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The Myc Family and the Metastasis Suppressor NDRG1: Targeting Key Molecular Interactions with Innovative Therapeutics

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Running Title Page

Targeting Interactions of Myc and NDRG1 with Therapeutics

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Abbreviations

17-AAG	17-N-allylamino-17-demethoxygeldanaMycin
AKL	Anaplastic lymphoma kinase
AML	Acute myeloid leukemia
AMPK	AMP-activated kinase
AR	Androgen receptor
CAK	CDK-activation kinase
CD47	Cluster of differentiation 47
CDK7	Cyclin-dependent kinase 7
Cx43	Connexin 43
DFO	Desferrioxamine
DKK3	Dickkopf WNT signaling pathway inhibitor 3
Dp44mT	Di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone
DpC	Di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone
DpT	Di-2-pyridylketone thiosemicarbazone
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal-transition
ENO1	Enolase 1
ERRFI	ERBB receptor feedback inhibitor 1
EZH2	The enhancer of zest homolog 2
GA	Geldanamycin
GLUT1	glucose transporter 1
GSK-3β	Glycogen synthase kinase-3β
H3K27me3	Trimethylation of Lys-27 in histone 3
H3K4	Histone 3 lysine 4
HAND2	Heart- and neural crest derivatives-expressed protein 2
HAUSP	Herpes virus-associated ubiquitin-specific protease
HK2	Hexokinase 2
HIF-1α	Hypoxia-inducible factor-1α
Hsp90	Heat shock protein 90
IL6/7	Interleukin 6/7
IRE	Iron response element
IRP2	Iron regulatory protein-2

ISL-1	Insulin gene enhancer protein ISL-1
LEF-1/TCF	Lymphoid enhancer factor-1/T-cell factor
LRP6	Low-density lipoprotein receptor-related protein 6
LSD1	Lysine-specific demethylase 1
MAT1	Ménage-à-trois 1,
MAX	Myc-associated protein X
MDM2	Mouse double minute 2
MGA	MAX gene-associated protein
MIG6	Mitogen-inducible gene 6
MNT	MAX-binding protein
MXI1	MAX interactor 1
NB	Neuroblastoma
NDRG1	N-Myc downstream-regulated gene 1
NHE III	Nuclease hypersensitive element III
NTRK1	Neurotrophic receptor tyrosine kinase 1
Nur77	Nuclear receptor 4A1
PD-L1	Programmed death-ligand 1
PFKM	Phosphofructokinase
PHOX2B	Paired-like homeobox
PIM1	Pro-viral integration site for Moloney murine leukemia virus-1
PLAGL2	Pleiomorphic adenoma gene-like 2
Pol II	Polymerase II
PRC2	Polycomb repressive complex 2
PSA	Prostate-specific antigen
RALT	Receptor-associated late transducer
SCF	SKP1-cullin1-F-box
Skp2	S-phase kinase-associated protein 2
SMYD2	The lysine methyltransferase, suppressor of variegation, enhancer of zeste, trithorax and myeloid-nervy-DEAF1 domain-containing protein 2
SP1	Specificity protein 1
T-ALL	T-cell acute lymphoblastic leukemia
TBX2	T-box transcription factor 2
TCP	Tranylcypromine
TfR1	Transferrin receptor 1
THZ1	*N*-[3-[[5-chloro-4-(1H-indol-3-yl)-2-pyrimidinyl]amino]phenyl]-4-[[(2E)-4-(dimethylamino)-1-oxo-2-buten-1-yl]amino]-benzamide
T-LBL	T-cell acute lymphoblastic lymphoma

USP7	Ubiquitin-specific protease 7
β-TrCP	β-transducin repeats-containing protein

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Abstract

Cancer is a leading cause of death worldwide resulting in ~10 million deaths in 2020. Major oncogenic effectors are the *Myc* proto-oncogene family that consists of three members including *c-Myc, N-Myc,* and *L-Myc.* As a pertinent example of the role of the Myc family in tumorigenesis, amplification of *MYCN* in childhood neuroblastoma strongly correlates with poor patient prognosis. Complexes between Myc oncoproteins and their partners such as hypoxia-inducible factor-1α (HIF-1α) and Myc-associated protein X (MAX) results in proliferation arrest and pro-proliferative effects, respectively. Interactions with other proteins are also important for N-Myc activity. For instance, the enhancer of zest homolog 2 (EZH2) binds directly to N-Myc to stabilize it by acting as a competitor against the ubiquitin ligase, SCF^{FBXW7}, which prevents proteasomal degradation. Heat shock protein 90 may also be involved in N-Myc stabilization since it binds to EZH2 and prevents its degradation. N-Myc downstream-regulated gene 1 (NDRG1) is down-regulated by N-Myc, and participates in the regulation of cellular proliferation *via* associating with other proteins, such as glycogen synthase kinase-3β (GSK3β) and low-density lipoprotein receptor-related protein 6 (LRP6). These molecular interactions provide a better understanding of the biological roles of N-Myc and NDRG1, which can be potentially used as therapeutic targets. In addition to directly targeting these proteins, disrupting their key interactions may also be a promising strategy for anti-cancer drug development. This review examines the interactions between the Myc proteins and other molecules, with a special focus on the relationship between N-Myc and NDRG1 and possible therapeutic interventions.

Significance Statement

Neuroblastoma (NB) is one of the most common childhood solid tumors, with a dismal 5-year survival rate. This problem makes it imperative to discover new and more effective therapeutics. The molecular interactions between major oncogenic drivers of the Myc family and other key proteins *e.g.*, the metastasis suppressor, NDRG1, may potentially be used as targets for anti-neuroblastoma drug development. In addition to directly targeting these proteins, disrupting their key molecular interactions may also be promising for drug discovery.

Table of Contents

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1. Introduction – Cancer and Neuroblastoma

Neuroblastoma (NB) is an embryonal tumor of the autonomic nervous system that occurs in very young children with a median age of diagnosis of 19 months (London et al., 2005). Unfortunately, it has been demonstrated that nearly 35% of NB cases occur before the age of one year (Ries, 1999; Alexander, 2000). As such, NB is the most common cancer diagnosed during the first year of life and has been divided into four stages (I to IV), which correlate with survival (Evans et al., 1971; Brodeur et al., 1988; Ries, 1999). Localized disease (stage I) has the best prognosis, whereas widespread disease (stage IV) has the worst (Evans et al., 1971; Brodeur et al., 1988). Unfortunately, the prognosis of children with advanced NB remains poor and new therapeutic approaches are urgently required.

Fifty percent of high-risk NB cases have amplified *MYCN* oncogene and increased N-Myc protein expression, which is considered to play a key role in determining NB aggressiveness (Brodeur et al., 1984; Seeger et al., 1985). The expression N-myc is established to be the major driver of NB (Brodeur et al., 1984; Seeger et al., 1985). This conclusion is also confirmed by the observation that genetic overexpression of *MYCN* leads to the development of NB, as well as other cancer-types (Zhu et al., 2012; Althoff et al., 2015). Further, high c-Myc protein levels have been demonstrated to associate with poor clinical outcomes in NB (Wang et al., 2015a). There is an inverse relationship between c-Myc and N-Myc expression (Zhe et al., 1999), demonstrating a complex interplay that underlines the importance of understanding their biological roles to enable rationale therapeutic development.

The current review focuses on the interactions between the Myc family, predominantly c-Myc and N-Myc, and a variety of other molecules that participate in their regulation and

function. These protein-protein interactions can be potentially targeted by pharmacological strategies to disrupt the function and regulation of Myc family proteins.

2. Overview of Myc Family Members – the Function of Myc Proteins in Cancer Cells

There are three members of the *Myc* proto-oncogene family, *c-Myc*, *MYCL*, and *MYCN*, which encode the c-Myc, L-Myc, and N-Myc proteins, respectively, and play key roles in cellular proliferation, apoptosis, and differentiation (Pelengaris et al., 2002; Adhikary and Eilers, 2005; Dang, 2012). The Myc family are at the crossroads of many growth-promoting signal transduction pathways and constitutes an immediate early response downstream of many ligand-membrane receptor complexes (Kelly et al., 1983; Armelin et al., 1984). All three Myc family members perform their transcriptional regulation function by forming Myc/MAX heterodimers that bind to the transcriptional regulatory region of many target genes (Pelengaris et al., 2002; Adhikary and Eilers, 2005; Meyer and Penn, 2008). Moreover, Myc family members can amplify transcription by interacting with the promoter regions of downstream target genes (Lin et al., 2012; Nie et al., 2012; Nie et al., 2020).

The proteins encoded by the three *Myc* genes participate in more than 15% of the transcription of the entire genome and participate in many different cellular processes, including ribosome biogenesis, protein translation, cell-cycle progression, and metabolism (Dang et al., 2006b; Meyer and Penn, 2008). As such, the Myc family plays a significant role in many biological functions, including proliferation and differentiation (**Fig. 1**) (Dang et al., 2006b; Meyer and Penn, 2008).

In normal cells, the expression of the Myc family is tightly controlled, while in cancer cells it is frequently dysregulated and enhanced *via* multiple mechanisms (Adhikary and Eilers, 2005; Meyer and Penn, 2008; Dejure and Eilers, 2017). The elevated expression of Myc family members is detected in both Myc-driven tumors and tumors driven by other oncogenes (Felsher and Bishop, 1999; Shachaf et al., 2004; Soucek et al., 2008; Annibali et al., 2014). To match the enhanced demand for anabolic metabolites, the dysregulation of *Myc* family gene expression can alter intermediary metabolism in cancer cells and induce metabolic stress. For example, enhanced expression of *Myc* causes a decrease in cellular ATP levels and activates AMP-activated kinase (AMPK) that functions as a metabolic sensor (Liu et al., 2012a; von Eyss et al., 2015).

There are many studies demonstrating that c-Myc and N-Myc play roles in the WNT/β-catenin signaling pathway (Zhang et al., 2012; Katase et al., 2018; Kong et al., 2018). Indeed, the expression of *N-Myc* activates WNT/β-catenin signaling by suppressing the expression of the key WNT inhibitor, Dickkopf WNT signaling pathway inhibitor 3 (DKK3) (Katase et al., 2018; Kong et al., 2018) (**Fig. 2**). Consequently, activation of WNT/β-catenin signaling results in the accumulation of cytoplasmic β-catenin that is then translocated to the nucleus (Kobayashi et al., 2000). In the nucleus, β-catenin binds to lymphoid enhancer factor-1/T-cell factor (LEF-1/TCF) and some other co-regulators to transcribe target genes such as *c-Myc* and *cyclin D1* (Khramtsov et al., 2010; Gekas et al., 2016). Further regulation of WNT/β-catenin signaling can also be achieved through glycogen synthase kinase-3β (GSK-3β) that phosphorylates β-catenin at Ser33, Ser37, and Thr41 (Liu et al., 2002). The GSK-3β-induced phosphorylation of β-catenin at these latter residues initiates the recruitment of ubiquitin E3 β-transducin repeats-containing protein (β-TrCP) that leads to β-catenin ubiquitination and its proteasomal degradation (Jiang and Struhl, 1998; Liu et al., 1999) (**Fig. 2**).

The activation of WNT/β-catenin signaling also induces *c-Myc* transcription, and the overexpression of *c-Myc* promotes HIF-1α expression at the post-transcriptional level (Zhang et al., 2012; Chen et al., 2013). The increased expression of HIF-1α also up-regulates N-Myc downstream-regulated gene 1 (NDRG1) expression (Vallée et al., 2018; Zhang et al., 2020b). The induction of NDRG1 in lung cancer cells leads to c-Myc stabilization *via* decreasing the phosphorylation of S-phase kinase associated protein 2 (Skp2), which is a component of RING E3 ubiquitin ligases involved in the proteasomal degradation of c-Myc (Wang et al., 2017c).

The overexpression of *c-Myc* also affects the expression of multiple proteins involved in the metabolism of iron (Wu et al., 1999) that is important for cellular growth and proliferation (Richardson and Ponka, 1997; Kwok and Richardson, 2002). Regarding this, c-Myc expression results in the up-regulation of iron regulatory protein-2 (IRP2) (Wu et al., 1999) that binds to the iron response elements (IRE) in the untranslated regions of *TfR1* mRNA and *ferritin* mRNA, to prevent degradation of *TfR1* mRNA and inhibit ferritin translation, respectively (Klausner et al., 1993; Hentze and Kühn, 1996). This effect results in increased cellular iron uptake from the serum iron transport protein, transferrin, by the TfR1 and decreased iron-storage by ferritin (Richardson and Ponka, 1997; Ponka et al., 1998) (**Fig. 3**). Overall, increased *c-Myc* expression results in an elevation of cellular iron levels (Klausner et al., 1993; Hentze and Kühn, 1996; Wu et al., 1999) that is essential for proliferation (Kwok and Richardson, 2002).

In summary, the *Myc* family is indispensable to cancer cell growth and plays a critical role in the regulation of tumor cell metabolism by their regulatory roles in transcription. This fact

emphasizes the importance of the Myc family as targets for drug discovery research. The sections below examine the functions of Myc family members, with a major focus on c-Myc and N-Myc.

2.1 c-Myc

The c-Myc protein regulates ~15% of gene expression and is considered to be significant in the pathogenesis of over 20% of human cancers (Dang, 1999). The *c-Myc* gene was initially discovered to be the cellular homolog of an avian retrovirus transforming gene, *v-Myc* (Sheiness and Bishop, 1979; Sheiness et al., 1980). The overexpression of *c-Myc* is found in many types of human cancer (Lee et al., 2015; Wang et al., 2016; Jung et al., 2017), with the protein demonstrating conserved topology in a range of species from *Xenopus* to human (Spencer and Groudine, 1991; Ma et al., 1992; Thompson, 1998).

The expression of c-Myc maintains proliferation and prevents cells from entering into the state of growth arrest in G_0/G_1 that is required for terminal differentiation (Freytag, 1988). As such, high c-Myc expression is necessary at later stages of embryonic development to support rapid cellular proliferation (Schmid et al., 1989). However, during the early stage, only a limited subset of dividing embryonic cells has high c-Myc levels (Pfeifer-Ohlsson et al., 1985; Hirvonen et al., 1990). This indicates that c-Myc may also contribute to cell migration or invasiveness during embryogenesis (Henriksson and Lüscher, 1996). Moreover, the expression of c-Myc has also been demonstrated to participate in the regulation of the anti-tumor immune suppression in cancer cells through regulating cluster of differentiation 47 (CD47) and programmed death-ligand 1 (PD-L1) (Casey et al., 2016).

The function of c-Myc is regulated by many post-translational modifications, including phosphorylation, ubiquitination, and protein-protein interactions (Salghetti et al., 1999; Kim et al., 2003; Yeh et al., 2004). It has been well characterized that c-Myc performs its transcriptional activity through forming a heterodimer with MAX (Grandori et al., 2000). In addition to MAX, c-Myc also participates in multiple interactions with other proteins, and these are discussed in detail in section 3 below.

2.2 L-Myc

Similarly to the other two *Myc* family members, *L-Myc* encodes a conserved basic helix-loop-helix leucine zipper (bHLHzip) protein that interacts with MAX (Blackwood et al., 1992). Unlike c-Myc and N-Myc, it is notable that L-Myc is the least efficient at promoting cellular transformation and transcription, and also has limited functions in maintaining embryonic viability (Hatton et al., 1996; Wasylishen et al., 2011). The distribution of L-Myc is also more restricted than c-Myc and N-Myc (Zimmerman et al., 1986). During embryogenesis, L-Myc is expressed in the nervous system, kidney, and lung, while in adults, L-Myc expression is only detected in the lung (Zimmerman et al., 1986).

Although the function of L-Myc in transformation and transcription is less pronounced than c-Myc and N-Myc, a unique function of L-Myc is its role in regulating the activation of T cells (Kc et al., 2014). The loss of L-Myc results in the failure of T cell activation during infections with the vesicular stomatitis virus and *Listeria monocytogenes* (Kc et al., 2014). This observation indicates that L-Myc may play a vital role as an immune cell regulator. Since L-Myc is not principally involved in the regulation of gene expression in cancer cells, this review will now focus on c-Myc and N-Myc.

2.3 N-Myc

In normal cells, N-Myc acts as a transcription factor involved in nervous system development during embryogenesis (Mathsyaraja and Eisenman, 2016). N-Myc is overexpressed in many types of cancer, including NB, medulloblastoma, neuroendocrine prostate cancer, and pancreatic cancer (Davis et al., 1999; Beltran et al., 2011; Northcott et al., 2012; Fielitz et al., 2016; Cassia et al., 2018). The gene expression profile in NB tumors with amplified *MYCN* is distinct to that observed in normal cells (Westermann et al., 2008), and suggests that N-Myc induces its oncogenic activity though altering gene expression.

Recently, N-Myc has also been demonstrated to induce an immunosuppressive environment that is beneficial to NB survival, with inhibition of N-Myc expression restoring the sensitivity of NB cells with amplified *MYCN* to natural killer cells (Raieli et al., 2021). The correlation between N-myc and immunosuppression is not only restricted to NB, but is also identified in other malignancies, including small cell lung cancer, rhabdomyosarcoma, Wilms' tumor, retinoblastoma, acute myeloid leukemia, and T-acute lymphoid leukemia (Rickman et al., 2018). These findings indicate that N-Myc is not only responsible for promoting proliferation, but also plays an important role in supporting tumor cell survival.

As mentioned above, key to the activity of c-Myc and N-Myc is its direct interaction with a number of proteins (Goda et al., 2003; Koshiji et al., 2004; Welcker et al., 2004; To et al., 2006; Conacci-Sorrell et al., 2014; Wang et al., 2022). These data suggest that a pharmacological strategy that targets these associated proteins may indirectly also result in alterations in N-Myc or/and c-Myc expression and function. The sections below will focus on proteins that have direct associations with N-Myc or/and c-Myc and are involved in the regulation of their expression and function.

3. Myc Proteins and their Protein Partners.

3.1 Myc-associated protein X (MAX)

MAX is a member of the basic helix-loop-helix leucine zipper (BR/HLH/LZ) family that can homodimerize or heterodimerize with other BR/HLH/LZ proteins (**Fig 4**), in particular with the Myc protein (Pelengaris et al., 2002; Adhikary and Eilers, 2005; Meyer and Penn, 2008; Cascón and Robledo, 2012; Suzuki et al., 2017). MAX is commonly expressed as protein isoforms that migrate at 21- and 22-kDa in SDS-PAGE gels (Blackwood et al., 1992). As shown in **Figure 4**, there is a BR/HLH/LZ motif located at the *N*-terminal of both *Myc* and *MAX* genes, which is required for DNA-protein interactions (Pelengaris et al., 2002; Adhikary and Eilers, 2005; Meyer and Penn, 2008). This BR/HLH/LZ motif is critical for heterodimer formation between Myc and MAX (Murre et al., 1989; Ferré-D'Amaré et al., 1994).

The binding of MAX to Myc is considered to activate the transcriptional activity of Myc (Cascón and Robledo, 2012). Since MAX can also form a heterodimer with other BR/HLH/LZ proteins, including MAX itself (Amati et al., 1992), the formation of the MAX/MAX homodimer in cells competitively inhibits Myc/MAX heterodimer formation (Cascón and Robledo, 2012). Competition between the MAX/MAX homodimer and Myc/MAX heterodimers is regulated by the phosphorylation of one or more serine residues in the *N*-terminus of MAX (Kato et al., 1992; Prochownik and VanAntwerp, 1993; Koskinen et al., 1994). These regulatory phosphorylation's of MAX prevent the binding of the MAX/MAX homodimer to target DNA, while they have no inhibitory effect on the

interaction of the Myc/MAX heterodimer with its target DNA (Berberich and Cole, 1992; Bousset et al., 1993).

MAX interactor 1 (*MXI1*) is a gene located in the cancer hotspot region of human chromosome 10 at 10q24-q25, with the MXI1 protein antagonizing the transcriptional activity of Myc by competing for MAX (Wechsler et al., 1994; Hurlin and Huang, 2006) (**Fig. 5**). There are several other MXD1 family members, namely MXD1, MXD3, and MXD4, as well as the MAX-binding protein (MNT), and MAX gene-associated (MGA) that can also form heterodimers with MAX (Hurlin et al., 1995; Hurlin et al., 1997; Meroni et al., 1997) (**Fig. 5**). The MXD family, MNT, and MGA generally antagonize the formation of the Myc/MAX dimer and repress the transcription of Myc/MAX target genes (Grandori et al., 2000) (**Fig. 5**). In fact, the binding of MXD/MAX dimer to DNA results in repression of its transcription (Ayer et al., 1995; Schreiber-Agus et al., 1995). Overall, the binding of the MXD family to MAX, as well as the binding of MXI1 to MAX have an opposite function to the binding of MAX to Myc, which results in the transcriptional activation of target genes.

Interestingly, similar to Myc (Burchett et al., 2021), it has also been demonstrated that MAX participates in the regulation of the circadian clock (Blaževitš et al., 2020). The circadian clock exhibits cellular regulatory functions related to cancer, including control of proliferation, cell death, DNA repair, and metabolic alteration. As such, MAX may play a role in cancer progression *via* its circadian regulatory function (Shafi and Knudsen, 2019). Due to the importance of MAX in regulating Myc (Cascón and Robledo, 2012; Suzuki et al., 2017), appropriate regulation of MAX is necessary for maintaining cellular proliferation, and thus, targeting the Myc/MAX interaction is a promising therapeutic modality (see section 6.2.1).

3.2 Hypoxia inducible factor-1α (HIF-1α) and HIF-2α

HIF-1α is a basic helix-loop-helix (bHLH) transcription factor that regulates gene expression under hypoxia to maintain ATP production by improving oxygen delivery and enhancing glycolysis (Wang et al., 1995; Koshiji et al., 2004). Knockdown of *c-Myc* expression decreases HIF-1α protein levels, while the overexpression of *c-Myc* promotes the expression of HIF-1α at the post-transcriptional level (Chen et al., 2013; Weili et al., 2019). HIF-1α also collaborates with dysregulated c-Myc levels to drive glycolytic gene expression *e.g.*, *glucose transporter 1 (GLUT1)*, *hexokinase 2 (HK2)*, *phosphofructokinase (PFKM)*, and *enolase 1 (ENO1)*, which participate in altered cancer cell metabolism leading to the Warburg effect (Kim et al., 2007; Dang et al., 2009) (**Fig. 6a**). The expression of these genes increases glucose uptake into cells and its metabolism to lactate. Additionally, c-Myc has been described to play a role in glutamine metabolism inducing its oxidation under tissue normoxia (Dang et al., 2009) (**Fig. 6a)**. As such, cooperation between c-Myc and HIF-1α may support tumor cell survival in solid cancers (Harris, 2002; Jain, 2005; Pries et al., 2009; Lu and Kang, 2010; Dang, 2012).

HIF-1α can indirectly or directly participate in the regulation of c-Myc function *via* various mechanisms (Goda et al., 2003; Koshiji et al., 2004; To et al., 2006; Conacci-Sorrell et al., 2014). In terms of indirect regulation of c-Myc by HIF-1α, it is known that HIF-1α can inhibit c-Myc during hypoxia due to the competitive binding of HIF-1α to MAX, which disrupts c-Myc/MAX complexes and leads to cell cycle arrest (**Fig. 6b)** (Goda et al., 2003; Koshiji et al., 2004; Gordan et al., 2007; Li et al., 2020). As another example of indirect regulation, HIF-1α competes against c-Myc by binding to specificity protein 1 (SP1) that is a transcriptional coactivator of c-Myc (Koshiji et al., 2005; To et al., 2006) (**Fig. 6**). Moreover,

HIF-1α also induces the expression of MXI1 under hypoxia, which is a competitor to c-Myc that binds to MAX, preventing c-Myc/MAX complex formation (Conacci-Sorrell et al., 2014).

Considering the direct regulation of c-Myc by HIF-1α, a physical interaction of HIF-1α has been demonstrated with c-Myc through its bHLH/PAS domains located in its *N*-terminus that leads to p21 expression and G_1 arrest (Koshiji et al., 2004). However, it is still unclear whether this physical interaction between HIF-1α and c-Myc has direct inhibitory effects on c-Myc activity (Li et al., 2020).

All the effects of HIF-1α on c-Myc can lead to the arrest of cellular proliferation under hypoxia (Goda et al., 2003; Koshiji et al., 2004; To et al., 2006; Conacci-Sorrell et al., 2014). Regarding NB cells, the stimulation of cellular proliferation induced by the overexpression of N-Myc, may override the inhibition of cell cycle progression by HIF-1α, enabling continued proliferation even under hypoxic conditions (Qing et al., 2010). Considering that c-Myc and N-Myc expression have an inverse relationship (Zhe et al., 1999), hypoxia-induced cell cycle arrest caused by the HIF-1α and c-Myc interaction may be less prominent in NB cells with amplified *MYCN* (Westermann et al., 2008). Further, N-Myc can also be up-regulated by hypoxia (Cangul, 2004), and potentially HIF-1α protein may directly or indirectly participate in the regulation of N-Myc expression. This speculation is supported by the observation that HIF-1α binds directly to c-Myc (Koshiji et al., 2004) to regulate its function and *c-Myc* expression inversely regulates N-Myc levels (Westermann et al., 2008).

Apart from HIF-1α, HIF-2α also interacts with c-Myc that prevents association of HIF-1α and c-Myc in hepatocellular carcinoma cells under mild chronic hypoxia (Mu et al., 2021). Unlike HIF-1α, this interaction between HIF-2α and c-Myc induces cellular proliferation

rather than inhibiting it (Goda et al., 2003; Koshiji et al., 2004; To et al., 2006; Conacci-Sorrell et al., 2014; Mu et al., 2021). Collectively, this evidence suggests that the interaction between Myc and HIF family members may play an important role in regulating cancer cell survival and proliferation under hypoxic conditions.

3.3 Enhancer of zest homolog 2 (EZH2)

EZH2 is an enzymatic catalytic subunit of polycomb repressive complex 2 (PRC2) that functions as a methyltransferase, which participates in altering gene expression by trimethylation of Lys-27 in histone 3 (H3K27me3) (Cao et al., 2002). The expression of EZH2 is critical to cells as it is involved in cell cycle progression, autophagy, apoptosis, and DNA damage repair (Yao et al., 2016; Ito et al., 2018; Nutt et al., 2020). Deficiency of EZH2 leads to the death of mice embryos *in utero*, suggesting the importance of EZH2 in embryo development (O'Carroll et al., 2001).

Due to its role in cell cycle progression, the overexpression of EZH2 is observed in many different tumors, including prostate cancer (Varambally et al., 2002), breast cancer (Bachmann et al., 2006), esophageal cancer (Qiu et al., 2020), gastric cancer (Gan et al., 2018), and anaplastic thyroid carcinoma (Pellecchia et al., 2020). The expression of EZH2 has been reported to prevent differentiation of NB cells *via* inhibition of neurotrophic receptor tyrosine kinase 1 (NTRK1) (Li et al., 2018). The importance of EZH2 in NB progression is demonstrated by the fact that N-Myc protein is stabilized by EZH2. This stabilization by EZH2 occurs *via* its binding to N-Myc as a competitor against the ubiquitin

ligase, SCF^{FBXW7}, which protects against ubiquitination and proteasomal degradation (Welcker et al., 2004; Wang et al., 2022).

Pharmacological targeting and inhibition of EZH2 activity or expression can be speculated to increase N-Myc degradation that would suppress WNT/β-catenin signaling and NB proliferation (Wang et al., 2018). An example of a EZH2 inhibitor under development is described in section 6.2.2.

3.4 Heat shock protein 90 (Hsp90)

Hsp90 is an essential molecular chaperone and accounts for 1-2% of cellular protein and can increase to 4-6% if cells are under stress (Prodromou, 2016). It is an ATP-dependent molecular chaperone involved in preventing the aggregation of misfolded proteins (Mayer and Le Breton, 2015). The chaperone function of Hsp90 in *vivo* cannot be achieved before its dimerization (Wayne and Bolon, 2007). Methylation of Lys615 on the Hsp90α chain promotes dimer formation, while the demethylation of Lys615 prevents dimerization (Abu-Farha et al., 2011). These alterations in methylation are mediated by the lysine methyltransferase, suppressor of variegation, enhancer of zeste, trithorax and myeloid-nervy-DEAF1 domain-containing protein 2 (SMYD2), and also the lysine-specific demethylase 1 (LSD1) (Abu-Farha et al., 2011).

Because of the essential role of Hsp90 in the response to extracellular stress, it is unsurprisingly up-regulated in cancer cells as a result of multiple stress stimuli, including nutrient deficiency, oxygen deficiency, *etc.* (Birbo et al., 2021). The increased levels of Hsp90 expression are associated with decreased response to treatment in breast cancer

patients, as well as poor overall survival rates (Pick et al., 2007; Cheng et al., 2012). In addition, Hsp90 may also be involved in N-Myc stabilization considering that in T cells, Hsp90 prevents the degradation of EZH2 protein (Huang et al., 2017) (**Fig. 7**).

Although Hsp90 has only been reported to function in EZH2 stabilization in T cells, it is intriguing to note that both Hsp90 and N-Myc protein levels are decreased after incubation of NB cells with drugs that specifically bind cellular iron (Fan *et al.*, 2001; (Sidarovich et al., 2015). Hence, it is tempting to speculate that the decrease in Hsp90 will prevent EZH2 stabilization, resulting in greater proteasomal degradation of N-Myc by FBXW7 (**Fig. 7**). Considering that targeting N-Myc is a promising strategy to treat NB, therapeutics that inhibit Hsp90 and EZH2 expression and/or activity could also be useful new agents for NB treatment (see Section 6.2).

3.5 Aurora-A

Similar to EZH2, the mitotic kinase, Aurora-A (also known as AURKA), is also able to prevent FBXW7-mediated proteasomal degradation of N-Myc by directly binding to both N-Myc and FBXW7 (Otto et al., 2009). Aurora-A belongs to the aurora family of serine/threonine kinases that consists of Aurora-A, Aurora-B, and Aurora-C, which share a highly conserved catalytic domain containing auto-phosphorylating sites (Yan et al., 2016). The overexpression of Aurora-A is observed in multiple cancers, including breast cancer (Sen et al., 1997), ovarian cancer (Gritsko et al., 2003), prostate cancer (Buschhorn et al., 2005), and lung cancer (Xu et al., 2014), *etc*. These observations indicate the significant role of Aurora-A in maintaining tumor growth.

There are many studies demonstrating that Aurora-A performs an oncogenic role in cancer cell proliferation and the inhibition of autophagy (Do et al., 2014; Yuan et al., 2015; Fu et al., 2016; Min et al., 2016; Xie et al., 2018; Heo et al., 2019). The inhibition of Aurora-A activity induces *in vitro* and *in vivo* stabilization and up-regulation of the tumor suppressor, p53, and its downstream target cyclin-dependent kinase inhibitor, p21 (Huck et al., 2010). Moreover, Aurora-A also activates WNT/β-catenin signaling *via* mediating the inhibitory phosphorylation of GSK-3β at Ser9 that prevents β-catenin accumulation and its nuclear translocation (Xie et al., 2017).

Considering the ability of Aurora-A to stabilize N-Myc in NB cells and the fact that N-Myc is responsible for NB progression, many Aurora-A inhibitors have been investigated (see section 6.2) and show anti-tumor activity against NB *via* down-regulating N-Myc expression (Faisal et al., 2011; Brockmann et al., 2013; Boi et al., 2021).

3.6 Lysine-specific demethylase 1 (LSD1)

LSD1 (also known as KDM1A) was the first identified histone demethylase and is a flavin adenine dinucleotide (FAD)-dependent amine oxidase superfamily member (Shi et al., 2004). The demethylation activity of LSD1 participates in the regulation of gene expression through histone 3 lysine 4 (H3K4) (Shi et al., 2004). This demethylation activity of LSD1 suggests its pivotal role in various processes such as cellular proliferation (Lv et al., 2012; Haines et al., 2018), the EMT (McDonald et al., 2011), and metabolism (Sakamoto et al., 2015). LSD1 also exhibits both activation and suppression functions on gene expression *via* its direct association with different proteins (Lee et al., 2005; Wang et al., 2007; Wang et al., 2009; Zhang et al., 2011; Amente et al., 2015; Kim et al., 2016; Lee et al., 2017; Gao et al., 2020). For instance, LSD1 demethylates HIF-1α at lysine 391 that protects against ubiquitin-

dependent degradation of HIF-1α and results in activation of HIF-1α target genes (Lee et al., 2017).

In NB cells, LSD1 is strongly expressed, which indicates it may be involved in NB pathobiology (Schulte et al., 2009). LSD1 binds to c-Myc in NB cells and triggers a transient demethylation of H3K4 that results in local DNA oxidation as a driving force in assembling the Myc-induced transcription initiation complex (Amente et al., 2010). In addition, LSD1 also cooperatively works with N-Myc as a critical co-factor in maintaining the repressive function of N-Myc on certain target genes *e.g.*, *p21* (Amente et al., 2015). The LSD1/N-Myc complex becomes co-localized at the *p21* promoter, which may suggest the LSD1/N-Myc complex-mediated inhibition of *p21* expression is at the transcriptional level (Amente et al., 2015). The expression of *NDRG1* is also inhibited by the LSD1/N-Myc complex through its binding to the *NDRG1* promoter (Ambrosio et al., 2017). Considering the essential role of LSD1 in cancer cells, the inhibition of this protein is becoming popular in anti-cancer drug discovery and is described in section 6.2.5.

3.7 Ubiquitin-specific protease 7 (USP7)

USP7, also known as herpes virus-associated ubiquitin-specific protease (HAUSP), was discovered in the late 1990s (Everett et al., 1997). It is a member of the deubiquitinating enzyme family that contributes to ubiquitin removal on client proteins and is involved in DNA repair, gene expression, protein localization, kinase activation, protein degradation, cell cycle progression, and apoptosis (Everett et al., 1997; Reyes-Turcu et al., 2009). As a key regulator of apoptosis, USP7 plays a critical role in maintaining the stability of p53 through its de-ubiquitination function (Li et al., 2004). Expression of USP7 decreases the half-life of p53 through deubiquitinating the p53 ubiquitin E3 ligase. The degradation of mouse double

minute 2 homolog (MDM2) and the up-regulation of p53 half-life can be achieved *via USP7* knockdown (Cummins and Vogelstein, 2004; Li et al., 2004; Qi et al., 2020).

It has been demonstrated that USP7 is also involved in the regulation of N-Myc stability and activity in NB (Tavana et al., 2016). USP7 directly binds to N-Myc to deubiquitinate it and prevent its proteasomal degradation (Tavana et al., 2016). Consistent with the oncogenic role of USP7 in N-Myc stabilization, USP7 was found to be overexpressed in NB patients with amplified *MYCN* and a poor prognosis (Brodeur et al., 1984; Schwab et al., 1984; Seeger et al., 1985; Liu et al., 2020b). An example of a novel therapeutic agent targeting USP7 is described in section 6.2.4.

4. Regulation of Myc Proteins – the Dynamic Balance of Myc Protein Levels in Cancer Cells

As discussed in the sections above, Myc proteins play a key role in regulating cell growth and metabolism (Freytag, 1988; Wu et al., 1999; Pelengaris et al., 2002; Adhikary and Eilers, 2005; Dang, 2012). Due to this, the Myc proteins are strictly regulated in cells to obtain controlled gene expression, metabolism, and proliferation. The following section will focus on molecules involved in the regulation of Myc proteins.

4.1 Cyclin-dependent kinase 7 (CDK7) and the super-enhancer are involved in *N-Myc* transcription

The super-enhancer of *MYCN* is vital for its transcriptional regulation and comprises multiple enhancers bound by various transcription factors that are important in cell-type-specific gene regulation (Hnisz et al., 2013; Lovén et al., 2013; Whyte et al., 2013). Inhibition of cyclin-dependent kinase 7 (CDK7) leads to the suppression of super-enhancer linked oncogenic

MYCN transcription in *MYCN*-driven NB (Chipumuro et al., 2014). Furthermore, this super-enhancer linked transcriptional suppression of *MYCN via* CDK7 inhibition is highly selective against NB cells with amplified *MYCN* compared to those with non-amplified *MYCN* (Chipumuro et al., 2014).

CDK7 is a component of the CDK-activation kinase (CAK) that performs several key functions including regulation of the cell cycle and transcriptional activation (Larochelle et al., 2007; Glover-Cutter et al., 2009; Schachter and Fisher, 2013; Wong et al., 2014). As a part of the CAK, CDK7 induces a T-loop phosphorylation once bound to cyclin H and ménage-à-trois 1 (MAT1), which is required for activation of CDKs 1, 2, 4, and 6 and subsequent cell cycle progression (Larochelle et al., 2007; Bisteau et al., 2013; Schachter and Fisher, 2013; Schachter et al., 2013; Wood and Endicott, 2018). Moreover, CDK7 also performs phosphorylation of RNA polymerase II (Pol II) at Ser5 and Ser7 to facilitate transcriptional initiation (Akhtar et al., 2009; Glover-Cutter et al., 2009; Wong et al., 2014). In addition to directly phosphorylating Pol II, CDK7 also phosphorylates Pol II Ser2 *via* activating CDK9 and this phosphorylation drives the elongation of the transcript (Larochelle et al., 2012). There are many studies demonstrating that CDK7 inhibition leads to the suppression of cancer cell proliferation indicating it is a potential target for anti-cancer drug development (Fisher, 2019).

The development of cancers resulting from aberrant transcription can be propelled by super-enhancers (Lovén et al., 2013; Chipumuro et al., 2014; Christensen et al., 2014; Kwiatkowski et al., 2014). Cancer cells can acquire super-enhancers through gene amplification, genetic translocation, or transcription factor overexpression, and thus, facilitate high-level expression of genes including *Myc* (Hnisz et al., 2013; Lovén et al., 2013).

More recently, transcription factors including Heart- and neural crest derivatives-expressed protein 2 (HAND2), insulin gene enhancer protein ISL-1 (ISL1), paired-like homeobox 2b (PHOX2B), GATA3, and T-box transcription factor 2 (TBX2) are harbored in super-enhancers and are essential for *MYCN* expression, as well as the survival of NB cells with amplified *MYCN* (Durbin et al., 2018; Helmsauer et al., 2020). The oncogenes driving super-enhancers were demonstrated to have a high sensitivity to CDK7 inhibition that suggests CDK7 is involved in the formation of super-enhancers or is one of its components (Durbin et al., 2018; Minzel et al., 2018). In fact, CDK7 inhibitors exhibit encouraging anti-cancer activity against many different types of cancer, including lung cancer, prostate cancer, colorectal cancer, breast cancer, and NB, and an example of one of these compounds is described in section 6.1.2 (Chipumuro et al., 2014; Wang et al., 2015c; Rasool et al., 2019; Wang et al., 2019a; Wang et al., 2020).

4.2 Transcriptional regulation *via* transcription factors

There are also other transcription factors that participate in the regulation of *MYCN* expression, such as SP1 (Tuthill et al., 2003), E2F (Strieder and Lutz, 2003), and pleiomorphic adenoma gene-like 2 (PLAGL2) (Zhao et al., 2020). These three transcription factors directly bind to the promoter of the *MYCN* gene to activate it (Liu et al., 2020b). Interestingly, the activated transcription of *MYCN* gene results in positive feedback to activate PLAGL2 expression through N-Myc binding to the *PLAGL2* promoter (Zhao et al., 2020). Since SP1, E2F, and PLAGL2 play critical roles in regulating *MYCN* expression, pharmacological strategies that inhibit their activity could lead to promising agents, especially for *MYCN*-driven cancers such as NB.

Transcription of *c-Myc* and the *MYCN* gene is also regulated by non-B DNA structures, which include single-stranded bubbles, Z-DNA, and G-quadruplexes (Levens, 2010). Of particular relevance to the current review, the G-quadruplex forming sequence silences the expression of *c-Myc* and *MYCN* through binding to its promoter and intron 1, respectively (Siddiqui-Jain et al., 2002; Trajkovski et al., 2012). The silencing function of G-quadruplex sequence in *c-Myc* and *MYCN* expression makes it an attractive candidate for anti-cancer drug development and is discussed further in section 6.1.1.

4.3 Regulation by non-coding RNA

Expression of *MYCN* is not only regulated through transcriptional regulation, but non-coding RNA is also involved in the post-transcriptional regulation of *MYCN* expression (Liu et al., 2020b). Indeed, the microRNA-204 (miR-204) directly binds to *MYCN* mRNA to suppress its expression and inhibits a subnetwork of oncogenes that strongly correlates to NB tumors with amplified *MYCN* and poor patient outcome (Ooi et al., 2018). Furthermore, miR-193b may also play a role in down-regulating *MYCN* expression, since *MYCN* is one of its targets (Roth et al., 2018). Additionally, the expression of miR-193b is suppressed in N-myc-overexpressing NB cells, which may be a compensatory response, although the mechanism remains unclear (Roth et al., 2018).

Non-coding RNA expression can also regulate c-Myc (Zhang et al., 2020a). For example, the long non-coding RNA, LINC01123, was demonstrated to enhance c-Myc expression in non-small cell lung cancer (Hua et al., 2019). Mechanistic dissection demonstrated that LINC01123 acted as a decoy to sequester the tumor suppressor microRNA, miR-199a-5p, from binding *c-Myc* mRNA, relieving its inhibitory effect on c-Myc expression. These studies demonstrated LINC01123 functioned as an oncogene in promoting glycolysis as well

as tumor growth through increasing c-Myc levels (Hua et al., 2019). Further, Zhang and colleagues reported that the long non-coding RNA, ARAP1-AS1, promotes tumorigenesis and metastasis through regulation of c-Myc translation (Zhang et al., 2020a). Hence, pharmacological targeting of non-coding genes could be another intriguing strategy for the development of anti-cancer pharmacopoeia.

4.4 Proteasomal degradation

4.4.1 Proteasome and cancer

Proteasomes are multi-enzyme complexes that maintain protein homeostasis (proteostasis) and important cellular functions through the degradation of misfolded, redundant, and damaged proteins (Njomen and Tepe, 2019). There are two different complexes involved in proteasome degradation, namely the 20S complex and the 26S complex, which participate in the ubiquitin-ATP independent and ubiquitin-ATP dependent proteolysis, respectively (Njomen and Tepe, 2019). The expression of Myc family proteins is regulated by the proteasomal system (Welcker et al., 2004; Popov et al., 2007; Choi et al., 2010; Farrell and Sears, 2014; Tavana et al., 2016). Importantly, there are many E3 ubiquitin ligases involved in ubiquitin-dependent proteasomal degradation of Myc (Sun et al., 2021). The following section will discuss the ubiquitin-mediated regulation of Myc family proteins.

4.4.2 Ubiquitination of the Myc family of proteins

Ubiquitination is the process that couples ubiquitin to target proteins by a cascade of reactions catalyzed by activating (E1), conjugating (E2), and ligating (E3) enzymes (Popovic et al., 2014). Since proteasomal degradation is involved in the regulation of Myc protein function in cancer cells (Welcker et al., 2004; Popov et al., 2007; Choi et al., 2010; Farrell

and Sears, 2014; Tavana et al., 2016), ubiquitination may also participate in Myc protein regulation.

There are three E3 ubiquitin ligases that participate in the ubiquitination of Myc proteins, including FBXW7, HUWE1, and TRUSS (Rickman et al., 2018). The dysfunction of these ubiquitin ligases can increase Myc stability and its protein levels that can support the increased metabolism of cancer cells. Previous studies have indicated overexpression of Myc protein in 70% of human cancers, while only 20% of these tumors have amplification of the *Myc* gene (Nesbit et al., 1999). This observation suggests the possibility there is inhibited Myc protein degradation in cancer cells. Approximately 30% of human cancer cells were reported to have a genetic deletion of *FBXW7* (Knuutila et al., 1999) and the mutational status of *FBXW7* in primary human tumors accounts for 6% of the overall mutation rate (Akhoondi et al., 2007; O'Neil et al., 2007). Further, the deubiquitinating enzyme, USP28, deubiquitinates Myc and is overexpressed in colon carcinoma (Popov et al., 2007).

Other proteins are also involved in the stabilization of Myc proteins through modulating ubiquitination (Wang et al., 2017c; Wang et al., 2022). For example, in lung cancer cells, NDRG1 stabilizes c-Myc through binding to Skp2, which is a E3 ubiquitin ligase that targets c-Myc for proteasomal degradation (Wang et al., 2017c). Additionally, EZH2 prevents N-Myc ubiquitination *via* competitively binding to N-Myc to prevent the interaction with FBXW7 (Wang et al., 2022).

In conclusion, the regulation of ubiquitination and proteasomal degradation maintains Myc protein levels in cells to support their demand for cellular growth and proliferation.

5. *NDRG* Family – the Role of NDRG1 in Cancer Cells and Its Functions via Associating with Other Proteins

The *N-Myc* downstream-regulated gene (NDRG) family consists of four members, including *NDRG1*, *NDRG2*, *NDRG3*, and *NDRG4* (Qu et al., 2002). Amongst all four members, there is approximately 57%-65% amino acid identity (Qu et al., 2002). The family is named after the discovery of the *NDRG1* gene that can be repressed by the expression of N-Myc (Li and Kretzner, 2003). However, not all NDRG family members are directly controlled by N-Myc or c-Myc (Melotte et al., 2010; Zhong et al., 2015).

The expression of the four NDRG family members varies depending on the species, tissues, or model organism examined (Okuda and Kondoh, 1999; Zhong et al., 2015). In *Xenopus tropicalis*, *NDRG1* expression was mainly observed in the forebrain in early development and was identified later in the cerebrum, while *NDRG2-4* was expressed in the developing brain and spinal cord (Zhong et al., 2015). In a mouse model, the expression of *NDRG3* was detected in the early embryo (embryonic day 9.5; E9.5), while NDRG1 and NDRG2 were identified in E12.5 and E13.5 respectively. Moreover, *NDRG1* was strongly expressed in the cerebral cortex, while *NDRG2* was specifically expressed around the ventricular zone in the cerebrum and spinal cord (Okuda and Kondoh, 1999). Since the NDRG family consists of four members, with each demonstrating its own specific regulation and function, this review mainly focuses on *NDRG1* due to its well-described metastasis suppressor role in some cancers, its crosstalk with other molecules (Park et al., 2020b), and its ability to be repressed by N-myc (Li and Kretzner, 2003)

5.1 NDRG1

NDRG1 acts as a potent metastasis suppressor in multiple tumor types (Le and Richardson, 2004; Kovacevic et al., 2008; Kobayashi et al., 2011; Liu et al., 2012b; Fang et al., 2014), with its epigenetic regulation by methylation suppressing its expression in prostate cancer (Li et al., 2015) and gastric cancer (Chang et al., 2021). Low levels of *NDRG1* expression have been detected in many tumors, including prostate, pancreatic, and colon cancers (Angst et al., 2006; Maruyama et al., 2006; Angst et al., 2011). Paradoxically, *NDRG1* can also be highly expressed in cancers of the kidney (Masuda et al., 2003), liver (Chua et al., 2007; Akiba et al., 2008; Yan et al., 2008), mouth (Chang et al., 2005), skin (Dang et al., 2006a), and uterine cervix (Song et al., 2008), where it has been suggested to play a role in promoting tumorigenesis.

Clinically, high levels of *NDRG1* expression in tumors are correlated with a better prognosis in patients suffering from prostate cancer (Bandyopadhyay et al., 2003), breast cancer (Bandyopadhyay et al., 2004), pancreatic cancer (Maruyama et al., 2006), and colorectal cancer (Strzelczyk et al., 2009). There are various stress stimuli that can induce *NDRG1* expression (Ellen et al., 2008; Fang et al., 2014), including iron depletion that up-regulates this metastasis suppressor *via* HIF-1α-dependent and independent mechanisms (Le and Richardson, 2004; Yuan et al., 2004; Lane et al., 2013; Lane et al., 2014). The HIF-1α-independent mechanism of up-regulating NDRG1 is at least partially mediated by eukaryotic initiation factor 3a (eIF3a) (Lane et al., 2013).

Studies have indicated that the expression of *NDRG1* and *NDRG2* may have an inverse relationship in some cancer cell types (Murray et al., 2004). Both NDRG1 and NDRG2 may be potentially phosphorylated on their core regions by SGK1, PKC, and AKT in certain cell

types (Burchfield et al., 2004; Murray et al., 2004; Melotte et al., 2010). The phosphorylation of NDRG1 at Thr346, Thr356, and Thr366 induced by SGK1 regulates tumor cell proliferation, differentiation, migration, and invasion (Melotte et al., 2010; Sahin et al., 2013). Furthermore, phosphorylation of NDRG1 by SGK1 primes it for phosphorylation by GSK3 (Murray et al., 2004). Studies have also demonstrated that connexin 43 (Cx43) may be essential for the phosphorylation of NDRG1 induced by AKT (Dunn and Lampe, 2014; Solan et al., 2019).

In addition to SGK1, there is also a relationship between NDRG1 expression and AKT activity in prostate cancer cells, where silencing *NDRG1* increases activated AKT (Dixon et al., 2013). Similarly, in placental cell, the knockdown of *NDRG1* significantly increased the activation of VEGF/AKT signaling, while the overexpression of NDRG1 suppressed PI3K/AKT signaling (Dai et al., 2020).

As a downstream target of N-Myc, NDRG1 is predictably involved in crosstalk with Myc family members. The expression of *N-Myc* represses *NDRG1* expression (Shimono et al., 1999) and in hemangioma endothelial cells, *NDRG1* expression positively regulated c-Myc protein levels, with *NDRG1* silencing leading to the down-regulation of *c-Myc* protein (Byun et al., 2018). Similar to Myc family members, NDRG1 performs at least part of its functions through directly associating with many other proteins (Liu et al., 2012b; Lu et al., 2015; Wang et al., 2017c; Menezes et al., 2019; Lim et al., 2021) and these interactions are discussed below.

5.2 Molecules that interact with NDRG1

5.2.1 Glycogen synthase kinase-3β (GSK-3β) and nuclear receptor 4A1 (Nur77)

NDRG1 directly interacts with GSK-3β and Nur77 to regulate β-catenin degradation in hepatocellular carcinoma cells (Lu et al., 2015). GSK-3 is an evolutionarily conserved serine/threonine kinase that consists of two highly homologous isoforms, GSK-3α and GSK-3β (Embi et al., 1980; Forde and Dale, 2007). GSK-3β can compensate for GSK-3α and this has been reported in studies examining knockout mouse models (Souder and Anderson, 2019). Relevant to this finding, GSK-3α knockout mice survived, while the loss of GSK-3β function was embryonically lethal (Hoeflich et al., 2000; MacAulay et al., 2007; Kaidanovich-Beilin et al., 2009). As a kinase, GSK-3β participates in the regulation of many cellular processes including, DNA repair, cell cycle, and tumor progression (Diehl et al., 1998; Harada et al., 2005; Kang et al., 2008; Luo, 2009). The overexpression of GSK-3β is observed in various cancers, such as colon, liver, ovarian, and pancreatic cancer, which show sensitivity to GSK-3β inhibition (Ougolkov et al., 2005; Shakoori et al., 2005; Luo, 2009; Zhou et al., 2012).

In hepatocellular carcinoma cells, GSK-3β directly interacts with NDRG1 that prevents the binding of GSK-3β to β-catenin, thereby protecting β-catenin from degradation and enhancing β-catenin translocation to the nucleus (Lu et al., 2015). In contrast, the suppression of NDRG1 expression in the same cell-type decreases β-catenin protein levels and nuclear accumulation, which further leads to the down-regulation of its target, cyclin D1 (Shtutman et al., 1999; Lu et al., 2015). The critical role of this interaction between NDRG1 and GSK-3β to hepatocellular carcinoma progression may also explain the fact that NDRG1 is overexpressed in hepatocellular carcinoma and is positively associated with aggressive tumor features such as vascular invasion and de-differentiation (Chua et al., 2007).

Similarly, NDRG1 also binds to Nur77 and disrupts the interaction between Nur77 and β-catenin, inhibiting Nur77-induced ubiquitination and proteasomal degradation of β-catenin (Sun et al., 2012; Lu et al., 2015). Taken together, the interaction of NDRG1 with both GSK-3β and Nur77 may explain the oncogenic function of NDRG1 in hepatocellular carcinoma. However, NDRG1 also plays anti-oncogenic roles in many cancer types, such as prostate, pancreatic, and colon cancers (Angst et al., 2006; Maruyama et al., 2006; Angst et al., 2011; Chen et al., 2012; Dixon et al., 2013; Kovacevic et al., 2016; Menezes et al., 2019). Further studies are needed to demonstrate whether NDRG1 also interacts with GSK-3β and Nur77 in tumor cells where NDRG1 is playing an anti-oncogenic role, and this would provide a better understanding of NDRG1 biology.

5.2.2 Low-density lipoprotein receptor-related protein 6 (LRP6)

It has been described above that NDRG1 can modulate WNT/β-catenin signaling *via* the regulation of β-catenin (Sun et al., 2012; Lu et al., 2015). In hepatocellular carcinoma cells, NDRG1 activates WNT/β-catenin signaling and plays an oncogenic role (Shtutman et al., 1999; Lu et al., 2015). In contrast, in breast cancer cells, NDRG1 inhibits WNT/β-catenin signaling through directly interacting with LRP6 (Liu et al., 2012b). LRP6 is a single-pass transmembrane receptor with an extracellular domain containing four tandem β-propeller/epidermal growth factor repeats, followed by three LDLR type A repeats (MacDonald and He, 2012). As an indispensable co-receptor of WNT ligands, the overexpression of LRP6 has been observed in many cancer-types including breast cancer, and leads to the enhancement of WNT/β-catenin signaling (Raisch et al., 2019).

The activation of the WNT/β-catenin signaling pathway requires the phosphorylation of LRP6 at multiple sites (Thr1479, Ser1490, and Thr1493) (Tamai et al., 2004; Davidson et al., 2005; Zeng et al., 2005). Examining breast cancer cells, the binding of NDRG1 to LRP6 prevented LRP6 phosphorylation at Ser1490 induced by WNT/β-catenin signaling, while having no effect on total LRP6 levels (Liu et al., 2012b). This inhibition of LRP6 phosphorylation at Ser1490 suggested that NDRG1 prevented the activation of WNT/β-catenin signaling (Liu et al., 2012b).

In prostate and colon cancer cells, NDRG1 expression also inhibited WNT/β-catenin signaling by a different mechanism (Wu and Pan, 2010; Jin et al., 2014). In fact, NDRG1 had no consistent effect on total GSK-3β levels or its activation, but decreased β-catenin phosphorylation at Ser33, Ser37, and Thr41 that is required for β-catenin degradation, while increasing non-phosphorylated β-catenin and total β-catenin (Wu and Pan, 2010; Jin et al., 2014). This NDRG1-mediated up-regulation of β-catenin did not activate WNT/β-catenin signaling as translocation of β-catenin to the nucleus was inhibited, with β-catenin being targeted to the plasma membrane (Jin et al., 2014). The translocation of β-catenin to the plasma membrane resulted in the colocalization with E-cadherin in adherens junctions that results in the inhibition of the EMT (Chen et al., 2012; Liu et al., 2012b; Jin et al., 2014; Lu et al., 2015).

From the analysis above, it can be suggested that NDRG1 behaves differently in the WNT signaling pathway depending on the cell-type, and can perform opposite functions resulting in either activation or inhibition of the WNT/β-catenin signaling pathway (Chen et al., 2012; Liu et al., 2012b; Jin et al., 2014; Lu et al., 2015). Further studies are needed to understand

the mechanism dictating the opposite functions of NDRG1 in different cancer cell-types so that this knowledge can be applied to developing innovative anti-cancer agents.

5.2.3 Mitogen-inducible gene 6 (MIG6)

Mig6 is a tumor suppressor that is also known as the receptor-associated late transducer (RALT), or ERBB receptor feedback inhibitor 1 (ERRFI), and is a cytoplasmic protein that functions as an epidermal growth factor receptor (EGFR) inhibitor by preventing asymmetric catalytic dimer formation (Wick et al., 1995; Anastasi et al., 2005; Xu et al., 2005; Zhang et al., 2007). The activity of MIG6 also includes its ability to increase the internalization and lysosomal degradation of EGFR (Ying et al., 2010). More recent studies suggest that MIG6 is involved in suppressing the activity of multiple membrane-bound tyrosine kinases, including c-Met (Park et al., 2020a), HER2, HER3, and HER4 (Zhong et al., 2021). In fact, Zhong and colleagues have suggested that MIG6 is a specific pan-HER inhibitor that can broadly suppress HER family members (Zhong et al., 2021).

The association between NDRG1 and MIG6 increases the half-life of MIG6 leading to its up-regulation in pancreatic cancer cells and this could play a role in the down-regulation of EGFR after NDRG1 overexpression (Menezes et al., 2019). Moreover, pharmacological-inducers of NDRG1 (Le and Richardson, 2004; Menezes et al., 2019) also up-regulate MIG6 and increase its co-localization with NDRG1 observed by confocal microscopy (Le and Richardson, 2004; Menezes et al., 2019). As such, the ability of pharmacological strategies to up-regulate NDRG1 is an emerging therapeutic modality and is discussed below in section 6.3

5.2.4 S-phase kinase-associated protein 2 (Skp2)

Skp2, also known as p45, is a key component of SKP1-cullin1-F-box (SCF) complex, which is one of the major categories of E3 ligases where the F-box protein is responsible for substrate recognition (Wang et al., 2012; Hnit et al., 2015). Skp2 induces K48-linked ubiquitination and proteasomal-mediated degradation of many substrates, including cyclin D (Yu et al., 1998), cyclin E (Yeh et al., 2001), and c-Myc (Kim et al., 2003). Furthermore, Skp2 is a direct transcriptional target of N-Myc in NB cells (Evans et al., 2015) and as such, could explain the inverse relationship between the expression of N-myc and c-Myc in NB cells (Zhe et al., 1999). That is, N-Myc up-regulates Skp2 that then degrades c-Myc through the proteasome.

NDRG1 was reported to directly interact with Skp2 resulting in the inhibition of Skp2 phosphorylation through the inactivation of CDK2 in lung cancer cells (Wang et al., 2017c). This event leads to the disrupted ubiquitination of c-Myc by Skp2 leading to the stabilization of c-Myc (Wang et al., 2017c). This finding indicates an oncogenic function of NDRG1 in lung cancer cells. In contrast, NDRG1 also functions as an anti-oncogenic effector in other tumor cell types such as breast, prostate, colon, and pancreatic cancer, with its expression correlating with better patient prognosis (Bandyopadhyay et al., 2003; Bandyopadhyay et al., 2004; Maruyama et al., 2006; Strzelczyk et al., 2009). In these latter cell-types, the interaction between NDRG1 and Skp2 remains unclear.

5.2.5 Androgen receptor (AR)

AR is a type I nuclear receptor and is one of the 49 members of the steroid receptor family of ligand-activated transcription factors, which play pivotal roles in organogenesis, differentiation, development, and a variety of physiological and pathological processes

(Evans, 1988; Tsai and O'Malley, 1994). The canonic functions of the AR are related to male physiology, such as differentiation of sex and sex-specific pathology (Quigley et al., 1995). It is also involved in tumor progression, particularly prostate cancer, and is considered to be the major tumorigenic driver in this disease (Heinlein and Chang, 2004). Activation of AR signaling supports the survival and growth of prostate cells, with nuclear AR staining in bone metastases of castration-resistant prostate cancer cells being correlated with worse outcomes (Ruizeveld de Winter et al., 1994; Crnalic et al., 2010).

The expression of *NDRG1* prevents AR activation and attenuates downstream AR signaling and prostate-specific antigen (PSA) levels (Lim et al., 2021). It has been demonstrated that NDRG1 directly binds to the Hsp90/AR complex that blocks ligand-mediated AR activation (Lim et al., 2021). This interaction inhibits EGF-mediated androgen-independent AR signaling *via* its effects on c-Jun, as well as the EGFR, PI3K, STAT3, and NF-κB signaling pathways (Lim et al., 2021). These results support the hypothesis that NDRG1 can be a key molecular target in androgen signaling to develop innovative therapies against prostate cancer and castration-resistant prostate cancer.

Lim and colleagues have observed a negative correlation between NDRG1 and PSA levels in patients that have relapsed (Lim et al., 2021). Combined with the fact that NDRG1 expression is negatively correlated with Gleason grade and metastasis in prostate cancers (Kurdistani et al., 1998; Bandyopadhyay et al., 2004; Caruso et al., 2004; Liu et al., 2011), NDRG1 could be a candidate biomarker for prostate cancer diagnosis.

5.2.6 Heat shock protein 90 (Hsp90)

As described previously, Hsp90 is an essential molecular chaperone that is involved in the cell stress response (Prodromou, 2016) and may indirectly prevent degradation of N-Myc protein through EZH2 stabilization (Welcker et al., 2004; Huang et al., 2017; Wang et al., 2022). In addition to EZH2, NDRG1 is also a client protein for Hsp90 (Banz et al., 2009). However, inhibiting Hsp90 activity neither affected the formation of Hsp90/NDRG1 complex nor NDRG1 stability, which may suggest the binding of Hsp90 to NDRG1 is not due to the canonical maturation and stabilization function of Hsp90 (Banz et al., 2009). The up-regulation of *NDRG1* transcription and phosphorylation can be achieved by inhibition of Hsp90 activity (Banz et al., 2009). As such, the use of Hsp90 inhibitors (see section 6.2.3) could be a useful therapeutic modality in some cancers to suppress NDRG1 activity.

5.2.7 Pro-viral integration site for Moloney murine leukemia virus – 1 (PIM1)

PIM1 is a proto-oncogene encoding a serine/threonine kinase (Nawijn et al., 2011; Narlik-Grassow et al., 2014). It is found to be constitutively active and does not depend upon post-translational modification for activation (Kumar et al., 2005; Qian et al., 2005). The overexpression of PIM1 has been detected in several tumor cell-types, including pancreatic cancer cells (Peng et al., 2013; Xu et al., 2016; Cheng et al., 2017). Recently, Ledet *et al.* have revealed that NDRG1 can be phosphorylated by PIM1 at serine 330 (pS330), which may lead to *NDRG1* destabilization (Ledet et al., 2021). This is also observed in LNCaP prostate cancer cells, where PIM1 overexpression resulted in decreased NDRG1 protein levels (Ledet et al., 2021).

PIM1 also regulates cell death, tumor growth, and chemotherapy response in breast cancer (Brasó-Maristany et al., 2016), and can be up-regulated by interleukin 6 (IL6) in breast cancer through activation of STAT3 signaling (Gao et al., 2019). The up-regulation of PIM1 is responsible for the EMT and stemness in breast cancer cells *via* the activation of *c-Myc* (Gao et al., 2019). These oncogenic functions are consistent with the role of PIM1 in down-regulating NDRG1 expression that is known to inhibit the EMT in prostate and colon cancer cells (Chen et al., 2012).

Investigations by Gao and colleagues provide evidence that the expression of PIM1 up-regulates *c-Myc* (Gao et al., 2019). In ovarian cancer, PIM1 participates in glycolysis and promotes proliferation by interacting with the Myc family (Wu et al., 2018). Furthermore, the interaction between the nuclear factor of activated T cells cytoplasmic 1 (NFATC1) and PIM1 is essential for promotion of prostate cancer cell migration and invasion (Eerola et al., 2019). PIM1 facilitates the EMT by phosphorylation of c-Myc to activate the transcriptional repressors, ZEB1, ZEB2, Snail1, Snail2, and Twist, while having no effect on Smad activation (Zhao et al., 2018) (**Fig. 8**).

Taken together, the various functions of PIM1 in tumor progression, including supporting tumor growth, and inducing the EMT, suggest that PIM1 is an attractive pharmacological target for cancer therapy. There are many studies examining the pharmacological targeting PIM1, and these have shown promising results (Merkel et al., 2012; Ogawa et al., 2012; Holder and Abdulkadir, 2014; Xie and Bayakhmetov, 2016; Zhao et al., 2017). However, drug strategies that specifically target the interaction between PIM1 and NDRG1 are currently poorly explored and could be important for inhibiting EMT.

6. Therapeutics targeting c-Myc and N-Myc

The Myc family members play a significant role in tumor pathogenesis and are overexpressed in many cancer cells (Dang, 1999; Davis et al., 1999; Beltran et al., 2011; Northcott et al., 2012; Fielitz et al., 2016; Cassia et al., 2018). Considering this, there are many compounds targeting the Myc family (Calabrese et al., 2018; Das et al., 2018; Hu et al., 2018) (**Fig. 9**), and some well-studied examples of these agents are described below.

6.1 Pharmacological inhibition of c-Myc and N-Myc transcription

6.1.1 G-quadruplex stabilizer

As discussed in sections 4.1 and 4.2, there are a variety of proteins involved in the transcriptional regulation of *c-Myc* and *MYCN* that can be potent pharmacological targets to inhibit their transcription (Siddiqui-Jain et al., 2002; Trajkovski et al., 2012; Chipumuro et al., 2014). For example, G-quadruplexes located at the nuclease hypersensitive element III (NHE III) region of the *c-Myc* and *MYCN* promoters lead to suppression of their mRNA and protein levels, which induces cytotoxicity especially to Myc-driven cancers (González and Hurley, 2010; Brown et al., 2011; Mathad et al., 2011; Calabrese et al., 2018; Das et al., 2018; Hu et al., 2018). Considering the silencing function of G-quadruplexes on *c-Myc* and *MYCN* expression (Siddiqui-Jain et al., 2002; Dutta et al., 2018; Psaras et al., 2021), there are a number of small molecules that stabilize the G-quadruplex to prevent *Myc* expression and cellular proliferation (Siddiqui-Jain et al., 2002; Dutta et al., 2018; Psaras et al., 2021).

These compounds include one pan-G4-stabilizer, namely CX-5461, that is in a current clinical trial for BRCA-mutated breast cancer (Xu et al., 2017). This agent down-regulates N-Myc protein levels and suppresses NB tumor growth (Taylor et al., 2019). More importantly,

the tumor inhibitory activity of CX-5461 is selective for *c-Myc* and NB cell-types with amplified *MYCN* compared to non-amplified NB cells (Taylor et al., 2019). However, these G-quadruplex stabilizing agents do not possess absolute selectivity for any specific gene (Felsenstein et al., 2016). As such, further studies are needed to develop G-quadruplex stabilizers with better selectivity for *Myc*-driven cancers.

6.1.2 CDK7 inhibitor

As another thearpeutic target, the super-enhancer of *MYCN* is also attractive. The experimental anti-cancer drug, N-[3-[[5-chloro-4-(1H-indol-3-yl)-2-pyrimidinyl]amino]phenyl]-4-[[(2E)-4-(dimethylamino)-1-oxo-2-buten-1-yl]amino]-benzamide (THZ1), is a covalent inhibitor of CDK7 that plays a key role in super-enhancer activity and demonstrates excellent inhibitory activity on NB proliferation (Chipumuro et al., 2014). This inhibition of proliferation was somewhat selective, with THZ1 being far more effective against NB cells with amplified *MYCN* compared to those without amplification (Chipumuro et al., 2014). This inhibitory activity of THZ1 has suggested to be mediated by the suppression of super-enhancer activity (Chipumuro et al., 2014). However, the clinical performance of THZ1 is limited by its short half-life (45 min in mouse plasma), and similar to G-quadruplex stabilizers, THZ1 may also unfortunately inhibit transcription in normal cells (Li et al., 2019a).

Collectively, transcriptional inhibition of *c-Myc* and *N-Myc* has achieved promising results in suppressing cancer cell proliferation and tumor growth (Chipumuro et al., 2014; Li et al., 2019a; Taylor et al., 2019). However, their selectivity against tumor cells relative to normal cells needs to be refined (Felsenstein et al., 2016; Li et al., 2019a).

6.2 Indirect pharmacological targeting based on Myc family interactions

Another pharmacological strategy is to target protein-protein interactions as well as the protein stabilization of the Myc family. As described in section 3, there are multiple proteins involved in direct interaction with the Myc family that have been targeted with pharmacopoeia including MAX, EZH2, Hsp90, Aurora-A, LSD1, and USP7, and these agents are described below.

6.2.1 Myc/MAX complex inhibitor

Since Myc proteins perform their oncogenic function through forming a heterodimer with MAX (Pelengaris et al., 2002; Adhikary and Eilers, 2005; Meyer and Penn, 2008), this interaction can be a promising pharmacological target to inhibit Myc oncoprotein activity. The small molecule, MYCMI-6 (**Fig. 9**), has been identified that demonstrates strong selective inhibition of the Myc/MAX interaction and has promising inhibitory activity against the proliferation of Myc-driven cancers, such as NB (Clausen et al., 2010; Choi et al., 2017; Castell et al., 2018). This agent blocks the formation of Myc/MAX heterodimer and leads to the suppression of Myc-driven transcription, which significantly induces cell death and decreases the proliferation of NB cells with amplified *MYCN* (Castell et al., 2018). However, MYCMI-6 and other heterodimer inhibitors cannot degrade N-Myc protein (Ambrosio et al., 2017).

6.2.2 EZH2 and Aurora-A inhibitors

Inhibitors of EZH2 (Wang et al., 2022) and Aurora-A (Otto et al., 2009) have been developed to decrease Myc protein levels (**Fig. 9**). These agents have been examined for their anti-cancer activity in Myc-driven cancers such as NB, with some entering clinical trials (Faisal

et al., 2011; Tavana et al., 2016; Bownes et al., 2021). For example, the EZH2 inhibitor, GSK343 (**Fig. 9**) (Bownes et al., 2021), and Aurora-A inhibitor, CCT137690 (**Fig. 9**) (Faisal et al., 2011), decrease NB cellular proliferation *in vitro* and prevent NB tumor growth *in vivo*. The compound, CCT137690, is more active against NB cells with amplified *MYCN* compared to cells with low *MYCN* expression (Faisal et al., 2011). Moreover, CCT137690 also has inhibitory activity against Aurora-B activity in NB cells, while simultaneously inhibiting Aurora-A, which is also a potent target for N-Myc-driven NB (Faisal et al., 2011; Bogen et al., 2015).

Other inhibitors have also been developed that specifically target Aurora-A activity and have shown promise. Some of these agents have entered clinical trials, including VX-680/MK-0475 (tozasertib) (Seymour et al., 2014), PHA-739358 (Danusertib) (Schöffski et al., 2015), and MLN8237 (Brunner et al., 2020). Unfortunately, clinical trials have established that Aurora-A inhibitors have unexpected adverse events such as neutropenia (Seymour et al., 2014), fatigue/asthenia, nausea, diarrhea, anorexia, vomiting, alopecia, constipation, and pyrexia (Schöffski et al., 2015). The combination of Aurora-A inhibitors with other anti-cancer drugs also has promising anti-tumor activity and has successfully entered phase I clinical studies as a new approach to NB therapy (DuBois et al., 2016).

6.2.3 Hsp90 inhibitors

Geldanamycin (GA) was first identified in 1970 from *Streptomyces hygroscopicus* as a benzoquinone annamycin antibiotic (DeBoer et al., 1970) that binds to the *N*-terminal of Hsp90 preventing its ability to bind ATP and perform its chaperone activity (**Fig. 10**) (Stebbins et al., 1997). Treatment with GA directly inhibits Hsp90 activity, and as a result, this leads to destabilization of its client protein resulting in proteasomal degradation (**Fig. 10**).

This suggestion was derived from studies where GA induced rapid depletion of HER2/Neu (ERBB-2), which can be rescued by proteasomal inhibitors (Mimnaugh et al., 1996). Similar results were also reported for epidermal growth factor receptor, cyclin-dependent kinase 4, polo-like kinase 1, *etc.* (Blagosklonny, 2002; Blagg and Kerr, 2006; Fukuyo et al., 2010).

Unfortunately, the clinical application of GA is restricted by its side effects, including substantial hepatotoxicity (Supko et al., 1995). To overcome these problems, multiple GA analogs have been prepared to decrease its side-effects and increase its pharmacological efficacy (Blagosklonny, 2002; Mitsiades et al., 2006; Senju et al., 2006; Williams et al., 2007; Trepel et al., 2010). One such GA analog, 17-N-allylamino-17-demethoxygeldanamycin (17-AAG), has been demonstrated to have broad anti-tumor activity and has entered phase III clinical trials (Blagosklonny, 2002; Mitsiades et al., 2006; Senju et al., 2006; Williams et al., 2007; Trepel et al., 2010). Further, 17-AAG showed better selectivity than GA with a 100-fold higher binding affinity to Hsp90 in tumor cells relative to normal cells (Kamal et al., 2003). In addition, 17-AAG also resulted in synergistic anti-cancer activity upon combination with other anti-cancer drugs (Vasilevskaya et al., 2003; Bagatell et al., 2005). For example, cisplatin was found to enhance the activity of 17-AAG in many tumor cell-types including, colon cancer and NB cells (Vasilevskaya et al., 2003; Bagatell et al., 2005). These studies confirmed the important role of Hsp90 in cancer cells and the effectiveness of Hsp90 inhibitors in cancer chemotherapy.

More recently, 17-AAG also has been shown *in vivo* and *in vitro* to protect against neurotoxicity of the FDA- approved anesthetic, sevoflurane *via* Hsp70-dependent inhibition of apoptosis, oxidative stress, and pro-inflammatory signaling pathways (Liu et al., 2020a).

These studies indicate that 17-AAG could potentially be used as a co-treatment to reduce the side effects of sevoflurane (Michel and Constantin, 2009; Miller et al., 2022).

Apart from 17-AAG, there are currently a number of Hsp90 inhibitors with anti-cancer activity that have also entered clinical trials (**Table 1**) (Trepel et al., 2010). As introduced previously, the Hsp90 inhibitors also can be used as part of a combination of anti-cancer drugs either to enhance its anti-cancer activity or decrease the side effects of other drugs. Considering the function of N-myc in NB cell progression (Brodeur et al., 1984; Seeger et al., 1985) and the interaction between Hsp90 and N-Myc (Huang et al., 2017; Wang et al., 2022), the application of Hsp90 inhibitors in NB cancer therapy may also be an important future therapeutic strategy.

6.2.4 USP7 inhibitor

USP7 is another pharmacological target that is also involved in stabilizing N-Myc protein reported to markedly suppress the growth of human NB cells with amplified *MYCN in vitro* and *in vivo* (Tavana et al., 2016; Ohol et al., 2020). The USP7 inhibitor, P22077 (**Fig. 9**), inhibits the deubiquitinase activity of USP7 on N-Myc that leads to its down-regulation (Tavana et al., 2016). Due to the function of USP7 in also decreasing p53 half-life, the inhibition of USP7 by P22077 also increases p53 expression to induce p53-mediated apoptosis that inhibits NB growth (Fan et al., 2013).

Taken together, the pharmacological strategies described above provide potential strategies regarding the potential of cocktail therapy for Myc-driven cancers.

6.2.5 LSD1 inhibitors

There are a number of natural and synthetic LSD1 inhibitors being investigated some of which are undergoing clinical trials (Wang et al., 2015b; Przespolewski and Wang, 2016; Yu et al., 2016; Zheng et al., 2016a; Zheng et al., 2016b; Li et al., 2017; Wang et al., 2017a; Wang et al., 2017b; Li et al., 2019b; Wang et al., 2019b). For example, tranylcypromine (TCP), is an irreversible LSD1 inhibitor (Binda et al., 2010) that has tumor suppression activity against bladder cancer and NB (Schulte et al., 2009) and has entered phase I clinical trials for acute myeloid leukemia (AML) (Fang et al., 2019). Regrettably, the adverse effects of this agent such as febrile neutropenia and thrombocytopenia make it necessary to develop safer LSD1 inhibitors (Watts et al., 2018).

6.3 Targeting cellular iron to down-regulate c-Myc and N-Myc and up-regulate NDRG1

Surprisingly, the depletion of cellular iron results in the potent down-regulation of both c-Myc and N-Myc expression (Kyriakou et al., 1998; Fan et al., 2001). The well-characterized iron chelator, desferrioxamine (DFO) (**Fig. 11**), has been used for decades for treating iron overload disease (Propper et al., 1977; Brittenham et al., 1994; Richardson and Ponka, 1998) and effectively inhibits c-Myc (Kyriakou et al., 1998) and N-Myc expression (Fan et al., 2001). Moreover, DFO has also been demonstrated to up-regulate both *NDRG1* mRNA and protein levels (Le and Richardson, 2004). Considering the critical role of c-Myc, N-Myc, and NDRG1 in cancer biology, cellular iron sequestration can be an attractive pharmacological strategy for cancer therapy.

Although promising outcomes for NB patients have been observed after treatment with DFO (Donfrancesco et al., 1990), this agent suffers several problems. These issues of DFO include

poor membrane permeability that leads to inactivity after oral administration, a short serum half-life (Buss et al., 2003; Millán et al., 2021), and side-effects at high doses (Blatt, 1994). Therefore, it is important to develop new anti-cancer therapeutics targeting cellular iron. A number of other iron-binding ligands have been examined for NB treatment, including Deferiprone (1,2-dimethyl-3-hydroxypyridin-4-one) (Blatt et al., 1989) and pyridoxal isonicotinoyl hydrazone analogs (Richardson and Ponka, 1994; Richardson et al., 1995). More recently, the di-2-pyridylketone thiosemicarbazone (DpT) class of iron-binding ligands have been developed (**Fig. 11**) (Yuan et al., 2004). These compounds and their related analogs effectively inhibit the proliferation of a broad spectrum of cancer cells *in vitro* and *in vivo* with marked selectivity (Yuan et al., 2004; Whitnall et al., 2006; Kovacevic et al., 2011; Lovejoy et al., 2012; Kovacevic et al., 2013; Jansson et al., 2015b; Kovacevic et al., 2016; Lim et al., 2020; Geleta et al., 2021; Dharmasivam et al., 2022), including neuroblastoma (Guo et al., 2016).

The first lead agent of the DpT class of agents, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT), and its analog, namely di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC) (Kovacevic et al., 2011; Lovejoy et al., 2012) induces marked up-regulation of the metastasis suppressor, NDRG1, in many tumor cell-types (Le and Richardson, 2004; Kovacevic et al., 2011). As described in Section 5, NDRG1 performs anti-oncogenic roles in cancer cells by interacting with other anti-oncogenic effector proteins, such as LRP6, MIG6, and AR (Liu et al., 2012b; Menezes et al., 2019; Lim et al., 2021). This evidence suggests the potential mechanism of anti-proliferative activity of Dp44mT and DpC against these cancer cell-types (Dharmasivam et al., 2022) by increasing anti-oncogenic interactions in these cancer cells.

As described above (see sections 5.2.1 and 5.2.4), NDRG1 may also perform an oncogenic role in some cancers, including hepatocellular carcinoma (Lu et al., 2015) and lung tumors (Wang et al., 2017c) by associating and inhibiting the anti-oncogenic activity of GSK-3β and Skp2, respectively. This up-regulation of NDRG1 expression induced by Dp44mT and DpC may potentially contribute to the interaction between NDRG1 and GSK-3β, as well as NDRG1 and Skp2, and this could be speculated to support proliferation. However, it is notable that DFO significantly inhibits the proliferation of hepatocellular carcinoma cells (Asperti et al., 2019), while Dp44mT and DpC also have marked tumor suppression activity against lung cancer cells *in vitro* and *in vivo* (Lovejoy et al., 2012; Dharmasivam et al., 2022). Indeed, while there are some reports that NDRG1 plays a pro-oncogenic role (Chua et al., 2007; Lu et al., 2015; Wang et al., 2017c), it is notable that potent NDRG1-inducing agents such as Dp44mT and DpC demonstrate very broad and potent anti-cancer activity against a wide variety of tumor cell-types (Yuan et al., 2004; Whitnall et al., 2006; Kovacevic et al., 2011; Lovejoy et al., 2012; Kovacevic et al., 2013; Jansson et al., 2015b; Kovacevic et al., 2016; Lim et al., 2020; Geleta et al., 2021; Dharmasivam et al., 2022). This comprehensive spectrum of anti-cancer efficacy could be related to the fact that these agents have multiple key targets in addition to NDRG1 that leads to pronounced anti-neoplastic activity (Jansson et al., 2015a; Wijesinghe et al., 2021).

7. Perspectives and Conclusions

Myc family members, including c-Myc and N-Myc, are both essential proteins involved in the regulation of cancer cell metabolism, progression, and proliferation (Pelengaris et al., 2002; Le and Richardson, 2004; Adhikary and Eilers, 2005; Kovacevic et al., 2008; Kobayashi et al., 2011; Dang, 2012; Liu et al., 2012b). The interactions between Myc family

proteins and their partners *e.g.*, Aurora-A and MAX are responsible for their stability and function, respectively (Pelengaris et al., 2002; Adhikary and Eilers, 2005; Meyer and Penn, 2008; Otto et al., 2009; Tavana et al., 2016; Wang et al., 2022) and can be potential targets for anti-cancer drug development, especially for Myc-driven cancers. Therapeutics that target the stability and function of Myc proteins have shown promising inhibitory activity against the proliferation of Myc-driven cancers, including NB (Faisal et al., 2011; Fan et al., 2013; Tavana et al., 2016; Castell et al., 2018; Bownes et al., 2021). Due to their different mechanisms of action, the combination of these drugs can also be promising for cancer therapies.

While the interactions of Myc with various protein partners are promising for cancer therapy, some have not been pharmacologically targeted, such as the interactions between HIF-1α and c-Myc (Koshiji et al., 2004). It has been demonstrated that slowly dividing cells under hypoxic conditions can escape or demonstrate resistance to cytotoxic anti-cancer drugs (Birner et al., 2000; Anjum et al., 2019). Since HIF-1α binds to c-Myc and induces p21 expression, G_1 arrest, and the inhibition of proliferation (Koshiji et al., 2004), this interaction could be the mechanism behind hypoxia-induced drug resistance and could be a promising pharmacological target to overcome hypoxia-induced drug resistance in cancer cells. Thus, knowledge regarding protein-protein interactions can be important for anti-cancer drug development, as this provides a rationale for "cocktail therapy" using drug combinations. Since the interaction between HIF-1α and c-Myc may contribute to drug resistance, the combination of anti-cancer drugs and HIF-1α inhibitors could lead to better patient outcomes.

In addition to the Myc family proteins, the metastasis suppressor, NDRG1, plays an important role in many cellular processes *via* interacting with other proteins (Banz et al.,

2009; Liu et al., 2012b; Lu et al., 2015; Wang et al., 2017c; Menezes et al., 2019; Ledet et al., 2021; Lim et al., 2021). These functions include its anti-oncogenic roles through direct binding to LRP6, MIG6, and the AR (Liu et al., 2012b; Menezes et al., 2019; Lim et al., 2021). Since the DpT class of drugs potently induces the up-regulation of NDRG1 in many different cancer cell-types (Kovacevic et al., 2011; Chen et al., 2012; Dixon et al., 2013; Kovacevic et al., 2013; Xi et al., 2017; Merlot et al., 2019; Park et al., 2020a), this could be one of their major mechanisms of anti-tumor activity (Liu et al., 2012b; Menezes et al., 2019; Lim et al., 2021).

In some tumors, NDRG1 has been reported to play an oncogenic role *via* interacting with other proteins, such as GSK-3β and Skp2 (Lu et al., 2015; Wang et al., 2017c; Zhao and Richardson, 2023). Currently, it remains unclear what dictates the interaction of NDRG1 with either pro-oncogenic or anti-oncogenic proteins. As such, a better understanding of protein-protein interactions of NDRG1 may provide further clues to its roles in cancer cell biology.

Considering promising future therapeutic directions, there have been a number of preclinical studies or clinical trials conducted based on targeting protein-protein interactions that have achieved promising results. For instance, a phase I clinical trial revealed that the combination of the Aurora-A inhibitor, alisertib, the topoisomerase I inhibitor, irinotecan, and the alkylating agent, temozolomide, showed a promising response rate compared to NB patients treated with alisertib alone (Bagatell et al., 2011; DuBois et al., 2016; Mossé et al., 2019). This review provides further ideas for targeting different protein-protein interactions involving the Myc family and NDRG1 by combining various agents, including the NDRG1-inducing thiosemicarbazones that show synergy with a broad variety of chemotherapeutics (Dharmasivam et al., 2022).

In summary, the interactions between Myc family proteins and their partners, as well as NDRG1 with its partners are critical for their biological functions in cancer cells in either oncogenic or anti-oncogenic roles. These interactions provide attractive targets for anti-cancer drug development. However, despite many agents being developed based on these protein-protein interactions, appropriate drug combinations in clinical trials or pre-clinical trials remain poorly explored. Additionally, there are some protein interactions that require further investigation, as they may also provide potential pharmacological targets for drug development or suggestions for drug combinations.

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Footnotes

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Table 1. Line drawings of the structures of common Hsp90 inhibitors, their clinical trial states, and routes of administration.

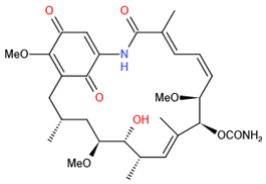
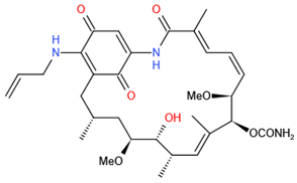
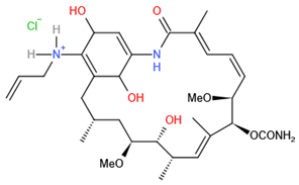
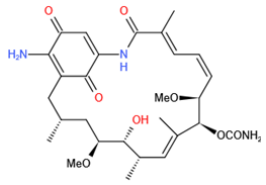
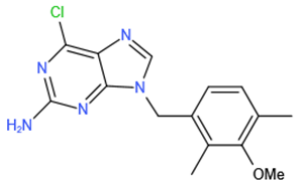
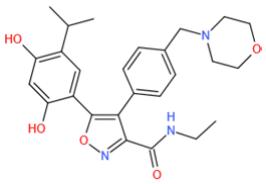
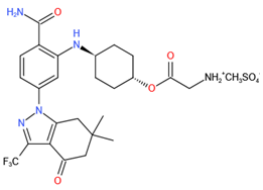
Name	Structure	Phase	Route
GeldanaMycin (GA)		-	-
TanespiMycin (17-AAG)		**III**	**Intravenous**
RetaspiMycin hydrochloride (IPI-504)		**II**	**Intravenous**
IPI-493		**I**	**Oral**
BIIB021 CNF2024		**II**	**Oral**
AUY922		**I/II**	**Intravenous**
SNX-5422 mesylate		**I**	**Oral**

Figure Captions

Figure 1. Functions of Myc proteins. Myc family members are involved in many cellular processes, including transcription, signal transduction, the cell cycle, protein synthesis, translation, metabolism, cell adhesion, the cytoskeleton, DNA repair, *etc*.

Figure 2. WNT signaling is involved regulation of Myc proteins. N-Myc can activate the WNT pathway by inhibition of DKK3, while c-Myc is a transcriptional target of β-catenin and is regulated by the WNT signaling pathway. Activated WNT signaling increases HIF-1α expression, which up-regulates NDRG1 and further decreases Skp2 phosphorylation, which leads to the stabilization of c-Myc in breast cancer cells

Figure 3. Iron metabolism is regulated by c-Myc. c-Myc participates in the iron metabolism of cancer cells through the up-regulation of the RNA-binding protein, iron regulatory protein 2 (IRP2). IRP2 inhibits the translation of the iron storage protein, ferritin, by binding to the 5' untranslated region of *ferritin* mRNA. In contrast, IRP2 binds to the 3' untranslated region of *TfR1* mRNA increasing its stability and translation. This up-regulation of TfR1 increases uptake of iron from the iron transport protein, transferrin. The overall effect of c-Myc expression is an increase in the levels of cellular iron that is essential for proliferation. Created with BioRender.com (2022).

Figure 4. Polypeptide structures of the three Myc family members and MAX.

Figure 5. Functions of MAX in cellular processes. MAX perform its biological functions *via* binding to a variety of different proteins. The binding of MAX to Myc family members

activates the target gene of Myc proteins, which can be blocked by the binding of MXI1, MNT, MGA, and MXD family members to MAX.

Figure 6. c-Myc and HIF-1α associations in regulating metabolism and proliferation. **(A)** c-Myc and HIF-1α participated in the regulation of glucose metabolism and stimulate the Warburg effect. Under normoxic conditions, c-Myc transcriptionally regulates glucose metabolism genes including, the *glucose transporter 1 (GLUT1)*, *hexokinase 2 (HK2)*, *phosphofructokinase (PFKM)*, and *enolase 1 (ENO1)*. These genes can also be regulated by HIF-1α under hypoxia conditions, to increase the transport of glucose into cells, its catabolism to pyruvate, and ultimately to lactate. Moreover, c-Myc is also reported to be involved in glutamine metabolism and can induce oxidation of glutamine under an adequate oxygen tension (Dang et al., 2009). Adapted from 'Cancer cell metabolism (nutrient-replete)', by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates. **(B)** The interactions between c-Myc and HIF-1α. Under normoxic conditions, c-Myc performs its functions in gene expression and DNA repair with its partners such as MAX and SP1. Under hypoxic conditions, HIF-1α inhibits c-Myc function through competitive binding to its molecular partners *e.g.*, MAX.

Figure 7. Hsp90 indirectly stabilizes N-Myc through EZH2 stabilization. Hsp90 binds to EZH2 to stabilize it, which binds to N-Myc in competition with FBXW7 and prevents the ubiquitination and proteasomal degradation of N-Myc.

Figure 8. NDRG1 and PIM1 are involved in the regulation of the epithelial mesenchymal transition (EMT). PIM1 interacts with Smad2 or Smad3 in the nucleus and subsequently phosphorylates these proteins to induce the expression of the E-cadherin

repressors, SNAIL and ZEB1. NDRG1 has been demonstrated to decrease the expression of SNAIL and ZEB1.

Figure 9. Pharmacological strategies aimed at inhibiting Myc activity through indirect mechanisms. There are many different pharmacological strategies targeting Myc indirectly. Inhibitors of CDK7 (*e.g.*, TZH1) inhibit Myc expression at the transcriptional level. Myc translation can also be blocked by inhibitors such as BEZ235, MK2206, and Rapamycin, whereas inhibitors of Aurora-A, EZH2, and USP7 can destabilize Myc at the post-translational level. Myc/MAX heterodimer formation can be inhibited by the agent, MYCMI-6. Created with BioRender.com (2022).

Figure 10. Mechanism of Geldanamycin (GA) in inhibiting Hsp90 function. GA blocks the binding of ATP to Hsp90, which is required for its client protein binding activity and further leads to the degradation of client proteins.

Figure 11. Line drawings of the chemical structures of: (A) DFO; **(B)** di-2-pyridylketone thiosemicarbazone (DpT) analogues; **(C)** Dp44mT; and **(D)** DpC**.**

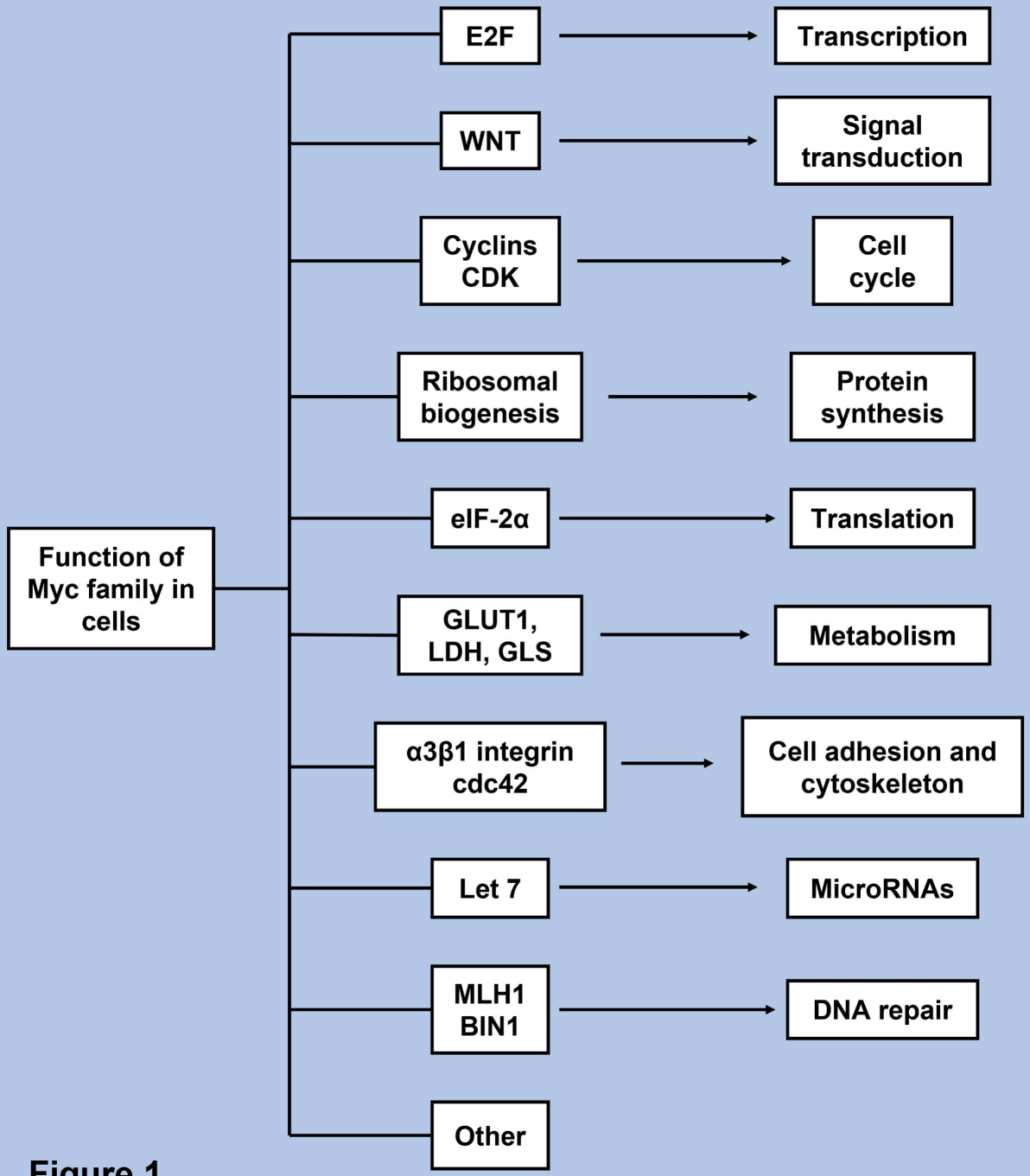

Function of Myc family in cells
E2F
Transcription
WNT
Signal transduction
Cyclins CDK
Cell cycle
Ribosomal biogenesis
Protein synthesis
eIF-2α
Translation
GLUT1, LDH, GLS
Metabolism
α3β1 integrin cdc42
Cell adhesion and cytoskeleton
Let 7
MicroRNAs
MLH1 BIN1
DNA repair
Other

Figure 1

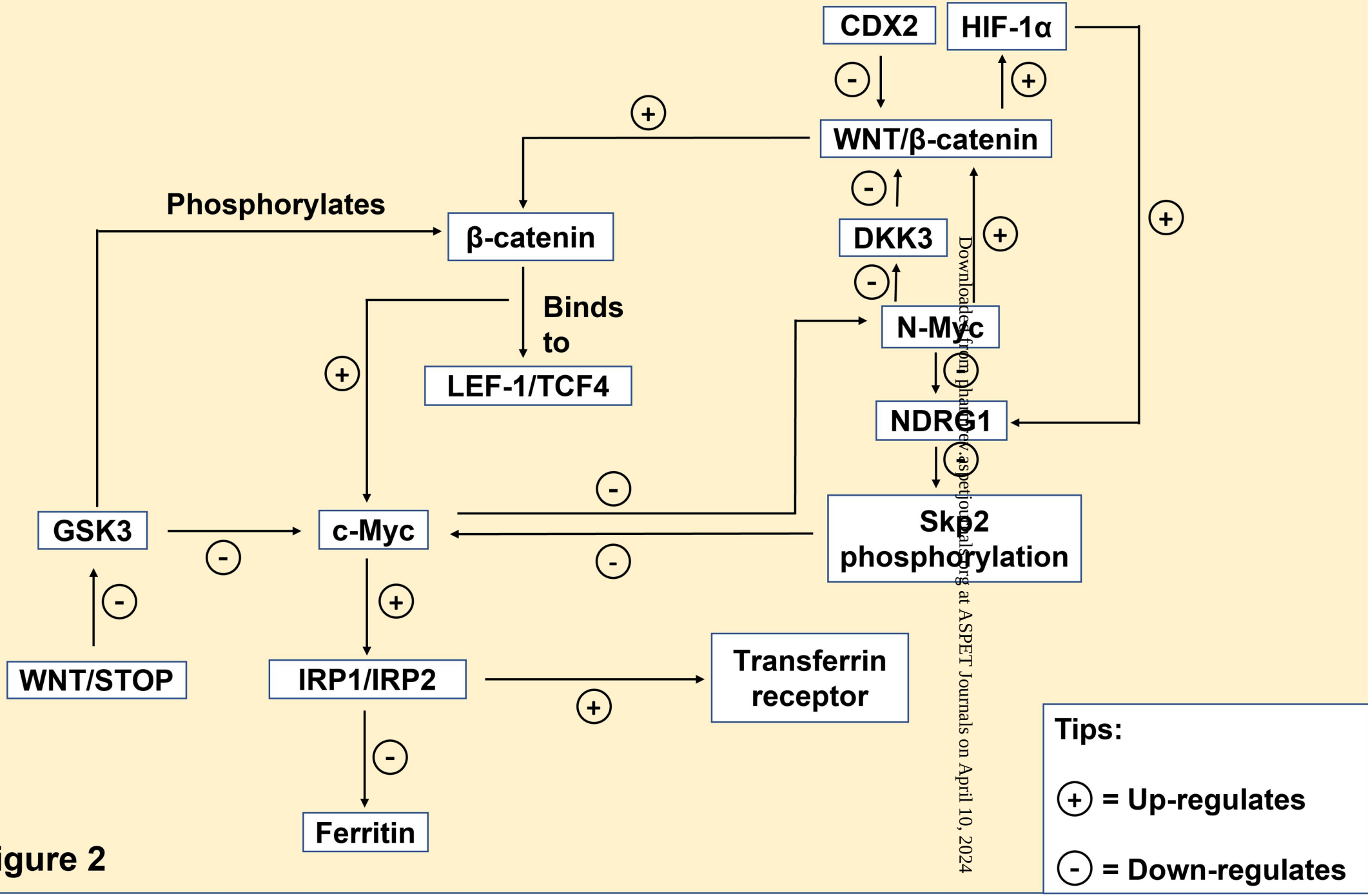


Figure 2

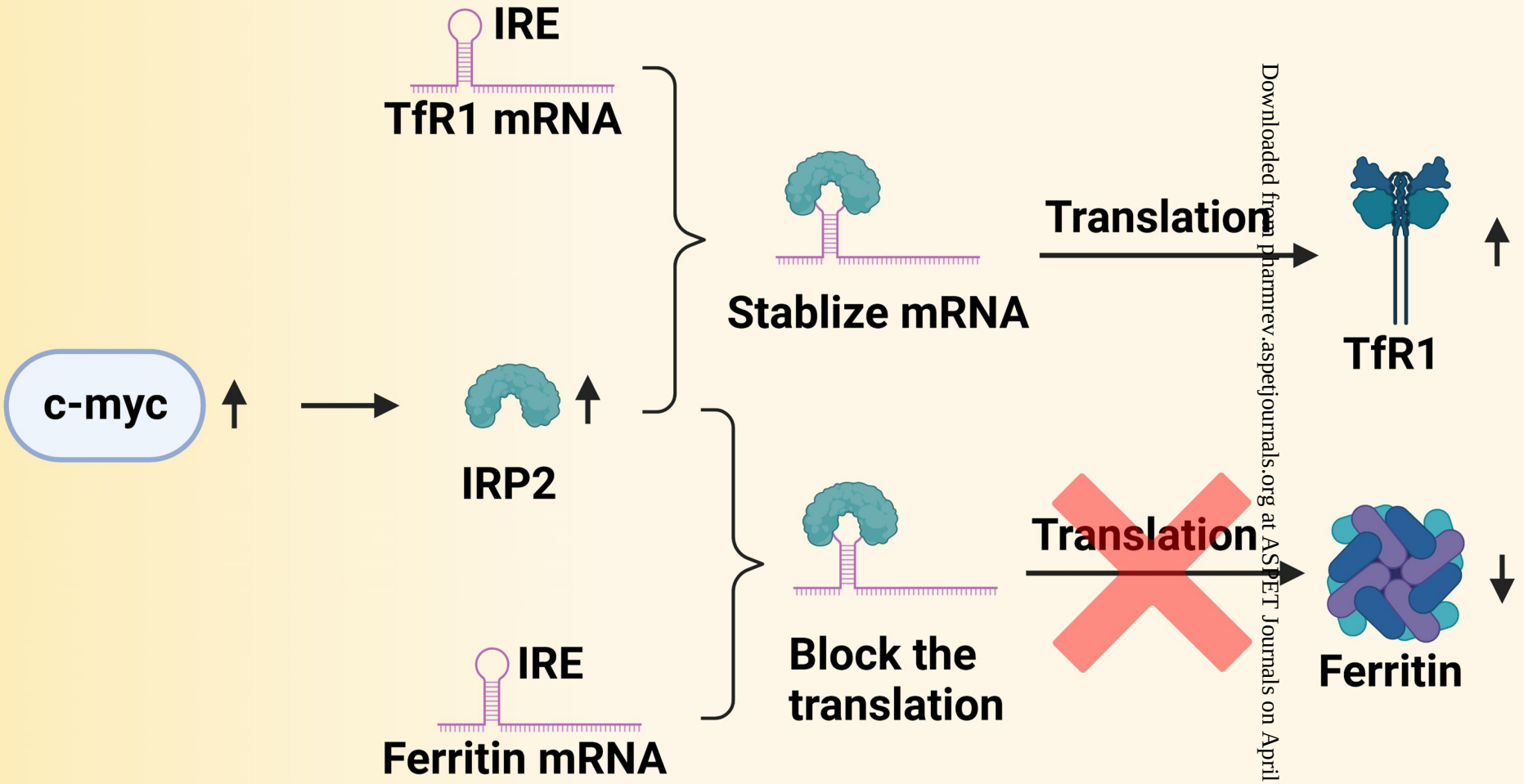


Figure 3

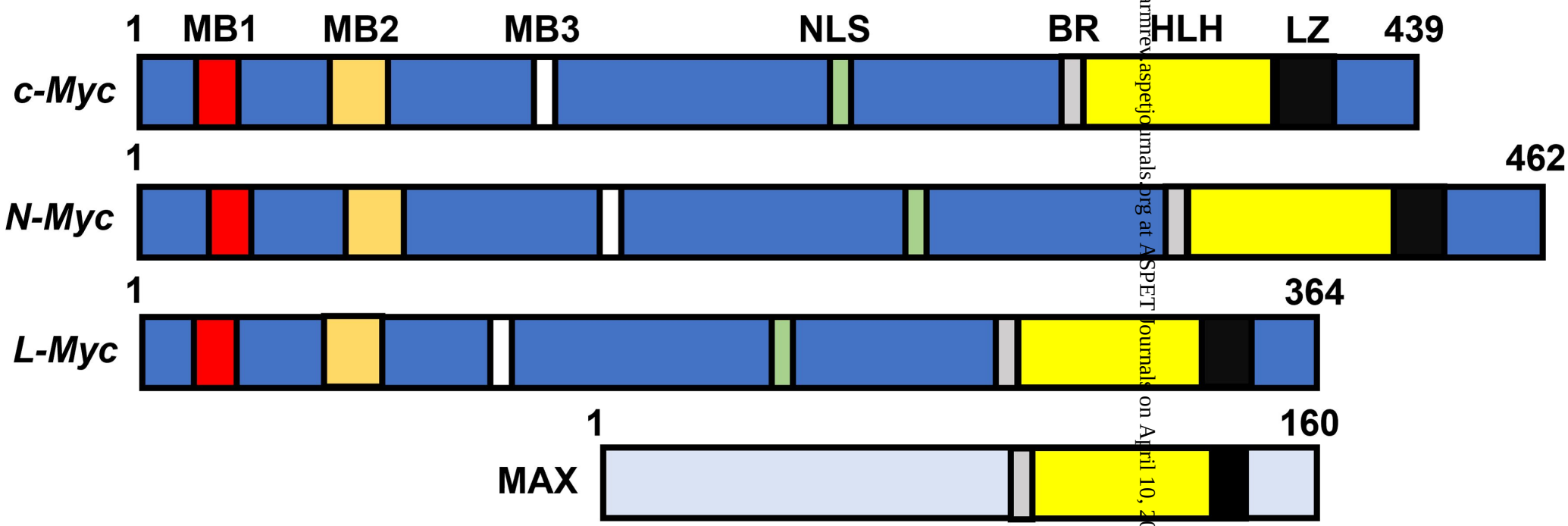


Figure 4

Figure 5

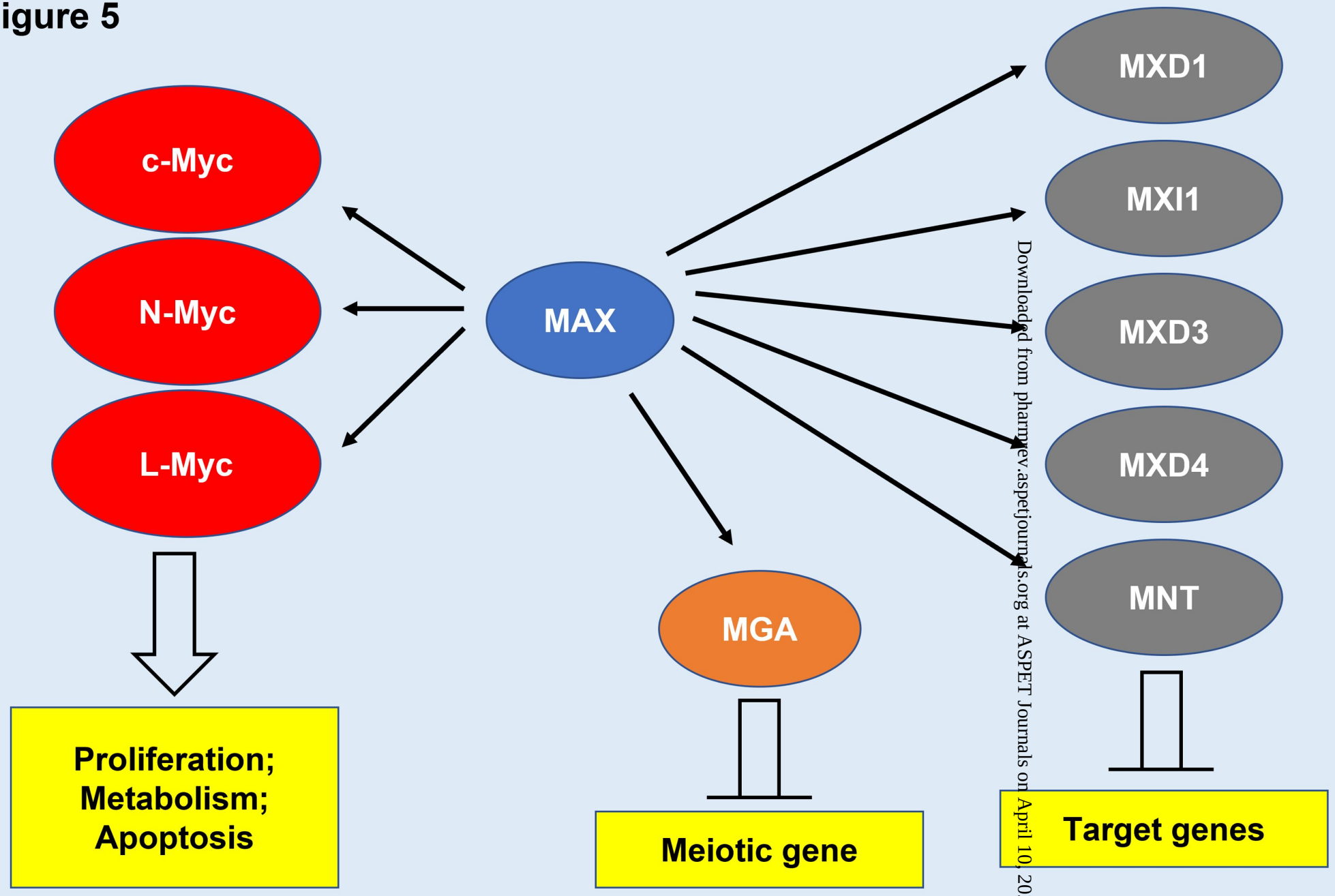


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Figure 6

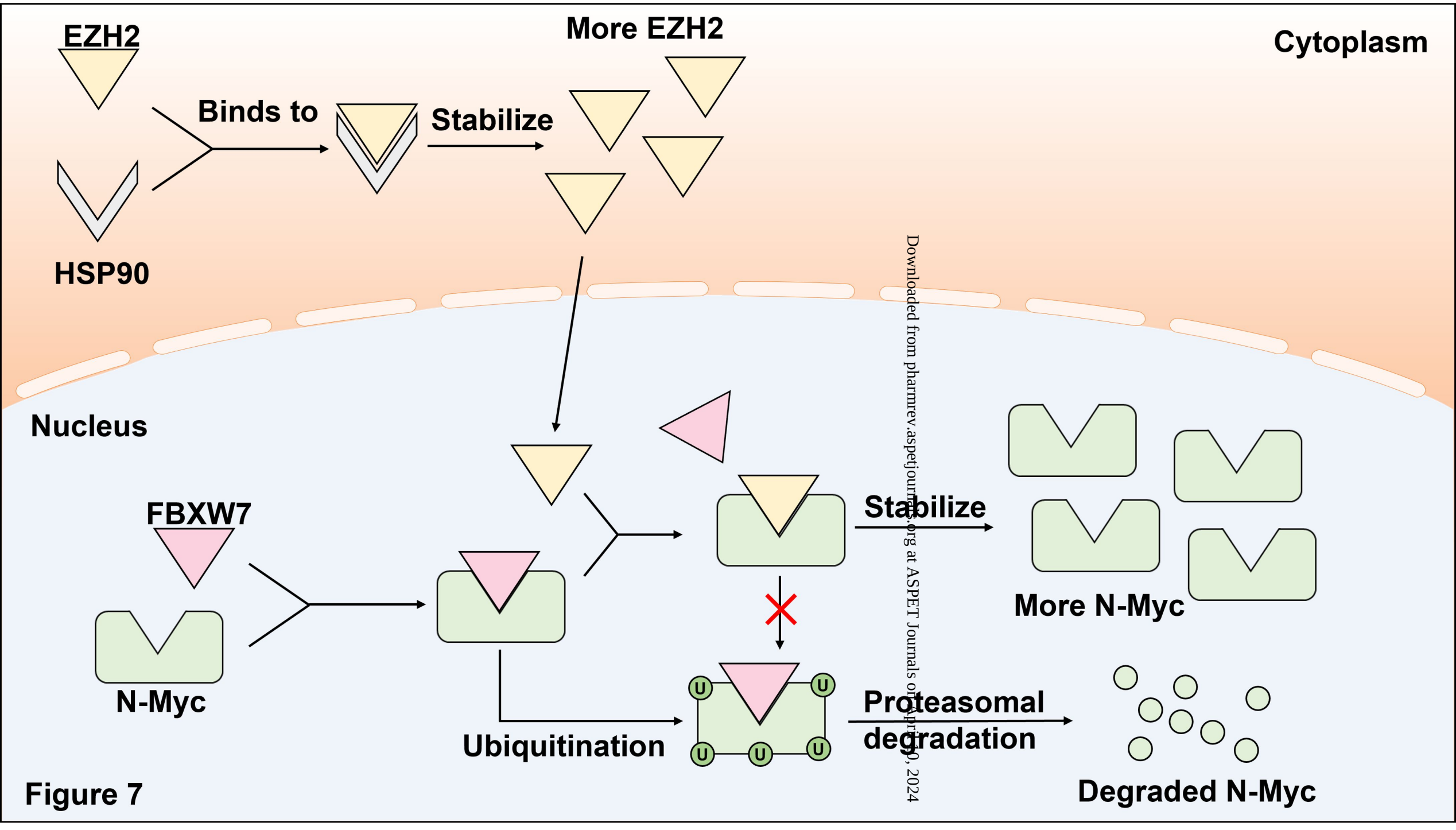


Figure 7

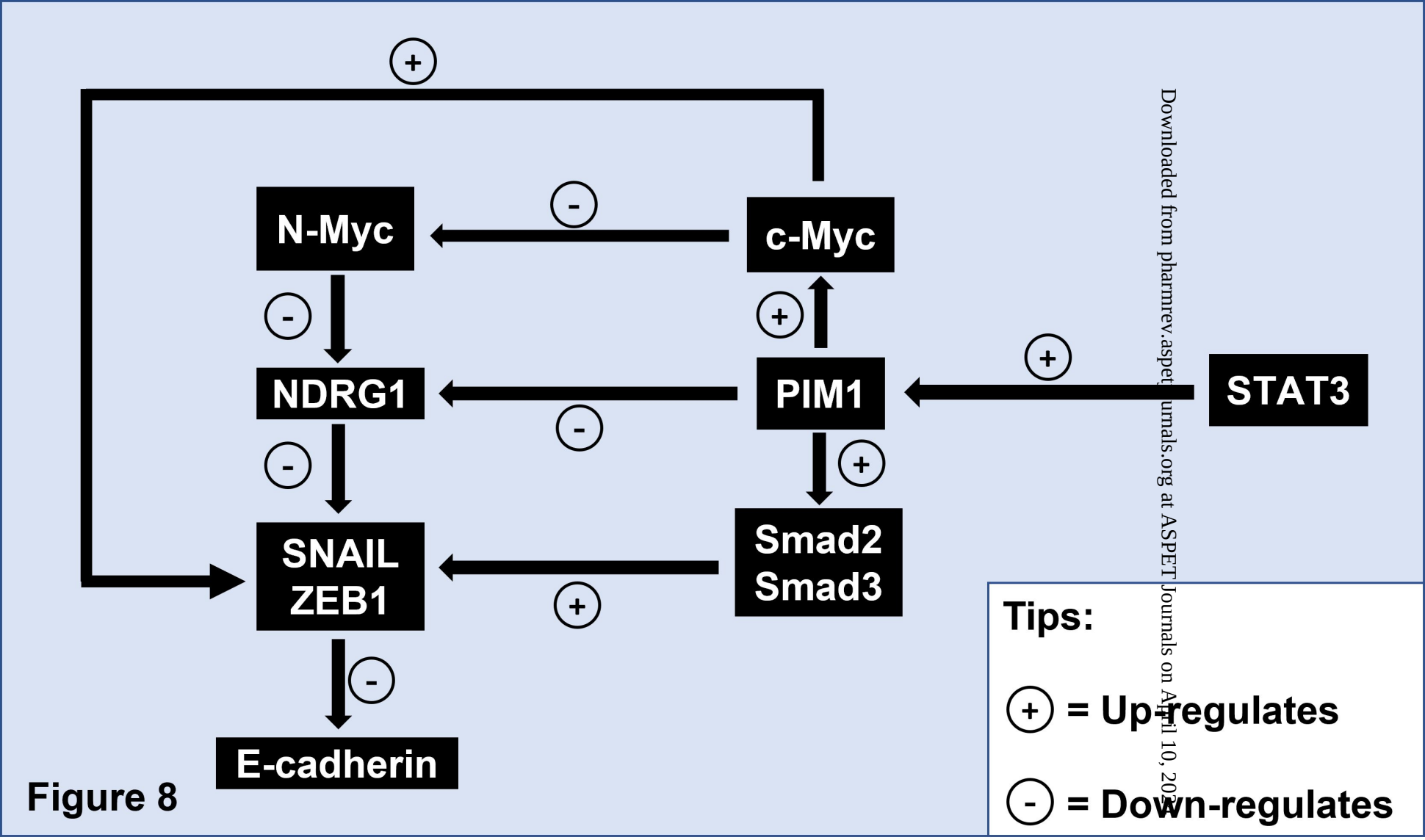
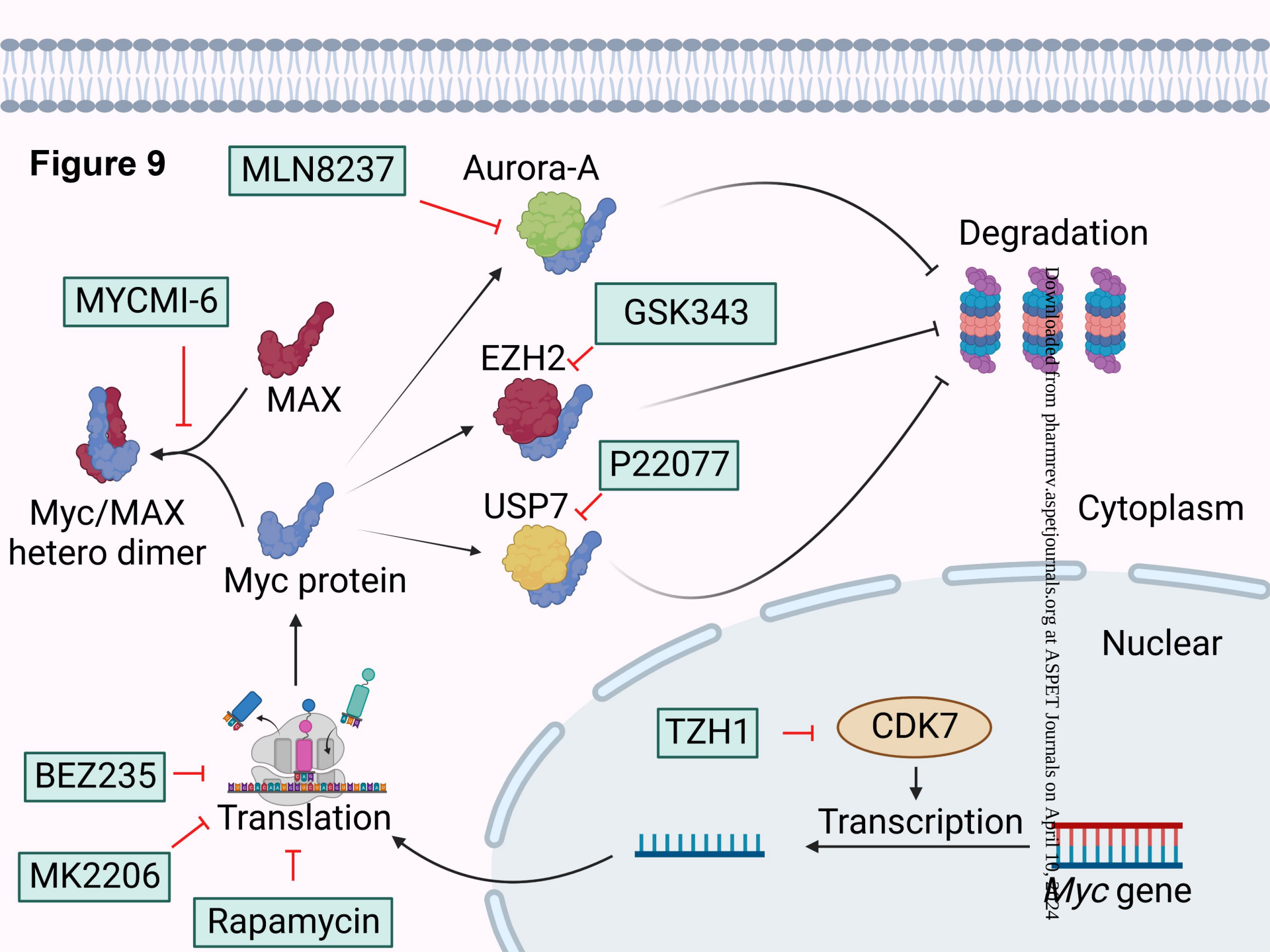


Figure 8



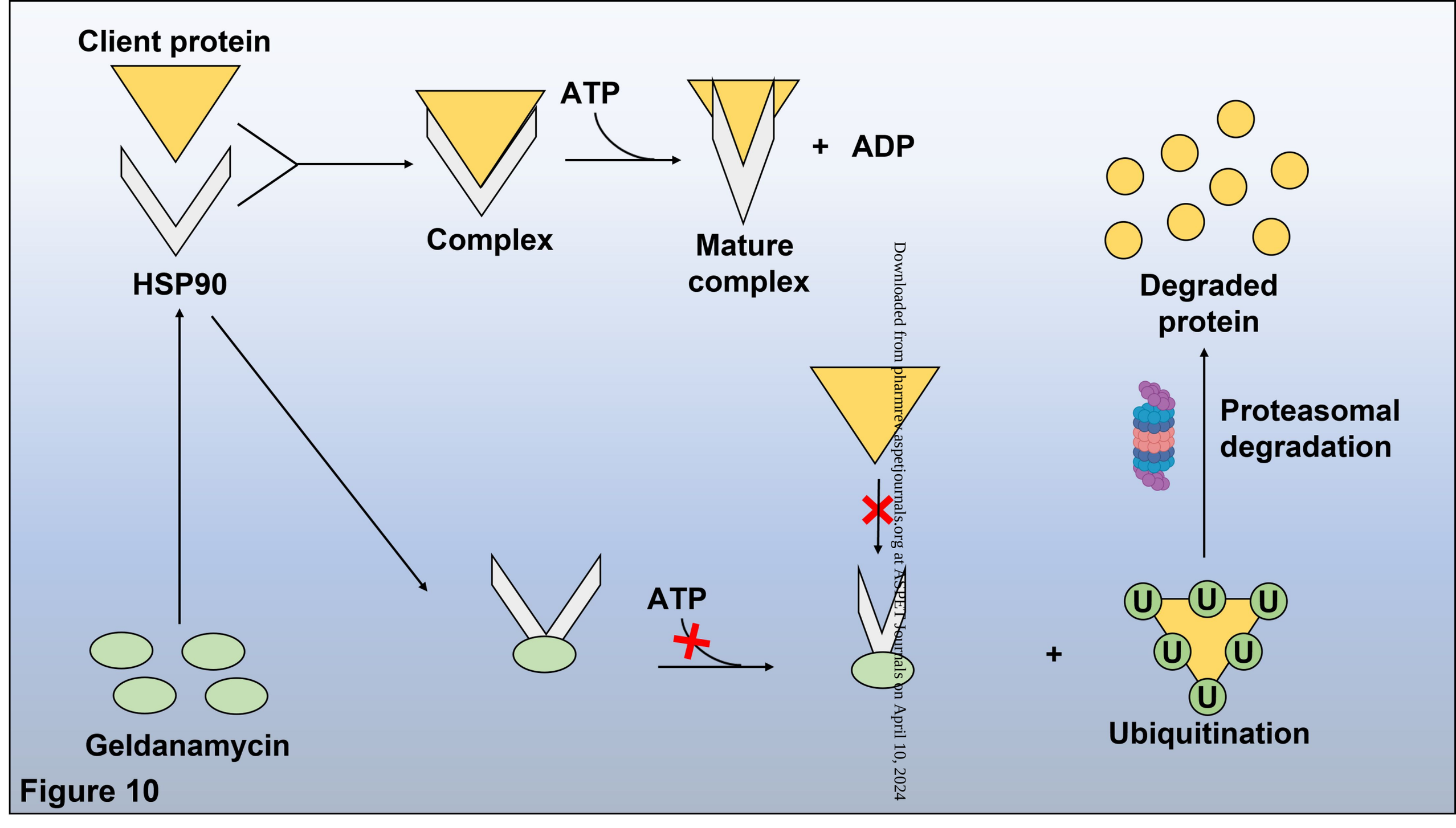
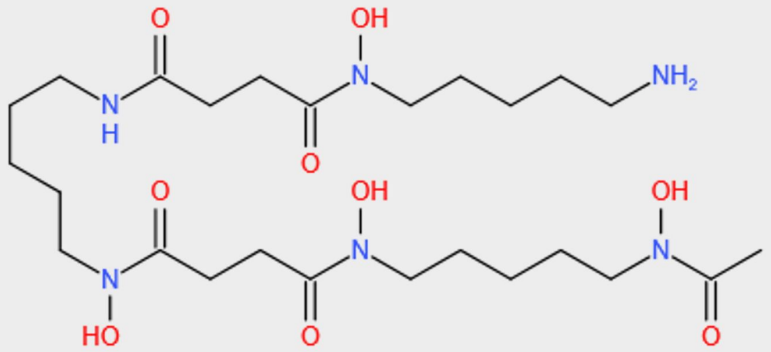


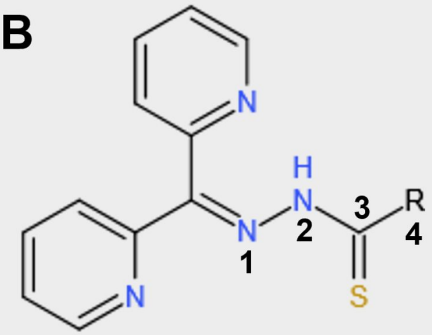
Figure 10

A



DFO

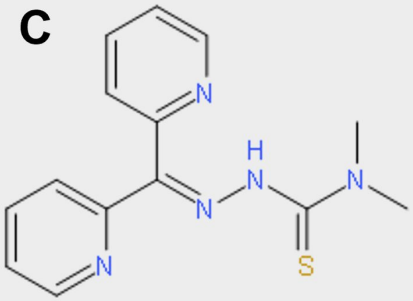
B



DpT:	R = NH_2
Dp4mT:	R = $NHCH_3$
Dp44mT:	R = $N(CH_3)_2$
Dp4eT:	R = NHC_2H_5
Dp4aT:	R = $NHC_2H_3{=}CH_2$
Dp4pT:	R = $NH(C_6H_6)$

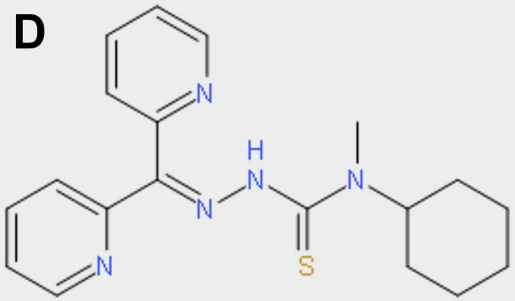
General structure of DpT analogs

C



Dp44mT

D



DpC

Figure 11