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To my family

Xi Qiao

Regulation of MYC activity by post-translational modifications

University of Turku, Faculty of Medicine, Department of Pathology, Turku Doctoral Programme of Molecular Medicine, Turku Centre for Biotechnology, Turku, Finland

ABSTRACT

MYC is a transcription factor that is often found deregulated in cancer. Post-translational modifications, including phosphorylation and ubiquitination, play important role in controlling MYC expression and activity.

This thesis investigates the *in vivo* effect of the cancerous inhibitor of protein phosphatase 2A (CIP2A)-regulated phosphorylation of MYC. Here, I find that phosphorylation of MYC at serine 62 (pS62MYC) recruits MYC to Lamin A/C-associated structures. In mouse model, CIP2A-mediated phosphorylation of MYC at S62 is connected to MYC-dependent initiation of proliferation, and support of intestinal regeneration in response to DNA damage.

The study also identified ubiquitin protein ligase E3 component n-recognin 5 (UBR5) as a novel E3 ligase for MYC. UBR5 promotes MYC degradation by ubiquitination. Functionally, UBR5 defines MYC-dependent phenotypes both in normal and in cancer cells. In *drosophila*, inhibition of UBR5/HYD causes MYC-overgrowth of wing imaginal discs. In cancer cells, UBR5 keeps MYC expression level below the apoptotic priming threshold. Taken together, these results give us further understanding of the role of post-translational modifications in regulating MYC activity in normal tissue growth and regeneration, as well as in cancer.

KEYWORDS: MYC, post-translational modification, CIP2A, UBR5, phosphorylation, ubiquitination, proliferation, apoptosis

Xi Qiao

Post-translationaaliset modifikaatiot transkriptiotekijä MYC:in aktiivisuuden säätelyssä

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TIIVISTELMÄ

MYC on transkriptiotekijä. Tietyillä post-translationaalisilla modifikaatioilla, kuten fosforylaatiolla ja ubikitinylaatiolla on tärkeä rooli MYC:in ilmentymisen ja aktiivisuuden säätelyssä. Tässä väitöskirjatyössä tutkittiin CIP2A-välitteisen MYC-fosforylaation *in vivo*-vaikutuksia. Havaitsimme että MYC proteiinissa oleva seriini62 aminohapon fosforylaatio aikaansaa MYC:in kulkeutumisen tumakalvon lamiini A/C:ta sisältäviin rakenteisiin sekä soluviljelmissä että hiiren kudoksissa. Hiirimallissa CIP2A:n todettiin olevan välttämätön DNA-vaurion jälkeiselle MYC seriini-62:n fosforylaatiolle, joka puolestaan tarvittiin suolipoimujen solujen jakautumiseen sekä suolen epitteelin uudistumiseen. Tässä tutkimuksessa tehtiin myös uusi havainto siitä, että UBR5 toimii E3-ligaasina MYC:lle ja edistää MYC proteiinin hajoamista. UBR5 määrittää MYC-riippuvaisia fenotyyppejä sekä normaaleissa soluissa että syöpäsoluissa. Banaanikärpäsessä UBR5/ HYD-inhibitio aiheuttaa MYC:in välityksellä siipien imaginaalilevyjen epänormaalia kasvua *in vivo*. Syöpäsoluissa UBR5 säätelee MYC:in ilmentymistasoa siten, että solut eivät joudu apoptoosiin. Saadut tulokset lisäävät ymmärrystämme post-translationaalisten modifikaatioiden merkityksestä MYC:in aktiivisuuteen normaalien kudosten kasvussa ja syövässä.

AVAINSANAT: MYC, post-translationaalinen modifikaatio, CIP2A, UBR5, fosforylaatio, ubikinitylaatio, solujen jakautuminen, apoptoosi

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ABBREVIATION

ATP	Adenosine triphosphate
B-HLH-LZ	Basic helix-loop-helix leucine zipper region
CHIP	Carboxy terminus of Hsp70-interacting protein
CIP2A	Cancerous inhibitor of PP2A
CPT	Camptothecin
DNA	Deoxyribonucleic acid
DBD	DNA-binding domain
ER	Estrogen receptor
ERK	Extra cellular signal regulated kinase
FBW7	F-box and WD repeat domain containing 7
GSK3	Glycogen synthase kinase 3
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
HUWE1	HECT, UBA And WWE domain containing 1
KO	Knock out
LAS	Lamin A/C associated structures
MAX	MYC associated factor X
MB	MYC box
MEF	Mouse embryonic fibroblast
MEK	Mitogen activated/Extracellular signal regulated kinase kinase
MIZ-1	MYC-interacting zinc finger 1
mRNA	Messenger ribonucleic acid

NLS	Nuclear localization signal
PI3K	Phosphatidylinositol 3 kinase
PLA	Proximity ligation assay
PP2A	Protein phosphatase 2A
PTM	Post-translational modification
S	Serine
Scr	Scrambled siRNA
SD	Standard deviation
SEM	Standard error of mean
siRNA	Short interfering RNA
SKP2	S-phase kinase associated protein 2
T	Threonine
TAD	Trans-activating domain
TF	Transcription factor
UBR5	Ubiquitin protein ligase E3 component n-recognin 5
UPS	Ubiquitin-proteasome system
USP28	Ubiquitin-specific protease 28
WT	Wild-type

LIST OF ORIGINAL PUBLICATIONS

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II. **Xi Qiao**, Ying Liu, Maria Llamazares Prada, Heidi Haikala, Kati Talvinen, Abhishekh Gupta, Laxman Yetukuri, Joanna W. Pylvänäinen, Juha Klefström, Annika Meinander, Tero Aittokallio, Pauliina Kronqvist, Ville Hietakangas, Martin Eilers, and Jukka Westermarck. Control of MYC-dependent apoptotic threshold by a co-amplified ubiquitin E3 ligase UBR5. (Submitted for publication. 2018)

1. INTRODUCTION

The transcription factor MYC plays an important role in cell proliferation, apoptosis, cell growth and tumorigenesis (Meyer and Penn 2008). Deregulated MYC expression leads to abnormal activity that is harmful for cells during both physiological and pathological processes. Thus, MYC expression needs to be tightly controlled to be nontoxic and beneficial for cells. Post-translational modifications (PTMs), including phosphorylation and ubiquitination, of the MYC protein are crucial mechanisms that regulate MYC expression and activity (Hann 2006; Farrell and Sears 2014). The phosphorylation status of threonine 58 (T58) and serine 62 (S62) in the N-terminus of MYC can affect MYC stability and activity. Specifically, phosphorylated T58 destabilizes MYC, whereas phosphorylated S62 stabilizes MYC (Sears 2004). Ubiquitination of MYC by E3 ubiquitin ligases also plays a critical role in controlling protein amount by promoting proteasomal degradation. Thus far, several E3 ligases have been identified as MYC E3 ligases to promote MYC ubiquitination.

Previous studies have shown that cancerous inhibitor of protein phosphatase 2A (CIP2A) stabilizes MYC in cultured cells by inhibiting the activity of protein phosphatase 2A (PP2A) that was shown to dephosphorylate MYC at S62 (Sears 2004; Junttila, Puustinen et al. 2007). Depletion of CIP2A significantly inhibits proliferation of cancer cells (Junttila, Puustinen et al. 2007; Come, Laine et al. 2009; Khanna, Bockelman et al. 2009; Laine, Sihto et al. 2013). However, *in vivo* evidence for CIP2A-mediated regulation of MYC phosphorylation is lacking. Ubiquitin protein ligase E3 component n-recognin 5 (UBR5) is an E3 ligase that targets dozen of proteins, but it has not been demonstrated to target MYC for ubiquitination thus far.

This thesis study investigates PTMs of MYC. Specifically, this study further investigates CIP2A-regulated phosphorylation of MYC at S62 in an *in vivo* mouse model. FBW7 is a well-studied E3 ubiquitin ligase that promotes MYC proteasomal degradation, which is

dependent on phosphorylation of MYC at T58 (Welcker, Orian et al. 2004). However, T58 phosphorylation is dispensable for some E3 ligases to mediate MYC proteasomal degradation (Farrell and Sears 2014; Chen, Zhou et al. 2016). Here we identifies and characterizes UBR5 as a novel E3 ubiquitin ligase that can promote MYC ubiquitination independently from phosphorylation of T58. Moreover, we explore the functional effects of UBR5-mediated MYC regulation. Finally, we demonstrate the relevance of UBR5 and MYC in breast cancer. Taken together, results in my thesis study provide more understanding on the significance of PTMs on MYC activity.

2. REVIEW OF THE LITERATURE

2.1 MYC

2.1.1 Identification of MYC

At the end of the 1970s, the *myc* gene was identified from an avian retrovirus MC29 that caused chicken tumors (Duesberg, Bister et al. 1977; Hu, Lai et al. 1979). The transforming sequence of the virus MC29 that is capable of inducing myelocytomatosis was named as *v-myc*. In 1982, the cellular homologue of this oncogenic gene was isolated and characterized as *c-myc* (Vennstrom, Sheiness et al. 1982). The *c-myc* gene encodes protein c-Myc (MYC henceforth) that is very conserved and commonly expressed in many species in both non-vertebrates and vertebrates (Walker, Boom et al. 1992).

In the following years after MYC was discovered, another two *myc* family members were reported. N-MYC was identified in neuroblastoma cells and is mainly expressed in the brain (Kohl, Kanda et al. 1983; Kohl, Gee et al. 1984; Zimmerman and Alt 1990). L-MYC was cloned and characterized in small cell lung cancer (Nau, Brooks et al. 1985).

2.1.2 Structure of MYC

The amino-terminus of MYC harbors a transcription activation domain (TAD), which is necessary for MYC transcriptional activity and for its cell transforming function (Kato, Barrett et al. 1990; Vervoorts, Luscher-Firzlaff et al. 2006). On the other hand, within the MYC carboxyl-terminus, there is a basic helix-loop-helix leucine zipper region (B-HLH-LZ) that functions as a DNA-binding domain (Blackwell, Kretzner et al. 1990; Vervoorts, Luscher-Firzlaff et al. 2006). MYC has several conserved domains, which are crucial for MYC activity and are termed MYC Box (MB) (Atchley and Fitch 1995;

Conacci-Sorrell, McFerrin et al. 2014). So far, MBI, II, III, and IV have been characterized (Fig. 1).

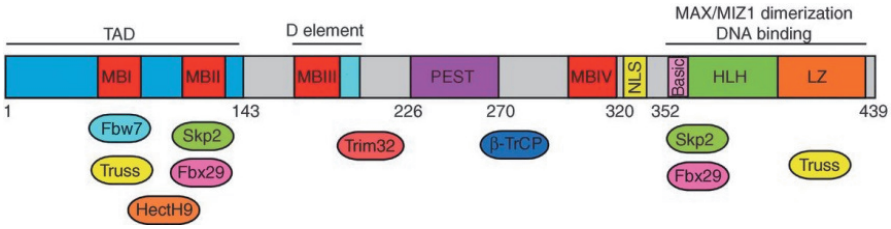

Figure 1: **Diagram of MYC structure**

MYC is composed of a TAD, four MBs, and a B-HLH-LZ region mediating DNA binding. The nuclear localization signal (NLS) is located close to the B-HLH-LZ domain, while the D element and PEST sequence are within central region of the protein. Known MYC E3 ligases and their interaction sites MYC are shown (Farrell and Sears 2014).

MBI and MBII lie in the TAD and provide binding sites for different MYC-interacting partners. MBI is important for regulating MYC stability. Within MBI, phosphorylation of T58 and S62 mediates ubiquitin-dependent proteasomal degradation of MYC by F-box and WD repeat domain containing 7 (FBW7) (Sears, Leone et al. 1999; Sears, Nuckolls et al. 2000; Welcker, Orian et al. 2004). As part of the TAD, MBII plays an essential role in MYC activity to promote cell transformation *in vitro*, to initiate tumorigenesis *in vivo*, and activate or repress transcription of MYC target genes (Stone, de Lange et al. 1987; Li, Nerlov et al. 1994; Hemann, Bric et al. 2005; Herbst, Hemann et al. 2005; Zhang, DeSalle et al. 2006). It is critical for MBII to bind cofactor TRRAP and mediate recruitment of histone acetyltransferase GCN5 to MYC, leading to the opening of the chromatin structure to promote transcription of MYC target genes (McMahon, Van Buskirk et al. 1998; McMahon, Wood et al. 2000). Like MBI, MBII is also important for MYC stability by promoting MYC proteasomal degradation via E3 ligases, such as SKP2 (Herbst, Salghetti et al. 2004). MBIII is less studied as compared to MBI and MBII. It has been reported that MBIII is needed for MYC function in transformation *in vitro* and it is involved in proteasomal degradation of MYC (Sears, Nuckolls et al. 2000; Herbst, Hemann et al. 2005). MBIII is also important for MYC-mediated transcriptional repression by binding to HDAC3 (Kurland and Tansey 2008). MBIV is involved

in MYC-induced apoptosis, and contributes to MYC transcriptional activity both on activated and repressed target genes (Cowling, Chandriani et al. 2006). The D element is a sequence that is required for ubiquitin-independent degradation of MYC. The PEST sequence is usually associated with short-lived proteins, and is enriched in proline (P), glutamic acid (E), serine (S) and threonine (T). PEST sequence is located in the central part of MYC that is required for MYC degradation without employing ubiquitination (Gregory and Hann 2000). The nuclear localization signal (NLS) is a classic sequence that is responsible for MYC nuclear localization. Depletion of NLS caused MYC to be distributed both in the nucleus and in the cytoplasm (Dang and Lee 1988). The basic region (BR) is necessary for sequence specific DNA-binding activity of MYC. Together with HLH-LZ domain, B-HLH-LZ region enables MYC to form heterodimer with MAX (MYC-associated factor X). MYC-MAX can recognize and bind to 'Enhancer box' (E-box, CACGTGT), to activate gene expression (Blackwood and Eisenman 1991). Additionally, the B-HLH-LZ region also provides a platform for MYC association with MIZ1 (MYC-interacting zinc finger protein 1), a well-known mechanism for MYC-regulated repression of gene transcription (Peukert, Staller et al. 1997).

2.1.3 Transcriptional regulation by MYC

MYC target genes

MYC's function as a transcription factor has well characterized. MYC is thought to regulate up to about 15% of genes (Li, Van Calcar et al. 2003). By transcriptionally regulating these target genes, MYC can influence cellular processes, for example, regulation of cell cycle checkpoint genes in cell cycle progression, regulation of β-integrin expression in cell adhesion and regulation of hexokinase II in metabolism (Dang, O'Donnell et al. 2006).

Transcriptional activation

In order to activate gene transcription, MYC dimerizes with its partner protein MAX through their respective B-HLH-LZ domains,

and the MYC/MAX dimer binds to specific DNA sequences, the E-boxes (CACGTG), which is located in the enhancer/promoter regions of target genes (Blackwell, Kretzner et al. 1990). Upon DNA binding, MYC is able to recruit many co-activators, such as p300 or the adaptor protein TRRAP, a core subunit of several complexes that contain the histone acetyltransferases (HAT) GCN5 or TIP60, through its N-terminal TAD (Meyer and Penn 2008; Luscher and Vervoorts 2012). The recruitment of HATs by MYC to its target promoters results in an increase of H3 and H4 acetylation that further opens the chromatin structure and enables transcriptional activation.

Transcriptional repression

MYC-mediated transcriptional repression occurs via the interaction with other transcription factors like MIZ1 or SP1 (Schneider, Peukert et al. 1997; Gartel, Ye et al. 2001). In this context, MYC can displace co-activators of MIZ1 and SP1, then recruits co-repressor, histone deacetylase (HDAC) complexes, leading to the local closing of chromatin structures, favoring transcriptional repression (Luscher and Vervoorts 2012). In addition, MYC/MIZ1 complexes can also recruit DNA methyltransferases as a means to repress transcription (Brenner, Deplus et al. 2005).

General amplifier of transcription

Instead of sequence-specific transcription factor, it was observed that MYC bound to promoter regions of active genes and amplified the expression of these genes (Lin, Loven et al. 2012; Nie, Hu et al. 2012). MYC bound preferentially to promoters that were already occupied by RNA Pol II (RNAPII) and active chromatin marks, and failed to bind to and induce the transactivation of silent genes.

2.1.4 Biological functions of MYC

2.1.4.1 MYC function in cell proliferation

MYC is a key regulator of mammalian cell proliferation by promoting the progression of the cell cycle (Bouchard, Staller et al. 1998). In eukaryotic cells, the cell cycle is divided into four phases: Gap1 (G1), Synthesis (S), Gap2 (G2), and Mitosis (M). In quiescent cells, MYC expression is virtually absent. In response to mitogenic stimulation or enforced expression of MYC, MYC is rapidly expressed, and then promotes cells to enter the G1 phase of cell cycle (Rabbitts, Watson et al. 1985). During the cell cycle, MYC is shown to be essential for G0/G1 to S phase progression and G1 seems to be shortened in the MYC-expressing cells. MYC participates in cell cycle by regulation of its target genes. For example, MYC abrogates the transcription of cell cycle checkpoint genes (*gadd45, gadd153*) and inhibits the function of cyclin-dependent kinase (CDK) inhibitors (Bretones, Delgado et al. 2015). In addition, MYC also promotes cell cycle progression by transcriptionally activating *cyclin D1, cyclin D2, cyclin E1, cdk4, cdc25a, e2f1* and *e2f2* (Meyer and Penn 2008). Furthermore, MYC enables cells to enter into S phase and initiate mitosis without external growth factor stimulation (Eilers, Schirm et al. 1991). In mice, MYC dependence is important for *in vivo* proliferation response during intestinal regeneration after DNA damage (Muncan, Sansom et al. 2006; Sansom, Meniel et al. 2007; Athineos and Sansom 2010).

In addition to cell proliferation, MYC can promote cell growth (accumulation of cell mass) by regulating genes involved in cell metabolism and protein synthesis (Iritani and Eisenman 1999; Schuhmacher, Staege et al. 1999). MYC function in cell growth is well-studied in a *drosophila* model, where the MYC orthologue, dMYC, is a central regulator of growth. Depletion of dMYC in cells in the wing imaginal disc results in smaller cell size. Overexpression of dMYC dramatically promotes cell growth, and increases cell size (Johnston, Prober et al. 1999; Gallant 2013).

2.1.4.2 MYC in apoptosis

The term apoptosis is derived from an ancient Greek word that means “dropping off,” which was used to describe leaves falling off from trees in autumn. It was first used to describe morphological features of cell death in 1972 (Kerr, Wyllie et al. 1972). Apoptosis is a process of programmed cell death that is crucial in physiological and pathological conditions. This conserved process is used to eliminate unwanted cells during embryonic development. In cancer, apoptosis is often inhibited which in turn results in increased cell survival and tumor development (Fernald and Kurokawa 2013). In response to apoptotic stimuli, a group of cysteine proteases called “caspases” can be activated, including ‘initiator’ caspases (caspase-2, -8, -9, or -10) and ‘executioner’ caspases (caspase-3 or -7). Various specific cellular substrates, like PARP, are cleaved following activation of caspases, leading to the morphological and biochemical changes seen in apoptotic cells (Li and Yuan 2008).

Deregulated MYC expression was found to drive cell apoptosis in the early 1990s. Several studies hinted that ectopic MYC expression could sensitize cells to undergo apoptosis. It was reported that co-expression of RAS and MYC caused more cell death in rodent fibroblasts than in cells only expressing RAS (Wyllie, Rose et al. 1987). In 1991, Neiman and his colleagues found that normal B lymphocytes with overexpressed MYC were more sensitive to apoptosis induction by radiation (Neiman, Thomas et al. 1991). It was shown that deregulated MYC expression induced apoptosis in serum-deprived fibroblasts and that the level of MYC expression correlated with the extent of the apoptotic response (Evan, Wyllie et al. 1992). Through decades of accumulated studies, it is currently evident that MYC-induced apoptosis mainly involves the BCL-2 family network and the p53 pathway.

BCL-2 was revealed to cooperate with MYC and abrogate MYC-induced apoptosis in tumorigenesis (Fanidi, Harrington et al. 1992). Similarly, acceleration of lymphomagenesis was found in transgenic mice that express both MYC and BCL-2 compared with transgenic mice that harbor only the MYC transgene (Strasser, Harris et al. 1990). Several studies showed that MYC drives apoptosis through

BCL-2 family member, BAX (Soucie, Annis et al. 2001; Juin, Hunt et al. 2002; Dansen, Whitfield et al. 2006), and that loss of BAX impairs the potentiation of apoptosis by MYC *in vivo* (Eischen, Roussel et al. 2001). Furthermore, MYC expression is required for the BAX conformational change that is in turn needed for the pro-apoptotic activation of BAX (Annis, Soucie et al. 2005). In addition, it was reported that MYC suppresses the expression of the anti-apoptotic protein BCL-X_L and hence sensitizes tumor cells to apoptosis (Eischen, Woo et al. 2001; Maclean, Keller et al. 2003). MYC also regulates pro-apoptotic protein BIM. In Eμ-Myc transgenic mice, elevated BIM protein expression was induced in the apoptosis-prone B lymphoid cells. When Eμ-Myc-expressing B lymphoid cells have a deficiency in BIM expression, the cells are refractory to apoptosis induced by cytokine deprivation or antigen receptor cross-linking (Egle, Harris et al. 2004). Recently, Muthalagu and colleagues found that BIM is a common specific requirement during MYC-induced apoptosis in multiple solid tissues (Muthalagu, Junttila et al. 2014). Furthermore, it was found that MYC mutants were unable to drive apoptosis because of a loss in ability to induce BIM expression (Hemann, Bric et al. 2005).

The tumor suppressor protein, p53, plays an important role in MYC-induced apoptosis. In 1994, Hermeking and colleagues found that p53 was required for MYC-induced apoptosis. In $p53^{-/-}$ cells expressing c-MycER, MYC-induced apoptosis was absent (Hermeking and Eick 1994). Overexpression of MYC promoted accumulation of $p19^{ARF}$ (p14ARF in human cells), which in turn activated p53 to regulate its apoptosis-relevant target genes (Zindy, Eischen et al. 1998). Conversely, several studies suggested that elevated MYC can induce apoptosis in the absence of p53, for example, by suppressing BCL-X_L. Thus, MYC-induced apoptosis may be independent on p53 (Hsu, Marin et al. 1995; Maclean, Keller et al. 2003).

It was found that threshold levels of MYC expression define functional outcome of MYC, including proliferation and apoptosis (Murphy, Junttila et al. 2008; Shachaf, Gentles et al. 2008; Berta, Baker et al. 2010; Levens 2013). Overexpression of MYC, rather than a low deregulated level of MYC expression, is required for

activation of apoptosis (Murphy, Junttila et al. 2008). Murphy and his colleagues generated a mouse model that can express low levels of MYC. In this model, MER fusion gene (Myc-oestrogen receptor), which encodes the MYC protein, is driven by a weak Rosa26 promoter and the abundance of MYC expression is controlled by gene dose. The mice harbor two deregulated *myc* alleles ($R26^{MER/MER}$) resulting in 2-fold increase in MYC expression compared to mice with one deregulated *myc* allele ($R26^{MER/WT}$). When compared to endogenous levels of MYC, $R26^{MER/WT}$ and $R26^{MER/MER}$ mice express 1.5-fold and 2-fold more MYC, respectively. In $R26^{MER/WT}$ mice, no increase in cell proliferation was detected, suggesting that 1.5-fold increased MYC expression was unable to induce proliferation. However, an overt increase in cell proliferation was detected in $R26^{MER/MER}$ mice. This indicated that the threshold of MYC expression required for inducing cell proliferation in mice is between 1.5-fold and 2-fold higher than the physiological levels of MYC. In both $R26^{MER/WT}$ and $R26^{MER/MER}$ mice, apoptosis was not observed in most tissues, and this indicated that the threshold level of MYC expression for activating apoptosis is higher than that for inducing cell proliferation. When MYC was expressed at 15-fold over physiological levels in pIns–$MycER^{TAM}$ mice, apoptosis was observed in the pancreas. This suggested that the threshold of MYC expression required for activating apoptosis is between 2-fold and 15-fold more than that of endogenous MYC. In addition to spontaneous apoptosis, high MYC expression sensitizes cells to many apoptosis inducers, such as DNA damage regents and antimitotic drugs. For example, high MYC expression sensitizes tumor cells to camptothecin (also known as CPT) that causes DNA damage (Arango, Mariadason et al. 2003; Frenzel, Zirath et al. 2011). A similar finding was also demonstrated in Murphy's work, where $R26^{MER/MER}$ mice harboring 2-fold increase in MYC expression, were sensitive to doxorubicin treatment (Murphy, Junttila et al. 2008). Recently, it was reported that MYC sensitizes lung, breast, ovarian, and colon cancer cells to taxol and other antimitotic drugs (Topham, Tighe et al. 2015).

2.1.4.3 Other MYC functions

Ectopic MYC expression strongly blocks cell differentiation in many cell types. Downregulation of MYC is necessary for cells to exit the cell cycle and undergo differentiation (Coppola and Cole 1986; Dmitrovsky, Kuehl et al. 1986). MYC can regulate cell metabolism by promoting up-regulation of proteins that are responsible for glucose up-take and for glycolysis (Dejure and Eilers 2017). By regulating angiogenic factors, like vascular endothelial growth factor (VEGF), MYC is also involved in angiogenesis (Baudino, McKay et al. 2002). MYC can induce cellular transformation by cooperating with the oncogene *RAS* (Land, Parada et al. 1983), and induce genomic instability and DNA damage (Kuzyk and Mai 2014).

2.2 MYC in cancer

MYC expression is tightly controlled under physiological conditions. Deregulation of MYC occurs in many human cancer types, and it is usually associated with poor outcomes (Dang, O'Donnell et al. 2006; McKeown and Bradner 2014). Deregulation of MYC can occur by different mechanisms. Chromosomal translocation of the *myc* gene is mainly observed in Burkitt's lymphoma, which places *myc* gene under the control of the immunoglobulin μ heavy chain enhancer, resulting in very high levels of mRNA synthesis (Taub, Kirsch et al. 1982; Dave, Fu et al. 2006). Gene amplification is a common marker of MYC deregulation, and this is mainly observed in solid tumors, such as breast cancer and ovary cancer (Meyer and Penn 2008; Kalkat, De Melo et al. 2017). Mutation in MYC has also been observed and has principally been linked to Burkitt's lymphoma, and the most frequent mutation is on T58 (Rabbitts, Hamlyn et al. 1983; Love, Sun et al. 2012; Schmitz, Young et al. 2012). On protein level, MYC can be stabilized in cancers by post-translational modifications that are reviewed in the following text.

2.3 Regulation of MYC expression

MYC expression can be governed at different levels from mRNA expression to protein stability regulated by PTMs.

2.3.1 Transcriptional control of MYC expression

Transcriptional regulation of the *myc* gene is triggered by several signal transduction pathways, including WNT, TGFβ, NF-κb, BCR-ABL1, and NOTCH (Levens 2010; Kalkat, De Melo et al. 2017). Some transcription factors, for example, CNBP, FBP and TCF can bind to the *myc* promoter and directly regulate *myc* gene transcription (Levens 2013). A BET bromodomain protein, called BRD4, was found to be a transcriptional co-activator of the *myc* gene, and depletion of BRD4 decreases MYC expression. Inhibitors of BRD4 have been shown to trigger efficient inhibition of *myc* transcription (Delmore, Issa et al. 2011; Posternak and Cole 2016), but these inhibitors also target other proteins (Perez-Salvia and Esteller 2017). MYC mRNA, which is tightly controlled, has been shown to be affected by several microRNAs (miRNA), including let-7, miR-34 and miR-145 (Kim, Kuwano et al. 2009; Sachdeva, Zhu et al. 2009; Cannell, Kong et al. 2010; Christoffersen, Shalgi et al. 2010). Translation of MYC mRNA is also regulated by multiple mechanisms. For example, AUF1 promotes translation of MYC mRNA, whereas, rapamycin inhibits MYC mRNA translation (Liao, Hu et al. 2007; Wall, Poortinga et al. 2008).

2.3.2 Post-translational modifications of MYC

The MYC protein is targeted by several different PTMs. These PTMs can affect MYC protein stability and also directly influence MYC activity.

2.3.2.1 Acetylation of MYC

MYC can interact with, and be acetylated by several histone acetyltransferase (HAT) enzymes, including GCN5, p300/CBP and Tip60 (Vervoorts, Luscher-Firzlaff et al. 2003; Patel, Du et al. 2004;

Faiola, Liu et al. 2005; Zhang, Faiola et al. 2005). CBP binds to the C-terminus of MYC and acetylates MYC in vitro, and this promotes MYC stabilization (Vervoorts, Luscher-Firzlaff et al. 2003; Faiola, Liu et al. 2005). Acetylation of MYC by GCN5 or TIP60 can also strongly increase MYC stability (Patel, Du et al. 2004). Interestingly, it is possible that acetylation of MYC competes with ubiquitination of MYC and thereby promotes MYC stability because both modifications target lysine residues (Vervoorts, Luscher-Firzlaff et al. 2006). It is currently unclear whether acetylated sites of MYC are required for binding of specific MYC-interacting partners.

2.3.2.2 Phosphorylation at Threonine 58 and Serine 62

Phosphorylation is an important PTM affecting MYC, and the best-studied phosphorylation events occur at T58 and S62 residues within N-terminal MBI box, which regulates MYC's stabilization and activity (Alvarez, Northwood et al. 1991; Henriksson, Bakardjiev et al. 1993; Hann 2006; Farrell and Sears 2014). S62 is phosphorylated by multiple kinases, for example, mitogen-activated kinase (MAPK, also known as ERK), CDK1, JNK and CDK2; Whereas T58 is a target of GSK3β (Henriksson, Bakardjiev et al. 1993; Hann 2006; Hydbring, Bahram et al. 2010). GSK3β-mediated T58 phosphorylation requires prior phosphorylation of S62 (Lutterbach and Hann 1994; Gregory, Qi et al. 2003). S62 phosphorylation can stabilize MYC, whereas phosphorylation at T58 can destabilize MYC (Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004). In some human cancer cell lines, it was observed that abnormal phosphorylation of T58 and S62 accounts for increased MYC stability (Malempati, Tibbitts et al. 2006; Arnold, Zhang et al. 2009).

Phosphorylation on both T58 and S62 affects the transcriptional activity of MYC. It was reported that mutations of T58 and S62 decrease Gal4-MYC activity in transactivation assays (Alvarez, Northwood et al. 1991; Gupta, Seth et al. 1993; Albert, Urlbauer et al. 1994). Serum-induced S62 phosphorylation was shown to dramatically increase Gal4-MYC activity (Seth, Alvarez et al. 1991; Lutterbach and Hann 1994). In addition, phosphorylation of S62 recruits MYC to the promoter of its target genes, for example, *γ-GCS* and *hTERT*, while mutation of S62 to an alanine (S62A) prevents

this recruitment (Benassi, Fanciulli et al. 2006; Hydbring, Bahram et al. 2010). On the other hand, T58 phosphorylation was shown to be important for regulation of the MYC target gene, BIM (Hemann, Bric et al. 2005). Both phosphorylation sites can influence MYC biological activity. S62A decreases MYC's transforming activity, but T58A increases MYC's transforming activity (Pulverer, Fisher et al. 1994; Chang, Claassen et al. 2000; Thibodeaux, Liu et al. 2009). Phosphorylation at T58 is involved in apoptosis, whereas phosphorylation at S62 plays role in cell cycle progression (Hann 2006). The significance of T58 and S62 phosphorylation sites was addressed in mouse models (Wang, Cunningham et al. 2011). Wang and her colleagues generated three conditional *myc* knock-in mice expressing nearly physiological levels of MYC-WT, MYC-T58A, or MYC-S62A. In these mice, expression of MYC-T58A, but not MYC-WT or MYC-S62A induced mammary carcinoma. Their works collectively show that MYC activity is differentially affected by T58 and S62 phosphorylation.

2.3.2.3 Other phosphorylation sites of MYC

In contrast to T58 and S62, other phosphorylation sites of MYC are less studied. In the PEST domain and around the BR domain, there are two clusters of amino acid residues, T247/T248/S249/S250/S252 and T343/S344/S347/S348, both of which can be targeted by casein kinase 2 (CK2) (Luscher, Kuenzel et al. 1989). Although CK2-dependent phosphorylation of clusters in the PEST domain has been suggested to increase MYC stability, the functional consequence for MYC when these sites are phosphorylated remains largely unclear (Channavajhala and Seldin 2002). On the other hand, it was observed that phosphorylation of the cluster around the BR domain may affect MYC's transformation capability, since mutation of these sites to alanine resulted in increased transforming activity (Wasylishen, Chan-Seng-Yue et al. 2013). In addition to the mentioned sites, phosphorylation of S71, S81, S164 and S293 residues has also been observed (Lutterbach and Hann 1994; Lutterbach and Hann 1997). Moreover, five tyrosine residues in the MYC N-terminus, Y12/16/22/32/74, can be phosphorylated by the tyrosine kinase Abl (Sanchez-Arevalo Lobo, Doni et al. 2013). Among these five tyrosine residues, the principal site is Y74.

Phosphorylation of MYC at Y74 represents only a small fraction of the whole cellular MYC, and this fraction of MYC localizes to the cytoplasm, not to the nucleus. The function of phosphorylated MYC at Y74 in the cytoplasm remains unknown.

2.3.2.4 Ubiquitination of MYC

Ubiquitination is a common protein modification that has proteolytic or non-proteolytic function in normal and pathological states. Ubiquitination is a multi-step process that transfers ubiquitin to target proteins by the ubiquitin proteasome system (UPS).

Ubiquitin-conjugating cascade

Ubiquitin is a highly conserved protein that consists of 76 amino acids (Swatek and Komander 2016). Ubiquitin can be covalently attached to substrates via three enzymes: ubiquitin-activating E1, ubiquitin-conjugating E2 and ubiquitin-protein ligase E3 (Fig.2). In human, there are two E1s that activate ubiquitin in an ATP-dependent reaction. In this process, ubiquitin is activated by the formation of a thioester bond between the cysteine located in the active center of the E1 and the glycine in the C-terminus of the ubiquitin. Next, the activated ubiquitin is transferred to the active site cysteine of one of the ~ 30 E2s. The activated ubiquitin is then transferred from the E2 enzyme to a lysine residue of the substrates by one of the ~ 600 E3s, which are responsible for the substrate specificity of the UPS (Senft, Qi et al. 2018).

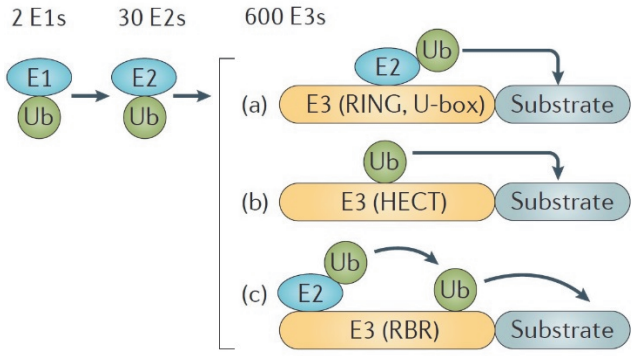

Fig.2 Illustration of the ubiquitination cascade

The cascade starts with ubiquitin activation by ubiquitin-activating enzyme E1. Subsequently, the activated ubiquitin is attached to ubiquitin-conjugating enzyme E2. Finally, one of the different types of E3 ligases transfers ubiquitin from E2 to the substrate protein directly (RING, U-box type ligases), or via a covalent ubiquitin-E3 ligase binding intermediate (HECT, RBR type ligases). The figure is cited from Senft et al., 2018.

After the first ubiquitin is attached to the substrate protein, the substrate is monoubiquitinated. Next, the three-step cascade is repeated and more ubiquitin molecules are transferred to the first ubiquitin to form a polyubiquitin chain. Seven internal lysine residues within ubiquitin, K6, K11, K27, K29, K33, K48, and K63, serve as attachment sites for ubiquitin, which subsequently leads to various polyubiquitin chains. Different polyubiquitin chains determine different functional outcomes (Ikeda and Dikic 2008). K48 or K11-linked polyubiquitin chains commonly lead to degradation of the labeled substrate via the 26S proteasome, which is a complex consisting of several proteins with proteolytic capacity. K63-linked polyubiquitin chains usually represents non-proteolytic ubiquitination, which is involved in many biological processes, for example, transcription regulation, DNA replication and DNA repair, whereas, K29-linked polyubiquitin chains play a role in protein degradation via lysosomal pathway (Kee and Huang 2015).

26S proteasome

The degradation of substrates labelled with polyubiquitin chains is carried out by the 26S proteasome, which can be located both in the

cytoplasm and the nucleus. The 26S proteasome is a multi-protein complex that consists of the 20S catalytic core and two 19S regulatory particles (Livneh, Cohen-Kaplan et al. 2016). The 20S core has a barrel-shaped structure composed of two outer α-heteroheptameric rings and two inner β-heteroheptameric rings. The two α-rings regulate the entry of substrates into the catalytic chamber, and remove degraded products from the complex. The two β-rings harbor active sites with different proteolytic specificities: the peptidyl-glutamyl-peptide hydrolyzing or caspase-like, the trypsin-like, and the chymotrypsin-like activity. The 19S regulatory particle is a multifunctional complex consisting of a base and a lid. The base has six regulatory particle AAA-ATPase (ATPase associated with diverse cellular activities) subunits, and is required for unfolding the substrates and opening the α-rings of the 20S core to allow translocation into the catalytic chamber for degradation. The lid also has different subunits, and the main function of the lid is to mediate deubiquitination of the substrate.

Classification of E3 ubiquitin ligases

E3 ubiquitin ligases are responsible for specificity and selectivity of the substrate. Approximately 600 E3 ligases have been identified thus far. According to their mode of ubiquitin ligation, E3 ligases can be divided into three groups: homologous to E6-associated protein C terminus (HECT) group, really interesting new gene (RING) and UFD2 homology (U‑box) group, and RING-in-between-RING (RBR) E3s group.

In humans, approximately 30 HECT E3 ligases have been found, including NEDD4, ITCH, TRIP12, HUWE1 and UBR5 (Scheffner and Kumar 2014). These E3 ligases have a HECT domain in their C-terminus that harbors three ubiquitination relevant regions: N-terminal lobe, C-terminal lobe and a flexible tether between N- and C- terminal lobes. The N-terminal lobe is required for binding E2 and determining the specificity of the substrates. The C-terminal lobe receives ubiquitin from E2. The catalytic cysteine residue sits in the C-terminal lobe. The flexible tether enables rotation of the N- and C-lobes when the enzymatic reaction is initiated. For HECT domain E3 ligases, there are two distinct steps for catalyzing substrate

ubiquitination. First, the HECT domain binds to E2, followed by transfer of ubiquitin from E2 to a cysteine residue located in the C-terminal lobe in the HECT domain. Next, ubiquitin is transferred to the lysine residue of the substrate that is recognized by the E3 N-terminal lobe (Berndsen and Wolberger 2014).

In humans, almost 95% of E3 ubiquitin ligases belong to the RING-type subgroup (Li, Bengtson et al. 2008). The RING domain is a Zn^{2+} coordinating domain that is composed of a series of spaced cysteine and histidine residues required for binding to E2. The U-box domain has the same RING fold, but no Zn^{2+} requirement. In contrast to HECT E3s, the RING E3s serve as a scaffold for E2 enzyme and substrate, and directly transfer ubiquitin from E2 enzyme to the substrate.

The third subgroup E3s consist of 14 RBR E3 ligases (Smit and Sixma 2014). RBR E3s have three domains: RING1 domain, RING2 domain, and in-between-RING (IBR). For ubiquitin transfer, the RING1 domain binds to ubiquitin-loaded E2 enzyme. RING2 domain is responsible for receiving ubiquitin from RING1 by a catalytic cysteine residue, and transfers the ubiquitin to the substrate by targeting the lysine of the substrate (Senft, Qi et al. 2018). Thus, RBR E3s have properties of both HECT-type and RING-type E3s, and have a hybrid function of HECT E3s and RING E3s.

E3 ubiquitin ligases of MYC

There are several E3 ubiquitin ligases that have been identified to target MYC. Ubiquitination by these E3 ligases can either promote MYC proteasomal degradation or affect MYC activity (Farrell and Sears 2014).

FBW7

FBW7 (F-box and WD40 repeat domain-containing 7) is a RING domain E3 ubiquitin ligase. FBW7 is the best characterized E3 ligases for MYC. In 2004, it was found that FBW7 can directly mediate MYC ubiquitination, and lead to proteasomal degradation of MYC (Welcker, Orian et al. 2004; Yada, Hatakeyama et al. 2004).

FBW7-mediated degradation of MYC is T58 phosphorylation dependent. The T58 residue of MYC located in MBI needs to be phosphorylated by GSK3β. Mutation of T58 disrupts the interaction between MYC and FBW7, abolishing MYC recognition by FBW7 (Gregory, Qi et al. 2003; Welcker, Orian et al. 2004). In human, FBW7 has three isoforms: FBW7α, FBW7β and FBW7γ with different cellular localizations. FBW7α is present in the nucleus, FBW7β is localized in the cytosol and FBW7γ is localized in nucleolus. Both FBW7α and FBW7γ, but not FBW7β promote MYC proteasomal degradation (Welcker, Orian et al. 2004). In addition to T58 phosphorylation, dephosphorylation at S62 by PP2A also participates in the process of MYC proteasomal degradation by FBW7 (Farrell and Sears 2014). Other proteins, such as Cyclin E, c-Jun, and Notch-1 were also identified as substrates of FBW7, (Wang, Inuzuka et al. 2012). FBW7 was evaluated as a tumor suppressor, since it negatively regulates oncoproteins that are often expressed in cancers (Welcker and Clurman 2008). FBW7 is mutated in a variety of tumors, including colorectal cancer, breast cancer, pancreatic cancer and lung squamous cell carcinoma (Welcker and Clurman 2008; Davis, Welcker et al. 2014).

β-TrCP

β-TrCP (β-transducin repeats-containing proteins) is also a RING-type E3 ligase. In contrast to FBW7, ubiquitination of MYC mediated by β-TrCP stabilizes MYC (Popov, Schulein et al. 2010). PLK1 phosphorylates MYC at S278-283 to create a phosphodegron that binds to β-TrCP, and mutation of serine residues can disrupt the interaction between MYC and β-TrCP. FBW7 forms K48-linked polyubiquitin chains on MYC, but β-TrCP assembles heterotypic K48- and K63-linked polyubiquitin that may stabilize MYC by preventing the assembly of K48-linked chains for FBW7-mediated proteasomal degradation. Overexpression of β-TrCP is observed in various cancers (Ougolkov, Zhang et al. 2004; Koch, Waha et al. 2005), and β-TrCP inhibits some tumor suppressors, for example, IkappaB and FOXO3 (Kroll, Margottin et al. 1999; Tsai, Chung et al. 2010). Therefore, β-TrCP was primarily identified as an oncogene.

HUWE1

HUWE1 (HECT, UBA and WWE domain containing 1) is a HECT domain E3 ligase. HUWE1 can interact with MYC at the TAD region and promote MYC ubiquitination (Adhikary, Marinoni et al. 2005; Inoue, Hao et al. 2013; Myant, Cammareri et al. 2017). In human cells, HUWE1 promotes ubiquitination by assembling K63-linked polyubiquitin chains on MYC, but this does not affect MYC stability, and depletion of HUWE1 in human cells does not reduce MYC expression (Adhikary, Marinoni et al. 2005). HUWE1-mediated ubiquitination of MYC can increase its transcriptional activity on target genes (Adhikary, Marinoni et al. 2005). In mice, HUWE1 regulates MYC stability through K48-linked polyubiquitin chains, and HUWE1 deficiency strongly increases MYC expression (Inoue, Hao et al. 2013; Myant, Cammareri et al. 2017).

Other E3 ubiquitin ligases for MYC

SKP2 (S-phase kinase associated protein 2) belongs to the RING-type E3 ligases. In 2003, two studies found that SKP2 regulates MYC ubiquitination and promotes MYC degradation (Kim, Herbst et al. 2003; von der Lehr, Johansson et al. 2003). In contrast to FBW7, SKP2 binds and recognizes MYC through MBII and HLH-LZ domains, but not MBI. U-box-containing E3 ligase CHIP (carboxyl terminus of Hsc70-interacting protein) was recently identified as an E3 ligase for MYC (Paul, Ahmed et al. 2013). The authors showed that CHIP-mediated MYC degradation is connected to decreased MYC transcriptional activity. In addition to E3 ligases described previously, there are several less characterized E3 ligases for MYC, including TRUSS, Fbx29, TRIM32 and NEMO (Koch, Zhang et al. 2007; Schwamborn, Berezikov et al. 2009; Choi, Wright et al. 2010; Kim, Yang et al. 2010).

2.4 UBR5

2.4.1 General information

The E3 ubiquitin ligase UBR5 (ubiquitin protein ligase E3 component n-recognin 5), also named as EDD (E3 identified by Differential Display), is the human orthologue of the *drosophila* melanogaster 'hyperplastic discs' gene (hyd) (Mansfield, Hersperger et al. 1994). UBR5 is a large protein, of ~310 kDa, and is expressed in many different cell types. There are several functional domains in UBR5, including HECT domain (Fig.3). Interestingly, unlike the HECT domain in other E3 ubiquitin ligases, ubiquitin is unable to form a non-covalent interaction with C-lobe of the UBR5 HECT domain. Instead, ubiquitin binds to ubiquitin-activation (UBA) domain of UBR5 in the N-terminus (Kozlov, Nguyen et al. 2007; Matta-Camacho, Kozlov et al. 2012). UBR5 harbors two NLS, one in the N-terminus and another one in the central part of protein. Between the two NLS sequences, there is an ubiquitin recognin box (UBR), which may be involved in N-end rule substrate recognition (Tasaki, Mulder et al. 2005). In the C-terminus, there is a MLLE/PABC domain (homologous to the C-terminal region of poly adenylation binding protein) adjacent to the HECT domain, which is a protein-protein interaction motif. Studies have shown that there is intramolecular interaction between the MLLE/PABC domain and the N-lobe of HECT domain, and that this interaction may play a role in the substrate selectivity and catalytic activity of UBR5 (Munoz-Escobar, Matta-Camacho et al. 2015). UBR5 has 477 potential phosphorylation sites among 2799 amino acids, and 24 sites were already identified by mass spectrometry (Bethard, Zheng et al. 2011). Candidate kinases for the phosphorylation of UBR5 include ATM, CHK and ERK2 (Eblen, Kumar et al. 2003; Kim, Kim et al. 2007; Matsuoka, Ballif et al. 2007). However, the functional effect of these phosphorylation sites has not been illustrated and further investigations are needed to understand whether UBR5 phosphorylation status influences UBR5 activity.

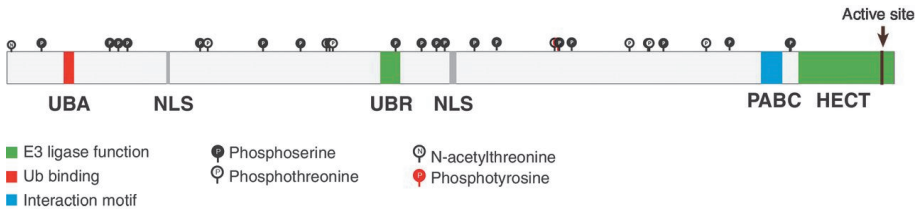

Fig.3. UBR5 functional domains and post-translational modification sites

Arrow shows the conserved catalytic cysteine for UBR5 ligase activity. UBA:Ubiquitin activation domain; NLS: nuclear localization sequence; UBR: Ubiquitin Recognin Box; MLLE/PABC: homologous to the C-terminal region of poly-adenylation binding protein (Shearer, Iconomou et al. 2015).

2.4.2 UBR5 in development

UBR5/HYD was first described as being important in *drosophila* development, and mutation of UBR5/HYD showed a series of developmental phenotypes, including imaginal disc overgrowth and larval lethality (Martin, Martin et al. 1977; Mansfield, Hersperger et al. 1994). The mechanism by which UBR5/HYD functions in *drosophila* development is not yet totally understood. One possibility is that UBR5/HYD negatively regulates the expression of Hedgehog and Decapentaplegic, which are essential for differentiation of the eye imaginal disc (Lee, Amanai et al. 2002). Another possibility is that UBR5/HYD controls the activation status of the transcriptional co-repressor Groucho/TLE by ubiquitination during Wnt signaling (Flack, Mieszczanek et al. 2017).

In mice, knockout of UBR5 leads to embryonic lethality around 10.5, and this embryonic death is independent from p53 status (Saunders, Hird et al. 2004). During development, UBR5-deficient embryos exhibited defects in yolk sac and allantoic vascular formation and failed chorioallantoic fusion. UBR5 was found to suppress the expression of angiogenic factor ACVRL, the deregulation of which, can result in abnormal vascular development (Chen, Yang et al. 2013). This may be one explanation for failed vascular formation in UBR5-deficient mouse embryos. Widespread apoptosis was also observed in UBR5-deficient embryos. The molecular mechanism of UBR5-deficiency induced apoptosis remains unclear.

2.4.3 UBR5 in cancer

Mutation of UBR5/HYD cause imaginal disc hyperplasia in *drosophila*, thus it was originally identified as a tumor suppressor (Mansfield, Hersperger et al. 1994). In human, *ubr5* was first identified and cloned from breast cancer cells (Callaghan, Russell et al. 1998). *Ubr5* gene is located in the long arm of chromosome 8 (8q22.3), close to the *myc* gene, 8q24.21. The studies showed that the *ubr5* gene is amplified and overexpressed in breast and ovarian cancers (Clancy, Henderson et al. 2003). Data from the cancer genome atlas (TCGA) reveals that *ubr5* gene is widely amplified also in other human cancers. Recently, studies from the Ma's laboratory demonstrated that *ubr5* is amplified and overexpressed in triple negative breast cancers, and UBR5 deficiency in tumors can cause abnormal epithelial to mesenchymal transition by inhibiting E-cadherin expression (Liao, Song et al. 2017). In ovarian cancer, UBR5 suppresses apoptosis and contributes to cisplatin resistance (Bradley, Zheng et al. 2014). In colorectal cancer, UBR5 promotes tumor progression by destabilizing the tumor suppressor ECRG4, and high expression of nuclear UBR5 protein indicates poor prognosis in patients (Wang, Zhao et al. 2017; Xie, Liang et al. 2017). A more convincing link between UBR5 and cancer comes from a study analyzing mantle cell lymphoma (MCL) samples from patients. In MCL, nonsynonymous mutations of *ubr5* were found in 18% of tumors with majority of mutations affecting the catalytic cysteine in the HECT domain, which may kill its ligase activity (Meissner, Kridel et al. 2013). In COSMIC analysis of published mutations in different cancers, many point mutations of *ubr5* gene are located in functional domains, including the HECT domain.

2.4.4 UBR5 substrates

UBR5 interaction partners have been identified, and a subset of these proteins have been validated as substrates for UBR5 ubiquitin ligase activity, including TopBP1, RNF168, ATMIN, CDK9, PEPCK and PAIP2 (Shearer, Iconomou et al. 2015).

2.5 PP2A-mediated MYC regulation

Protein phosphatase 2A (PP2A) is a heterotrimeric serine-threonine protein phosphatase that is composed of a catalytic subunit, a scaffold subunit, and a regulatory B subunit. The regulatory B subunit family member, B56α is specific for mediating the dephosphorylation of MYC at S62, resulting in MYC destabilization (Yeh, Cunningham et al. 2004; Arnold and Sears 2006).

2.5.1 CIP2A

CIP2A (cancerous inhibitor of PP2A) was identified as an interaction partner and inhibitor of the tumor suppressor PP2A in human cancer cells (Junttila, Puustinen et al. 2007). Overexpression of CIP2A was reported in multiple human cancers, including breast cancer, ovarian cancer, lung cancer, colon cancer and prostate cancer (Khanna and Pimanda 2016). CIP2A contributes to cellular transformation of Ras-induced mouse embryonic fibroblasts (MEF) and of human embryonal kidney fibroblasts, and promotes cancer cell proliferation (Junttila, Puustinen et al. 2007; Come, Laine et al. 2009; Ventela, Come et al. 2012; Gao, Xu et al. 2017). Mechanistically, CIP2A can interact with and stabilize MYC by inhibiting PP2A activity and therefore blocking the PP2A-dependent dephosphorylation of MYC at S62 and subsequent MYC destabilization (Junttila, Puustinen et al. 2007). Several studies have validated that this CIP2A-mediated MYC regulatory pathway exists both in cancer cells and normal cells (Khanna, Bockelman et al. 2009; Kerosuo, Fox et al. 2010; Liu, Wang et al. 2011; Niemela, Kauko et al. 2012).

CIP2A is expressed at low levels in most normal human tissues except in testis (Junttila, Puustinen et al. 2007; Liu, Wang et al. 2011; Huang, Wei et al. 2012; Ventela, Come et al. 2012). In mice, a hypomorphic mutation of CIP2A is nontoxic and does not cause clear anatomical phenotypes, but leads to defects in sperm production (Ventela, Come et al. 2012). In proliferative fetal and pubertal testes, CIP2A is co-expressed with the proliferation marker protein Ki67, but not in the non-proliferating juvenile testis, suggesting that CIP2A stimulates sperm production by promoting proliferation. In addition to the testis, high expression of CIP2A was

found in the neurogenic areas in mouse embryos as well as in the adult mouse brain (Kerosuo, Fox et al. 2010). CIP2A promotes cell proliferation and self-renewal of neural progenitor cells isolated from the lateral ventricle wall of mouse embryos. Furthermore, CIP2A can promote MYC expression in neural progenitor cells, and it seems that the functional effect of CIP2A on cell proliferation and self-renewal is linked to MYC expression (Kerosuo, Fox et al. 2010).

3. AIM OF THE STUDY

The purpose of this study was to investigate the effect of the post-translational modifications on MYC activity. Phosphorylation and ubiquitination are two main post-translational modifications of MYC, which play important role in MYC activity. Previous work has shown that CIP2A regulated phosphorylation of MYC at S62 and promoted cell proliferation in cultured cells. UBR5 is an E3 ligase that mediates ubiquitination of proteins. However, MYC was not identified as a substrate of UBR5.

The specific aims of this study:

I. To investigate the *in vivo* evidence for the effect of CIP2A-regulated phosphorylation on MYC activity

II. To characterize the role of UBR5 as a possible E3 ubiquitin ligase for MYC ubiquitination

III. To find the functional influence of UBR5-mediated regulation on MYC activity

4. MATERIALS AND METHODS

4.1 Materials

All the product information for cell lines, plasmids, antibodies, chemicals siRNA sequence and other relevant materials can be found in original publications **I** and **II**.

4.2 Methods

Cell culture and transfection (**I**, **II**)

Hela, HEK293, HCC38, HCC1937 and MDA-MB-231 cell lines were obtained from American Type Culture Collection. Osteosarcoma-MYC-off cell line was a nice generous from Dean Felsher (Stanford University). Hela, HEK293, Osteosarcoma-MYC-off and MDA-MB-231 cell lines were cultured in DMEM (Sigma). HCC38 and HCC1937 cell lines were cultured in RPMI (ATCC-modified vision, Thermo Fisher Scientific). All growth mediums were supplemented with 10% heat-inactivated FBS (Gibco), 2 mmol/L L-glutamine, and penicillin (50 units/mL)/streptomycin (50 mg/mL) and all cell lines were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

GFP-UBR5, GFP-UBR5-ΔHECT, Flag-UBR5, and Flag-UBR5-ΔHECT were gifts from Darren Saunders & Charles Watts (Henderson, Russell et al. 2002; Gudjonsson, Altmeyer et al. 2012). V5-MYC, V5-MYC^{T58A} and V5-MYC^{S62A} have been described previously (Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004; Arnold and Sears 2006; Junttila, Puustinen et al. 2007). HA-ubiquitin is a kind gift from professor Lea Sistonen (Åbo Akademi University). Plasmids were transfected with Lipofectamine® 2000 Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. After 48 hours transfection, cell lysate was collected.

Small interfering RNA (siRNA) transfections were performed with Oligofectamine™ Transfection Reagent (Thermo Fisher Scientific) following to the manufacturer's protocol. Three days after transfections, cells were harvested for relevant analysis.

Western blot (**I**, **II**)

Cultured, siRNA transfected and/or treated cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 0.5 % DOC, 0.1 % SDS, 1% NP-40, and 150mM NaCl) with protease and phosphatase inhibitors. The lysate was sonicated, added with 6X SDS loading buffer, boiled and resolved by 4-20% precast protein gels. Proteins were transferred to PVDF membranes. Membranes were blocked in 5% Milk-TBS-Tween 20 for 30 minutes under RT, and then incubated with primary antibodies overnight at 4°C. Secondary antibodies were incubated in 5% Milk-TBS-Tween 20 for 1 hour under room temperature, and developed by ECL western blotting substrate. Densitometric analysis of the blots was performed using ImageJ.

Immunofluorescence staining of cells (**I**, **II**)

Cells plated on chambered coverslip (80826, Ibidi) were transfected with relevant siRNA. After 72 hours transfection, the cells were fixed with 4% paraformaldehyde 15 minutes under room temperature, and then cells were permeabilized with 0.5% Triton X-100 in PBS on ice for 5 minutes. Next, the cells were blocked by 10% normal goat serum diluted in PBS for 30 minutes, and followed by incubating the primary antibodies overnight at 4°C. Subsequently, cells were washed with PBS and incubated with secondary antibodies for 1 hour under room temperature. After secondary antibody incubation, the cells were washed with PBS and nuclei were stained with DAPI in PBS at RT for 10 min. Images were acquired with confocal microscope (LSM780, Carl Zeiss).

Proximity Ligation Assay (**I**, **II**)

The PLA assay was performed according to the manufacturer's protocol (Duolink, Sigma). Briefly, cells plated on coverslips were

transfected with relevant siRNA. After 72 hours transfection, the cells were fixed with 4% paraformaldehyde 15 minutes under room temperature, and then cells were permeabilized with 0.5% Triton X-100 in PBS on ice for 5 minutes. Next, cells were blocked with blocking solution, and incubated in a pre-heated humidity chamber for 30 min at 37°C, followed by incubating the primary antibodies (in blocking solution) overnight at 4°C. Subsequently, cells were washed with buffer A, and the PLA probe was incubated in a pre-heated humidity chamber for 1 hr at 37°C, followed by ligase reaction in a pre-heated humidity chamber for 30 minutes at 37°C. Next, amplification polymerase solution for PLA was added, followed by incubating the cells in a pre-heated humidity chamber for 100 min at 37°C. After amplification, the coverslips were washed with buffer B, and mounted with DAPI. PLA signal was detected by using a confocal microscope (LSM780, Carl Zeiss).

RNA isolation and real-time PCR analysis (**I**, **II**)

Total RNA was isolated from by using RNAeasy kit (Qiagen) according to the manufacturer's protocol. cDNA was synthetized using 1 µg of DNAse I treated RNA using MMLV RNase H minus reverse transcriptase (Promega) and random hexamer primers (Promega). Real-time PCR analysis of cDNA samples was performed with specific primers and probes designed by using Assay Design Center (Roche).

Cell fractionation (**I**)

For fractionation of cellular proteins to cytoplasmic, soluble nuclear and insoluble nuclear fractions, cells were resuspended in 400 µl buffer A (Hepes pH 7.9 10 mM, KCl 10 mM, EDTA 0.1 mM, EGTG 0.1 mM, NP-40 0.1 %, MgCl2 1.5 mM) and incubated for 15 min at 4°C in rotation. The supernatant (cytoplasmic fraction) was collected, and the pellet was resuspended in 125 µl buffer B (Hepes pH 7.9 20 mM, NaCl 150 mM, EGTA 0.25 mM, $MgCl_2$ 1.5 mM, Glyserol 10 %) followed by rotation at 4°C for 20 min. The supernatant was collected as nuclear soluble fraction. The pellet was resuspended in 250 µl RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, DOC 0.5 %,

SDS 0.1 %, NP-40 1%), sonicated and supernatant was collected as insoluble nuclear fraction. In order to analyze proteins that are associated with chromatin, insoluble nuclear pellet was suspended into buffer containing Tris-Hcl 7.5 50mM, 150mM NaCl and 2mM $MgCl_2$, and incubated in the presence of 50U/ml Benzonase, on ice for 40 minutes.

Caspase3/7 activity assay (**II**)

Caspase3/7 activity was measured by luminescence based method, which utilizes a substrate containing Caspase3 and Caspase7 target peptide DEVD, named Caspase-Glo® 3/7 assay (G8091, Promega). The assay was performed following the manufacturer's protocol in white polystyrene 96-well plates (Nunc, Thermo Fisher Scientific Inc.) and luminescence was measured with Perkin Elmer Victor-2 Plate Reader (PerkinElmer Inc.).

Colony formation assay (**II**)

The optimized numbers of cells were seeded in 12-well plates directly or after 24h transfection for siRNA transfected cells until formation of colonies. After 24 hours seeding, colonies were treated with indicated concentration of chemical drugs for another 24 hours. Cell colonies were fixed with cold methanol and stained with 0.2% crystal violet solution (made in 10% ethanol) for 15 minutes at room temperature each. Excess stain was removed by repeated washing with PBS. Plates were dried and scanned with Epson perfection V700 scanner. Quantifications were performed with ColonyArea ImageJ plugin1 and graphs were plotted using the area % values.

Analysis of mutual exclusivity of MYC and UBR5 on single cell level (**II**)

To analyze MYC and UBR5 expression levels on a single cell level, we generated an automated image analysis macro for the open source software FIJI (Schindelin, Arganda-Carreras et al. 2012). This macro was used to analyze cells in 20 histological samples. In brief the macro defines a single cell by creating a mask and applying a

color threshold on a merged (MYC-green and UBR5-red) image after background subtraction and image smoothing. The created mask is further processed by applying a watershed function and followed by a selection of a minimum particle size, to precisely distinguish individual cells. Every separated cell is then given an ID and both signal levels, MYC (green) and UBR5 (red), are measured and displayed.

The measurement output of the macro was further analysed using Microsoft Excel. First we normalized the signal intensities in both channels against the maximum green/red signal in the dataset. The difference of these values was calculated and if the signal intensity was more than 10% higher in one channel than in the other, it was defined as dominant, and the cell was marked as “green” or “red”. Differences in signals intensities within the ±10% were considered equal and cells were marked as “yellow”. Finally, the number of “green”, “red” and “yellow” cells was calculated.

Other methods

The other relevant methods employed in this thesis can be found in attached original publications I and II.

5. RESULTS

5.1 CIP2A-regulated phosphorylation of MYC at serine 62 is important for regenerative proliferation *in vivo* (I)

5.1.1 Characterization of the spatial distribution of CIP2A-MYC interaction (I)

MYC is a nuclear protein. CIP2A is mainly localized in the cytoplasm and perinuclear region, but nuclear immunohistochemical staining of CIP2A has also been reported in several studies (Katz, Jakymiw et al. 2010; Bockelman, Lassus et al. 2011; Ren, Li et al. 2011). In order to understand spatial localization of the CIP2A-MYC interaction, we examined the subcellular distribution of CIP2A by immunofluorescence staining and cell fractionation in HeLa cells. In line with the cell staining results, the majority of CIP2A was found in the cytoplasmic fraction, but a small fraction of CIP2A was localized in the nucleus, and especially in the insoluble nuclear fraction together Lamin A/C (I, Fig. 1A and B). We then investigated the relationship between CIP2A and Lamin A/C. By PLA (proximity ligation assay), we found CIP2A to be associated with Lamin A/C (I, Fig.1C). Interestingly, the interaction signals not only localized in the nuclear lamina, but were also detected inside the nucleus. This is consistent with our immunofluorescence staining of Lamin A/C and other studies that show Lamin A/C associated structure (LAS) is dynamic in the nuclei (Kind, Pagie et al. 2013). Like CIP2A-Lamin A/C association, most PLA signals of MYC-Lamin A/C were detected in the nuclear lamina, but intranuclear signals were also clearly observed (I, Fig. 1D). Moreover, PLA dots of CIP2A-MYC association co-localized with Lamin A/C (I, Fig. 1E-a-b). Since MYC phosphorylated at S62 (pS62MYC) plays an important role in MYC stability and activity (Hann 2006; Luscher and Vervoorts 2012), we examined pS62MYC and CIP2A by PLA. We found that pS62MYC is associated with CIP2A in a similar pattern at nuclear lamina and

inside the nucleus as seen with total MYC (I, Fig. 1E-c-d), indicating that the form of MYC that associates with CIP2A is phosphorylated at S62. Interestingly, pS62MYC cell staining showed a lamina-enriched punctuate pattern similar to pS62MYC-CIP2A PLA signals, but this punctuate pattern was not observed with total MYC cell staining (I, Fig. 1E and 1G). This further suggests that the form of MYC that is associated with CIP2A at the LAS is pS62MYC. Treatment with increasing salt concentrations released both pS62MYC and MYC from the insoluble to the soluble nuclear fraction (I, Fig. 1H), indicating that the association of pS62MYC with LAS is mediated by an ionic interation. By using DNase (Benzonase) to digest DNA, it demonstrated that CIP2A and the majority of pS62MYC binds to the proteinaceous component of the LAS (I, Fig. 1I and J).

5.1.2 Phosphorylation of MYC at serine 62 promotes its association with LAS (I)

Based on the above results, we speculated that phosphorylation of MYC at S62 may drive MYC to LAS, and CIP2A may be required to retain pS62MYC at this location. In order to validate this, we analyzed the partitioning of wild type MYC and two MYC mutants between the soluble and insoluble nuclear fractions. In our previous study, we found that mutant S62AMYC is unable to interact with CIP2A, but mutant T58AMYC, a functional mimic of pS62MYC, can interact with CIP2A (Junttila, Puustinen et al. 2007; Wang, Cunningham et al. 2011). In contrast to wild type MYC, S62AMYC showed less accumulation in the insoluble nuclear fraction, whereas T58AMYC, with CIP2A binding-capacity, accumulated efficiently in the insoluble nuclear fraction (I, Fig. 2A and B). This is in line with PLA results that pS62MYC is the form of MYC to associate with LAS (I, Fig. 1E-1G). Furthermore, in PLA results, T58AMYC-Lamin A/C association signals were more than S62AMYC-Lamin A/C (I, Fig. 2C). These results demonstrate that phosphorylation on serine 62 promotes MYC to associate with LAS. To address the role of CIP2A in the accumulation of MYC on LAS, we examined the subnuclear distribution of pS62MYC and total MYC in HeLa cells transfected with CIP2A siRNA or scrambled siRNA under low salt buffer to

preserve MYC's association with Lamin A/C. The result showed that depletion of CIP2A inhibits pS62MYC expression in the Lamin A/C-enriched insoluble nuclear fraction, but total MYC expression was less inhibited (I, Fig 2D-lanes 2 and 4, and 2E, insoluble). Moreover, pS62MYC in the soluble nuclear fraction is insensitive to CIP2A depletion (I, Fig 2D-lanes 1 and 3, 2E, soluble). Under high salt buffer, pS62MYC elutes from the insoluble fraction to the soluble fraction (I, Fig. 1H). We treated cell with high salt buffer, and found that the CIP2A-sensitive pool of MYC was the pS62MYC eluted from the insoluble fraction (I, Fig 2D-lanes 5 and 7, 2E). Taken together, the above results demonstrate that phosphorylation at S62 of MYC drives its association with LAS, and CIP2A selectively supports the expression of the LAS-associated pool of pS62MYC.

5.1.3 CIP2A interacts with MYC *in vivo* and promotes intestinal regeneration in response to DNA damage (I)

In order to investigate the effect of CIP2A-mediated MYC phosphorylation in vivo, we chose an intestinal regeneration mouse model. In this mouse model, intestinal regeneration is totally dependent on MYC amount in response to DNA damage, which can be employed to address the MYC-dependence of the in vivo proliferation response (Muncan, Sansom et al. 2006; Sansom, Meniel et al. 2007; Athineos and Sansom 2010). In this model, CIP2A showed similar immunopositivity in the intestinal crypt as MYC (I, Fig.4A). By PLA, we also detected CIP2A-MYC association in intestinal crypt cells (I, Fig.4B-D). In addition, PLA signals were reduced in intestinal sections of $CIP2A^{HOZ}$ mice compared to wild type mice (I, Fig. 4E and F).

Our previous study showed that $CIP2A^{HOZ}$ mice have normal growth, weight development, and lifespan (Ventela, Come et al. 2012), whereas, MYC is crucial to maintain normal crypt structure in the adult intestine (Muncan, Sansom et al. 2006). Thus, it seems that CIP2A-MYC interaction may be not essential for intestinal crypt homeostasis. By comparing WT and $CIP2A^{HOZ}$ mice, we found that CIP2A deficiency did not cause aberrant proliferation and differentiation of intestinal crypts, or gross change in crypt

architecture (I, Fig. S4), suggesting that CIP2A is dispensable for normal intestinal crypt function.

In response to DNA damage, we found that CIP2A expression was increased in regenerating intestinal tissues (I, Fig. 5A and B), which is similar to MYC induction (Ashton, Morton et al. 2010; Athineos and Sansom 2010). To determine the contribution of increased CIP2A to MYC-dependent intestinal regeneration, we studied crypt regeneration caused by irradiation. By comparing WT and CIP2A^{HOZ} mice, we found that intestines in CIP2A^{HOZ} mice showed attenuated regenerative response and less proliferative cells in regenerating crypts than WT mice (I, Fig. 5C-F). In addition, the absence of CIP2A didn't cause increased apoptosis (I, Fig. 5G). A similar phenomenon was also found in response to cisplatin-induced DNA damage (I, Fig. 5H-J).

Next, we examined whether CIP2A-regulated phosphorylation of MYC at S62 correlates with the different phenotypes of intestinal regeneration described above. In untreated intestines from control and CIP2A^{HOZ} mice, there was not obvious difference in total MYC expression (I, Fig. 6A). In regenerating crypts, the immunohistochemical staining of MYC in CIP2A^{HOZ} crypts is nearly on the same levels as in WT crypts, and *myc* mRNA were expressed at equal levels in regenerating CIP2A^{HOZ} and WT crypts (I, Fig. 6B-D). However, by immunostaining, it was shown that pS62MYC expression was notably inhibited in CIP2A^{HOZ} crypts in response to irradiation, and this was correlated with the induction of proliferation assessed by Ki67 co-staining (I, Fig. 6E and F). We examined MYC target genes that were involved in intestinal regeneration in irradiated WT and CIP2A^{HOZ} intestines (Sansom, Meniel et al. 2007; Athineos and Sansom 2010). Of the genes analyzed, all were significantly downregulated in regenerating guts from CIP2A^{HOZ} mice compared with controls (I, Fig. 6G). We also confirmed impaired MYC recruitment to the *tiam1* promoter upon CIP2A inhibition by siRNA in cultured cells (I, Fig. S5). Taken together, these results collectively demonstrate that CIP2A promotes MYC-dependent intestinal regeneration caused by DNA damage, and that CIP2A selectively supports the expression of pS62MYC *in vivo*.

5.1.4 Confirmation of the role of serine 62 phosphorylation in proliferation induction *in vivo* (I)

In order to validate our main conclusion that the expression of pS62MYC is important for proliferation induction *in vivo*, we used T58AMYC and S62AMYC mutants in an *in vivo* setting. Since T58AMYC has enhanced S62 phosphorylation (Lutterbach and Hann 1994; Sears, Nuckolls et al. 2000), we speculated that T58AMYC has more potential to rescue the effects of losing endogenous MYC in the intestinal regeneration model than wild type MYC and S62AMYC. We generated the mice expressing WT, T58A, or S62A allele of MYC at subphysiological levels by B-napthoflavone stimulation in the absence of endogenous MYC (I, Fig. S6A). These transgenes were transcriptionally expressed at the same level (I, Fig. S6B), whereas both T58AMYC and S62AMYC showed significantly higher protein expression in regenerating intestine than WT MYC (I, Fig. 7A). In contrast to WT MYC and S62AMYC, the T58AMYC mutant showed an obvious increase in phosphorylation at S62 in regenerating crypts despite the similar protein expression levels of T58AMYC and S62AMYC (I, Fig. 7B and C). Irradiation of WT mice resulted in a robust regenerative response 72 hours later, as evidenced by large crypts that stained positively for Ki67 (I, Fig. 7D and S6C). However, deletion of *myc* (*myc*$^{fl/fl}$) suppressed crypt regeneration, and subphysiological expression of WT *myc* (*Rosa*$^{myc/+}$) failed to drive regeneration in response to irradiation (I, Fig. 7D and S6C), which is consistent with previous studies (Ashton, Morton et al. 2010). In line with our other results that phosphorylation at S62 defines MYC effect on proliferation, T58AMYC mutant was more competent than WT MYC or S62AMYC to compensate for the loss of endogenous *myc* both in the regeneration response and in driving cell proliferation in response to irradiation (I, Fig. 7D, E and S6C). Furthermore, PLA results showed that T58AMYC, but not S62AMYC, associates with both CIP2A and Lamin A/C in regenerating intestinal tissues (I, Fig. 7F). This is consistent with our *in vitro* results that show S62 phosphorylation is a determining factor for whether MYC interacts with both CIP2A and Lamin A/C (I, Fig. 2A-C). Finally, we confirmed that expression of T58AMYC rescued the expression of a number of CIP2A-regulated MYC target genes that were downregulated in *myc*$^{fl/fl}$ intestine (I, Fig. 7G).

In summary, phosphorylation at S62 promotes MYC driving *in vivo* proliferation and DNA damage-induced crypt regeneration, and association of pS62MYC with both CIP2A and LAS is linked to MYC-mediated proliferation induction.

5.2 UBR5 mediates proteasomal degradation of MYC (II)

5.2.1 UBR5 regulates MYC protein stability (II)

FBW7 is a well-studied E3 ubiquitin ligase that destabilizes MYC and promotes MYC proteasomal degradation, which is dependent on phosphorylation of MYC at T58 (Welcker, Orian et al. 2004). To identify other E3 ubiquitin ligases for MYC, we performed an siRNA screen against 591 ubiquitin ligases in U2OS cells stably expressing the MYCT58A mutant that is resistant to FBW7-mediated MYC destabilization (Welcker, Orian et al. 2004). After 48 hours transfection, we treated cells for 3.5 hours with the protein synthesis inhibitor cycloheximide (CHX) to analyze MYC protein stability, and immunofluorescence detection of MYC was used as a read-out in a high-content imaging based assay (II,Fig.1A). We found that UBR5 can affect MYCT58A expression. MYCT58A is unable to interact with and is not recognized by FBW7 (Gregory, Qi et al. 2003; Welcker, Orian et al. 2004). To validate the independent function of UBR5 from FBW7, we examined whether MYCT58A can interact with UBR5. By using co-immunoprecipitation, we found that MYCT58A retains the ability to bind to UBR5 similarly to wild type MYC (II, Fig. 1B). In addition, UBR5 overexpression decreased MYCT58A expression (II, Fig. 1C). Conversely, siRNA-mediated depletion of UBR5 trigger a significant increase in MYC expression (II, Fig. 1D). By immunofluorescence staining, we found that intense MYC staining was observed in small fraction of control cells, whereas UBR5 inhibition synchronized the cell population to be more homogenous in high MYC expression (II, Fig. 1E). In addition, increased MYC expression caused by depletion of UBR5 was also validated in other cell lines (II, Fig. 1F). The above-mentioned results demonstrate that UBR5 suppresses MYC expression. Next, we examined whether UBR5 regulates MYC on the mRNA levels. We

found that there is only a slight increase in MYC mRNA when UBR5 was depleted, whereas there was a robust increase in MYC protein expression, indicating that UBR5 regulates MYC mainly at the protein level (II, Fig. 1G). The tetracycline regulatory system (Tet-off) was used to control MYC expression in response to doxycycline addition. In the presence of doxycycline, the tetracycline transactivating protein changes conformation and is unable to bind to the seven tetO sequence to activate MYC transcription (Jain, Arvanitis et al. 2002; Wu, van Riggelen et al. 2007). We detected MYC expression in a UBR5 depleted osteosarcoma-MYC-off cell line derived from transgenic mice. MYC expression was increased upon UBR5 depletion when ectopic MYC mRNA expression was induced by gradual removal of doxycycline from osteosarcoma-MYC-off cells (II, Fig.1H). This further confirmed that UBR5 regulates MYC at the protein level. Finally, we performed CHX chase experiment in HeLa cells. We found that depletion of UBR5 increased MYC stability (II, Fig. 1I). Taken together, these data suggest that UBR5 negatively regulates MYC stability independently from FBW7 and T58 phosphorylation.

5.2.2 UBR5 is a novel E3 ubiquitin ligase for MYC (II)

Consistent with co-immunoprecipitation result, we further confirmed UBR5-MYC association by PLA, and the association was mostly disrupted by depletion of MYC with siRNA, suggesting this association was specific (II, Fig. 2A). UBR5 is a HECT domain E3 ubiquitin ligase and mediates protein ubiquitination, leading substrates to be degraded via the ubiquitin-proteasome system (UPS). Next, we explored the potential mechanism of UBR5-mediated destabilization of MYC. We treated cells overexpressing UBR5 and MYC with proteasome inhibitor MG132. We found that overexpression of UBR5 reduced MYC level, and that MG132 could block this UBR5 effect on MYC reduction, indicating that UBR5 promotes MYC degradation via the proteasome pathway (II, Fig. 2B and C). The conserved cysteine at position 2768 in the HECT domain is critical for UBR5 E3 ligase activity (Honda, Tojo et al. 2002). Substitution of this cysteine with an alanine incapacitates UBR5-mediated MYC regulation. It was further confirmed that UBR5

promotes MYC degradation via its E3 ligase activity (II, Fig. 2B and C). Moreover, we confirmed that only overexpression of wild type UBR5, not C2768A mutant can promote ubiquitination of MYC, and depletion of UBR5 can inhibit MYC ubiquitination (II, Fig. 2D and E). Together, these results demonstrate that UBR5 is a new E3 ligase that can mediate MYC ubiquitination, leading to proteasomal degradation of MYC.

5.2.3 UBR5 promotes tissue growth in a MYC-dependent manner in *drosophila* (II)

We utilized *drosophila melanogaster* as a model system to explore the physiological relevance of UBR5-mediated regulation of MYC. RNAi-mediated depletion of the *drosophila* UBR5 ortholog, HYD in *drosophila* S2 cells led to elevated levels of *drosophila* MYC (dMYC) protein, whereas the mRNA level of dMYC was not affected (II, Fig. 3A-C). *Drosophila* allows the use of somatic recombination to generate clones of mutant tissue in an otherwise heterozygous background. Clones of $hyd^{K3.5}$ mutant generated during mid larval development (72 h after egg laying) led to a phenotype clearly visible in the adult wings. Wings of animals with *hyd* mutant clones were irregular, lacking the normal flat wing morphology (II, Fig. S3). Intriguingly, this irregular wing phenotype was strongly suppressed by simultaneous knockdown of dMYC in the mutant clones (II, Figure 3D). The use on the MARCM system to generate mutant clones allowed us to GFP mark and visualize the tissue morphology of the wing imaginal discs, the larval wing precursors (Lee and Luo 1999). While control and MYC RNAi clones appeared morphologically normal, *hyd* mutant clones had a round morphology with clusters of cells growing out from the epithelial plane (II, Fig. 3E and F), which was confirmed by quantification of the clone roundness (II, Fig. 3G). Furthermore, knockdown of dMYC suppressed the roundness morphology of the *hyd* mutant clones (II, Fig. 3E-G). Taken together, in *drosophila*, suppression of dMYC expression is a critical part of growth control by UBR5 *in vivo*.

5.2.4 UBR5 suppresses MYC-induced apoptosis in cancer cells (II)

In cancer cells, increased MYC expression can induce cell proliferation or apoptosis priming (Pelengaris, Khan et al. 2002; Murphy, Junttila et al. 2008; Nieminen, Eskelinen et al. 2013; Topham, Tighe et al. 2015). In HeLa cells, we found that UBR5 depletion decreases colony growth (II, Fig. 4A), suggesting that increased MYC resulting from UBR5 loss may cause apoptosis priming. This was further confirmed by increased levels of cleaved PARP and induced caspase activity in UBR5-depleted cells (II, Fig. 4B and C). According to published studies, cells with high MYC expression is sensitive to antimitotic drugs and drugs that destroy DNA replication, such as taxol and camptothecins (Rohban and Campaner 2015; Topham, Tighe et al. 2015). We found that UBR5 depletion strongly sensitizes cells to taxol or camptothecin, but did not cause massive cell death (II, Fig. 4D). Importantly, PARP cleavage, increased caspase activity and hypersensitivity to drug treatment in UBR5 depleted cells can be rescued by co-depletion of MYC (II, Fig 4E-H). In osteosarcoma-MYC-off cells, depletion of UBR5 was unable to induce PARP cleavage in the absence of MYC (II, Fig. 4I). Recently, BIM was found to be primary mediator of MYC-induced apoptosis (Muthalagu, Junttila et al. 2014). To investigate whether the MYC-BIM axis is responsible for apoptosis sensitization in UBR5-depleted cells, we examined whether BIM is regulated by UBR5. Indeed, BIM expression is increased upon UBR5 depletion, and this was rescued by co-depletion of MYC (II, Fig. 4J). Furthermore, co-depletion of BIM can rescue the induction of apoptosis caused by UBR5 depletion (II, Fig. 4K). In summary, the above data demonstrate that MYC expression is a prerequisite for apoptosis priming induced by UBR5 inhibition.

5.2.5 Transcriptional changes of UBR5 inhibition is consistent with UBR5-mediated MYC expression (II)

The results above show that UBR5 suppresses MYC expression and demonstrate a functional relationship between UBR5 and MYC in both normal and cancer cells. We then asked whether transcriptional pattern of MYC target genes is correlated with UBR5-regulated MYC

expression upon inhibition of UBR5. We performed an RNA-sequencing analysis of HeLa cells depleted of MYC or UBR5 (II, Fig. 5A). By gene set enrichment analysis (GSEA), we found that the transcriptional changes of MYC target genes are consistent with the up-regulated MYC expression upon UBR5 depletion (II, Fig. 5B). These results show that up-regulated MYC expression by UBR5 depletion reflects up-regulated MYC transcriptional activity.

5.2.6 UBR5 dominates MYC expression at a single cell level in breast cancer tissues (II)

To find relevance between UBR5 and MYC in cancers, we analysed *ubr5* and *myc* mRNA expression in 672 cell lines from over 20 different cancer types. Our analysis showed a weak, but significant correlation between UBR5 and MYC mRNA expression (II, Fig. 6A, Pearson correlation 0.21, $p<0.01$). Among these cancer types, ovarian, lymphoid, breast and pancreatic cancer types showed significant positive correlation (II, Fig. 6A). Interestingly, both *ubr5* and *myc* genes are located in the long arm of chromosome 8 (II, Fig. 6B), suggesting that co-amplification of these two genes may provide a means to control MYC protein level. Examination of TCGA cancer patient amplification data for *ubr5* and *myc* in the four cancer types in which there was evidence for mRNA co-expression (II, Fig. 6A), revealed that amplification of *ubr5* alone is a very rare event, and that particularly in breast cancers, there seems to be a genetic pressure to co-amplify both *ubr5* and *myc* (II, Fig. 6C). As the breast cancer samples had the highest prevalence of co-amplification of *ubr5* and *myc*, we examined UBR5 and MYC protein expression in breast cancer tissues. We optimezed the IHC staining so that intensities from 0 to +++ were reliably observed with both UBR5 and MYC antibodies (II, Fig. 6D). In 78% of the samples, the percentage of the staining positive cells (+,++ or +++) was greater for UBR5 than for MYC (II, Fig. 6D and E). In several staining tumour areas, we observed mutually exclusive patterns for maximal staining intensities of UBR5 and MYC in the same individual cell (II, Fig.6F), where more intense UBR5 staining is correlated with weaker MYC staining, and vice versa. Based on this observation, we asked whether UBR5 dominates MYC expression at the single cell level. In order to

validate this, we performed dual immunofluorescence staining of UBR5 and MYC in the same breast cancer tissue samples. We categorized stained individual cells into three groups (II, Fig. S5E): $MYC^{high}/UBR5^{low}$ (Green), $UBR5^{high}/MYC^{low}$ (Red), and $MYC^{high}/UBR5^{high}$ (Yellow). The samples with most of the cells representing either UBR5 or MYC dominant protein expression pattern are shown in Fig. 6G. In those samples in which the overall staining pattern was not predominantly either red or green (II, Fig. 6G, the last row), the individual cells were however dominantly found to be either $MYC^{high}/UBR5^{low}$ or $UBR5^{high}/MYC^{low}$ (II, Fig. 6H and S5E). By quantitation, we confirmed the mutual exclusivity of maximal UBR5 or MYC expression intensity in individual cells (II. Fig.6H, I). Only 9% of cells showed equal UBR5 and MYC expression intensities, whereas, most cells displayed that UBR5 expression dominated over MYC expression at the single cell level (II, Fig. 6I).

These data demonstrate that UBR5 dominates MYC protein expression in the majority of breast cancer cells in vivo.

5.2.7 Depletion of UBR5 sensitizes *ubr5/myc* co-amplified breast cancer cells to apoptosis (II)

In order to investigate the functional effect generated by UBR5-mediated MYC expression in *ubr5/myc* co-amplified breast cancer cells, we analysed the correlation between UBR5 essentiality index (zGARP score) and *ubr5/myc* gene copy number by using data from a breast cancer cell line drop-out screen (Marcotte, Sayad et al. 2016). A significant correlation was observed between zGARP score and *ubr5/myc* gene copy numbers (II, Fig.7A). Since HCC1937 and HCC38 were among the most UBR5-dependent breast cancer cell lines harbour *ubr5/myc* amplification in the drop-out screen, we chose them to test functional relevance of UBR5. Depletion of UBR5 induced MYC expression and MYC-dependent PARP cleavage (II, Fig. 7B and C). Furthermore, UBR5 depletion led to MYC-dependent sensitisation to cell killing caused by camptothecin analogues, irinotecan and topotecan (II, Fig. 7D and E). In addition, we also

found similar MYC-dependent sensitisation to taxol-induced cell killing upon UBR5 depletion in HCC38 cells (II, Fig. 7F and G).

Finally, taking into account degree of heterogeneity in MYC positivity in breast cancer tumour tissues (II, Fig.6H), we wanted to assess whether increased apoptosis sensitivity of UBR5-depleted breast cancer population could be linked to synchronization of breast cancer cell population to become more uniformly MYC positive, as was initially observed in Hela cells (II, Fig. 1E). Indeed, whereas in scrambled siRNA transfected cell populations only few individual cells expressed MYC at the levels that could be envisioned apoptosis priming, frequency of cells expressing high MYC protein levels was clearly increased upon UBR5 depletion (II, Fig.7H and I). In conclusion, UBR5-mediated MYC suppression plays a crucial role in suppressing apoptosis priming in *ubr5/myc* co-amplified breast cancer cells (II, Fig. 7J). It also indicates that inhibition of UBR5 could promote heterogeneous cell populations to express MYC more uniformly at levels that may trigger apoptosis priming.

6. DISCUSSION

6.1 CIP2A promotes accumulation of pS62MYC on Lamin A/C associated nuclear structures (I)

MYC is involved in many physiological and pathological processes. Post-translational modifications play important roles in regulating MYC stability and activity. Among different post-translational modifications, phosphorylation of MYC is well-studied. Previous studies showed that phosphorylation at S62 enhances MYC stability and promotes accumulation of MYC activity (Lutterbach and Hann 1994; Sears, Leone et al. 1999; Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004). S62 can be dephosphorylated by PP2A, resulting in MYC destabilization (Yeh, Cunningham et al. 2004; Arnold and Sears 2006). Before initiation of my doctoral study, our lab found that CIP2A, as an endogenous inhibitor of PP2A, inhibits PP2A-mediated dephosphorylation of S62 in vitro, leading to increased MYC stability (Junttila, Puustinen et al. 2007).

Here, we further investigated the regulation of MYC phosphorylation at S62 by CIP2A. We revealed that phosphorylation of MYC at S62 promoted MYC recruitment to LAS, and that CIP2A selectively supported the expression of pS62MYC. This is consistent with previous studies about CIP2A and MYC subcellular localization. By immunofluorescence staining and immunohistochemistry, CIP2A was found to be localized to the perinuclear region and inside nucleus (Soo Hoo, Zhang et al. 2002), a result that was recapitulated in our immunofluorescence staining. Though MYC is mainly a nuclear protein, it was already demonstrated very early that MYC associated with a complex residual nuclear structure (Eisenman, Tachibana et al. 1985). By cell fractionation, we found CIP2A was localized to the Lamin A/C-enriched insoluble nuclear compartment, and in the following experiment, we examined the relation between CIP2A, MYC and Lamin A/C. However, the insoluble nuclear fraction also contains other lamina proteins, for example, Lamin B1 and Lamin B2. Therefore, we cannot exclude that other lamin proteins

may also participate in CIP2A-regulated MYC phosphorylation. Lamins are the major components of the nuclear lamina. By Lamins, nuclear lamina can interact with the regions of chromatin that are defined as Lamina-associated domains (Dechat, Adam et al. 2010; van Steensel and Belmont 2017). Thus, in addition to proteins, DNA may provide a platform for CIP2A to bind and regulate MYC. By removing DNA from the insoluble nuclear fraction, we found that CIP2A and most of the pS62MYC are enriched in the proteinaceous component of LAS where the regulation of CIP2A on pS62MYC happens. In mouse model, we validated that CIP2A supports pS62MYC expression, and that this is important to activate MYC target genes to drive proliferation and intestinal regeneration. In addtion to cultured cells, T58AMYC, exhibiting enhanced serine 62 phosphorylation, associates with CIP2A and Lamin A/C in vivo, but not S62AMYC. The association of Lamin A/C with chromatin at nuclear lamina generally contributes to gene transcriptional repression, whereas, intra-nuclear association of Lamin A/C with chromatin may be involved in gene activation, which may be caused by histone modifications (Naetar, Ferraioli et al. 2017; van Steensel and Belmont 2017). Our results show that the association between CIP2A, Lamin A/C and MYC mainly occurs at nuclear lamina. Since the domains on lamina-associated chromatin are transcriptionally repressive, more research is required to address that how MYC regulates genes in these domains. One possibility is that a subset of genes, ~5%-10% in these chromatin domains, are not transcriptionally repressive, and MYC target genes may be included in this subset of genes (van Steensel and Belmont 2017). MYC mediates chromatin remodelling by inducing global histone modification that can cause genome-wide changes of transcription (Amati, Frank et al. 2001; Berger 2002; Knoepfler 2007). Another possibility is that expression of pS62MYC at LAS may be important for inducing MYC-mediated histone modifications to change the transcriptionally repressive status of genes in lamina-associated chromatin, and finally leading to transcription of a subset of MYC target genes.

Importantly, our results indicated that CIP2A regulates MYC not on a global level, but selectively regulates Lamin A/C-associated pool of pS62MYC. Deletion of MYC caused embryonic lethality in mice,

whereas deficiency of CIP2A didn't have a clear toxic effect on mouse development (Davis, Wims et al. 1993; Ventela, Come et al. 2012). This may be explained by our conclusion here that CIP2A spatially regulates the MYC that is localized to LAS. Taken together, in the future work, it will be interesting to dissect whether the interaction between Lamin A/C, CIP2A and MYC is direct or indirect, and examine the phosphorylation status of MYC and the activity of MYC in the absence of Lamin A/C.

6.2 CIP2A-mediated phosphorylation of serine 62 is essential for MYC induced proliferation (I)

Though MYC is essential for proliferation induction in different cell types (Kelly, Cochran et al. 1983; de Alboran, O'Hagan et al. 2001; Trumpp, Refaeli et al. 2001), the role of post-translational modification that defines MYC activity in proliferation *in vivo* has not been identified. Here, we found that CIP2A-regulated phosphorylation of MYC on S62 is important for MYC-induced proliferation in response to DNA damage by using a mouse model, but CIP2A or pS62MYC did not influence basal cellular proliferation of intestinal crypts. In addition to MYC, CIP2A also regulates phosphorylation of AKT, which drives cancer cell proliferation (Khanna, Pimanda et al. 2013). Recently, it was reported that CIP2A can coordinate mTORC1 activity and MYC stability to promote cell proliferation (Puustinen, Rytter et al. 2014). Therefore, we cannot exclude that other CIP2A-regulated proteins may also play roles in proliferation induction in our intestinal regeneration mouse model after DNA damage.

In cultured cells, we showed that CIP2A stabilizes MYC by inhibiting PP2A activity to dephosphorylate MYC on S62. However, in our *in vivo* mouse model, it was shown that loss of pS62MYC caused by CIP2A depletion did not decrease total MYC expression in response to DNA damage (I, Fig. 6B). We speculated that this may be influenced by the biological context, i.e. the differences between *in vitro* cell models and *in vivo* mouse models, in which the role of phosphorylation on S62 is fundamentally different. Another

explanation is that cells may express more non-phosphorylated S62 MYC to compensate for loss of pS62MYC.

Uncontrolled cellular proliferation is one hallmark of cancer (Hanahan and Weinberg 2011). The role of MYC in promoting cancer cell proliferation is well-known, but it is still challenging to target MYC because of unacceptable toxicity caused by global inhibition of MYC (Prochownik and Vogt 2010; McKeown and Bradner 2014). Our results showed that CIP2A selectively supports the pool of MYC that is phosphorylated at S62 in LAS, which is essential for MYC activity for cell proliferation *in vivo* in response to DNA damage. Thus, targeting CIP2A may provide an indirect way to inhibit MYC activity and cancer cell proliferation.

6.3 UBR5 promotes MYC ubiquitination and MYC proteasomal degradation (II)

Different E3 ubiquitin ligases have been identified for MYC, including phosphorylation-independent and phosphorylation-dependent ones (Farrell and Sears 2014). In this study, we identified UBR5 as an E3 ligase for MYC that suppresses MYC expression by promoting proteasomal degradation of MYC.

As an E3 ligase for MYC, UBR5's interaction with MYC was confirmed by co-immunoprecipitation and PLA. We tried to map the interaction area between UBR5 and MYC by GST pull-down, but failed. UBR5 is a large protein (309kDa) with several domains. We failed to purify UBR5 protein and its fragments. In ubiquitination assay, we confirmed that UBR5 promotes MYC ubiquitination, leading to proteasomal degradation. Ubiquitin can form different polyubiquitin chains, and K48-linked polyubiquitin chains were found to be mainly responsible for proteolysis, and other polychain types may influence protein activity during biological processes (Kwon and Ciechanover 2017). In future work, it will be interesting to identify which type of polyubiquitin chain is involved in MYC ubiquitination by UBR5, and this is important for understanding downstream functional outcomes on MYC.

Here we found that overexpression of UBR5 can suppress both v5-wild type MYC, and v5-T58A MYC, which is resistant to FBW7-mediated proteasomal degradation. Meanwhile, double depletion of FBW7 and UBR5 caused additive stabilization of MYC (II, Fig S1D and S1E). These results indicated that the regulation of UBR5 on MYC ubiquitination is FBW7- and pT58-independent. It will be interesting to examine whether UBR5 regulates FBW7, and provide direct evidence that UBR5 can promote T58AMYC ubiquitination by ubiquitination assay in future work.

As mentioned above, dozens of E3 ubiquitin ligases have been identified for MYC (Farrell and Sears 2014). The question is that why does MYC need different E3 ligases to regulate its expression? It is possible that there are different pools of MYC in the cells, which are regulated by different E3 ligases and that in response to specific signals, the relevant E3 ligase will be employed to target the relevant pool of MYC. For example, FBW7 may target nucleolus pool of MYC (Welcker, Orian et al. 2004); SKP2 may regulate MYC in the context of gene transcription activation on chromatin (von der Lehr, Johansson et al. 2003) and one E3 ligase may be responsible for the CIP2A-sensitive pool of MYC localized to LAS.

Taken together, my studies identify UBR5 as a novel E3 ubiquitin ligase for MYC.

6.4 UBR5/HYD regulates tissue growth in a MYC-dependent manner in *drosophila* (II)

UBR5/HYD plays an important role in *drosophila* development. Mutation of UBR5/HYD can cause overgrowth in larval imaginal discs (Mansfield, Hersperger et al. 1994; Flack, Mieszczanek et al. 2017). Loss of MYC expression in wing imaginal discs in *drosophila* leads to decreased cell growth and smaller cell size, whereas overexpression of MYC increases cell growth (Johnston, Prober et al. 1999). In this study, we found that inhibition of UBR5/HYD caused wing imaginal disc overgrowth, leading to irregular wing morphology, and this phenotype was suppressed by co-depletion of MYC. In line with our results from human and mouse cell lines, it suggests that

UBR5 may be an evolutionary conserved endogenous suppressor of MYC. We didn't examine the apoptosis, proliferation, either other phenotypes in UBR5/HYD mutant, and it will be interesting to do this in the further study.

6.5 UBR5 suppresses MYC-induced apoptosis in cancer cells (II)

The links between increased MYC expression and apoptosis have been reported in the past decades, including the association of MYC overexpression with apoptosis priming that is a determinant of cancer cell fate in response to cytotoxic treatments (Murphy, Junttila et al. 2008; McMahon 2014; Sarosiek, Fraser et al. 2017). In this study, we investigated the role of the UBR5-MYC axis in apoptosis. We found that UBR5 suppresses apoptosis in a MYC-dependent manner. In UBR5-depleted cells, we did not see massive cell death, but observed high expression of the apoptosis marker, cleaved-PARP (II, Fig4, A and B). This indicated that depletion of UBR5 triggered apoptosis in a portion of cells, and shifted other cells towards apoptosis priming that sensitizes cells to pro-apoptotic signals, like camptothecin and taxol. Though several mechanisms have been identified to explain how MYC controls apoptosis (McMahon 2014), BIM was recently demonstrated as the primary mediator of MYC-induced apoptosis (Muthalagu, Junttila et al. 2014). In our case, depletion of UBR5 induced BIM expression, and this was rescued by co-depletion of MYC. Induction of apoptosis in UBR5-dpeleted cells can be rescued by depletion of BIM. Taken together, these results demonstrate that increased MYC expression is a prerequisite for apoptosis priming induced by inhibition of UBR5.

Controversially, UBR5 has been reported as both an oncogene and a tumor suppressor (Callaghan, Russell et al. 1998; Clancy, Henderson et al. 2003; Bolt, Stossi et al. 2015; Shearer, Iconomou et al. 2015; Liao, Song et al. 2017). Here, our identification of MYC as a UBR5 substrate may provide an explanation for the enigmatic role of UBR5. UBR5-mediated suppression of tissue growth via MYC is consistent with its tumor suppressor role in *drosophila*. In cancer

cells, UBR5 governs MYC expression at levels that maximally support proliferation, but not yet prime cells for apoptosis induction (II, Fig 7J), suggesting a pro-tumorigenic role for UBR5.

Though the importance of MYC thresholds in controlling the balance between proliferation and apoptosis has been demonstrated in several previous studies (Pelengaris, Khan et al. 2002; Murphy, Junttila et al. 2008), endogenous mechanisms controlling MYC protein expression thresholds both in physiological and pathological conditions remain to be elucidated. Our study here demonstrates that UBR5 can control MYC protein levels, and tresholds for MYC-induced growth and apoptosis.

6.6 Relevance of UBR5 and MYC in breast cancer (II)

Amplification of *ubr5* and *myc* genes was found in different cancer types (Clancy, Henderson et al. 2003; Meyer and Penn 2008). Our results indicate *ubr5* amplification as a novel co-dependency for *myc* amplification in human solid cancers. Based on amplification frequencies of *ubr5* and *myc* in four of the studied MYC-dependent cancer types, it is clear that there is no selective pressure to amplify *ubr5* alone in any of these cancers. In addition, *myc* amplification without *ubr5* amplification is a rare event in breast and ovarian cancers; and the *myc*/*ubr5* co-amplification frequency correlates with essentiality of UBR5 in breast cancer cells (II, Fig.7A). On the other hand, lack of *ubr5* amplifications in lymphoid cancers could be explained by a prevalent function of FBW7 in lymphomas (Reavie, Buckley et al. 2013), consistently with our results that FBW7 and UBR5 function independently as MYC ubiquitin ligases.

Results in my study may help in understanding the functional relevance of MYC protein expression for tumor heterogeneity. In our conclusion, we think that most of the individual cancer cells in breast tumor tissues harbor MYC levels that can efficiently support their proliferation (II, Figs. 4A, 4D, 4G, 6D, 6F, 6G), but do not yet prime them to apoptosis induction by drugs that preferentially kill high MYC expressing cancer cells (Arango, Mariadason et al. 2003; Topham, Tighe et al. 2015).

It is possible that the intra-tumoral heterogeneity in MYC protein expression at single cell level translates to heterogenic therapy responses. In support of this, we show that increased MYC positivity in a cancer cell population correlates with degree of cell killing by several drugs. Based on our data, we speculate that UBR5 inhibition could synchronize the tumor cells to more uniformly express MYC protein at levels that would sensitize cells to cancer drugs.

7. SUMMARY

Post-translational modifications of MYC play important role in regulating MYC activity. Prior to this study, we found CIP2A regulated MYC phosphorylation at S62 and stabilized MYC in cultured cells. However, the *in vivo* effect of CIP2A-mediated phosphorylation of MYC remained elusive. The work presented here demonstrates that CIP2A supports the expression of MYC that is phosphorylated at S62 to initiate proliferation during intestinal generation in a mouse model in response to DNA damage. Furthermore, we found that recruitment of pS62MYC to LAS by CIP2A is required during this process.

In this thesis work, we further identified UBR5 as a novel E3 ligase for MYC. UBR5 promotes MYC ubiquitination, leading to proteasomal degradation of MYC. For functional effect, UBR5 governs the threshold of MYC-induced growth and apoptosis. The results also give an explanation for the controversial role of UBR5 as an oncogene and a tumor suppressor.

Altogether, my thesis work shows the significance of post-translational modifications of MYC in the regulation MYC activity.

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