 treatment (50 nM, 18 h). Rectangles with purple outlines: CoT NMT substrates, ellipses: non-*N*-myristoylated interactors.

annotated, using STRING pathway enrichment analysis as a guide where necessary. Application of this analysis workflow for the high MYCN samples (**Figure 4.7**) revealed four clusters which were depleted upon NMTi treatment; these were all associated with NMT substrates. The largest cluster by far were proteins associated with RC1, consistent with the global proteomics results in the previous chapter, and this was again linked to depletion of NDUFAF4. In addition, SFKs were depleted, which as previously mentioned mediate pro-proliferative and pro-survival pathways and whose *N*-myristoylation status have been associated with cancer progression^{77,371}. Also depleted were Gai proteins and components of the Ragulator complex (LAMTOR proteins), consistent with previous studies of NMT biology and the global proteomics experiment^{95,146,147}. Notably, non-NMT substrates were identified in the RC1 and Ragulator clusters, indicating generalised effects on these complexes. Although a lack of such interactors in the Src kinase or Gai signalling clusters was seen, this does not rule out wider effects on these signalling pathways; such interactions may be missing due to weaker or more transient binding, which would be consistent with the signalling functions of these proteins. However, only one cluster was enriched in high MYCN cells, which related to vesicle-mediated transport, particularly of GLUT4. In normal physiology, proteins in this cluster mediate the translocation of GLUT4 to the plasma membrane to promote cellular glucose uptake in response to insulin³⁷². Taken together, these data support the global proteomics analysis suggesting that a major effect of NMTi is energetic dysfunction, likely through direct effects on RC1.

When this analysis was applied to the low MYCN results, more clusters were identified as a direct result of the higher number of proteins used for STRING analysis (**Figure 4.8**). Similar to the high MYCN results, multiple proteins relating to RC1 were depleted upon

NMTi, as were clusters relating to Gai, Src and Ragulator proteins. However, multiple clusters were uniquely affected in low MYCN samples. Vesicle-mediated trafficking was robustly depleted, and indeed the largest affected cluster consisted of SNARE proteins, whose primary function is to mediate vesicle fusion to membranes³⁷³. Other affected clusters relating to vesicular transport included TMED proteins, which function in the early signalling pathway of the ER/Golgi³⁷⁴, Rab proteins, which are implicated in all steps of vesicular transport³⁷⁵ and COPII coat proteins, which mediate anterograde transport from the ER to Golgi³⁷⁶. ARF downregulation perturbs vesicle trafficking in senescent cells¹⁵⁰ and ARF proteins are also depleted in this dataset. However, ARF-regulated vesicular transport does not appear in this analysis despite co-ordinately regulating vesicle transport as they are not high-confidence physical interactors of each other with the exception of ARF1/6. This is a limitation of this study. Apart from these clusters, integrins, which regulate cell adhesion and proliferation, were also depleted; this may be a negative feedback response to Src kinase depletion, which are major mediators of integrin function³⁷¹. Depletion of $\alpha 6$ and $\beta 4$ integrin subunits could also inhibit apoptosis, as the heterodimer $\alpha 6 \beta 4$ promotes apoptosis in wild-type p53 contexts, as is the case here³⁷⁷. Mirroring the global proteomic results, Rho GTPase signalling was also downregulated, and specifically the RAC1 and CDC42 GTPases, again possibly to prevent apoptosis.

Enriched clusters in low MYCN cells upon NMT inhibition also represented several distinct themes. Again, clusters relating to vesicular transport, such as clathrin-mediated transport and the conserved oligomeric Golgi (COG) complex, which functions in retrograde transport at the Golgi³⁷⁸, were identified, in this case probably as a compensatory response to restore homeostasis. Ribosomal proteins, tRNA ligases and initiation factors were also enriched, altogether indicating an increase in translation at the ER membrane. As such an increase was not seen in the global proteomics analysis, this is likely a selectively upregulated ER translational program. ER-associated ribosomes translate membrane, organellar or secreted proteins³⁰⁶ and this is consistent with the hypothesis that NMT inhibition disrupts membrane proteomes. This is also supported by enrichment of proteins involved in ubiquitin-dependent protein catabolism such as E3 ligases and deubiquitinating enzymes, perhaps to support membrane-anchored proteasome function²⁶⁴.

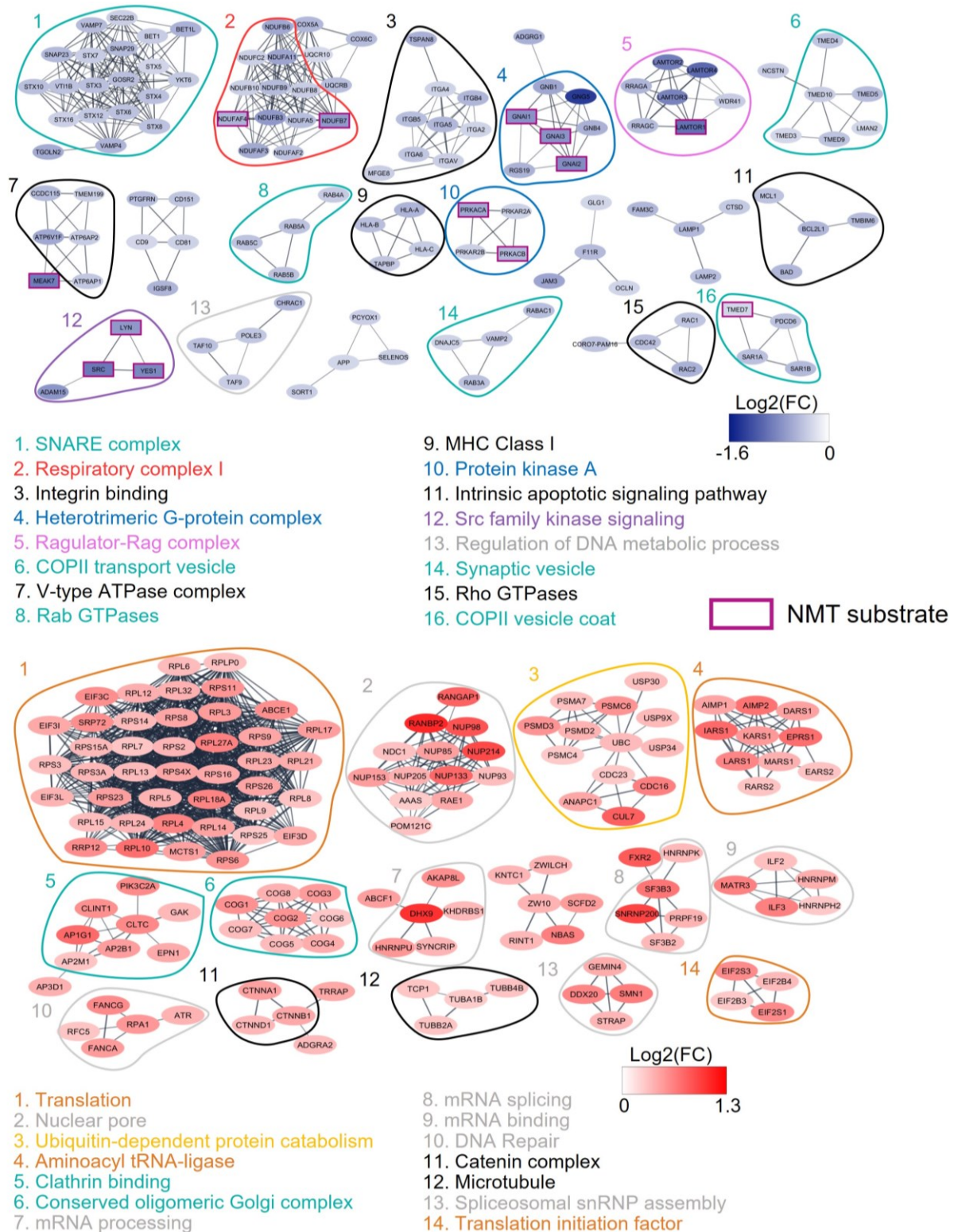


Figure 4.8 The effects of NMTi in detergent fractions in low MYCN cells in the pilot study. Results of STRING analysis for significantly affected ($p < 0.05$, $|\log_2 FC| > 0.26$) proteins with negative (top) and positive (bottom) fold changes in low MYCN detergent-enriched membrane fractions after IMP1088 treatment (50 nM, 18 h). Rectangles with purple outlines: CoT NMT substrates, ellipses: non-*N*-myristoylated interactors.

Overall, this analysis identified common effects on RC1, the Ragulator complex and Src and Gai signalling in the detergent fraction in both high and low MYCN cells; these likely represent primary effects of NMT inhibition. Enrichment of GLUT4 membrane trafficking in high MYCN cells further indicated energetic dysfunction, whereas many more protein interaction networks were affected in low MYCN cells, especially those relating to vesicular transport, protein translation and ubiquitin-dependent protein catabolism. These may be selectively affected in low MYCN cells due to a greater reliance of these cells on the associated pathways or NMT substrates; multiple Golgi proteins were also more sensitive to NMT inhibition in the YnMyr profiling experiment in low MYCN cells (**Figure 2.11**), or as a pro-survival adaptive response. However, as previously mentioned, these extra identifications are also a direct result of identifying more significantly affected proteins in the low MYCN state. To verify these effects, a second biological replicate of detergent-enriched membrane proteomes was prepared, this time as a time-course to obtain insights in a time-resolved manner.

4.4 A time-course study of NMT inhibition in detergent-enriched membrane fractions

4.4.1 Correlation of biological replicates

To prepare the time-course experiment, SHEP21N cells were first induced into the respective MYCN states and then treated with DMSO or 50 nM IMP1088 for 6, 18 or 36 hours. The 36 h timepoint was assayed to investigate NMTi-induced cellular states in both MYCN states at a timepoint which induced cell death only in high MYCN cells. PCA plots showed that for all MYCN and timepoint combinations, samples separated by NMTi treatment status (**Appendix Figure 19**). However, clustering of the 18 h timepoints with the pilot experiment using Pearson coefficients revealed only a low degree of similarity between the two replicates (**Appendix Figure 20**). Samples clustered primarily by replicate, except the low MYCN results in the pilot study which were highly dissimilar to all other samples. This suggests differences introduced during sample preparation contributed to the changes seen in these samples. Therefore, in subsequent analyses, pathways that were either seen in both pilot and time-course data or consistently observed across timepoints were considered as higher confidence effects.

Application of Triton X-114 phase separation to
investigate the effects of NMTi at membranes

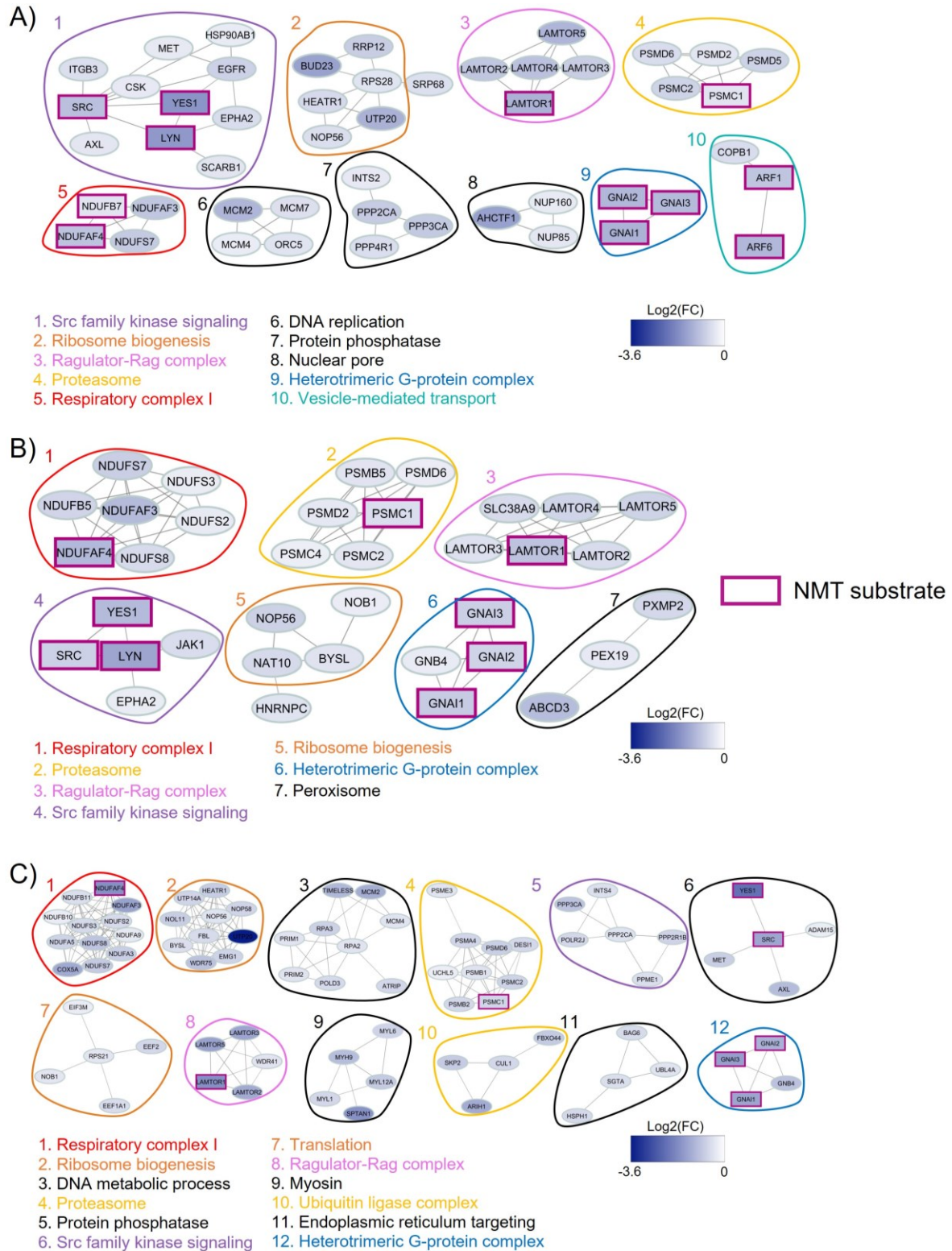


Figure 4.9 Depleted clusters in high MYCN cells in the time course study. Results of STRING analysis for significantly depleted ($p < 0.05$, $\log_2 FC < -0.26$) proteins in detergent-enriched membrane fractions from high MYCN cells after treatment with 50 nM IMP1088 for 6 h (A), 18 h (B) or 36 h (C). Rectangles with purple outlines: CoT NMT substrates, ellipses: non-*N*-myristoylated interactors.

Application of Triton X-114 phase separation to
investigate the effects of NMTi at membranes

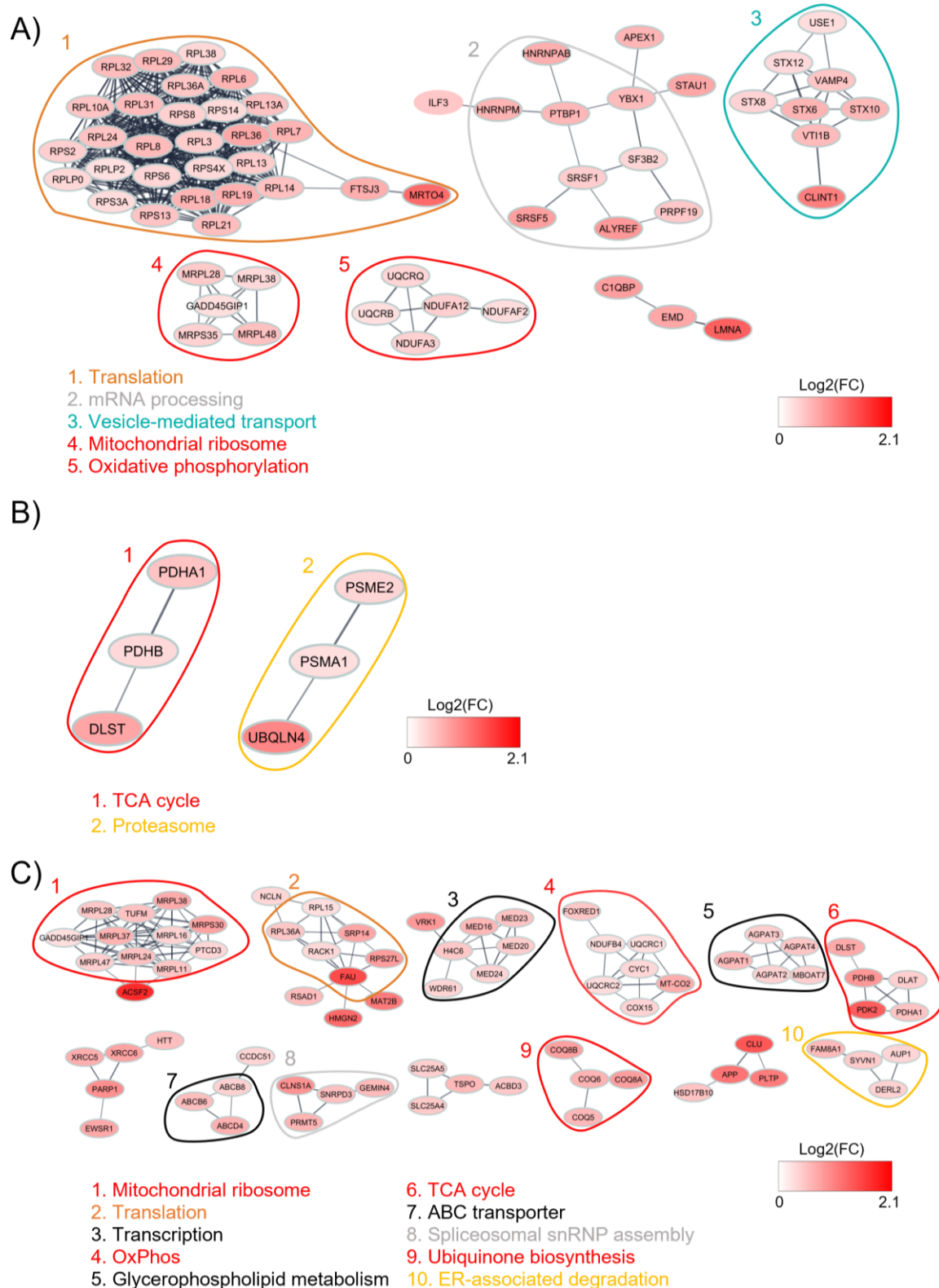


Figure 4.10 Enriched clusters in high MYCN cells in the time course study. Results of STRING analysis for significantly enriched ($p < 0.05$, $\log_2\text{FC} > 0.26$) proteins in detergent-enriched membrane fractions from high MYCN cells after treatment with 50 nM IMP1088 for 6 h (A), 18 h (B) or 36 h (C).

4.4.2 Effects of NMTi in high MYCN cells

Effects were already apparent at 6 h (**Figure 4.9, Figure 4.10**), with multiple clusters involving NMT substrates. All clusters depleted in the pilot study were also depleted here, alongside ARF (vesicle-mediated transport) and proteasomal proteins. While effects on the proteasome were not seen in the pilot study, a subset of proteasomes are anchored at membranes by *N*-myristoylated PSMC1 and the PSMC1 G2A mutant is embryonic lethal in mice, indicating biological importance of this modification²⁶⁴. In addition, nucleolar-localised ribosomal biogenesis (RiBi) and rRNA processing proteins were depleted. This was surprising as nucleolar-localised proteins were depleted in detergent fractions relative to global proteome lysates. In addition to supporting protein synthesis, ribosomal biogenesis is highly energy intensive and depletion may therefore reflect responses to energetic stress³⁷⁹. Clusters enriched in the detergent fraction at this timepoint (**Figure 4.10A**) related to translation, similar to the low MYCN pilot study, oxidative phosphorylation through upregulation of ETC components or the mitochondrial ribosome (mitoribosome) and vesicle-mediated transport.

IMP1088 treatment for 18 and 36 h also depleted similar clusters (**Figure 4.9B, C**) and notably the cluster corresponding to RC1 grew in size at each timepoint, revealing a time-dependent effect of NMTi. However, only two small clusters were enriched at 18 h, which related to the TCA cycle and proteasomal function respectively (**Figure 4.10B**). Increased flux through the TCA cycle may represent a compensatory response upon RC1 dysfunction. Indeed, DLST, which was upregulated here, has been found to support neuroblastoma growth through OxPhos upregulation³⁸⁰. Meanwhile, at 36 h, upregulated clusters were again highly associated with oxidative phosphorylation, with upregulation of mitoribosomal proteins, ETC components and synthesis of ubiquinone, the main respiratory electron shuttle, identified (**Figure 4.10C**).

4.4.3 Effects of NMTi in low MYCN cells

Contrary to high MYCN SHEP21N cells, only a few clusters were depleted upon 6 h of NMT inhibition (**Figure 4.11A**) and only the Src and Gai signalling clusters were associated with NMT substrates. Similar to the high MYCN results, increases in ribosomal proteins was identified alongside clusters related to splicing and tRNA aminoacylation (**Figure 4.12A**). Despite no depletion of a RC1 associated cluster, mitoribosomal proteins

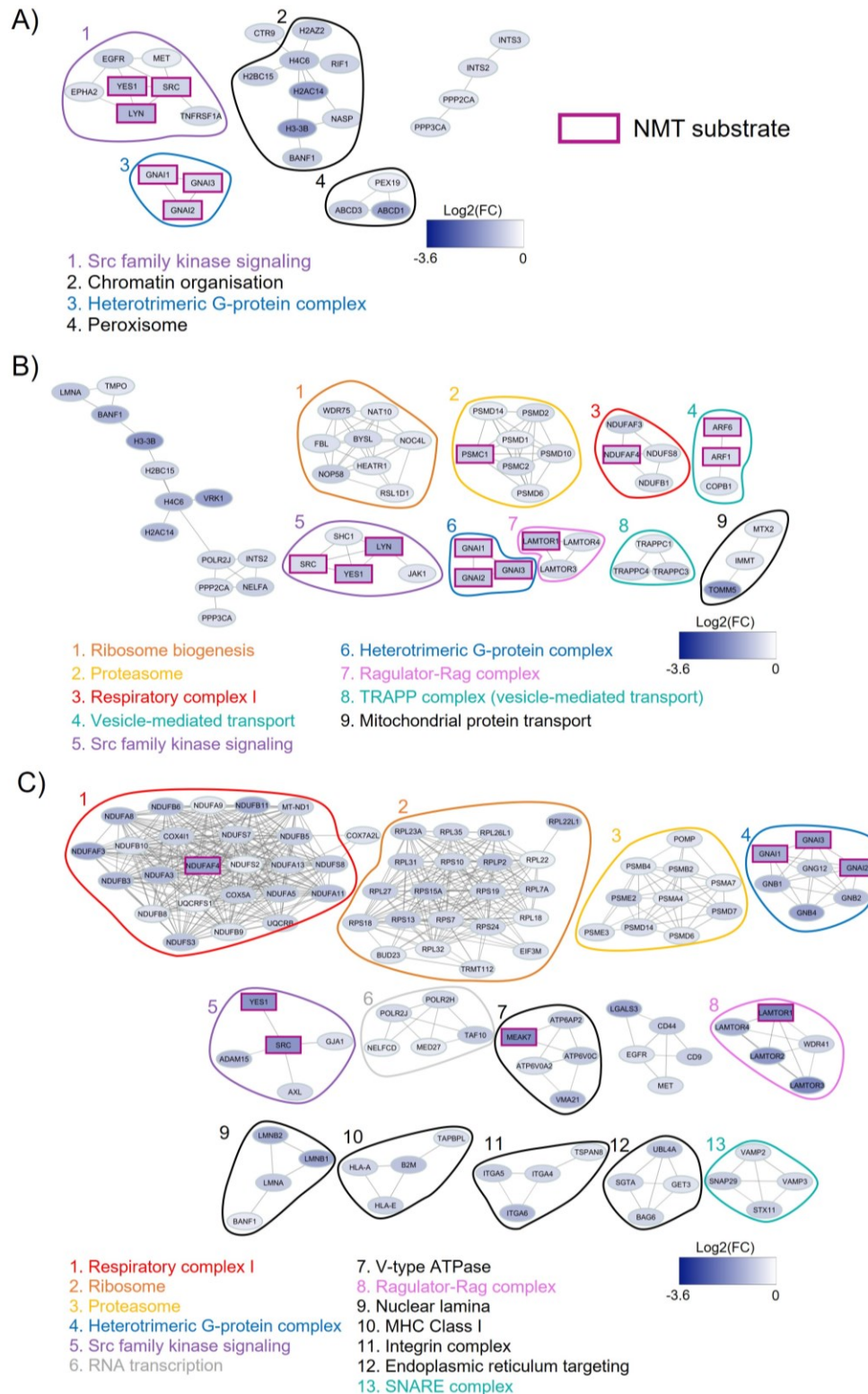


Figure 4.11 Depleted clusters in low MYCN cells in the time course study. Results of STRING analysis for significantly depleted ($p < 0.05$, $\log_2FC < -0.26$) proteins in detergent-enriched membrane fractions from low MYCN cells after treatment with 50 nM IMP1088 for 6 h (A), 18 h (B) or 36 h (C). Rectangles with purple outlines: CoT NMT substrates, ellipses: non-*N*-myristoylated interactors.

Application of Triton X-114 phase separation to
investigate the effects of NMTi at membranes

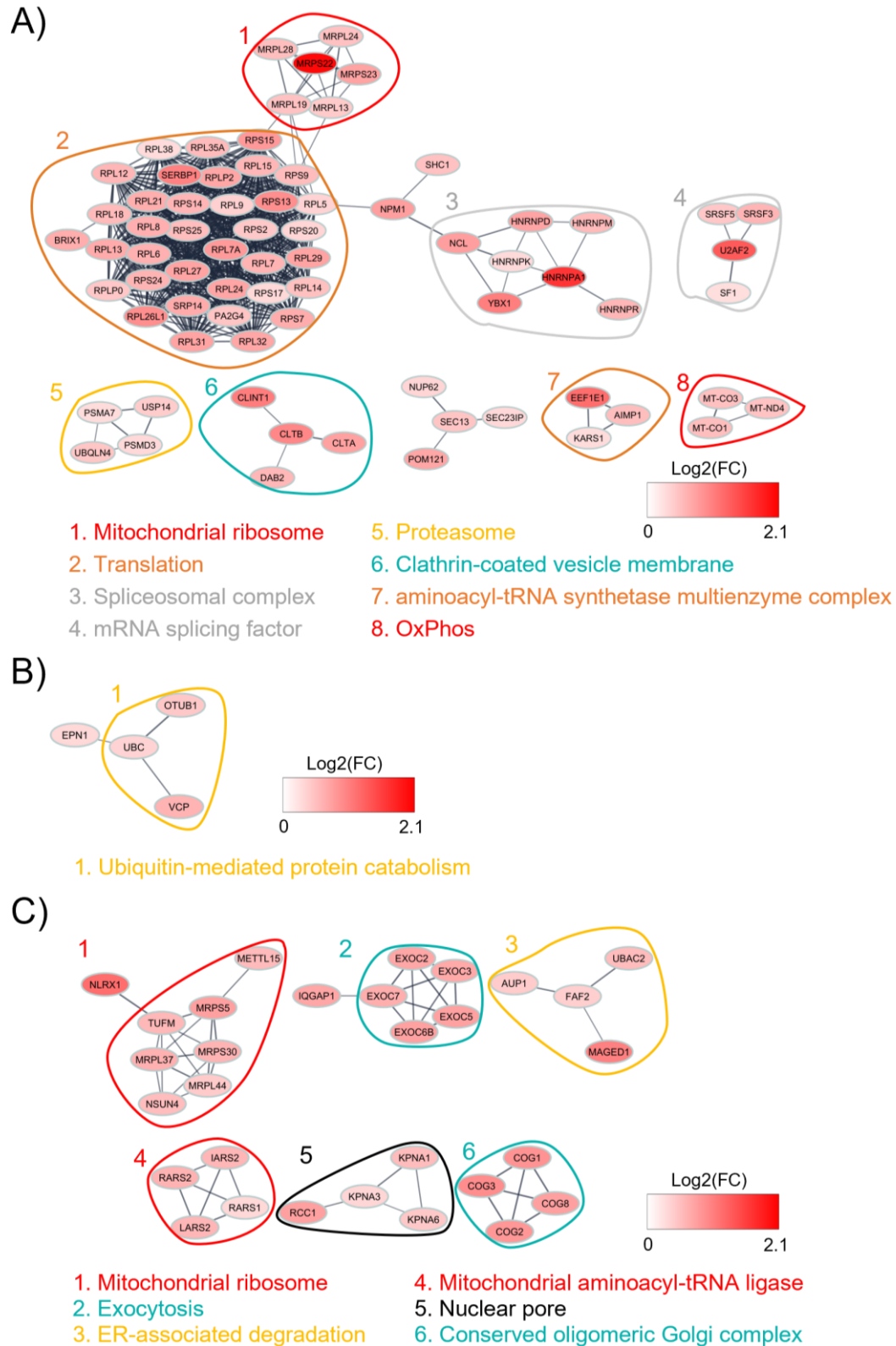


Figure 4.12 Enriched clusters in high MYCN cells in the time course study. Results of STRING analysis for significantly enriched ($p < 0.05$, $\log_2 FC > 0.26$) proteins in detergent-enriched membrane fractions from low MYCN cells after treatment with 50 nM IMP1088 for 6 h (A), 18 h (B) or 36 h (C).

and mitochondrially translated proteins were both enriched at this timepoint. This is therefore potentially a response for general energetic effects of NMTi at this timepoint, although it is notable that the RC1 assembly factor DMAC1 is already over two-fold depleted ($\log_2FC = -1.35$) at this timepoint. Subsequent late-stage misassembly of RC1 without loss of membrane localisation, for example by accumulation of a previously observed 600 kDa intermediate subcomplex²⁹³, may therefore induce mitoribosomal upregulation at this timepoint. While MRPS36, which was previously annotated to be a mitochondrial ribosomal protein, was also highly depleted ($\log_2FC = -0.86$), mitoribosomal upregulation should not be a direct response to its depletion as it does not appear to be a true mitoribosomal protein, instead operating in the TCA cycle³⁸¹. Enrichment of nodes related to clathrin-coated vesicle transport and proteasomal processing were also identified. The clusters depleted after 18 h of NMTi treatment (**Figure 4.11B**) resembled those depleted in high MYCN cells after 6 h treatment (**Figure 4.9A**). This indicates that low MYCN cells generally respond slower, perhaps due to reduced overall protein synthesis rates. Contrary to the pilot experiment, only one cluster was enriched at this timepoint (**Figure 4.12B**), which was again associated with ubiquitin-mediated protein processing. After 36 h of treatment (**Figure 4.11C**), RC1 was now the largest depleted complex, and uniquely a robust depletion in ribosomal proteins was observed, pointing towards a differential translational response in low MYCN cells. Enriched clusters at this timepoint (**Figure 4.12C**) matched previously observed themes, most notably mitochondrial translation, vesicle transport through the upregulation of the COG complex and ER protein homeostasis.

4.4.4 Summary of affected clusters identified

Overall, the time-course in the high MYCN cells revealed several findings. Multiple clusters were depleted by NMTi across all timepoints and were associated with NMT substrates, including those previously identified also in the pilot study, suggesting these are legitimate primary effects of NMT inhibition: Loss of Src and Gai signalling, and the Ragulator and RC1 complexes at the membrane. In addition, loss of proteasomal subunits was identified at all timepoints, associated with the NMT substrate PSMC1. Ribosome biogenesis also appeared to be depleted at all timepoints, suggesting this may also be a significant effect of NMT inhibition, although this requires verification due to its nucleolar localisation. Enriched clusters strongly represented various functions relating to oxidative

phosphorylation, probably as a compensatory mechanism to the depletion of RC1. Protein clusters involved in protein homeostasis were also enriched, indicative of altered membrane proteomes due to mislocalisation or degradation of NMT substrates, or loss of membrane-anchored proteasomes. The effects on OxPhos appear to be particularly prominent, consistent with the results of the whole proteome analysis. A large accumulation of ribosomal proteins was also identified in the detergent fraction at 6 h, suggesting induction of an early, ER-focused translational response.

Similarly, analysis of changes in low MYCN samples revealed that the Src, Gai, Ragulator, RC1 and proteasome clusters were also consistently depleted, although effects on the latter three only began to appear after 18 h of NMTi treatment under the conditions used. On the other hand, enrichment of OxPhos related clusters, as well as ER-associated proteostasis and vesicle-mediated transport across these timepoints suggests cellular compensation and biological relevance of these functions. For example, upregulation of the COG complex at 36 h, which was not seen in high MYCN cells, may contribute to preservation of Golgi function and contribute to cell survival. However, continuity in the specific identified proteins associated with the latter themes in particular was not observed. This may represent differential temporal timescales of activation (and deactivation) although this also requires validation. There is again a major enrichment of ribosomes in the detergent fraction after 6 h of treatment, although they are depleted after 36 h, suggesting adaptation of the translational response not seen in high MYCN cells.

While the above processes have been highlighted by this analysis, it is clear that overall NMT inhibition induces a plethora of simultaneous effects which span multiple important pathways. Indeed, the polypharmacology of NMTi likely underlies their robust anticancer properties. However, the significant effects on OxPhos via RC1 loss observed in the proteomics experiments suggested that this could be a major single contributor to cell death in these cells. If so, the selectivity of NMTi for high MYCN cells may be explained by their greater dependence on this pathway. Indeed, likely compensatory enrichment of OxPhos-related clusters was more prevalent in high MYCN over low MYCN cells upon extended (36 h) treatment with IMP1088. Therefore, the functional relationship between NMTi and OxPhos, and the causal effects of loss of NDUFAF4 *N*-myristoylation was further investigated.

4.5 Validation of the effects of NMTi on OxPhos

4.5.1 NMTi induces oxidative phosphorylation defects in selectively in high MYCN cells

First, the effects of IMP1088 on mitochondrial respiration in SHEP21N cells was functionally validated by use of the Mito Stress Test assay, which measures the oxygen consumption rate (OCR) upon sequential treatment with various respiratory modulators. Changes in the OCR after such treatment allows quantification of multiple respiratory parameters. High and low MYCN SHEP21N cells were therefore pre-treated with 100 nM IMP1088 for 18 h and mitochondrial parameters measured. MYCN expression increased respiratory parameters as expected²¹⁹, however IMP1088 treatment significantly affected respiration only in the high MYCN condition (**Figure 4.13**). In these cells, NMTi significantly

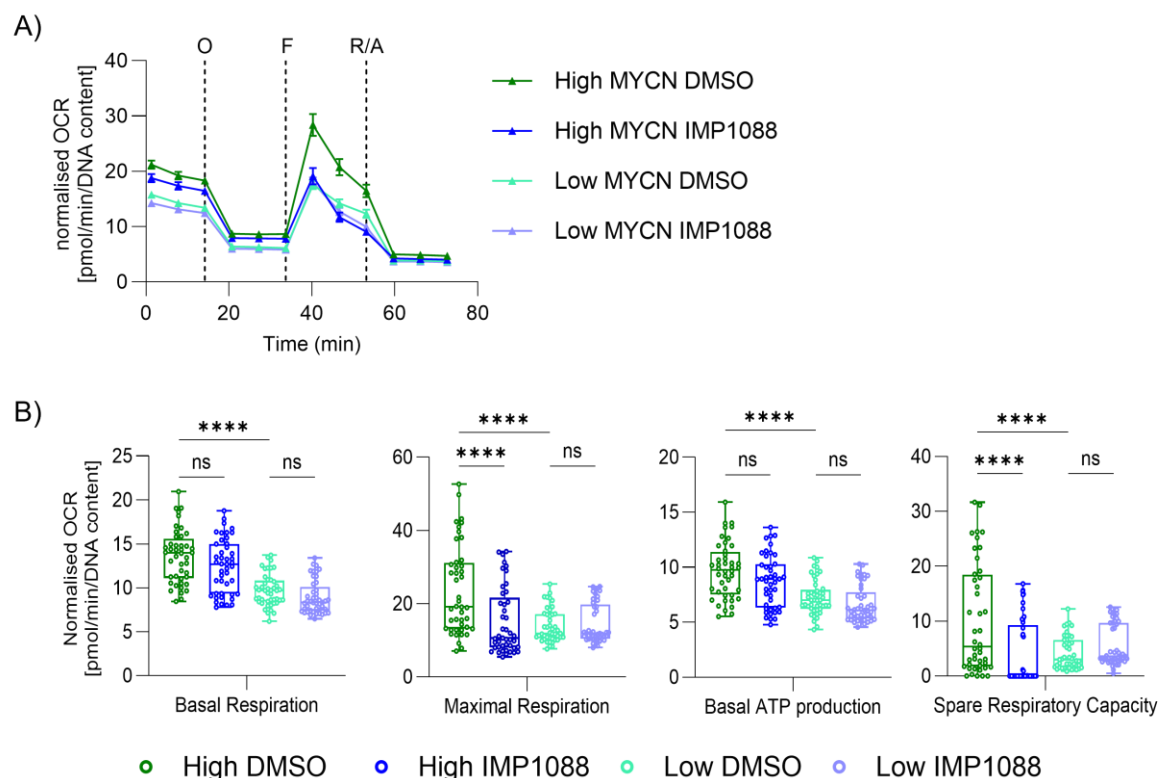


Figure 4.13 18 h treatment with IMP1088 affects mitochondrial respiration only in high MYCN cells. A) Normalised OCR of SHEP21N cells (high and low MYCN) upon treatment with DMSO or IMP1088 (100 nM, 18 h). **B)** Mitochondrial respiratory parameters calculated from **A**. O: oligomycin; F: FCCP, R/A: Rotenone/Antimycin A

lowered the maximal respiration and spare respiratory capacity, which was completely abolished for many of the treated cells. A downshift of basal respiration, although not significant, was also observed. RC1 depletion, as observed in the proteomics data, should significantly lower the basal respiratory rate. The unexpectedly non-significant effects may be due to compensatory increased activity of the other complexes in the respiratory ETC to sustain ATP generation by OxPhos. Similar effects have been seen upon dNDUFAF4 KD in *Drosophila*, which disrupts RC1 activity but post-translationally upregulates respiratory complex II and IV activity³⁸². However, one implication of the often-total loss of spare respiratory capacity is that mitochondrial ATP generation after NMT inhibition may not be able to supply the energetic demands of the cell. As a low, non-significant effect on basal ATP generation was seen, this implies loss of mitochondrial fitness, saturation of the usual cellular mechanisms which activate mitochondrial respiration and an increased energetic demand of the cell as a result of other NMTi-induced effects.

Overall, these results indicate that at the time frame just before cell death, high MYCN cells suffer from reduced respiratory parameters and energetic stress, whereas low MYCN cells do not at this same timepoint. These results are also consistent with the degree of RC1 depletion in membrane fractions at this timepoint.

4.5.2 NMTi induces loss of NDUFAF4 selectively in high MYCN contexts

Next, the effect of IMP1088 on NDUFAF4 was verified. High and low MYCN SHEP21N cells were treated with 100 nM IMP1088 for 18 h, total protein lysates generated and NDUFAF4 levels probed by western blotting (**Figure 4.14**). MYCN expression correlated with a ~3-fold increase in NDUFAF4 levels, as expected from the transcriptomics data, in which MYCN upregulated NDUFAF4 mRNA levels by >2-fold. To confirm a direct effect, publicly available ChIP-Seq data for MYCN in SHEP21N cells was re-examined. This revealed that MYCN binds to the promoter region of NDUFAF4, confirming it as a direct target of MYCN²⁰² (**Appendix Figure 21**). At this timeframe, IMP1088 induced significant loss of total NDUFAF4 in the high but not low MYCN state, consistent with the degrees of depletion of NDUFAF4 in membrane fractions at this timepoint. The loss of NDUFAF4 seen is likely due to proteasomal degradation of the non-*N*-myristoylated proteoform as previously shown⁶⁶ and may not significantly occur in low MYCN cells due to a lower synthesis rate. In total, the above results are consistent with a model in which MYCN increases NDUFAF4 levels to promote aerobic ATP generation. Upon NMT inhibition, loss

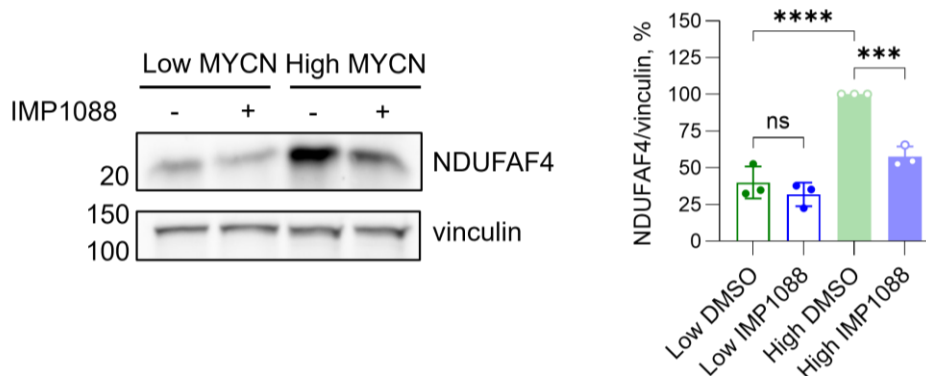


Figure 4.14 18 h treatment with IMP1088 reduces NDUFAF4 levels only in high MYCN cells. Western blot and quantification of total NDUFAF4 in SHEP21N cells (high and low MYCN) treated with IMP1088 (100 nM, 18 h). One representative biological replicate is shown. Three biological replicates were used to quantify NDUFAF4 changes.

of lipidation of newly synthesised NDUFAF4 induces its proteasomal degradation. High MYCN cells therefore cannot meet their energetic requirements and in conjunction with other NMTi-induced effects, die. Low MYCN cells are not affected at the same timepoint perhaps due to lower protein synthesis rates, and also may be less dependent on OxPhos (and other affected pathways), contributing to survival at longer timepoints. Notably, these results are also comparable to those identified in the P493-6 model, indicating at least partially conserved mechanisms across MYC paralogues⁶⁶.

4.6 Investigating the specific role of NDUFAF4 *N*-myristoylation on NMTi-mediated OxPhos disruption and cell death

4.6.1 Development of non-*N*-myristoylatable NDUFAF4 mutants

Having shown that NMTi-induced mitochondrial dysfunction is concomitant with loss of NDUFAF4 and occurs just prior to cell death in high MYC cells, the causal contribution of NDUFAF4 *N*-myristoylation to mitochondrial dysfunction and NMTi cytotoxicity in neuroblastoma was further investigated. Previously, an A3P mutation in NDUFAF4 has been associated with loss of NDUFAF4 expression and RC1 assembly defects to cause Leigh's syndrome and developmental delay in human patients³⁸³. Work in the Tate group further identified that this mutant is not *N*-myristoylated, indicating the importance of this modification alone⁶⁶.

To assess the effects of loss of *N*-myristoylation for endogenous NDUF4F protein in the neuroblastoma context, a CRISPR-Cas9 knock-in approach was used to generate the G2A mutant in SHEP21N cells to maintain model continuity (**Figure 4.15**). In addition, the parental SHEP line has no copy number changes in chromosome 16, where *NDUF4F* is located³⁸⁴, increasing the probability of a homozygous edit. In addition to the G2A mutant, generation of a cell line with the silent NDUF4F G2G mutation was also attempted. This cell line would serve as a control for the NDUF4F mutation but minimise phenotypic differences which may be a result of the CRISPR-Cas9 process. Guide RNAs and donor templates were designed using the ThermoFisher TrueDesign application and cells were transfected as described in the method section. To assess the efficiency of the CRISPR protocol, negative (no sgRNA) and mock (no donor template) controls were also prepared. Amplicon sequencing of the NDUF4F locus revealed that the protocol was successful, with ~2.5% uptake of the desired base edit in the total cell population of both G2A and G2G conditions, whereas edits at this locus were not enriched in the mock controls. Single cell clones were then isolated. Clones were first screened for the presence of the desired modification using PCR, which identified one clone possessing the G2A edit homozygously (**Figure 4.16A**) but no single cell clones with the G2G modification. Subsequently, successful gene editing was confirmed for the NDUF4F G2A candidate

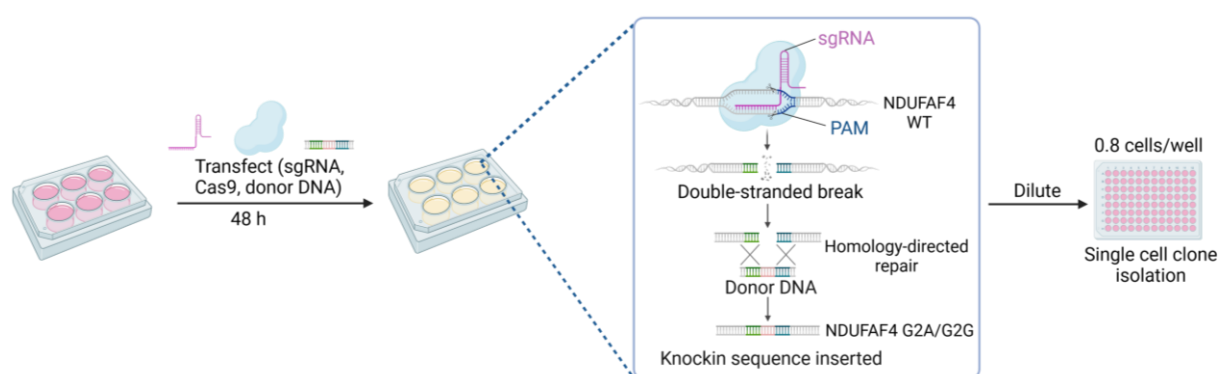


Figure 4.15 Schematic of the CRISPR-Cas9 knock-in approach to generate the NDUF4F G2A and G2G mutants. SHEP21N cells were transfected with sgRNA, Cas9 and donor DNA containing the G2A or G2G mutation template respectively. The sgRNA guides the Cas9 protein to the desired PAM site (here, the start of the NDUF4F coding sequence) allowing it to make a double-stranded break. The donor DNA is then used as a template for homology-directed repair to incorporate the desired mutation. 48 h after transfection, single cell clones were isolated, expanded and screened for the mutation by PCR.

Application of Triton X-114 phase separation to
investigate the effects of NMTi at membranes

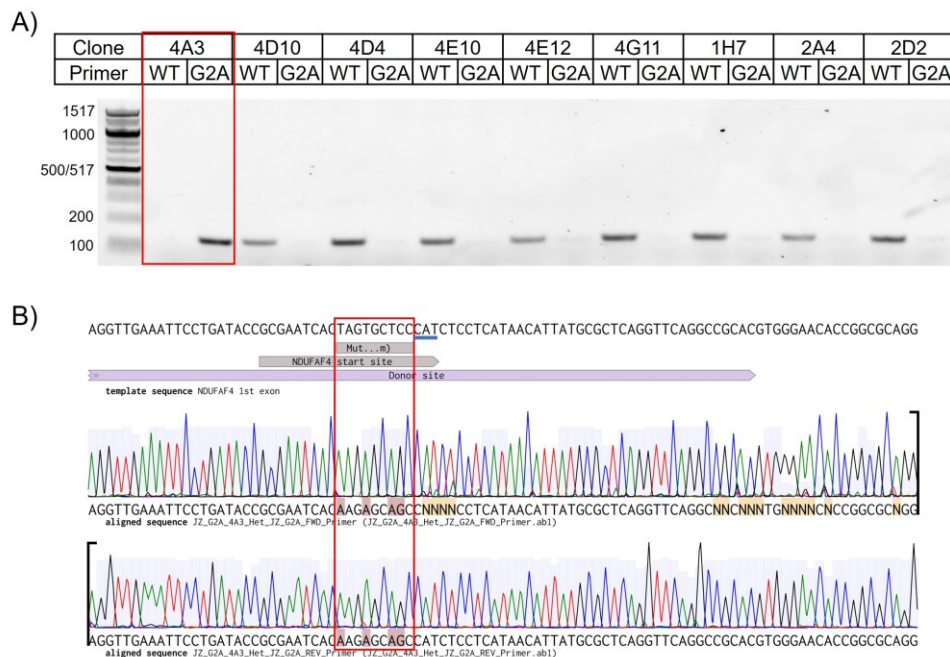


Figure 4.16 Identification of a homozygous NDUF4 G2A mutant. A) Genomic DNA (gDNA) was extracted from single cell clones. gDNA was amplified by PCR using primers against the wild type and target mutation sequence. Clone 4A3 was successfully amplified by the G2A primer and not the wild type primer indicating homozygous knock-in. **B)** Sanger sequencing results for clone 4A3 in the region surrounding the NDUF4 start site. The start codon is underlined in blue. Red box, the target mutation site with the desired base changes also highlighted in red.

by Sanger sequencing (**Figure 4.16B**). As no NDUF4 G2G clone was isolated, a clone which underwent the CRISPR-Cas9 editing protocol but did not show changes in the target sequence (**Appendix Figure 22**) was used in subsequent phenotypic assays as a control cell line (henceforth referred to as control), with the rationale that these cells should at least partially control for cellular changes due to the gene editing protocol.

4.6.2 Initial validation and assessment of the impact of the G2A mutation in NDUF4

With the NDUF4 G2A and control cell lines in hand, further validation of these cell lines was first carried out. All western blots were prepared by Dr. Anne Schuhmacher. Initial western blots revealed that the MYCN switching ability of these cells was intact (**Figure 4.17A**). Assessment of the effects of NMTi on global *N*-myristoylation by YnMyr labelling also revealed no major differences, with NMT activity severely diminished by 100 nM IMP1088 and completely abolished by 1 μ M IMP1088 (**Figure 4.17B**).

Application of Triton X-114 phase separation to
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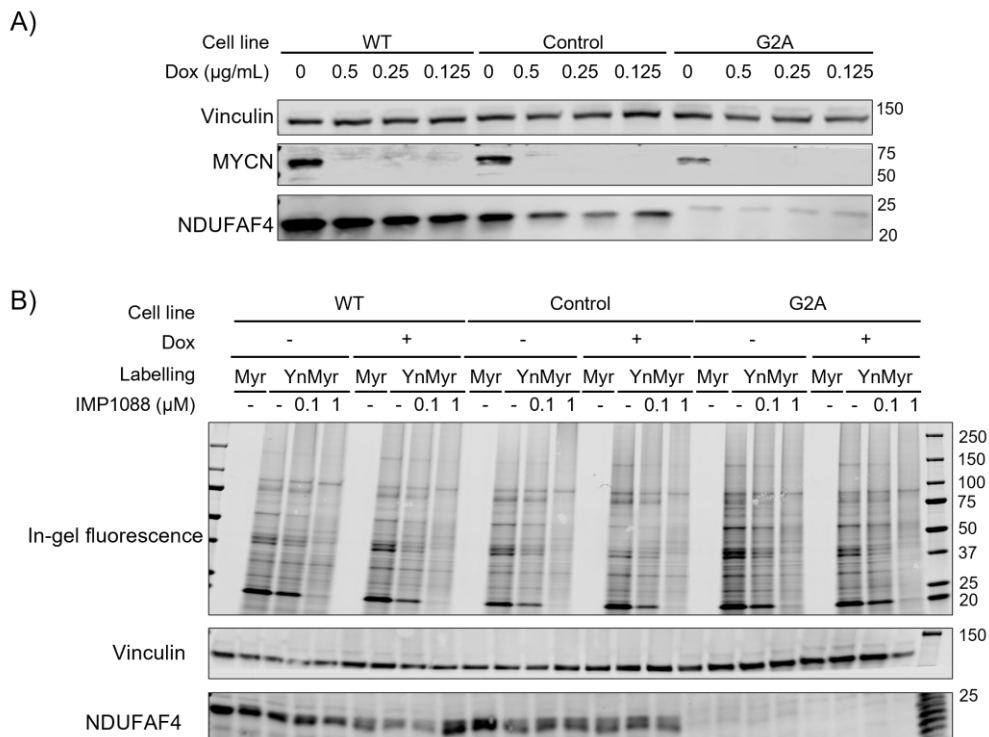


Figure 4.17 Initial validation of generated monoclonal cell lines reveals severe impacts on NDUFAF4 expression. Western blot and in-gel fluorescence results in high or low MYCN SHEP21N cells (parental, control and NDUFAF4 G2A). **A)** Blots for MYCN and NDUFAF4 in cells treated with doxycycline (0-0.5 µg/mL, 24 h). The MYCN switching ability of these cells is retained, but the G2A mutant abolishes NDUFAF4 expression. **B)** In-gel fluorescence readout of YnMyr metabolic labelling in cells treated with IMP1088 (0, 0.1, 1 µM) for 18 h. Global *N*-myristoylation responds similarly to IMP1088 in each cell line.

However, a major difference was observed in the NDUFAF4 blot, in which the G2A mutation induced a severe loss of total NDUFAF4 levels, confirming that the myristoyl group alone was essential for NDUFAF4 expression and therefore by extension RC1 assembly and function. To further explore the mechanisms behind the loss of NDUFAF4 expression, cells were first treated with 10 µM of the proteasomal inhibitor MG132 for 24 h. Although the G2A mutant was not expected to be a substrate of the glycine N-degron pathway, in contrast to the non-*N*-myristoylated A3P mutant, other cellular mechanisms could direct it to proteasomal degradation, for example through the Ac/N-end rule pathway following N-terminal acetylation or by triaging by UBQLN1 as has been shown for other mitochondrial proteins³⁸⁵. However, proteasomal inhibition was not sufficient to rescue expression of the G2A mutant (**Figure 4.18A**). Regulation does appear to be on a post-transcriptional level however as RT-qPCR revealed no depletion of NDUFAF4 mRNA

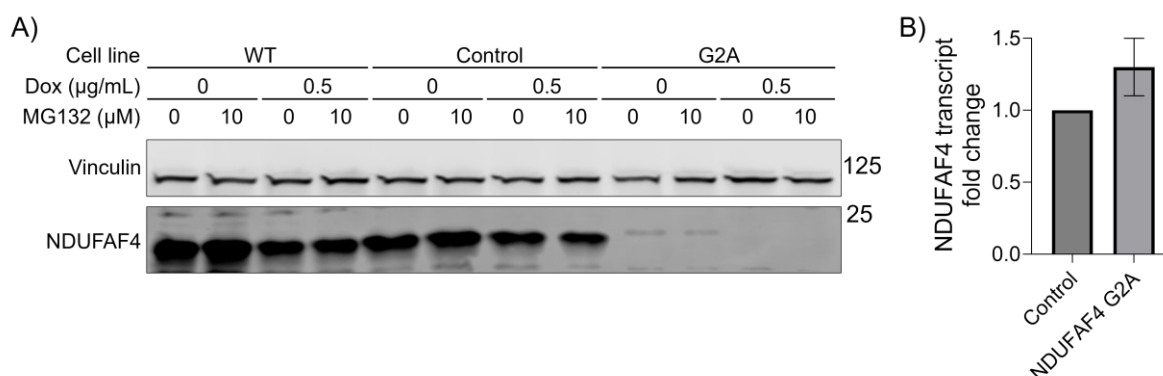


Figure 4.18 Loss of the NDUF4 G2A mutant protein is post-transcriptional but not proteasomal. (A) Western blot for NDUF4 in high or low MYCN SHEP21N cells (parental, NDUF4 G2A-mock and NDUF4 G2A) with or without MG132 treatment (10 µM, 24 h). **(B)** RT-qPCR results for NDUF4 transcripts in control and NDUF4 G2A cells. Fold changes were calculated relative to control cells using the $\Delta\Delta C_t$ method.

in NDUF4 G2A cells relative to control cells (**Figure 4.18B**). Such regulation may therefore manifest as non-proteasomal degradation, such as autophagy, or compromised NDUF4 translation.

4.6.3 Loss of NDUF4 N-myristoylation induces OxPhos defects but abolishes further changes induced by NMT inhibition

Next, the effect of the G2A mutant on mitochondrial parameters and the effects of NMT inhibition was measured. Cells were prepared prior to the assay by Erika Bernardini. The G2A mutant severely lowered the basal respiration and abolished the spare respiratory capacity of SHEP21N cells, consistent with the contribution of NDUF4 to RC1 activity (**Figure 4.19**). Notably, IMP1088 treatment did not induce further changes in respiratory parameters in NDUF4 G2A cells in contrast to the control cells. This observation implies that the effects of NMT inhibition on mitochondrial respiration are strongly mediated by effects on RC1, given that the G2A mutation abolishes NDUF4 expression which in turn ameliorates RC1 assembly²⁹³. However, because minimal functional respiratory complex I will be present in these cells even before NMTi treatment, this result does not suggest that the effects of NMTi are mediated solely by impacts on NDUF4 N-myristoylation in contexts with functional RC1 and NDUF4 i.e. pharmacological NMT inhibition in cancer. In these contexts, effects on NDUF4 will occur concurrently with other RC1-associated NMT substrates such as DMAC1 which may induce additive effects on RC1.

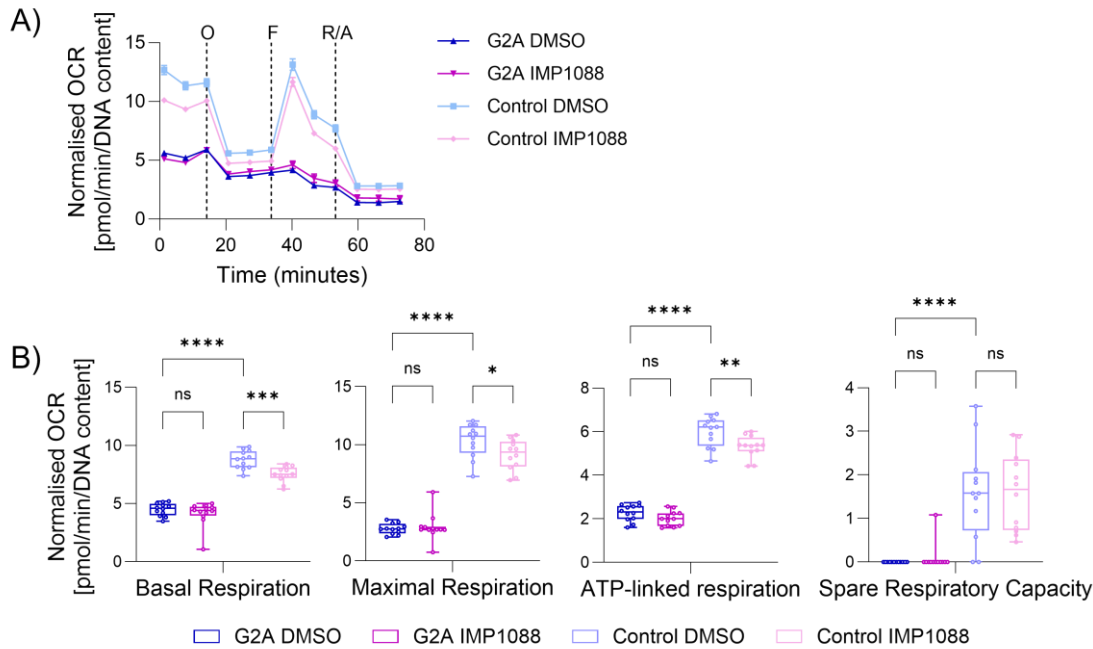


Figure 4.19 The NDUFAF4 G2A mutant induces OxPhos defects and prevents further NMTi-induced effects on respiration. A) Normalised OCR of high MYCN SHEP21N cells (NDUFAF4 G2A and control) upon treatment with DMSO or IMP1088 (100 nM, 18 h). **B)** Mitochondrial respiratory parameters calculated from **A**. O: oligomycin, F: FCCP, R/A: Rotenone/Antimycin A.

While respiratory parameters were affected by NMTi in control cells, surprisingly the impact of NMTi in these cells was different to those seen in the parental SHEP21N cells (**Figure 4.19**, compared to **Figure 4.13**). In particular, the decrease in maximal respiration, although still significant, was much smaller and the spare respiratory capacity was not significantly affected. One contributing factor to this observation was that the maximal respiration measured in the DMSO treated control cells was relatively low, which then contributes to minimising the changes in maximal respiration and spare respiratory capacity, and this may be an artefact due to the assay design. To measure maximal respiration, cells are treated with FCCP, a proton gradient decoupler. Importantly, the effects of FCCP are concentration dependent and needs to be titrated for maximum effect for each cell line. It is therefore possible that the CRISPR protocol changed the optimal FCCP concentration for the generated cell lines. That the effects of the G2A mutation is not strongly phenocopied by NMTi in the control cell line in this experiment may also be due to broad effects of NMTi on other pathways controlling respiration, such as AMPK signalling, to induce compensatory increases in maximal OCR not seen in the G2A mutant, or an adaptive re-wiring of the cell away from OxPhos in the mutant cell line. In either case,

further re-optimisation and validation of these results are needed, especially given that the maximal OCR was significantly affected by NMTi in the parental cell line (**Figure 4.13**). Furthermore, to account for clonal effects, multiple other control and G2A mutant cell lines should be generated and examined; such effects cannot be accounted for in the above experiment as only one clone of each cell line was used. Ultimately however, these initial results indicate that loss of NDUFAF4 *N*-myristoylation is sufficient to induce OxPhos defects as expected and that the effects of NMTi on OxPhos could be centred on RC1.

4.6.4 The NDUFAF4 G2A mutant does not alter sensitivity to NMTi

Finally, the cytotoxicity of NMTi was assessed in the generated cell lines using the Incucyte assay, and it was found that both NDUFAF4 G2A and control cells were equally sensitive to IMP1088 (**Figure 4.20A, Appendix Figure 23**). Dose-response curves were highly similar in both high and low MYCN contexts and <50% cell death was observed in low MYCN cells as expected. While the IC₅₀ values for IMP1088 were similar between the NDUFAF4 G2A and control cell lines, these were ~double the IC₅₀ value obtained in Chapter 2. However, this was likely due to a change in the FBS used to culture these cells. Indeed, repeating the cytotoxicity assay in parental SHEP21N cells yielded a similar dose-response graph and IC₅₀ value as the generated cell lines. Furthermore, the time required to induce cell death was not affected by the NDUFAF4 G2A mutation in either MYCN state (**Figure 4.20B**). Taken together, these results show that loss of NDUFAF4 (*N*-myristoylation) by itself is not a major mechanism through which NMT inhibition induces

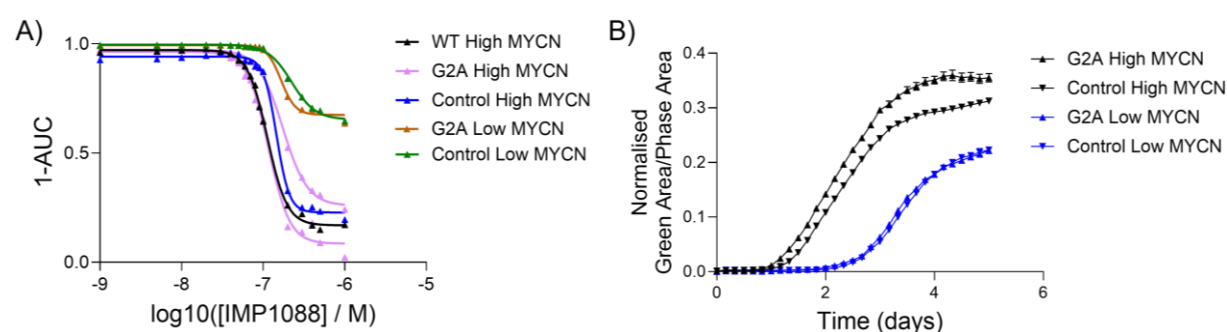


Figure 4.20 The NDUFAF4 G2A mutation does not affect sensitivity to IMP1088. A) Dose-response curves for high and low MYCN SHEP21N cell lines (parental, NDUFAF4 G2A and control). Individual biological replicates are shown. **B)** Cytotoxicity of 1 μM IMP1088 in SHEP21N NDUFAF4 G2A and control cells. Cell lines are coloured by MYCN status. Values are normalised against the puromycin AUCs to allow cross-comparison.

cell death in these cells. These results also indicate that an increased reliance of MNA neuroblastomas on OxPhos does not cause their increased sensitivity to NMTi. Instead, the simultaneous disruption of multiple pathways, as observed in the TX-114 fractionated proteomics study, underlies the cytotoxic effects of NMTi. In this case, it is possible that disruption of OxPhos does still contribute to NMTi-induced cell death, although only partly, and that its contribution in the NDUFAF4 G2A cell line is masked by the plethora of other effects of NMT inhibition, which ultimately induce cell death during the same timeframe.

4.7 Chapter conclusions and further work

In this chapter, an investigation of the effects of NMTi on membrane proteins was carried out, with subsequent validation of the contribution of NDUFAF4 *N*-myristoylation to observed responses. To enrich for the membrane proteome, TX-114 phase partitioning was used and suitability of this protocol for proteomics was first confirmed. Furthermore, changes in protein abundance in detergent fractions are not just due to abundance changes but also localisation changes, as revealed by comparison with both aqueous and global samples. Indeed, the major effect of NMT inhibition for some substrates was to induce mislocalisation without significant degradation.

A pilot study revealed that NMTi affects multiple clusters of interacting proteins in detergent-enriched membrane fractions and initially identified many more effects in low MYCN samples (**Figure 4.7**, **Figure 4.8**). However, generation of a second biological replicate (as part of a time-course experiment, section 4.4) revealed poor correlation of the initial low MYCN dataset with the rest of the samples, suggesting some effects identified may be also due to non-biological variation. Nevertheless, examination of these datasets revealed several insights, which are discussed in detail below.

First, major common primary effects of NMT inhibition under these experimental conditions appear to be loss of RC1, the Ragulator complex and Gai and Src signalling at the membrane through the NMT substrates NDUFAF4, LAMTOR1, GNAI1-3 and Src family kinases respectively. Additionally, depletion of membrane-anchored proteasomes through loss of PSMC1 *N*-myristoylation may be a primary effect, although this is a lower confidence conclusion given that it was not seen in the pilot study. However, these data do not exclude significant contributions from the many other NMT substrates affected, which may not be detected due to low total proteome coverage or assay/analysis design

(see below). These effects are independent of MYCN state but require longer NMTi treatment times to develop in low MYCN cells, perhaps due to generally lower protein turnover rates. This may particularly be the case for RC1, as RC1-associated NMT substrates were much more NMTi responsive in the YnMyr proteomics in high MYCN contexts. Encouragingly, analysis of an analogous experiment in P493-6 cells (prepared by Dr Josephine Walton and Silvia Vannini) identified similar clusters depleted in detergent fractions in high MYC P493-6 cells, but reduced effects in low MYC P493-6 cells, indicating that these main effects were conserved across MYC paralogues (**Appendix Figure 24**). It is worth re-iterating here however that NMTi are not expected to have fully conserved effects in all cell contexts but will have cell-type specific effects depending on the different pathways present.

Greatly affected upon NMT inhibition in this study was OxPhos; in particular, loss of RC1 was time-dependent and one of the most prominent effects seen. Furthermore, likely compensatory upregulation of clusters involved in mitochondrial ATP generation was observed across multiple timepoints, further indicating that this was an important affected process. These may be direct effects arising from defects in RC1 assembly and the analysis suggests this may be mediated by depletion of the NMT substrate NDUFAF4; NDUFB7 is less robustly affected by NMTi, whereas DMAC1 is not included due to limitations in this analysis, again discussed later. NMTi could further indirectly affect OxPhos through inhibition of mTORC1 signalling, which promotes translation of both RC1 and mitoribosomal proteins³⁸⁶. Depletion of MRPS36 may also play a role through its function in assembling the oxoglutarate dehydrogenase complex in the TCA cycle³⁸¹, or ARF proteins through their role in protein sorting³⁰⁹; these NMT substrates were also significantly depleted in detergent fractions.

Another common effect of NMT inhibition appears to be early activation of an ER-associated translational program without global translation upregulation, suggesting that membrane or organellar proteins are highly affected. However, loss of membrane-associated ribosomes is seen in the low MYCN samples at later timepoints, indicating a divergence of translational response. Elucidation of this translational program and identifying if there are differences in this program depending on MYCN status would further reveal how these cells adapt to NMT inhibition. This could be achieved using ribosome sequencing (Ribo-Seq), which sequences ribosome-protected mRNA fragments and therefore indicates which transcripts are being actively translated; positional data also

gives an indication of ribosomal stalling on transcripts and therefore whether these programs are successfully executed³⁸⁷. This may be especially relevant as disruption of protein homeostasis was also identified, for example by enrichment of clusters assigned to ER-associated degradation. As the ER, along with the Golgi, is key to protein sorting and targeting, functional disruption of this compartment could induce additional effects in other compartments. Upregulation of the COG complex in low MYCN cells, which was identified in both the pilot and time-course experiment, may improve ER-Golgi function and promote survival.

One function that was significantly depleted in high MYCN cells at all timepoints but minimally in low MYCN cells was ribosomal biogenesis. This may arise from the fact that many ribosomal biogenesis factors are upregulated by MYCN, but may be only be basally expressed in low MYCN cells³⁸⁸. Ribosome biogenesis is highly energy intensive, and it may therefore be downregulated in high MYCN states to reduce cellular energy demand. However, this would be in direct conflict with the anabolic effects of MYCN and this conflict may contribute to cell death. For example, inhibition of ribosomal biogenesis may result in activation of the impaired ribosomal biogenesis checkpoint, as observed in MYC-driven lymphoma models³⁸⁹. Visual examination of the ribosomal biogenesis cluster identifies UTP20, a subunit of the U3 small subunit processome which synthesises 18S rRNA as very highly depleted (although narrowly missing the significance threshold, this protein was highly depleted at the 18 h timepoint also; $\log_2FC = -1.7$). Therefore, one hypothesis would be that NMT inhibition leads to the downregulation of UTP20 to decrease ribosomal biogenesis. However, UTP20 is also known to localise to the plasma membrane⁴⁴ where its function is as of yet unknown. The depletion seen may therefore represent depletion of UTP20 specifically from the plasma membrane, impacting functions other than ribosomal biogenesis. Furthermore, the observation of ribosomal biogenesis was unexpected given its nucleolar localisation and the membrane-centric focus of TX-114 enrichment. Any follow up experiments of this finding must therefore begin with careful validation, including of loss of ribosomal biogenesis through measurement of nascently synthesized rRNA levels or 45S pre-rRNA levels and direct assessment of the effects of NMT inhibition on UTP20 localisation by microscopy.

While these findings generally represent bona-fide effects of NMTi on cellular state, several restrictions of the study design must be acknowledged when considering the hypotheses drawn from this study. First, as mentioned, this protocol successfully enriches

for membrane-associated proteins, but pure, intact membranes are not isolated in this protocol; instead, mixed membrane micelles are enriched. Therefore, further validation is required to categorically confirm that the effects seen in the proteomics are membrane-localised, although lack of similar effects identified in the global proteome do support this hypothesis. Such verification is especially important for complexes which have multiple subcellular localisations such as the proteasome or ribosome and for biological processes which are not canonically membrane localised as is the case for ribosomal biogenesis. In addition to immunofluorescence experiments to confirm localisation, alternative approaches such as organelle-targeted proximity labelling or subcellular fractionation by density gradient ultracentrifugation represent powerful alternatives. This latter method would be particularly powerful as it allows the generation of cellular spatial proteomes with high resolution of multiple subcellular niches simultaneously³⁹⁰. This provides resolution of localisation effects not accessible by the TX-114 method, which only enriches a crude membrane fraction. Additionally, the use of other detergents, such as digitonin, was not explored in this study but would be recommended. As digitonin permeabilises the plasma membrane through its interaction with cholesterol to obtain membrane fractions³⁹¹, fractions obtained using this detergent should be more representative of the membrane proteome. This would be expected to extend to the membrane protein/cytosolic protein interactions identified, which could have been affected by the phase partitioning step in the TX-114 method. One potential drawback of using digitonin, however, is that the poor permeabilization of cholesterol-poor, organellar membranes would lead to luminal protein contamination and complication of downstream interactomic analysis, necessitating first the optimisation of the lysis conditions.

There were also some limitations due to the analysis method used, which prioritised proteins with high-confidence PPIs to increase confidence in the hits identified. First, the method relies on the STRING database to assign PPIs, however this is not always accurate. For example, multiple RC1 proteins identified here, including NDUFS7, were strongly co-enriched with DMAC1 in an affinity purification experiment, a common method to identify protein interactors²⁹³. However, according to the STRING database, DMAC1 has no high-confidence interactors, and therefore its possible contributions are not represented in this analysis despite being significantly and strongly depleted in these experiments. This issue can be applied more generally for the assignment of cluster annotations, which assumes the validity of the underlying databases such as KEGG and

GO terms. Additionally, biological functions which are controlled by protein families which do not necessarily interact will be missed in this analysis. Finally, possibly significant contributions from individual proteins with individually high fold changes will not be represented in this analysis. These latter two limitations are due to the focus of this analysis on physical complexes and can be addressed by altering the analysis parameters, such as clustering proteins by functional annotation instead of physical interactions or manual inspection of the most highly affected proteins, whereas the former may be mitigated at least partly by the integration of other protein interaction databases, such as BioGRID³⁹², IntAct³⁹³ and hu.MAP³⁹⁴ to determine PPIs.

To validate some of the findings in this proteomics study, the effect of NMTi on OxPhos was further studied as well as the importance of NDUFAF4 *N*-myristoylation specifically. Initial functional assays and western blots confirmed that IMP1088 downregulated respiratory parameters after 18 h treatment and that this was associated with loss of NDUFAF4 expression, although only in high MYCN cells. Similar results were identified in the MYC-regulatable P493-6 cell line⁶⁶, confirming applicability across MYC paralogues, as well as recently in other lymphoma cell lines^{152,156}. That effects are not seen in low MYCN cells at this timepoint again may reflect protein synthesis rates and could be confirmed by Ribo-Seq or pulsed SILAC experiments⁶⁶. The proteomics data however indicated that low MYCN cells also experienced RC1 defects likely leading to OxPhos dysfunction upon prolonged NMT inhibition. Whether low MYCN cells experience functional OxPhos dysfunction at later timepoints (e.g. 48 h, just before these cells die) was not investigated but could be assessed by similar methods. Additionally, a limitation of the metabolic studies in this chapter was the use of standard culture media, which contains supraphysiological levels of multiple metabolites, including glucose, and therefore only poorly represents physiological cellular conditions. For example, the concentration of glucose in the tumour microenvironment is low relative to normal levels³⁹⁵, and trace elements such as selenium present in the body may not be in the media. This leads to the media-dependent rewiring or saturation of multiple metabolic pathways and can mask actual metabolic alterations in response to stimuli. For example, increased concentrations of glucose, as used here, reduces cellular oxidative phosphorylation and alters glycolysis³⁹⁶. Therefore, to ensure that the metabolic effects identified here are also physiologically relevant, these studies should be repeated in low glucose or physiologically relevant media, such as Plasmax. Furthermore, cells could be cultured in media containing

galactose instead of glucose, which causes a glycolysis to oxidative phosphorylation switch in cells³⁹⁷, to better reveal the effects of NMTi on OxPhos.

To investigate the effects of *N*-myristoylation blockade on endogenous NDUFAF4, a G2A knock-in mutant was generated in SHEP21N cells. This confirmed that the myristoyl group is essential for NDUFAF4 expression in a post-transcriptional manner, although its loss is not mediated by proteasomal degradation in contrast to non-*N*-myristoylated NDUFAF4 generated by the A3P mutation. One possible regulatory mechanism is defective translation of the modified NDUFAF4 sequence as a result of the introduced edits. However, this would be unexpected, as the G2A edit introduces a +5C (relative to the starting AUG codon), which has been associated with favourable translation³⁹⁸. To assess this hypothesis, multiple methods could be used. First, translation of NDUFAF4 transcripts can be profiled using a recently described method, FLARIM, which profiles ribosome-mRNA interactions of endogenous transcripts by microscopy³⁹⁹. In this protocol, ribosomes and mRNAs are targeted with oligonucleotide probes which only produce a fluorescence signal when proximal to each other. Alternatively, nascent chain tracking methods can be used to monitor translation, in which nascent protein synthesis and expression of an engineered reporter mRNA is measured⁴⁰⁰⁻⁴⁰³. Other methods such as Ribo-Seq, although more global, can also provide translational information. Alternatively, the mutant protein may be degraded by autophagy, also a major method of cellular protein catabolism. Indeed, vesicular shuttling of mitochondrial proteins for lysosomal degradation has been observed upon induction of oxidative stress⁴⁰⁴. While RC1 proteins were specifically excluded from these vesicles in this study, this may be because oxidative stress was induced through respiratory complex III inhibition. Degradation of NDUFAF4 by this mechanism could be confirmed using autophagy inhibitors such as bafilomycin A1⁴⁰⁵.

Regardless of the mechanism of NDUFAF4 depletion, testing of the generated cell lines revealed that mitochondrial respiration is significantly lower in NDUFAF4 G2A cells but is also NMTi insensitive. This observation implies that the effects of NMT inhibition on mitochondrial respiration are mainly mediated by its effects on RC1, which would be consistent with this respiratory complex being dominantly depleted in membrane fractions, although it is possible that metabolic adaptations to the genetic edit (e.g. a metabolic switch to increased glycolysis) occur which lower the cellular reliance on OxPhos. However, this mutation does not influence NMTi cytotoxicity. This result indicates that loss of NDUFAF4 *N*-myristoylation is not a major single contributor to loss of cell viability or

increased sensitivity in high MYCN cells. While it is possible that a different NMT substrate is central to the effects of NMTi, this appears unlikely, given that depletion of RC1 was consistently one of the most significant effects seen. Instead, simultaneous and synergistic pleiotropic effects of NMT inhibition is likely the ultimate cause of NMTi-induced cell death in these cells, which may also be a general feature of NMTi⁶. The separate contributions of individual pathways or NMT substrates to this phenotype, at least in the MNA neuroblastoma context, will therefore be extremely difficult to deconvolute through modulation of single NMT substrates in isolation.

Chapter 5. Conclusions and future directions

5.1 Thesis conclusions

Together, the studies presented in this thesis have built upon the original observation that MYC deregulation sensitises cancer cells to NMT inhibition, expanding this relationship experimentally to MYCN in the neuroblastoma context and delineating the effects of NMTi in both high and low MYCN contexts. Notably, this manifests as a decrease in both the concentration and time required to induce cell death in high MYCN cells. NMTi may therefore be a viable treatment for MNA neuroblastomas, with the *in vitro* results also indicating that MYC-deregulated neuroblastomas could also benefit from NMTi, although perhaps with refinement of the dosing schedule and intensity. This result suggests a novel indication of NMTi in which an actionable therapeutic window may exist.

The use of multiple -omics techniques to assess the responses of high and low MYCN cell to NMT inhibition has revealed mechanistic insights in NMTi MoA and have also highlighted the importance of matching the technique used to the compound of interest. Effects on the transcript level did not appear to accurately represent the primary effects of NMTi on the cell, which may mostly be due to their effects on a co-translational modification. This result is likely generalisable to other drugs acting in a co- or post-translational manner. However, the effects of NMTi on both the transcriptome and proteome were generally consistent across MYCN status, indicating that low MYCN cells do not strongly invoke specific resistance or pro-survival mechanisms upon NMT inhibition. The effects of NMTi on the global proteome do appear to be more reflective of NMTi primary effects but were characterised by a generally low effect size, suggesting that under the conditions used, the global proteome is not significantly remodelled. However, the effects of NMTi appear to primarily manifest as an effect on local protein (complex) concentrations at membranes, instead of overall protein levels, as suggested by the TX-114 fractionation proteomic experiments. These consistently exhibited greater effects on proteins in the detergent-enriched membrane fraction not fully explainable by total protein abundance changes alone and identified multiple clusters of physically interacting proteins, including with NMT substrates, which were co-ordinately affected and likely represent the significant effects of NMT inhibition.

Interestingly, when the effects of NMT inhibition *in vivo* were assessed by proteomics, it was shown that NMTi induces strong immune infiltration into the tumours, which may be associated with the anti-tumour effects of NMTi *in vivo* beyond the intrinsic susceptibility of tumour cells to NMTi. It would be interesting to further characterise this phenotype and investigate how and the extent to which this phenotype contributes to *in vivo* NMTi efficacy through the characterisation of infiltrating immune cells or the use of vaccination assays.

Finally, the generation of a NDUFAF4 G2A knock-in cell line to investigate the importance of *N*-myristoylation on an individual protein has also highlighted the complexity of NMTi MoA. While the effects of NMTi on NDUFAF4 *N*-myristoylation and OxPhos do not appear to be a major mechanism of NMTi-induced cell death in isolation, effects on RC1 were highly prominent in the -omics analyses presented in this thesis, as well as in other analyses of NMTi treatment or NMT1 KO^{66,152}. It is therefore likely that the higher potency of NMTi in high MYCN cells is not due to a reliance or impact on this, or any other, individual pathway. Therefore, an emerging working model is that upon NMT inhibition, neuroblastoma cells die through the simultaneous and synergistic dysfunction of multiple biological processes modulated by NMT substrates. *MYCN* amplification sensitises neuroblastomas to this effect through the increased synthesis (and turnover) of NMT substrates and increasing the general cellular dependence on affected pathways, a model consistent with findings also in other cancer subtypes⁶.

5.2 Future directions

The efficacy of NMTi in MNA neuroblastoma represents a novel method to address unmet need in this disease. However, drug avoidance and development of resistance are common issues in cancer treatment, and these should be further investigated, for example through the generation of NMTi-resistant cell lines, or characterisation of the influence of the adrenergic/mesenchymal cell state on sensitivity to NMTi. Furthermore, identification and validation of possible synergistic combination treatments, such as those with ferroptosis inducers, should be further carried out, to increase the therapeutic window and efficacy of NMTi.

To further increase insights on the effects of the global proteome, application of recently reported methods which increase the resolution of mechanistic analyses, such as aggregation analysis of proteome footprints³¹² or investigation the dose-dependent effects of NMTi³⁵⁵, would likely be insightful. In addition, by expanding such studies to other cancer

cell types, cell lineage specific or agnostic effects may also be identified. To further deconvolute the mechanistic effects of NMTi, validation and subsequent follow up of the affected clusters in the TX-114 fractionated proteomics as well as further identification of the spatial effects of NMTi at higher resolution, would be valuable. While loss of NDUFAF4 *N*-myristoylation does not confer a survival benefit against NMTi treatment, this has not been investigated in a systematic manner across all NMT substrates. Such a screen could now feasibly be carried out using the recently described prime editing method to introduce the G2A mutation in all NMT substrates⁴⁰⁶. Such a screen may reveal functional consequences of inhibiting *N*-myristoylation of individual substrates, even in the absence of cell viability effects upon NMTi challenge due to the pleiotropic effects of NMTi, if also combined with higher dimensional phenotypic readouts such as Cell Painting⁴⁰⁷.

Furthermore, the role of dysregulated Myr-CoA and lipid metabolism to NMTi sensitivity has not been well characterised to date, although recent reports indicate that such metabolic pathways are relevant to NMT biology^{84,151}. Interestingly, myristic acid is one of the most highly upregulated fatty acids upon MYCN expression²¹⁶. Therefore, it is possible that MYCN also sensitises neuroblastomas to NMTi through effects on lipid metabolism, and that targeting such pathways will also synergise with NMTi.

Overall, advances in our understanding of the MoA of NMTi and factors affecting sensitivity will primarily be fuelled by the clinical requirements of NMTi. One NMTi so far has demonstrated tolerability in a Phase I trial, with a Phase II trial ongoing in DLBCL patients. Improved mechanistic understanding of NMTi MoA will identify and prioritise the subset of cancers most responsive to NMTi. Finally, while a majority of work so far, including the studies in this thesis, have focused on NMTi as systemic single agent small molecules, the development of NMTi-ADCs by Myricx Bio may further unlock the potential of NMTi in oncology applications including neuroblastoma, as recently demonstrated, by widening the therapeutic window and/or the range of cancers in which NMTi are efficacious.

Chapter 6. Materials & Methods

6.1 General methods

6.1.1 Cell culture

All neuroblastoma cell lines were obtained from the Chesler laboratory at the Institute of Cancer Research and STR profiled at the Francis Crick Institute. P493-6 cells were obtained from the Calado laboratory at the Francis Crick Institute. CHP-212, IMR5, Kelly and SHEP21N cells were cultured in RPMI-1640 (Gibco, 11875101). BE(2)C, SHEP, SKNAS, SKNSH cells were cultured in high glucose DMEM (Gibco, 41966029). Th-MYCN cells were cultured in DMEM/F12-Glutamax (Gibco, 31331028) supplemented with B27 (Gibco, 17504044), 0.1 mM non-essential amino acids (Gibco, 11140050), 50 μ M β -mercaptoethanol (Gibco, 31350010), 5 μ g h-EGF (Gibco, PHG0315), 7.5 μ g b-FGF (PeproTech, 100-18B) and 1% penicillin-streptomycin (Sigma, P4333). P493-6 cells were cultured in DMEM-Glutamax (Gibco, 61965026) supplemented with 10 mM HEPES (Gibco, 15630080), 1 mM sodium pyruvate (Gibco, 11360070), 0.1 mM non-essential amino acids, 25 μ M β -mercaptoethanol and 1% penicillin-streptomycin. Th-MYCN media was supplemented with 15% fetal bovine serum (FBS) (Gibco, 41966029). All other media was supplemented with 10% FBS or tetracycline-free FBS (Gibco, A4736201) in the case of SHEP21N and P493-6 cells. Cells were cultured in humidified 37 °C incubators with 5% CO₂.

6.1.2 SHEP21N MYCN modulation

SHEP21N cells were treated with 0.5 μ g/mL doxycycline (Sigma, D9891) for 24 h to turn off MYCN expression.

6.1.3 Cell lysis

Unless stated otherwise, cells were lysed using 1% (v/v) Triton X-100 (Sigma, T8787) and 0.1% (w/v) sodium dodecyl sulphate (SDS) (Sigma, 71736) in PBS pH 7.4 supplemented with cOmplete protease inhibitor (Roche, 11873580001). Phosphatase inhibitor (Sigma, P5726) was added for experiments assessing phosphorylation. Cells were washed once with Dulbecco's phosphate buffered saline (DPBS) and ice-cold lysis buffer added to plates. Cells were lysed on-plate by scraping and the sample centrifuged (17,000 $\times g$,

10 min, 4 °C) to remove debris. Protein concentrations were determined using the DC Protein Assay Kit II (Bio-Rad, 5000112) and normalised across samples in lysis buffer (1-2 mg/mL).

6.1.4 SDS-Page

Samples were prepared using 4x Laemmli sample buffer (BioRad 1610747) supplemented with 10% β -mercaptoethanol, boiled for 5 min at 95 °C and 10-26 μ L (10-40 μ g protein) loaded onto 10%, 12% or 4-15% (w/v) SDS-PAGE gels. Proteins were separated at 180 V for 1 h in Tris-glycine-SDS buffer (Bio-Rad, 1610732). Gels were subsequently imaged for in-gel fluorescence (see section 6.1.9) and protein loading visualised by Coomassie staining (Expedeon, ab119211) or proteins transferred to nitrocellulose membranes for western blot.

6.1.5 Western Blot

After SDS-Page, proteins were transferred to nitrocellulose membranes (0.45 μ m pore size) (Amersham, 10600003) by wet or semi-dry transfer (Bio-Rad). Wet transfer: Proteins were transferred onto membranes using Towbin buffer (25 mM Tris, 190 mM glycine, 20% (v/v) methanol, pH 8.3) at 100 V for 1 h. Semi-dry transfer: Protein transfer was carried out using the “mixed molecular weight” protocol (25 V, 2.5 A, 7 min) using the Trans-Blot Turbo system (Bio-Rad, 1704150). Protein transfer was assessed by Ponceau staining (Sigma, P7170) for 5 min. Membranes were blocked for 1 h in 5% semi-skimmed milk (Sigma, 70166) or 3% bovine serum albumin (BSA) (Sigma, A7906) in Tris-buffered saline (50 mM Tris, 50 mM NaCl, pH 7.4) containing 1% (v/v) Tween-20 (TBST). Membranes were incubated with primary antibodies in blocking solution at 4 °C overnight. The next day, membranes were washed with TBST (3x 10 min) and incubated with secondary antibody in blocking solution for 1 h at room temperature (rt). Membranes were washed again with TBST (3x 10 min) and imaged. Membranes were incubated with Immobilon Crescendo Western HRP substrate (Merck, WBLUR0100) and chemiluminescence imaged using an ImageQuant LAS4000 (GE Healthcare) or a LI-COR Odyssey (LI-COR). Images were processed using Image J (NIH Bethesda) and Imperia Studio (LI-COR). A list of antibodies used and their dilutions is given in **Table 6.1**.

6.1.6 Chloroform-methanol protein precipitation

For 1 volume of sample, proteins were precipitated by the addition of 4:1:2 volumes of MeOH:CHCl₃:H₂O. Samples were vortexed and centrifuged (5,000 × *g*, 2 min, rt) yielding a biphasic mixture with a solid protein layer at the interphase. The top layer was removed, 500 µL MeOH added, the protein resuspended by vortexing, and the sample centrifuged (17,000 × *g*, 4 min, rt) to yield a protein pellet. The MeOH wash was repeated twice in total, and the pellet air-dried for 5 minutes. The pellet was resuspended in 0.1 volumes of 2% (w/v) SDS in 50 mM HEPES pH 8.0 by vortexing, and the sample diluted with 0.9 volumes of 50 mM HEPES pH 8.0 for a final SDS concentration of 0.2%.

6.1.7 SP4 precipitation

For 1 volume of sample, proteins were precipitated by the addition of 4 volumes MeCN and gently vortexed (400 rpm) for 5 s. Samples were centrifuged (4,000-17,000 × *g*, 4 min, rt) to obtain a protein pellet. The supernatant was removed, and the pellet was washed with >10 volumes 80% (v/v) EtOH without disturbing the pellet. The sample was then centrifuged (4,000-17,000 × *g*, 4 min, rt) and the supernatant removed. The 80% EtOH wash was repeated three times in total. After the final wash, samples were resuspended in either 50 mM HEPES pH 8.0 or digestion buffer (6.25 ng/µL of trypsin (Promega, V5111) in 25 mM ammonium bicarbonate), if the sample was intended for trypsinisation, or 0.2% (w/v) SDS in 50 mM HEPES pH 8.0 if samples were intended for pull-down. For pull-down samples, pellets were completely resolubilised using probe sonication. Samples were placed in a beaker containing ice to prevent heat-mediated protein degradation by sonication. The pellet was then resolubilised using a Fisherbrand 505 Sonicator (Fisher Scientific) using 5 second on/off pulses at 20% amplitude for 2 min. Foam from samples after sonication was removed by centrifugation (17,000 × *g*, 4 min, rt).

6.1.8 Click reaction

Lysates (1-2 mg/mL) with alkyne-tagged proteins of interest were prepared by metabolic labelling with YnMyr as described below (section 6.2.2). A click master mix was prepared by mixing 10 mM labelling reagent (AzT/AzRB), 50 mM CuSO₄, 50 mM TCEP and 10 mM TBTA in a 1:2:2:1 ratio (final concentrations 100 µM, 1 mM, 1 mM and 100 µM) in this order, vortexing after every addition. To 100 µL of lysate, 6 µL of click master mix was

added and the sample mixed vigorously (1000 rpm) at rt for 1 h. The reaction was then quenched by the addition of 0.01 sample volumes of 0.5 M EDTA solution (Sigma, 03690) and vortexed briefly.

6.1.9 In-gel fluorescence

After processing by click reaction using AzT as the labelling reagent, samples were precipitated (chloroform-methanol) and proteins (10 µg/lane) separated by SDS-PAGE. Fluorescence was imaged using a Typhoon FLA 9500 imager (GE Healthcare) using 532/568 nm excitation/emission (ex/em) for TAMRA and 635/670 ex/em for the ladder, or a LI-COR Odyssey using the 520 nm channel.

6.1.10 Triton X-114 pre-condensation

3 mL of TX-114 (Sigma, X114) was added to 47 mL of DPBS, clarified on ice and vortexed. The solution was then incubated at 37 °C overnight to induce phase separation and the aqueous phase discarded. Fresh DPBS was added to the lower detergent phase up to 50 mL and the above procedure repeated four times in total. The resulting detergent phase (12% TX-114) was depleted of hydrophilic components and was stored at 4 °C in the dark until used.

6.1.11 Public data

ChIP-Seq data for MYCN in SHEP21N cells was downloaded from GSE80154²⁰². CRISPR screening data (Public Release 24Q2) for neuroblastoma cells was downloaded from DepMap^{48,50}. siRNA screening data was obtained from reference ²⁵⁶.

6.2 Cellular assays

6.2.1 Incucyte cytotoxicity assay

2000-4000 cells in 100 µL media were seeded into 96 well plates (Greiner, 655180) and allowed to attach overnight. The next day, 100 µL of media containing Sytox Green (ThermoFisher, S7020) and 2X concentration of the indicated compounds were added to the cells in technical triplicate. Sytox Green was used at 250 nM final concentration except for IMR5 cells where 100 nM was used to minimise the effects of Sytox Green on cell proliferation. The final DMSO concentration was 0.1% for NMTi assays and 0.15% for

ferroptosis assays. Bubbles were removed from wells and plates monitored using the IncuCyte S3 platform (Sartorius). Images (4 per well) were acquired on the green and phase channels using a 10x objective every 4 h for 120 h except where otherwise indicated. Images were analysed using the default Incucyte software (versions 2020B-2022B). Analysis settings were modified for each cell line depending on the software's ability to distinguish cells from background and manually checked for each assay. For analysis of phase images, segmentation was usually kept at 1, hole fill set to 600 μm^2 and minimum area set to 230 μm^2 to filter out cellular debris. For analysis of the green channel, the default Top-Hat segmentation was used, radius usually kept at 100 μm and the GCU threshold as 8. Confluency was defined as phase area and normalised cell death was defined as green area divided by phase area. Data were analysed in Prism 10 (GraphPad). To calculate IC50s, the area under the curve (AUC) for each NMTi concentration was calculated and normalised by the puromycin AUC. Dose-response curves were generated by plotting 1-normalised AUC against $\log_{10}[\text{NMTi}]$ and absolute IC50s calculated constraining the baseline to 0. Extrapolated IC50s were calculated by constraining the bottom values of the IC50 curve to those obtained by IMP1088 in the same cell line, where applicable. To compare individual cytotoxicity curves between experiments, green/phase values were normalised by the puromycin AUC. Data in all plots are represented as mean $\pm$ s.e.m. Only errors larger than the symbol are plotted.

6.2.2 Assessment of NMT target engagement by YnMyr metabolic labelling

Cells were plated in 6-well plates and grown until 70% confluent. Cells were then treated with 100 nM or 1 μM of IMP1088 or DMSO for 30 min, followed by 20 μM YnMyr or DMSO for 18 h. Cells were lysed and processed as in sections 6.1.8 and 6.1.9. After in-gel fluorescence imaging, western blots for ARL1 were optionally carried out as in section 6.1.5. The presence of a higher MW form of ARL1 represents YnMyr-labelled and clicked ARL1; loss of this band indicates loss of NMT cellular activity.

6.2.3 Western blot analysis of MYC, MYCN and NDUFAF4 expression upon NMTi

Cells were plated in 6-well plates or 100 mm dishes and grown until 70% confluent, then treated with DMSO or 100 nM IMP1088 for 18 h. Cells were then lysed and 10 μg protein subjected to SDS-PAGE and Western blotting as described in sections 6.1.4 and 6.1.5.

6.2.4 Western blot analysis of ER stress upon NMTi

SHEP21N cells were plated in 100 mm dishes and grown until 70% confluent. Cells were then treated with DMSO, 5 mg/mL tunicamycin, 100 nM thapsigargin or 2 µg/mL Brefeldin A for 18 h, or 100 nM IMP1088 for 18, 36 or 60 h. Cells were then lysed, protein concentrations normalised to 2 mg/mL and 20 µg protein (40 µg protein for phosphoproteins) processed by western blotting against the indicated proteins. IMR5 cells were processed similarly, except they were also treated with 1 µM IMP1088 for 18, 36 or 60 h.

6.2.5 Western blot analysis of Triton X-114 membrane fractionation

P493-6 cells were lysed either as in section 6.1.3 or as for Th-MYCN cells in section 6.6.2. For cells lysed by the latter method, TX-114 based phase separation was carried out after debris removal as described in section 6.6.5. 10 µg of protein from each fraction (global proteome, membrane, and aqueous fractions) were then processed by western blotting against the indicated proteins.

6.2.6 Western blot analysis of proteasomal degradation of NDUFAF4

SHEP21N cells (parental, NDUFAF4 G2A and G2A-mock) were induced into the respective MYCN conditions and treated with DMSO or 10 µM MG132 for 24 h. Cells were then lysed and processed by western blotting against NDUFAF4.

6.2.7 Cell-Titer Glo assay

25,000 Th-MYCN cells were seeded into white 96-well plates and incubated with 0-1 µM of IMP1320 for 14-20 h (n=6 per condition). Each plate represented a separate timepoint. To measure cell viability, the CellTiter-Glo 3D kit (Promega, G9681) was used according to manufacturer's instructions. The cell plate was removed from the incubator and equilibrated to room temperature for 30 min. An equal volume of CellTiter-Glo 3D Reagent was added to the cells and the plate mixed on a plate shaker (800 rpm) for 5 min followed by incubation at rt for a further 30 min. Luminescence was recorded on a CLARIOstar Plus (BMG Labtech). Data were analysed with Prism.

6.2.8 Oxygen consumption rate (OCR) measurements

Oxygen consumption rate was measured using a Seahorse XFe96 extracellular flux analyser (Agilent) according to manufacturer's protocols. SHEP21N cells were first induced into the respective MYCN states. The day before the assay, 30,000 cells were plated in a 96-well Seahorse cell culture plate (Agilent) and allowed to adhere for 8 h. Cells were then treated with DMSO or 100 nM IMP1088 for 18 h. A Seahorse cartridge (Agilent) was hydrated in 200 μ L XF calibrant solution (Agilent) overnight in at 37 °C. The day of the assay, media was removed from the wells, cells washed twice with Seahorse XF RPMI medium (unbuffered RPMI without phenol red) containing 10 mM glucose and 2 mM L-glutamine (Agilent) and 180 μ L of this Seahorse media was added to the cells. The plate was then incubated in a non-CO₂ incubator at 37 °C for 45-60 min before assay start. Injection ports A, B and C were filled with 10X solutions of 20 μ L of oligomycin (15 μ M), 22 μ L of carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) (5 μ M) and 25 μ L of rotenone/antimycin A mixture (5 μ M each) with 25 μ M Hoechst 33342 (ThermoFisher, H1399) respectively. OCR data was obtained using Wave software (Agilent). After the Seahorse assay, DNA content in each well was assessed using a CLARIOstar Plus using an endpoint fluorescence intensity assay (top read) using the Hoechst pre-set settings, a gain and focus of 1400/2.1 mm, 2 flashes and using a 15 x 15 (3 mm) matrix scan at 37 °C. OCR values for each well was normalised to DNA content and the data analysed with Prism.

6.2.9 RT-qPCR

SHEP21N NDUFAF4 G2A and G2A-mock cells were grown to confluency in a 6-well plate, lysed and cellular RNA extracted using the RNeasy Plus Mini kit according to manufacturer's instructions. RNA was adjusted to 100 ng/ μ L and the RT-qPCR reaction carried out using the Luna Universal One-Step RT-qPCR Kit (NEB, E3005) according to manufacturer's instructions and run on a Stratagene Mx3005P (Agilent). 2 μ L of RNA was used per 20 μ L reaction. All reactions were carried out in triplicate and relative transcript expression was determined using the $\Delta\Delta$ Ct method using the housekeeping gene *GAPDH* as a control. Primers were designed using the PrimerQuest tool from IDT and are shown in **Table 6.2**.

6.3 Assessment of IMP1320 *in vivo*

6.3.1 Ethical approval

All animal experiments were approved by The Institute of Cancer Research Animal Welfare and Ethical Review Body and performed in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 and the UK National Cancer Research Institute guidelines for the welfare of animals in cancer research. Mice were maintained on a regular diet in a pathogen-free facility on a 12 h light/dark cycle with unlimited access to food and water.

6.3.2 Assessment of IMP1320 efficacy in the Th-MYCN syngeneic mouse model

1 million Th-MYCN tumour cells were injected subcutaneously into the right flank of 70-100 day old male and female 129SvJ mice and allowed to establish a tumour. Mice bearing tumours with a mean diameter of ~5 mm were treated with IMP1320 (25 mg/kg), or vehicle (PB buffer, pH 7.4) via intraperitoneal injection QD, using a 3 days on, 4 days off cycle for 3 cycles. Tumor volumes were plotted until half the control mice reached the ethical endpoint. Studies were terminated when the mean diameter of the tumor reached 15 mm. Tumor volumes were measured by Vernier caliper across two perpendicular diameters, and volumes were calculated according to the formula $V = \frac{4}{3}\pi [(d1+d2)/4]^3$; where d1 and d2 were the 2 perpendicular diameters.

6.4 Generation of the CRISPR-Cas9 knock-in NDUFAF4 cell lines

6.4.1 Design of sgRNA, primers and donor DNA

The sgRNA, donor DNA and sequencing primers to generate NDUFAF4 G2A and G2G mutant cell lines were designed using the ThermoFisher TrueDesign portal (<https://apps.thermofisher.com/apps/genome-editing-portal>) and ordered from ThermoFisher. To generate each mutant, the endogenous sequence of the first nine bases after the AUG start codon (GGAGCACTA) was changed to GCTGCTCTT (G2A) or GGGGCTCTT (G2G). Silent mutations of amino acids 3 and 4 were incorporated to improve specificity of diagnostic PCR primers targeted to the mutation site. Diagnostic PCR primers were designed using the OligoAnalyzer Tool from IDT (<https://eu.idtdna.com/calc/analyzer>) and PCR Primer Stats tool from GenScript

(https://www.genscript.com/sms2/pcr_primer_stats.html). All RNA and DNA sequences used are reported in **Table 6.3**.

6.4.2 SHEP21N transfection

150,000 SHEP21N cells were seeded in a 6-well plate and allowed to adhere overnight. The next day, cells were transfected according to previously established protocols. Cas9 protein (10 µg, 2 µL), sgRNA (60 pmol, 6 µL), donor DNA (90 pmol, 9 µL) and 20 µL lipofectamine Cas9 Plus reagent (ThermoFisher) were mixed in 125 µL OptiMEM (Gibco, 31985062). 7.5 µL lipofectamine CRISPRMAX was mixed in 125 µL OptiMEM. The two solutions were mixed together and 250 µL transfection mixture was added to each well. In addition to G2G and G2A knock-in conditions, a mock sample (no donor DNA) and negative control sample (no sgRNA) were also prepared to assess protocol efficiency. 48 h after transfection, cells were trypsinized. For the G2G and G2A knock-in conditions, half of each cell population was diluted to 8 cells/mL by limiting dilution and 100 µL added to each well of a 96-well plate. Four 96-well plates were prepared per condition. For the rest of the cells, and for the mock and negative control samples, cells were washed with DPBS, pelleted, and stored at -80 °C until further processed by extraction of gDNA.

6.4.3 Single-cell clone growth

One week after limiting dilution, wells were assessed for single cell clonal populations. Positive wells were trypsinized and reseeded in the 96-well plate to evenly distribute cells across the well in regular SHEP21N media. Cells were transferred to 48-well plates when confluent and then subsequently to 6-well plates. Once confluent in 6-well plates, cells were trypsinized and half of the cell population frozen for long term storage. The other half were washed with DPBS, pelleted, and stored at -80 °C until further processed by extraction of gDNA.

6.4.4 gDNA extraction

Cells pellets were thawed on ice and resuspended in 100 µL ice-cold PBS. gDNA was then extracted using the NEB Monarch Genomic DNA Purification Kit (NEB #T3010S) according to manufacturer's instructions. gDNA concentration and quality were assessed on a Nanodrop One (ThermoFisher).

6.4.5 PCR screening

gDNA extracted from single clones were screened by PCR for incorporation of the desired base edits. For every cell line, a 144 bp (wild type) or 141 bp (G2A/G2G mutant) region was amplified using forward primers targeted against the edit site in a 25 μ L PCR reaction using 30 cycles. The Q5 Hot Start High-Fidelity 2X Master Mix (NEB, M0494) was used and primer annealing temperatures were set to 67 °C as calculated using the online NEB T_m calculator (<https://tmcalculator.neb.com>). Primer sequences are shown in **Table 6.3**. 1 μ L (>75 ng/mL) or 2 μ L (<75 ng/mL) of gDNA was used as a template. After PCR, 5 μ L of sample was diluted with 1 μ L 6X Gel Loading Dye (NEB, B7024). 2% (w/v) agarose gels were hand-casted by melting agarose in 1X Tris-acetate-EDTA buffer in a microwave until fully dissolved. GelRed (Merck, SCT123) was added (1:10000 v/v, 1X final concentration) to the liquid gel and the gel set. Samples (5 μ L) were loaded onto the gel and run in 1X TAE buffer at 100 V for 30 min. DNA was imaged using a Typhoon FLA 9500 imager using 518/605 ex/em.

6.4.6 Sequencing

gDNA extracted from cell populations in section **6.4.2** or selected single cell clones in section **6.4.3** were processed for sequencing. A 332 bp region including the NDUFAF4 start site was amplified using the sequencing primers in a 50 μ L PCR reaction using 35 cycles. 2 μ L of gDNA (102-134 ng) was used as a template. Primer sequences are shown in **Table 6.3**. After PCR, DNA was purified using the QIAquick PCR Clean-Up kit (Qiagen, 28104) and submitted to Genewiz for amplicon sequencing (cell populations) or Sanger sequencing (single cell clones). Sanger sequencing data was analysed in Benchling.

6.5 Transcriptomic sample preparation protocol

SHEP21N cells were induced into high and low MYCN states, plated onto 100 mm dishes, and cultured until 70% confluent. Cells were then treated with 50 nM IMP1088 or DMSO for 2, 6 or 18 h in quadruplicate. Cells were lysed on-plate and cellular RNA extracted using the RNeasy Plus Mini kit (Qiagen, 74136) according to manufacturer's instructions. RNA quality was assessed using the Nanodrop One (ThermoFisher) using A260/280 and A260/230 ratios and purified RNA submitted to the Francis Crick ASF for library preparation and sequencing. cDNA libraries were prepared using the NEBNext Ultra II

Directional PolyA mRNA kit (NEB, E7760L) and sequenced on a NovaSeq 6000 (40 million reads per sample, paired end 100 bp reads). Raw fastq files were processed by the Francis Crick BABS facility. Read alignment was carried out using STAR against the human reference transcriptome GRCh38 and raw gene counts obtained using RSEM. This raw gene count matrix was processed in R using the DESeq2 package for differential expression analysis and to generate a normalised gene count matrix using default settings. MYCN status, treatment condition and timepoint were used as factors.

6.6 Proteomic sample generation

6.6.1 YnMyr enrichment proteomic sample generation

Cells were pre-treated with DMSO or 100 nM IMP1088 for 30 min, followed by 20 mM YnMyr or myristic acid for 18 h and subsequently lysed. Conditions were prepared in triplicate (IMR5) or quadruplicate (SHEP21N).

6.6.2 Global proteomic sample generation (*in vitro* samples)

SHEP21N cells were induced in their respective MYCN states, treated with 50 nM IMP1088 or DMSO for 18 h and then lysed. Th-MYCN tumour cells *in vitro* were treated with 10 nM IMP1320 or DMSO for 18 h and lysed. For the latter experiment, cell pellets were collected, washed twice with DPBS, and resuspended in ice-cold 2% (w/v) condensed TX-114 in PBS pH 7.4 with cOmplete protease inhibitor. Cells were lysed by shaking (800 rpm) at 4 °C for 30 min and cell debris removed by centrifugation (17,000 × g, 5 min, 4 °C). This protein lysate was then precipitated by chloroform-methanol and resuspended in 0.2% SDS in 50 mM HEPES pH 8.0. All conditions were prepared in triplicate.

6.6.3 Proteomic sample generation (*in vivo* samples)

Tumours were lysed using a FastPrep 24 (MP Biomedicals) using Lysing Matrix A (MP Biochemicals, 1169100-CF) in 500 µL of 1X SrtA lysis buffer (150 mM NaCl, 10 mM CaCl₂, 50 mM Tris pH 7.5 supplemented with cOmplete protease inhibitor). Samples were lysed using two runs (30 sec at 6.5 m/s per run) 5 minutes apart with samples cooled on ice between runs and lysates centrifuged (17,000 × g, 10 min, 4 °C). 350 µL of the supernatant was removed for target engagement analysis by Sortase A and 25 µL of 7% Igepal CA-630

(Sigma, I8896) in 1X SrtA lysis buffer was added to the remaining 150 μ L. The tubes were shaken (800 rpm) for 10 min, then centrifuged ($17,000 \times g$, 10 min, 4 $^{\circ}$ C). 100 μ L of this lysate was removed for processing for global proteomics. Lysates (for both proteomic workflows) were centrifuged again ($17,000 \times g$, 5 min, 4 $^{\circ}$ C) to remove residual fat, protein concentrations determined (DC protein concentration assay) and normalised to 1 mg/mL.

6.6.4 TX-114 fractionated proteomics sample generation

After induction into the desired MYCN state, 10 million SHEP21N cells were seeded in a T175 flask and allowed to attach overnight. Cells were then treated with 50 nM IMP1088 or DMSO for 6-36 h. Cells were lysed as for Th-MYCN cells in section **6.6.2** except that TX-114 based phase separation was carried out after debris removal. Every condition was prepared in triplicate.

6.6.5 TX-114 based phase separation

Cell lysates were incubated at 37 $^{\circ}$ C for 10 min, then centrifuged ($17,000 \times g$, 10 min, rt). The top phase was removed and kept as the aqueous fraction. The detergent phase was washed with 0.1% precondensed TX-114 in PBS pH 7.4 with cOmplete protease inhibitor and clarified on ice. The above steps were repeated three times in total except the wash fractions were discarded. After the washes, the proteins in each phase were precipitated by chloroform-methanol and resuspended in 0.2% SDS in 50 mM HEPES pH 8.0.

6.7 Proteomic sample preparation

6.7.1 Peptide desalting (stage tipping)

Peptide desalting was carried out as previously described¹¹¹. Homemade stage tips were prepared by placing three SDB-XC membranes inside a 200 μ L pipette tip. Stage tips were activated using 150 μ L MeOH followed by equilibration using 150 μ L H₂O. Acidified samples were transferred to the stage tips, washed with 150 μ L H₂O and peptides eluted in 60 μ L of 60% MeCN. Stage tips were centrifuged ($2,000 \times g$, 2 min, rt) after each step to ensure all liquid passed through. Samples were evaporated at 45 $^{\circ}$ C until dry in a Savant SPD1010 vacuum concentrator (ThermoFisher) and stored at -80 $^{\circ}$ C until submission. All solvents used were proteomics-grade.

6.7.2 TMT loading check

To ensure consistent loading across all samples, 0.25 µg (5 µL) of TMT-labelled peptides for each sample were first multiplexed into one protein LoBind Eppendorf (Eppendorf, 0030108116) and desalted on stage tips. Dried peptides were reconstituted in 10 µL of 2% (v/v) MeCN and 0.5% (v/v) TFA in H₂O by sonication (3 min), vortexing (5 min) and sonication again (5 min) and filtered through Durapore (0.1 µm) filter tips into MS vials. 3 µL of peptide was injected into a QExactive Orbitrap mass spectrometer coupled to an Ultimate 3000 LC (ThermoFisher) using an Easy Spray Nano-source. Data was obtained using data-dependent acquisition.

6.7.3 NeutrAvidin bead dimethylation

For the *in vivo* SrtA proteomic profiling experiment, NeutrAvidin beads were first dimethylated¹¹². Beads were washed with 100 mM triethylammonium bicarbonate (TEAB) pH 8.0 five times, then treated with 5 bead volumes of 100 mM TEAB containing 0.2% (w/v) formaldehyde and 25 mM sodium cyanoborohydride (NaBH₃CN). The mixture was incubated with shaking at rt for 2 h, the supernatant removed, and beads washed twice with 1% ethanolamine in 100 mM TEAB. Beads were then washed with 50 mM HEPES pH 8.0 thrice and stored at 4 °C until used the next day.

6.7.4 YnMyr pull-down profiling

IMR5 samples: 500 µg protein taken forward for proteomics as previously described¹¹¹. Proteins were ligated to AzRB as described in section 6.1.8 and proteins precipitated by chloroform-methanol. NeutrAvidin agarose beads (Pierce, 29201) (30 µL per 500 µg sample) were washed twice with 0.2% (w/v) SDS in 50 mM HEPES pH 8.0 and resuspended in 1 volume of the same buffer. Samples were centrifuged (17,000 × *g*, 4 min, rt), added to NeutrAvidin beads and incubated with shaking (1000 rpm, 3 h, rt). The supernatant was removed and beads washed thrice with 0.2% (w/v) SDS in 50 mM HEPES pH 8.0, twice with 1% (w/v) SDS in 50 mM HEPES pH 8.0, thrice with 50 mM HEPES pH 8.0 and resuspended in 30 µL of 50 mM HEPES pH 8.0. Beads were centrifuged (3,000 × *g*, 3 min, rt) between each wash. 3 µL of 55 mM TCEP and 165 mM CAA in 50 mM HEPES pH 8.0 was added to each sample and incubated for 10 min at rt. Proteins were then digested using 5 µg trypsin per sample shaking overnight (1000 rpm,

37 °C). Samples were then centrifuged ($3,000 \times g$, 3 min, rt), the supernatant kept and acidified to 0.5% (v/v) TFA (Sigma, T6508) (pH<3). Samples were desalted by stage tipping (section 6.7.1), dried peptides reconstituted in 10 μ L of 2% (v/v) MeCN and 0.5% (v/v) TFA in H₂O by sonication (3 min), vortexing (5 min) and sonication again (5 min) and filtered through Durapore (0.1 μ m) filter tips into MS vials. 1 μ L of sample was injected into a QExactive Orbitrap mass spectrometer coupled to an Ultimate 3000 LC (ThermoFisher) using an Easy Spray Nano-source. Data was acquired using data-dependent acquisition.

SHEP21N samples: SHEP21N cells (high and low MYCN) lysates (1.5 mg/mL) were prepared as for IMR5 cells above. For the optimisation experiment, 100, 250, 500, 750, 1000 μ g protein was taken forward; for the main experiment, 100 μ g was taken forward. Proteins were ligated to AzRB as described in section 6.1.8 and proteins precipitated by SP4. NeutrAvidin agarose beads (1:10 bead:sample v/v ratio) were prepared as above. For bead volumes <30 μ L, control agarose beads (Pierce, 26150) were added to the NeutrAvidin beads up to 30 μ L to improve sample handling. Samples were added to the beads and incubated overnight with end-to-end mixing at 4 °C. The supernatant was removed, and beads washed twice with 1% (w/v) SDS in 50 mM HEPES pH 8.0 and thrice with 50 mM HEPES pH 8.0. Samples were resuspended in 1 bead volume of 50 mM HEPES pH 8.0, and 0.5 bead volumes of 40 mM TCEP and 160 mM CAA in 50 mM HEPES pH 8.0 respectively was added to each sample and incubated for 10 min at rt. Beads were washed twice with 50 mM HEPES pH 8.0 and resuspended in 1 bead volume of 50 mM HEPES pH 8.0. 0.1 μ g trypsin was added to each sample and incubated with shaking overnight (1000 rpm, 37 °C). Samples were centrifuged ($7,000 \times g$, 4 min, rt) and the supernatant kept. Beads were washed with 50 μ L of H₂O and the wash combined with the previous supernatant. Peptides were acidified to pH<3 (0.5% formic acid final concentration) and acidified peptides filtered through a Durapore filter plate (Merck) and frozen at -20 °C until submission. For submission, peptide solutions were thawed on ice and half of the sample loaded onto Evotips (Evosep, EV2011) following manufacturer's instructions. Samples were analysed on a timsTOF HT (Bruker) coupled to an Evosep One (Evosep). Data was obtained using data-dependent acquisition.

6.7.5 Global proteomics profiling

LFQ protocol: Protein lysates were normalised to 1 mg/mL and 50 μ g protein taken forward for proteomic sample preparation. First, cysteines were reduced and alkylated by the

addition of 0.5 volumes of 40 mM TCEP and 160 mM CAA in 50 mM HEPES pH 8.0 to each sample and incubation for 5 min at 70 °C with shaking (800 rpm). Next, proteins were precipitated by SP4. Protein pellets were then resuspended in 125 µL digestion buffer and incubated with shaking overnight (1000 rpm, 37 °C). The next day, peptide concentration was determined using the Pierce Quantitative Fluorometric Peptide Assay (ThermoFisher, 23290), 5 µg of peptides aliquoted out and evaporated at 45 °C until dry in a Savant SPD1010 vacuum concentrator. Peptides were stored at -80 °C until sample submission. Peptides were reconstituted in 20 µL of 2% (v/v) MeCN and 2% formic acid in H₂O by sonication (3 min), vortexing (5 min) and sonication again (5 min) and 3 µL (0.75 µg) of peptides injected into an Orbitrap Eclipse coupled to an Ultimate 3000 RSLCnano HPLC (ThermoFisher).

TMT protocol: This refers to the *in vivo* global proteomics study. Proteins were precipitated by chloroform-methanol and resuspended in 0.2% SDS in 50 mM HEPES pH 8.0 (50 µL, 1 mg/mL). 5 µL of 55 mM TCEP and 165 mM CAA in 50 mM HEPES pH 8.0 was added to each sample and incubated for 45 min at rt. Proteins were precipitated by SP4, resuspended in 50 mM HEPES pH 8.0, and digested using 0.5 µg trypsin overnight (1000 rpm, 37 °C). The next day, peptides were quantified and 2.5 µg peptide (diluted to 0.1 µg/mL) per sample was taken forward for TMT labelling using a TMT 10plex (ThermoFisher, 90110, lot number WF319143). 1/20th of a TMT vial was dissolved in 25 µL MeCN, mixed with the corresponding sample and incubated for 2 h at rt shaking gently. As only 8 samples were processed, the 130N channel and 129N channels were omitted as these are associated with the highest ion coalescence⁴⁰⁸. The reaction was quenched with 1 µL of 5% hydroxylamine in 50 mM HEPES pH 8.0. A TMT loading check was carried out and TMT labelled peptides for all samples were multiplexed into one LoBind Eppendorf. The samples were combined such that the TMT labelling for each channel was equal. Peptides were fractionated using the Pierce High pH Reversed-Phase Peptide Fractionation Kit (ThermoFisher, 84868) and peptides evaporated at 45 °C until dry in a Savant SPD1010 vacuum concentrator. Dried peptides were reconstituted in 15 µL of 2% (v/v) MeCN and 2% formic acid in H₂O and 5 µL of peptide injected into an Orbitrap Eclipse mass spectrometer coupled to an Ultimate 3000 RSLCnano HPLC using an Easy Spray Nano-source. Data was obtained using data dependent acquisition.

6.7.6 Sortase A proteomic profiling

150 μ L of protein lysate (1 mg/mL) was taken forward for sample preparation. 175 μ M biotin-ALPET peptide and 0.2 μ M SrtA pentamutant¹¹² were added to each sample and incubated overnight at 4 °C with shaking (600 rpm). The reaction was quenched with 1.5 μ L of 0.5 M EDTA, proteins precipitated by SP4 and resuspended in 0.2% (w/v) SDS in 50 mM HEPES pH 8.0. Samples were added to 225 μ L of derivatised beads and incubated for 2 h at rt with mild shaking (800 rpm). The samples were then centrifuged (7000 rpm, 4 min, rt) and the beads washed twice with 0.2% (w/v) SDS in 50 mM HEPES pH 8.0, thrice with 50 mM HEPES pH 8.0 and resuspended in 50 μ L of 50 mM HEPES pH 8.0. Samples were partially digested with 0.4 μ g LysC (Promega, VA1170) for 1 h at 37 °C with shaking (800 rpm), the beads pelleted by centrifugation (7000 rpm, 4 min, rt) and the supernatant taken to a new Eppendorf. Cysteines were reduced and alkylated by the addition of 0.5 volumes of 40 mM TCEP and 160 mM CAA for 10 min at rt and proteins further digested by 0.1 μ g trypsin overnight (1000 rpm, 37 °C). The next day, peptides were taken forward for TMT labelling using a TMT 10plex (lot number WF319143) as above. Following TMT labelling, samples were processed following the same protocol as for the *in vivo* global proteomic experiment.

6.8 Proteomic processing protocols

As various instruments and analysis methods were used in this thesis to process proteomic experiments, reflecting changes in the instrumentation accessible to the Tate group over the past four years, **Table 6.4** describes the quantification method and mass spectrometer associated with each proteomic experiment.

6.8.1 LC-MS conditions: QExactive

Peptides (1 μ L) were loaded onto a 100- μ m internal dimension $\times$ 2-cm Acclaim PepMap 100 Precolumn (ThermoFisher Scientific) and separated using a 75- μ m internal dimension $\times$ 50-cm, 2- μ m particle EASY-Spray HPLC C18 analytical column (ThermoFisher Scientific). Analytical solvents A: 0.1% FA and B: 80% acetonitrile plus 0.1% FA. Peptides were separated at a 250 nL/min flow rate by a 70 min analytical gradient which was 5%–35% B over 45 minutes, rising to 99% B over 5 minutes, followed by a 20 minute wash at 99% solvent B. Analytical column was held at 45°C. Data were acquired in data-dependent

acquisition mode with the following settings: MS1 acquisition settings: 350–1650 m/z window, 70,000 resolution, 10^6 AGC target, 20 ms maximum injection time; MS2 acquisition settings: 17,500 resolution, quadrupole isolation at an isolation width of m/z 2, high-energy collisional dissociation fragmentation (NCE 25) with fragment ions scanning in the Orbitrap from m/z 120, 10^5 AGC target, 120 ms maximum injection time. Dynamic exclusion was set to ± 10 ppm for 20 seconds. MS2 fragmentation was triggered on precursors 10^2 counts and above. Up to 10 of the most abundant isotope patterns with a charge of +2 to +7 were selected for fragmentation.

6.8.2 LC-MS conditions: timsTOF HT

Peptides were separated using an 8cm x 150 μ m, 1.5 μ m analytical column (Evosep) using the Evosep 60SPD workflow. Analytical solvents A: 0.1% FA and B: acetonitrile plus 0.1% FA. Column was held at 40°C. Data was acquired in data dependent acquisition PASEF mode, the following settings were used: m/z range from 100 m/z to 1700 m/z, ion mobility range from $1/K_0 = 1.30$ to 0.85 Vs/cm² using equal ion accumulation and ramp times in the dual TIMS analyser of 100 ms each. The collision energy was lowered as a function of increasing ion mobility from 59 eV at $1/K_0 = 1.6$ Vs/cm² to 20 eV at $1/K_0 = 0.6$ Vs/cm². Isolation width was lowered as a function of decreasing m/z from 3 m/z at 800 m/z to 2 m/z at 700 m/z. Active exclusion was applied for 0.4 min. Each cycle consistent of 4 PASEF ramps (total cycle time 0.53 sec) with 2.75 ms measuring time allowed for each selected precursor.

6.8.3 LC-MS conditions: Orbitrap Eclipse

For LFQ data (all data run on the Eclipse except for *in vivo* proteomic experiments), peptides (3 μ L, 0.25 μ g/ μ L) were loaded on to a trap column (Acclaim PepMap 100 75 μ m x 2cm nanoViper; ThermoFisher Scientific) using loading buffer (2% MeCN, 0.05% TFA). Peptides were separated using an Acclaim PepMap RSLC C18 50 cm column (ThermoFisher, ES803) using an EasySpray source using a gradient of 2-40% buffer B in the first 93 min rising to 95% buffer B for 1 minute followed by a 10 min wash at 95% buffer B at a flow rate of 275 nl/min. The analytical column was held at 40°C. Analytical solvents A: 5% DMSO, 0.1% FA and B: 75% MeCN, 5% DMSO and 0.1% FA. Data was obtained in data-dependent mode. Survey scans were acquired by the Orbitrap Eclipse at a resolution of 120,000 at m/z 200. Scans were acquired from 350 to 1500 m/z with the

following parameters: RF Lens 30%, AGC Target 400,000, normalised AGC target of 100% and maximum injection time 50 ms. Monoisotopic Precursor Selection (MIPS) was set to peptide. Dynamic exclusion was enabled with an exclusion duration of 20 s after 1 times, with a mass tolerance of 10 ppm. The minimum intensity was set to 10,000. Precursors with charge states 2-6 were selected for MS/MS with an isolation window of 1.2 m/z, AGC Target of 10,000, normalised AGC Target of 100% and a maximum injection time of 100 ms. Precursor ions were fragmented by HCD with a collision energy of 30%. MS2 Scans were obtained in the ion trap at a rapid scan rate and obtained in centroid mode.

For *in vivo* TMT samples, the protocol contained the following changes. First, the chromatography was changed to a gradient of 2-30% buffer B in the first 85 min, followed by 30-40% buffer B in the next 8 min, rising to 95% buffer B in the next minute and finally 10 min at 95% buffer B at a flow rate of 275 nl/min. The column was held at 40°C. Analytical solvents A: 0.1% FA and B: 80% MeCN, 0.1% FA. Secondly, selected parameters were changed for the mass spectrometer: The MS1 scan range was 380-1500 m/z, the minimum intensity was set to 50,000 and a precursor fit error of 50% with a fit window of 1.2 was implemented. For MS/MS settings, the isolation window was set to 0.7, the AGC target to 100,000 and a maximum injection time of 86 ms. Scan Range Mode was defined as First Mass which was set to 110. The normalised AGC Target was 200%. Precursor ions were fragmented with a collision energy of 38%. Scans were obtained in the Orbitrap with a resolution of 50,000 at 200 m/z.

6.8.4 MaxQuant data analysis

Raw data obtained from the Eclipse and QExactive were processed using MaxQuant (version 1.6.10.43) using the in-built Andromeda search engine. The MS/MS spectra were matched against the human reference proteome with isoforms (UniProt, accessed January 2022). Cysteine carbamidomethylation was defined as a fixed modification; methionine oxidation, N-terminal acylation and N-terminal *N*-myristoylation were set as variable modifications. “Trypsin/P” was chosen as the digestion mode and a maximum of two missed cleavages allowed. Label-free quantification was selected with “Fast LFQ” enabled. Both “unique and razor peptides” were used for protein quantification. “Match between runs” was enabled. All other settings were kept as default. To check TMT labelling efficiency, TMT labels on lysines and N-termini were set as variable modifications. For the

in vivo data quantified using TMT, reporter ion MS2 quantification was used with the relevant TMT10plex lysine and N-terminal labels set as isobaric labels, accounting for the isotopic correction factors.

6.8.5 FragPipe data analysis

ddaPASEF Bruker .d files were processed using FragPipe (version 22.0) using the inbuilt “LFQ MBR” workflow. Default settings, other than MaxLFQ min ions was set as 1, were used. Bruker .d files were searched using MSFragger (version 4.1) with the following parameters: strict trypsin digestion; up to 2 missed cleavages allowed; precursor ion tolerance: 20 ppm; trimming of protein N-terminal methionine; methionine oxidation, N-terminal acetylation and glycine labelling by the YnMyr-AzRB adduct as variable modifications; cysteine carbamidomethylation as a fixed modification; Human database (UniProt, downloaded 13 July 2023 with decoys (50%) and contaminants added with the inbuilt FragPipe tool. MSFragger search results were processed using Percolator (version 3.5) for peptide-spectrum match validation, followed by Philosopher (version 5.1.1) for protein and FDR filtering. Label-free quantification values were calculated using the MaxLFQ algorithm using IonQuant (version 1.10.27) with match between runs enabled and min ions set as 1.

6.9 Data analysis of -omics experiments

6.9.1 Proteomic data analysis

Processed proteomic data were analysed with Perseus (version 1.6.14.0). Protein groups were filtered against contaminants, proteins identified only by site and reverse annotations. Protein intensities (normalised LFQ or TMT) were transformed using a base 2 logarithm. TMT intensities were normalized by median subtraction. TMT labelling efficiencies were calculated to be >75%. Protein groups were filtered to allow one missing value across the replicates in each condition for the two-condition comparison of interest. For YnMyr pull-down proteomics, protein groups were filtered to require three valid values across replicates in a single condition, and missing values were imputed from a downshifted normal distribution (width=0.3, downshift=1.8). A two-tailed student's t-test was then performed to determine abundance changes and significance. Volcano plots were plotted in Prism.

6.9.2 GSEA

For mRNA-Seq data, the normalised gene count matrix as a .gct file was used as an input for the GSEA application (version 4.3.2, Broad Institute). Gene identifiers were collapsed using the Ensembl_Gene_ID chip and ranked using the Signal2Noise metric. Sample annotation was uploaded as a .cls file. For proteomics data, proteins were first pre-ranked using $\log_2\text{FC} \times \log_{10}(\text{p-value})$ as the ranking metric. Gene sets were automatically downloaded from MSigDB (Broad Institute) and GSEA was run against the human Hallmark gene sets and the KEGG_MEDICUS and KEGG_LEGACY subsets of the Canonical Pathway gene sets (mRNA sequencing) or the mouse Cell type Signature gene sets (proteomics). In both cases, 1000 permutations over the gene sets and a weighted enrichment statistic was used. Gene sets with more than 500 or less than 15 members were excluded. The significance threshold for enriched gene sets was set as $\text{FDR} < 0.05$.

6.9.3 EnrichmentMap analysis

The results of the GSEA on the mRNA-Seq data was analysed using the EnrichmentMap plugin (version 3.4.0) in Cytoscape (version 3.10.1). Significantly enriched gene sets were clustered based off similarity in gene membership, with the default edge connectivity of 0.375 (50/50 Jacard overlap combination metric) used. Gene clusters were identified using AutoAnnotate (version 1.5.1) using MCL clustering, and the cluster membership and annotated function further refined based on manual inspection.

6.9.4 Ingenuity Pathway Analysis

After initial proteomic analysis in Perseus, protein $\log_2\text{FC}$ and p-values were exported and uploaded into the IPA application. To carry out ORA, a “Core Analysis” was carried out against the Ingenuity Knowledge Base reference set. Both direct and indirect relationships were considered. The thresholds used for proteins to be included in the analysis were set to $p < 0.05$ and $|\log_2\text{FC}| > 1.2$ for *in vitro* experiments or $|\log_2\text{FC}| > 0.585$ *in vivo* experiments given the larger effect size of NMTi in this model. The significance threshold for enriched Canonical Pathways was set to $\text{adj. p-value} < 0.01$.

6.9.5 Contaminant analysis

Contaminant analysis was carried out in Skyline (MacCoss Lab Software) using the method described in reference ³⁶⁷.

6.9.6 Protein localisation analysis

GO enrichment was conducted using PantherDB³⁶⁸ as a statistical overrepresentation test. Overrepresentation of gene sets represented in the “GO cellular component complete” was assessed using Fischer’s exact test and p-values corrected using Benjamini-Hochberg FDR.

6.9.7 STRING PPI analysis

Significantly differentially regulated proteins ($p < 0.05$ and $|\log_2FC| > 0.26$) were analysed by the stringApp plugin (version 2.0.1) in Cytoscape. To generate protein interaction networks, only high confidence interactions (confidence score > 0.7) within the physical subnetwork were considered. For low MYCN samples in the pilot experiment and the 36 h timepoints, the resulting network was clustered by MCL clustering using the default inflation value of 3 to aid interpretation. Functions associated with clusters were based on manual annotation and guided by the in-built STRING pathway enrichment analysis tool where necessary.

6.10 Key resource tables

Target	Species	Clone	Dilution	Supplier	Code
ARL1	Rabbit	Polyclonal	1:1000	Proteintech	16012-1-AP
ATF4	Rabbit	Monoclonal	1:1000	Cell Signaling	11815
BiP	Rabbit	Monoclonal	1:1000	Cell Signaling	3177
CHOP	Mouse	Monoclonal	1:1000	Cell Signaling	2895
eIF2 α	Rabbit	Polyclonal	1:1000	Cell Signaling	9722
GAPDH	Rabbit	Polyclonal	1:1000	Abcam	Ab9485
IRE-1 α	Rabbit	Monoclonal	1:1000	Cell Signaling	3294
MYC	Rabbit	Monoclonal	1:1000	Cell Signalling	5605
MYCN	Mouse	Monoclonal	1:1000	Sigma-Aldrich	OP-13
Na ⁺ /K ⁺ ATPase	Mouse	Monoclonal	1:1000	Abcam	Ab7671
NDUFAF4	Rabbit	Polyclonal	1:1000	ABclonal	A14345
PERK	Rabbit	Monoclonal	1:1000	Cell Signaling	5683
pS51 eIF2 α	Rabbit	Polyclonal	1:1000	Cell Signaling	9721
pS724 IRE-1 α	Rabbit	Monoclonal	1:1000	Abcam	Ab243665
pT980 PERK	Rabbit	Monoclonal	1:1000	ThermoFisher	MA5-15033
Rab27a	Rabbit	Monoclonal	1:1000	Cell Signaling	69295
Vinculin	Rabbit	Monoclonal	1:10,000	Abcam	ab129002
Mouse IgG (H+L), HRP conjugate	Goat	Polyclonal	1:10,000	Advansta	R--05071-500
Rabbit IgG (H+L), HRP conjugate	Goat	Polyclonal	1:10,000	Advansta	R-05072-500

Table 6.1 List of antibodies and their dilutions used in this study.

RT-qPCR primer	Sequence
NDUFAF4 1 (FWD)	CATTGGAAGGGACCTGAGAAA
NDUFAF4 1 (REV)	AGAGCTAAACTCACACACTCAC
GAPDH (FWD)	CCCTTCATTGACCTCAACTACA
GAPDH (REV)	ATGACAAGCTTCCCGTTCTC

Table 6.2 Primers used for RT-qPCR.

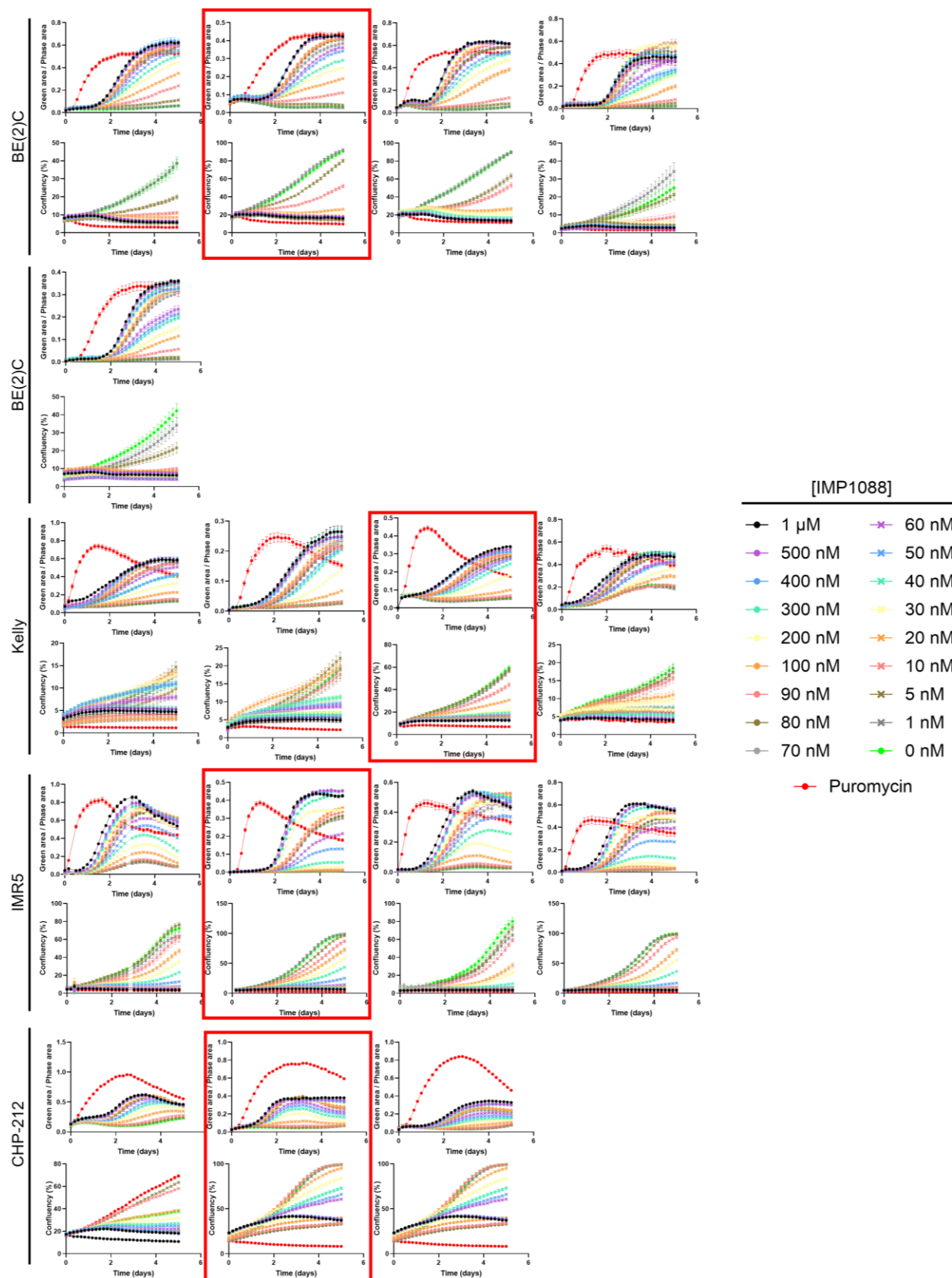
DNA/RNA species	Sequence
NDUFAF4 sgRNA	CGCATAATGTTATGAGGAGA
Donor DNA – G2A template	GTGCGGCCTGAACCTGAGCGCATAATGTTATGAGGAGATGGCT
	GCTCTTGTGATTTCGCGGTATCAGGAATTTCAACCTAGAGAA
Donor DNA – G2G template	GTGCGGCCTGAACCTGAGCGCATAATGTTATGAGGAGATGGG
	GGCTCTTGTGATTTCGCGGTATCAGGAATTTCAACCTAGAGAA
Sequencing primer (FWD)	GGGGCTCCCTTTTAAATGCC
Sequencing primer (REV)	ACGACTAATCTGCTCTCGCA
WT diagnostic primer (FWD)	GAGGAGATGGGAGCACTAGT
G2A diagnostic primer (FWD)	GAGATGGCTGCTCTTGTGATT
G2G diagnostic primer (FWD)	GAGGAGATGGGAGCACTAGT

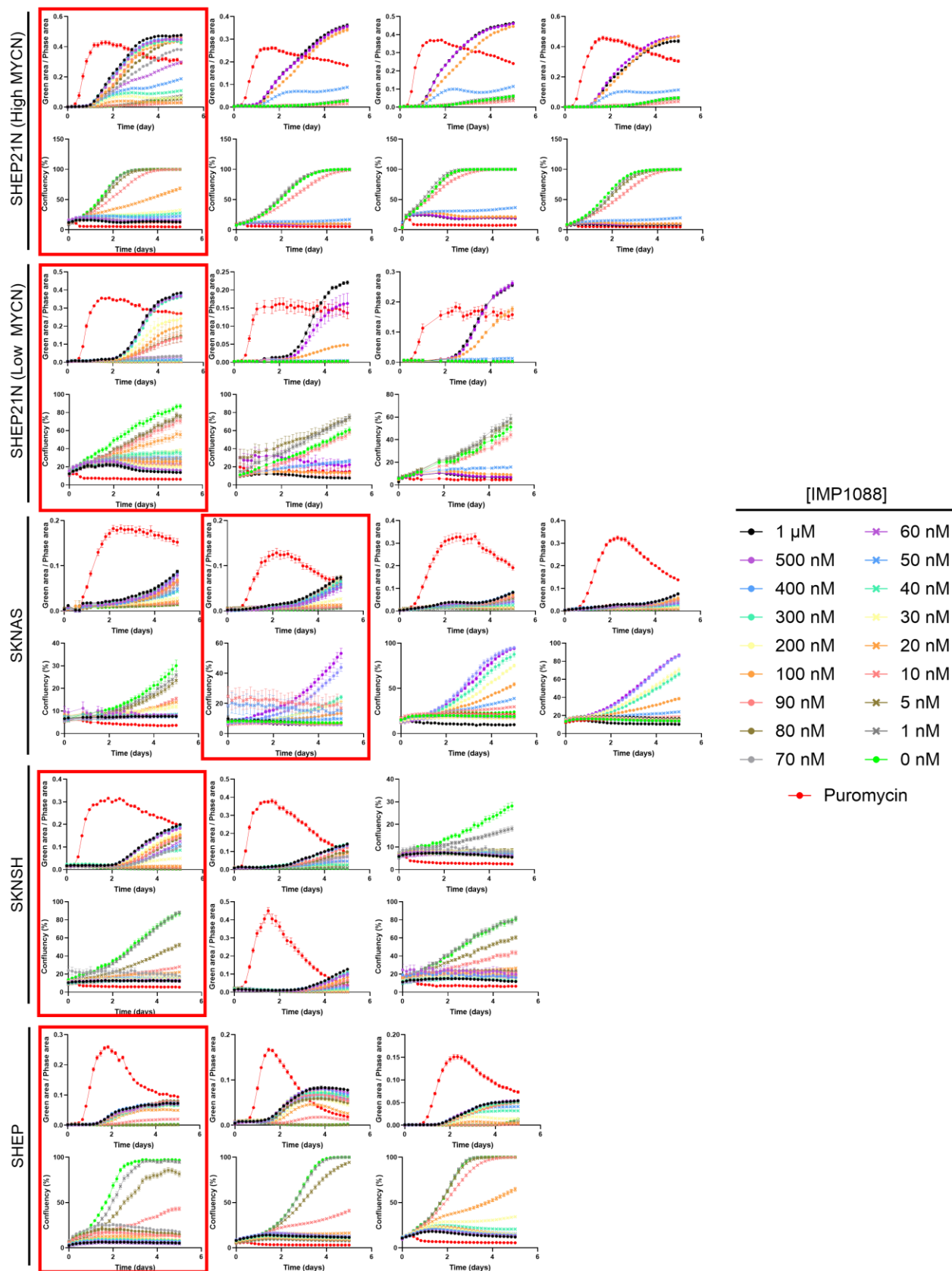
Table 6.3 sgRNA, DNA templates and primers used to generate NDUFAF4 G2A CRISPR-Cas9 knock-ins.

Experiment	Mass spectrometer	Analysis method
IMR5 YnMyr pulldown	Q-Exactive	LFQ
SHEP21N YnMyr pulldown	TimsTOF HT	LFQ
SHEP21N global proteome	Eclipse	LFQ
Th-MYCN <i>in vivo</i> SrtA pulldown	Eclipse	TMT
Th-MYCN <i>in vivo</i> global proteome	Eclipse	TMT
Th-MYCN <i>in vitro</i> global proteome	Eclipse	LFQ
SHEP21N TX-114 fractionated proteomes	Eclipse	LFQ

Table 6.4 Proteomic experiments in this study and their analysis method. The mass spectrometer and quantification method for each proteomic experiment is shown here. All data were acquired using DDA methods.

Chapter 7. Appendix

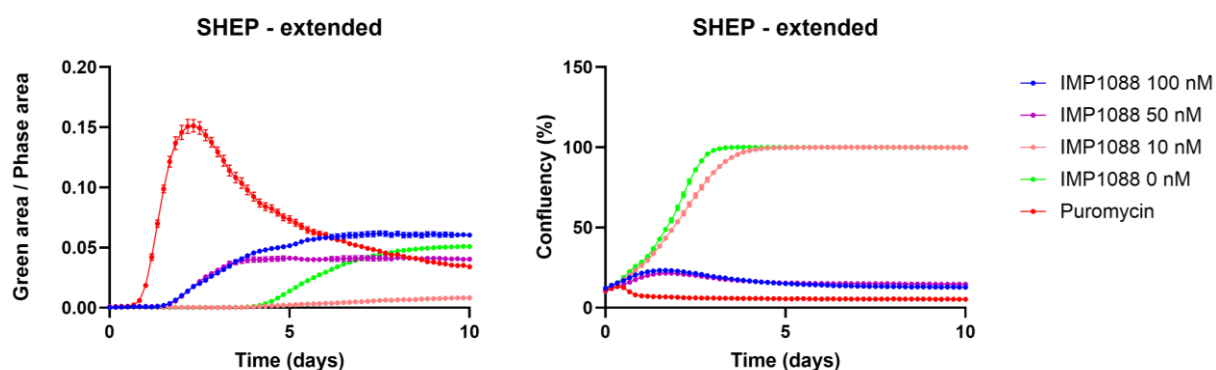




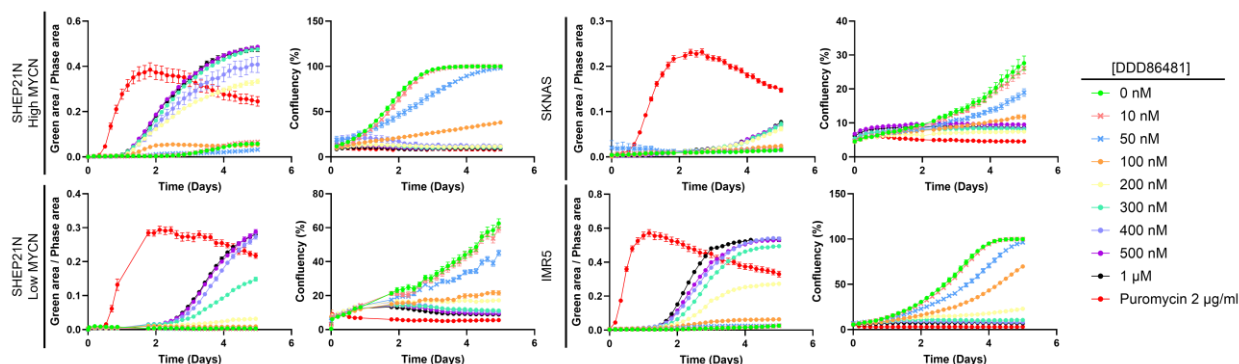
Appendix Figure 1 Raw cytotoxicity and proliferation plots for IMP1088 Incucyte assays. Related to **Figure 2.2** and **Figure 2.3**. Individual cytotoxicity (green area/phase area) and proliferation (phase area) plots to determine IMP1088 IC50s in the neuroblastoma cell line panel. Red box, the dataset corresponding to the representative dose-response plots in **Figure 2.2B**.

Cell line	MYCN amplification	MYC status	ALK status	p53	1p deletion	11q deletion	17q gain
IMR5	Yes	Low	WT	WT	Yes	Yes	Yes
BE(2)C	Yes	Low	WT	C135F	Yes	No	No
Kelly	Yes	Low	F1174L	P177T	Yes	Yes	Yes
CHP212	Yes	Low	WT	WT	Yes	No	Yes
SHEP	No	Low	F1174L	WT	No	No	Yes
SKNAS	No	High	WT	H168R	Yes	Yes	Yes
SKNSH	No	Med	F1174L	WT	No	No	Yes

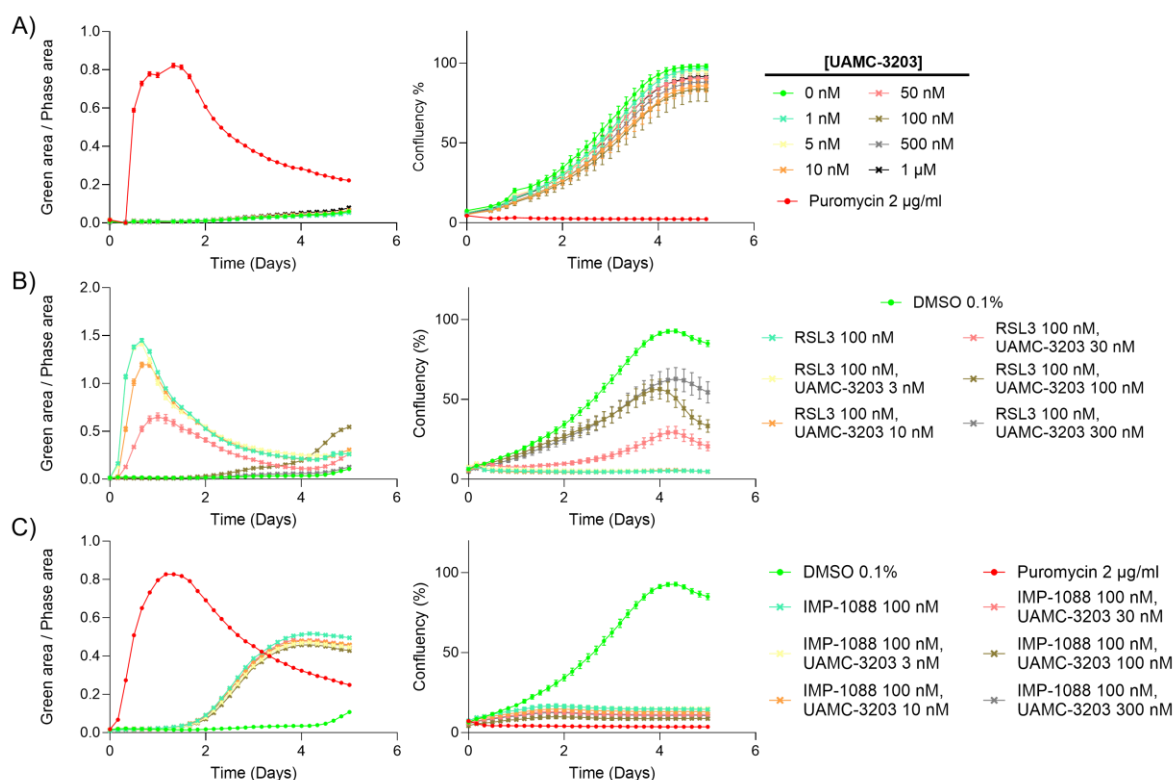
Appendix Table 1 Genetic background of the neuroblastoma cell line panel. Common genetic alterations of the neuroblastoma cell lines used in this study. The SHEP21N line is derived from the SHEP line and therefore is expected to have the same genetic background unless novel alterations have been acquired *in vitro*. Information on the genetic status of these cell lines was obtained from references ^{185,409-414}.



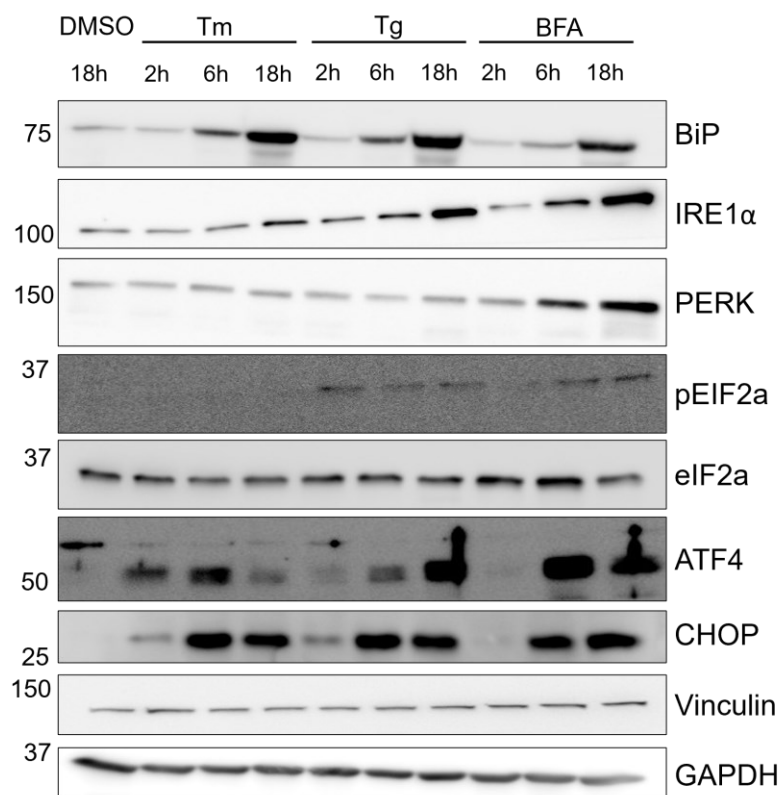
Appendix Figure 2 Effects of IMP1088 on SHEP cells over 10 days. Cytotoxicity and proliferation plots of SHEP cells upon prolonged treatment with IMP1088.



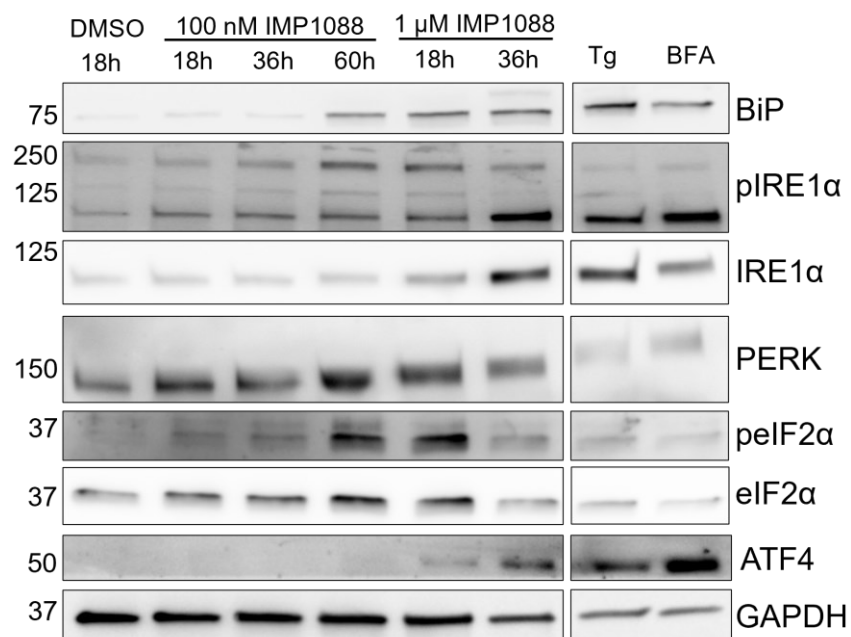
Appendix Figure 3 Sensitivity of neuroblastoma cell lines to DDD86481. Related to Table 2.1 and Figure 2.4. Individual cytotoxicity and proliferation plots of neuroblastoma cells upon treatment with DDD86481, used to calculate the reported IC50 values.



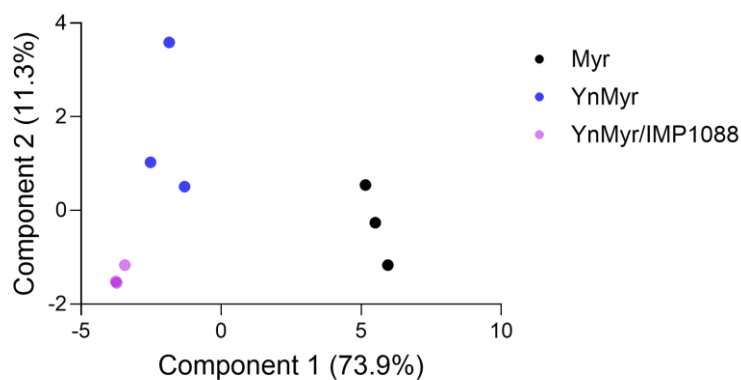
Appendix Figure 4 Validation of UAMC-3203 as a tool compound to inhibit ferroptosis. Cytotoxicity and proliferation plots of IMR5 cells. **(A)** Cells were treated with UAMC-3203 as a single agent to assess the effects of this compound on IMR5 viability. **(B)** Cells were treated with 100 nM RSL3 and increasing concentrations of UAMC-3203 to inhibit ferroptosis. **(C)** Cells were treated with 100 nM IMP1088 and increasing concentrations of UAMC-3203 to inhibit ferroptosis.



Appendix Figure 5 Optimisation of treatment conditions with known UPR inducers. Western blots for proteins involved in the UPR in high MYCN SHEP21N cells upon treatment with 5 mg/mL tunicamycin, 100 nM thapsigargin or 2 µg/mL Brefeldin A for 2-18 h.



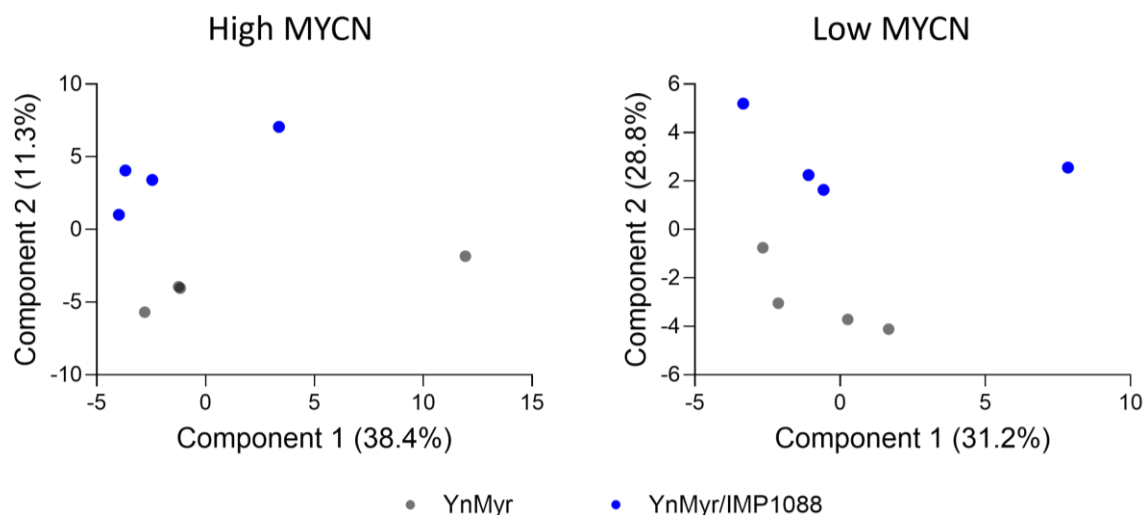
Appendix Figure 6 The UPR is not majorly activated in IMR5 cells upon NMT inhibition. Western blots for proteins involved in the UPR in IMR5 cells upon treatment with 100 nM or 1 μM IMP1088 for 18-60 h. The positive control blots shown are from the same membrane.



Appendix Figure 7 IMR5 proteomic samples separate based on treatment condition. PCA plot for the IMR5 samples analysed by YnMyr proteomics.

Proteins	Sequence
AKAP12	GAGSSTEQR
CHCHD3	GGTTSTR
FMNL2	GNAGSMDSQQTDFR
GNAI2	GCTVSAEDK
GNAI3;GNAI1	GCTLSAEDK
GORASP2	GSSQSVEIPGGGTEGYHVL
HCCS	GLSPSAPAVAVQASNASPPSGCPMHEGK
PPM1G	GAYLSQPNTVK
PRKAB1	GNTSSER
PRKAB2	GNTTSDR
PRKACA	GNAAAAK
PRKACB	GNAATAK
PSMC1	GQSQSGGHGPGGGK
TESC	GAAHSASEEVR

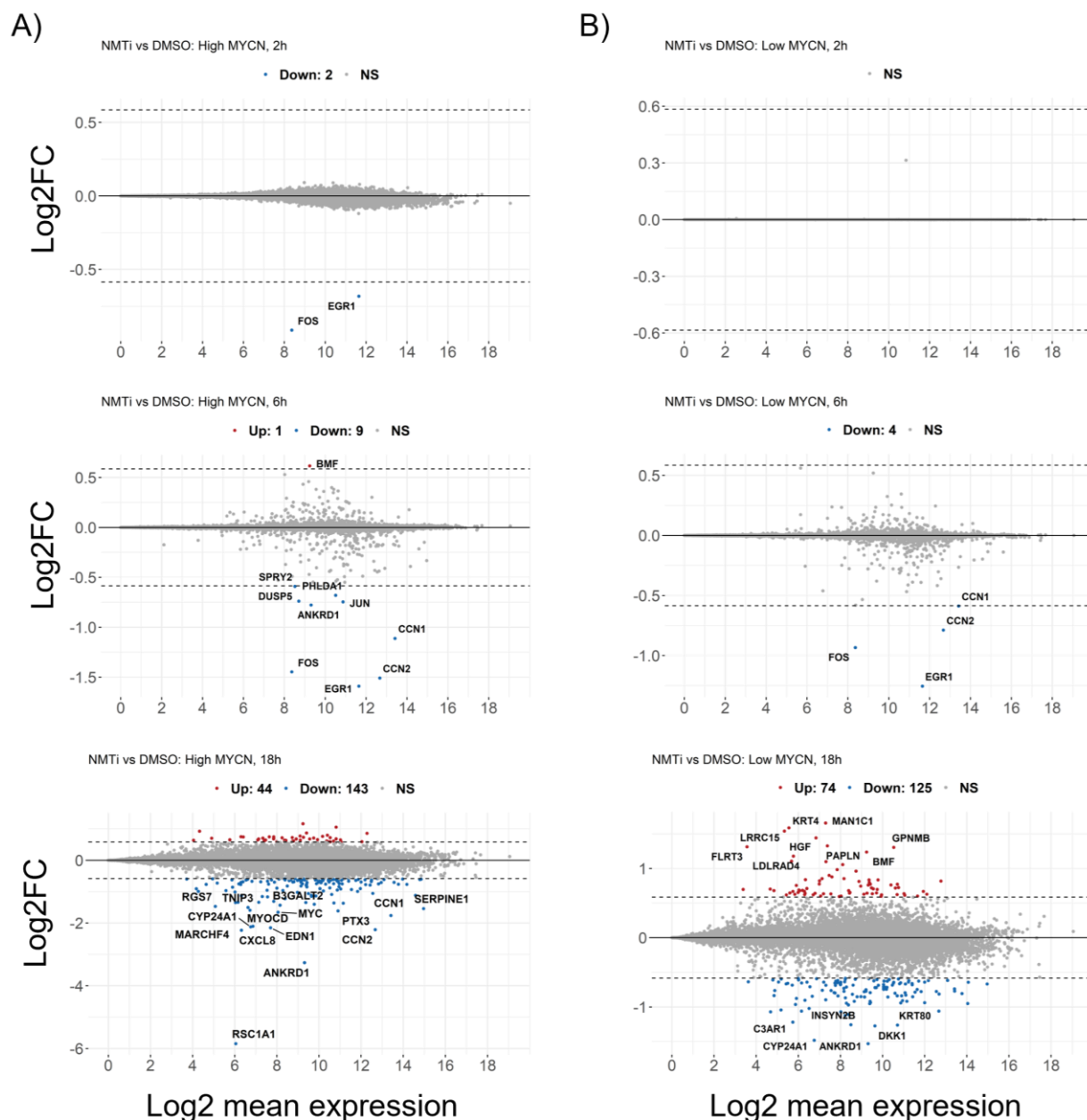
Appendix Table 2 Proteins for which the YnMyr-labelled peptide was directly identified by MS/MS in IMR5 cells.



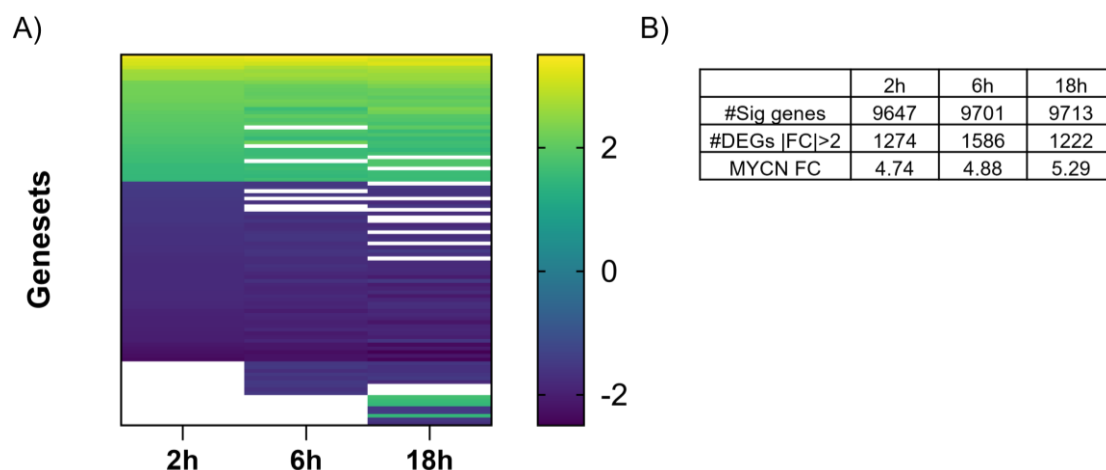
Appendix Figure 8 SHEP21N samples separate by treatment condition. PCA plot for the SHEP21N samples analysed by YnMyr proteomics.

Proteins	Modified Sequence
PPM1G	GAYLSQPNTVK
NIBAN1	GGSSASSQLDEGK
CHCHD3	GGTTSTR
PRKACA	GNAAAAK
PRKACB	GNAATAK
PSMC1	GQSQSGGHGPGGGK
GORASP2	GSSQSVEIPGGGTEGYHVL

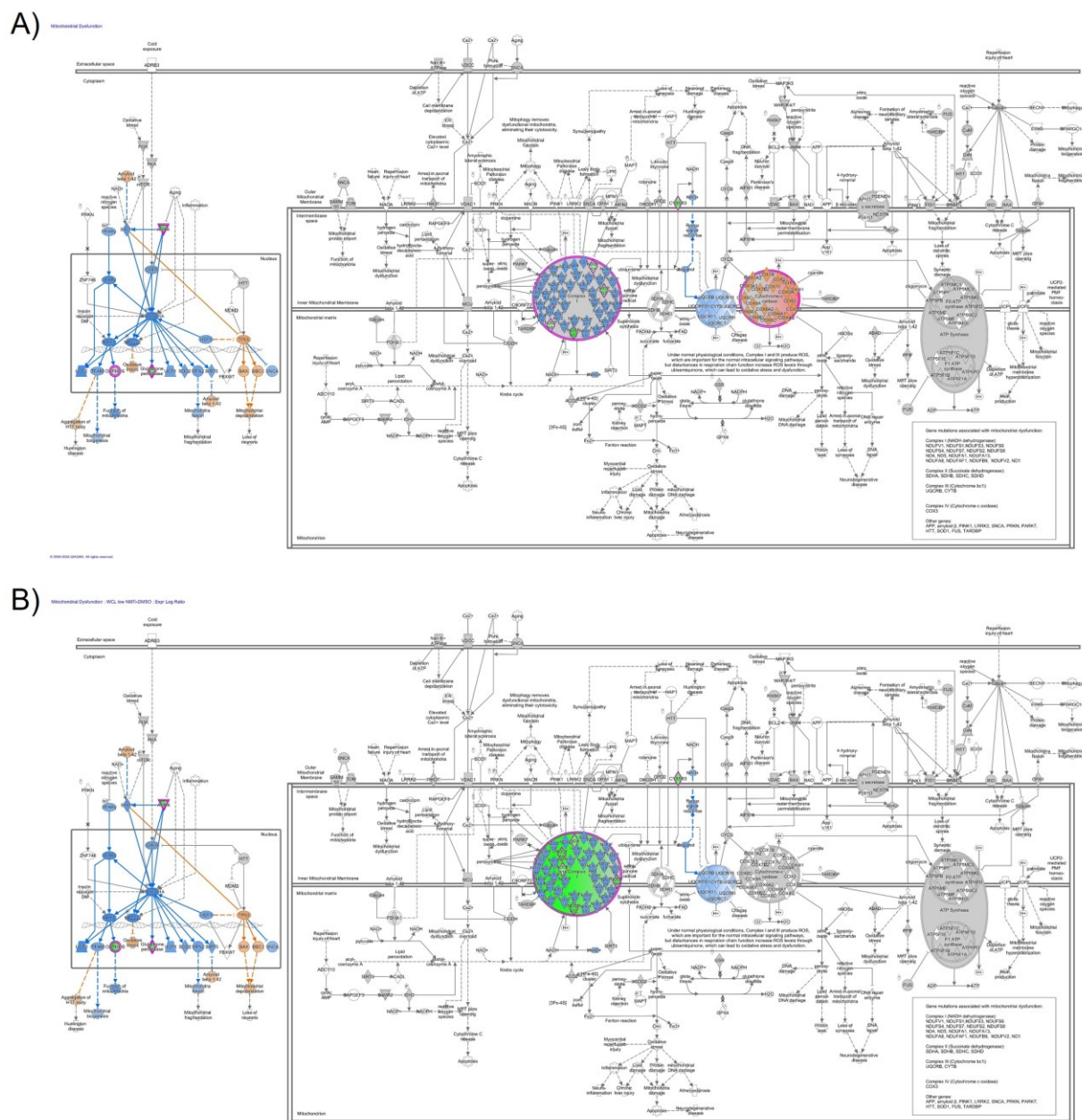
Appendix Table 3 Proteins for which the YnMyr-labelled peptide was directly identified by MS/MS in SHEP21N cells.



Appendix Figure 9 Effects of IMP1088 on transcripts in SHEP21N cells. Related to **Table 3.1** and **Figure 3.4**. MA plots for all protein coding genes upon treatment with IMP1088 (2, 6, 18 h) in high **(A)** and low **(B)** MYCN SHEP21N cells. Coloured dots indicate genes with a fold change of at least 1.5 and adj. p-value<0.05.



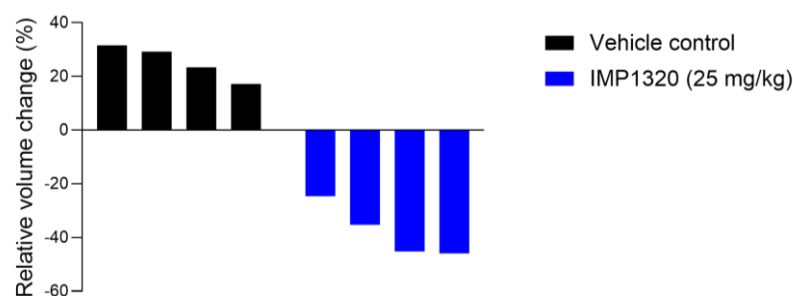
Appendix Figure 10 Similar effects of MYCN expression are observed at each timepoint. (A) Heatmap of the NES scores for gene sets significantly (FDR q-value < 0.05) affected by MYCN expression in SHEP21N cells at each timepoint as determined by GSEA. White indicates that the gene set was not significantly enriched by GSEA at that timepoint. (B) Summary statistics of genes affected by doxycycline treatment (MYCN expression) in the SHEP21N cells.



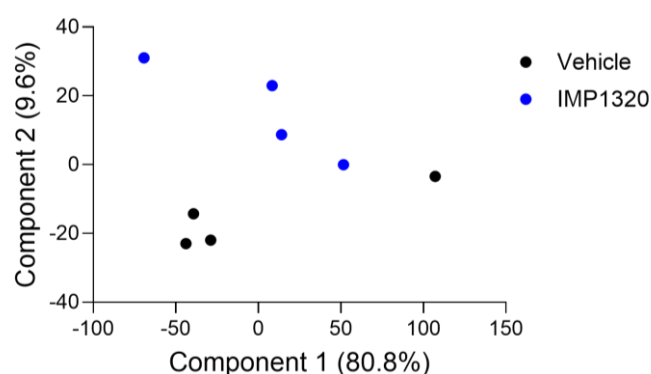
Appendix Figure 11 IMP1088 induces mitochondrial dysfunction in SHEP21N cells. Effects of IMP1088 on the Mitochondrial Dysfunction Canonical Pathway as predicted by IPA analysis in high MYCN **(A)** and low MYCN **(B)** SHEP21N cells. Blue and orange indicates predicted repression and activation; green indicates observed downregulation, purple outlines also indicate significant observed effects.



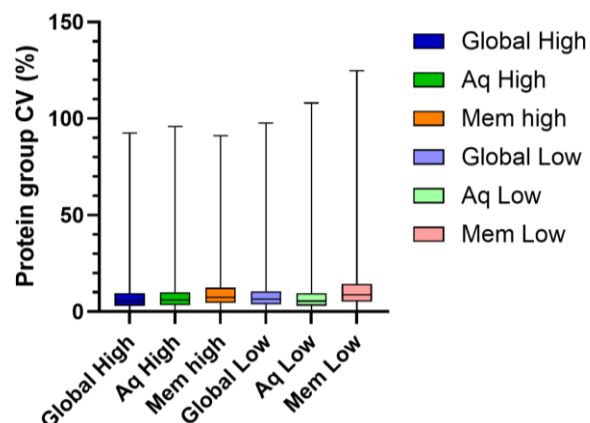
Appendix Figure 12 Canonical Pathways are more significantly affected in high MYCN cells than low MYCN cells. Heatmap comparison of the $-\log(\text{FDR } q\text{-values})$ for significantly affected ($\text{FDR} < 0.01$) Canonical Pathways as indicated by IPA analysis in high and low MYCN SHEP21N cells treated with IMP1088. A high value indicates higher significance.



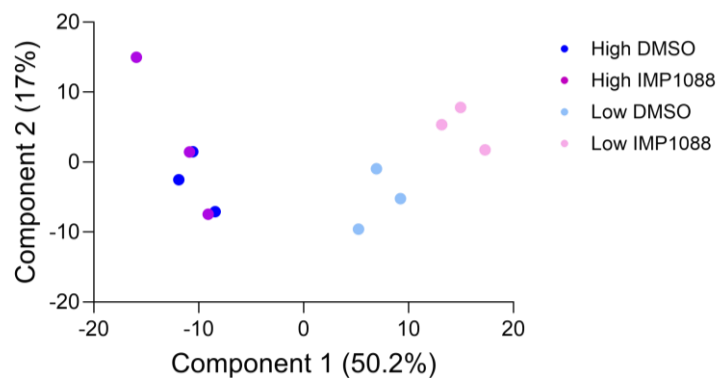
Appendix Figure 13 Effect of IMP1320 after three days in Th-MYCN syngeneic mice. Waterfall plot showing relative changes in tumour volume after 3 days of IMP1320 treatment. Each bar represents an individual mouse.



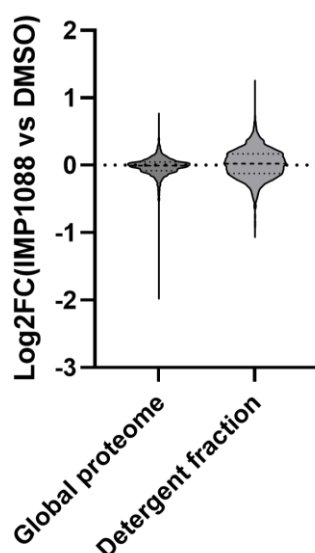
Appendix Figure 14 Clustering of tumours extracted from Th-MYCN mice treated with vehicle or IMP1320. PCA plot for the samples analysed by SrtA proteomics.



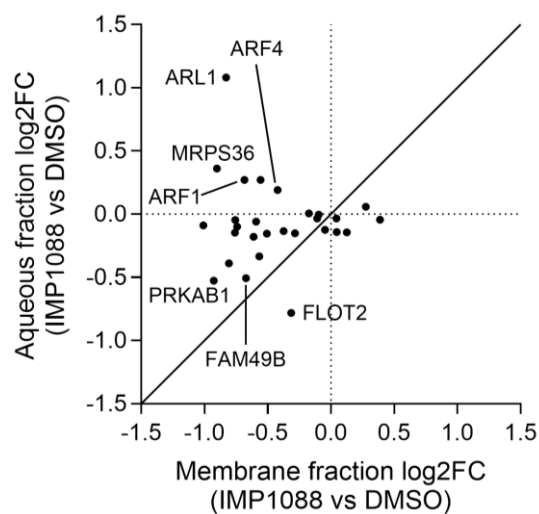
Appendix Figure 15 Protein quantification is generally reproducible in each fraction. Box plot of the CV values for protein groups in the pilot and global proteomic samples.



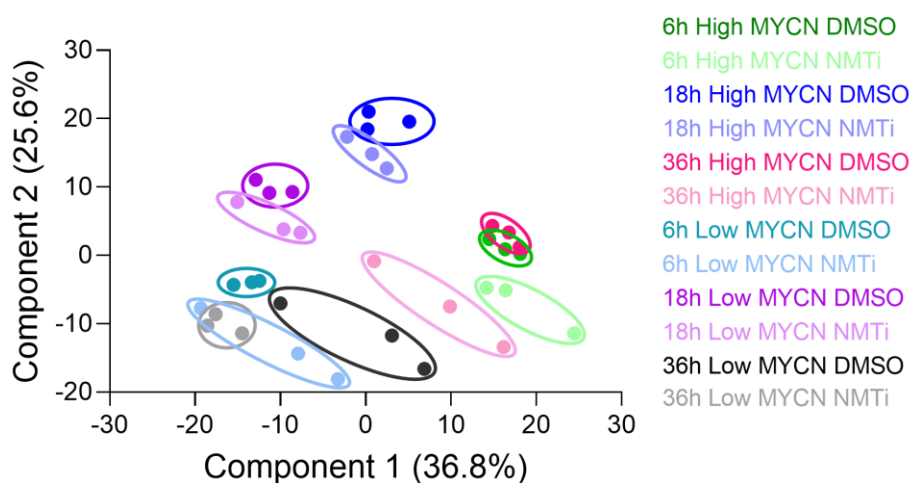
Appendix Figure 16 Clustering of SHEP21N pilot proteomic detergent fractions. PCA plot for the SHEP21N detergent fractions in the pilot study.



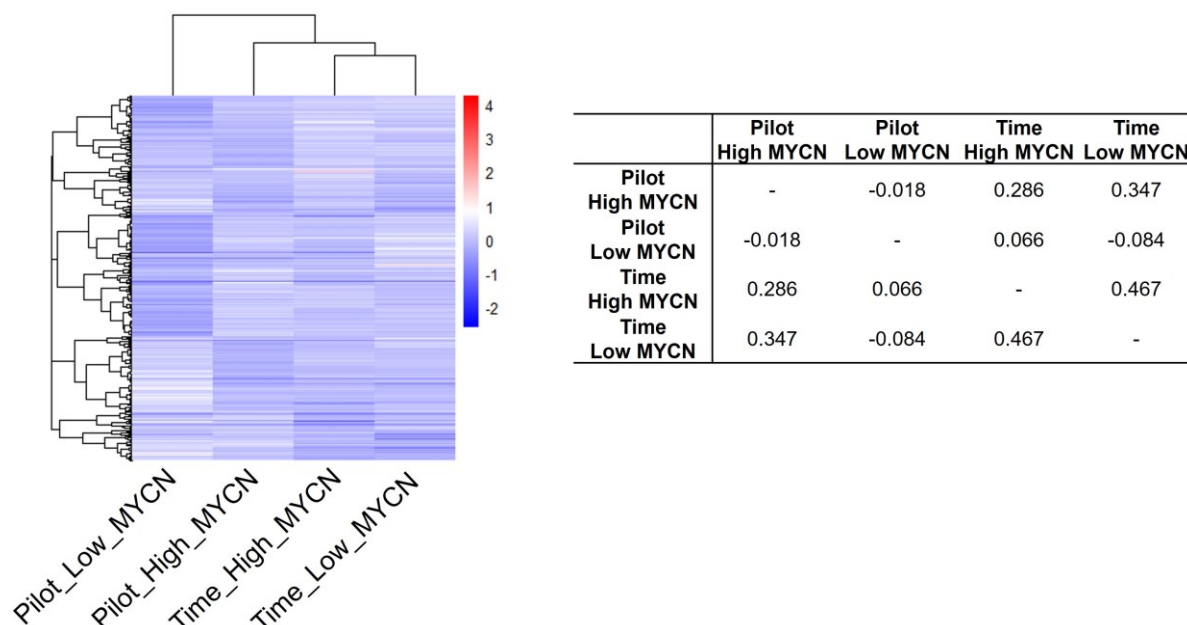
Appendix Figure 17 Protein fold changes in detergent fractions are more pronounced than for global abundance changes. Violin plots for the distribution of protein fold changes in high MYCN detergent and global proteomic samples.



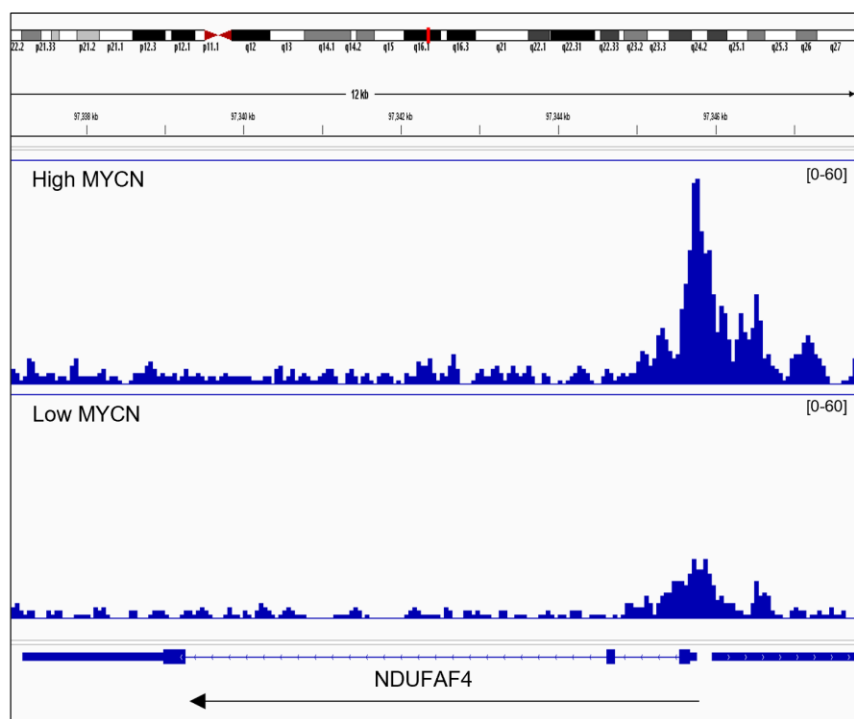
Appendix Figure 18 NMT substrates are both destabilised and mislocalised by NMTi in low MYCN cells. Comparison of protein fold changes for NMT substrates in aqueous and detergent fractions isolated from low MYCN cells after 18 h of 50 nM IMP1088 treatment.



Appendix Figure 19 Clustering of SHEP21N time course detergent fractions. PCA plot for all detergent fractions in the time course study.



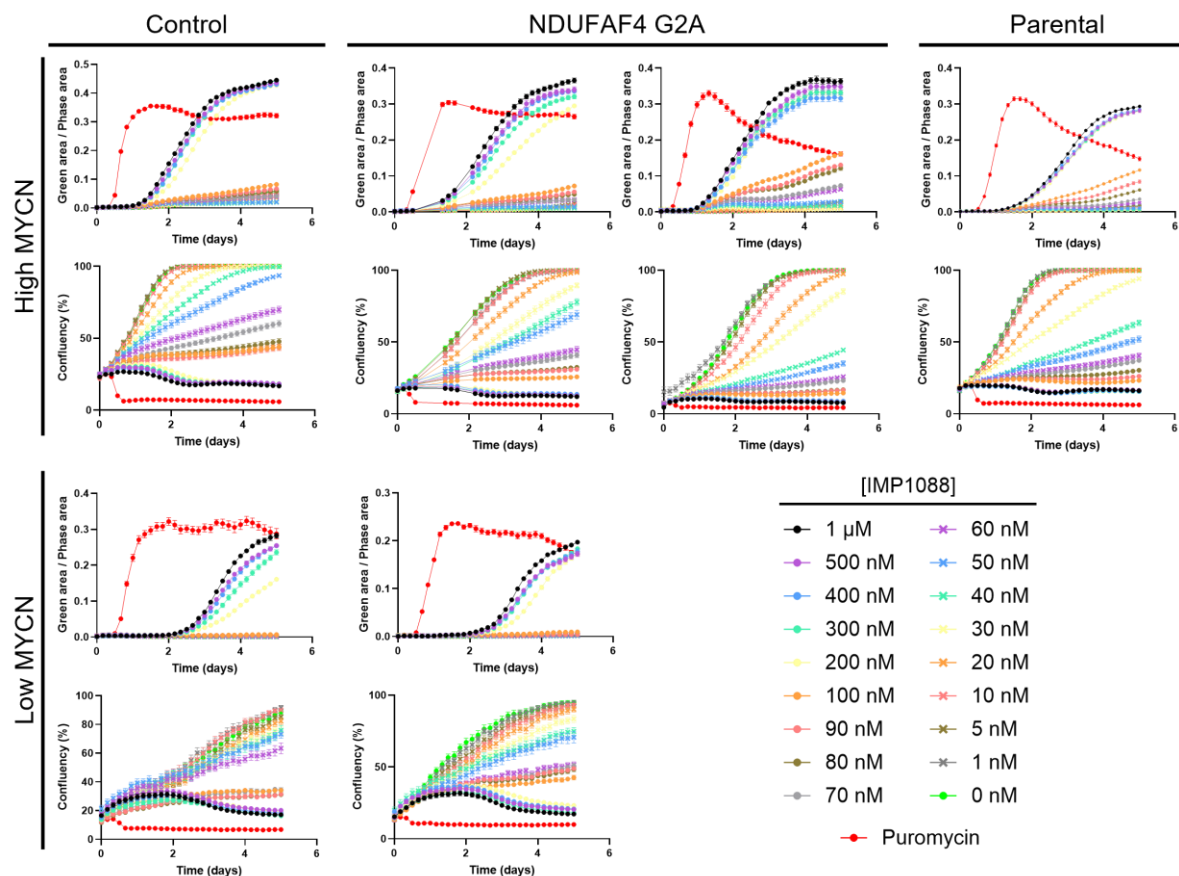
Appendix Figure 20 Fold changes seen in the low MYCN pilot samples are the most dissimilar from the other samples after 18 h of IMP1088 treatment. Hierarchical clustering by Pearson's correlation coefficient of the detergent fractions after 18 h treatment with 50 nM IMP1088. The Pearson correlation coefficients for each sample are indicated in the table (left). Time: Time course



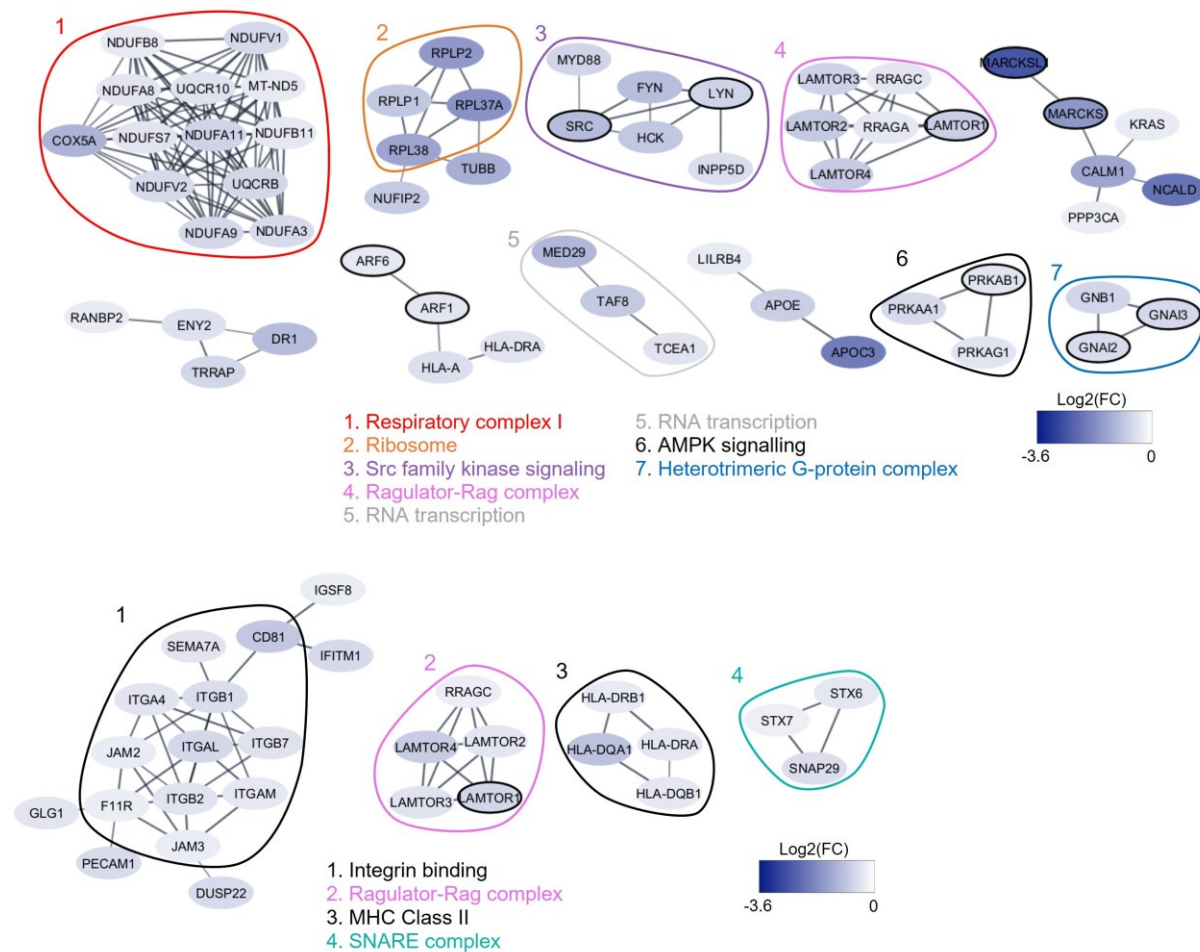
Appendix Figure 21 NDUFAF4 is a direct transcriptional target of MYCN. ChIP-Seq tracks for MYCN binding to the NDUFAF4 locus in high (top) and low (bottom) MYCN SHEP21N cells. Data were downloaded from GSE80154²⁰².



Appendix Figure 22 The NDUFAF4 site is not affected by the CRISPR-Cas9 protocol in SHEP21N control cells. Sanger sequencing results for the control monoclonal cell line in the region surrounding the NDUFAF4 start site. The start codon is underlined in blue.



Appendix Figure 23 Raw cytotoxicity and proliferation plots for IMP1088 Incucyte assays in control, NDUFAF4 G2A and parental SHEP21N cells. Related to **Figure 4.20**. Individual cytotoxicity (green area/phase area) and proliferation (phase area) plots for the experiments assessing the impact of NDUFAF4 *N*-myristoylation on IMP1088 cytotoxicity in the SHEP21N genetic background.



Appendix Figure 24 The effects of NMTi in detergent fractions in P493-6 cells. Results of STRING analysis for significantly depleted ($p < 0.05$, $\log_2\text{FC} < -0.26$) proteins in detergent-enriched membrane fractions from high (top) and low (bottom) MYC P493-6 cells after treatment with 100 nM IMP1088 for 24 h. Black outlines indicate CoT NMT substrates.

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