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# CHARACTERIZATION OF F-BOX PROTEINS AND THEIR TARGET SUBSTRATES IN CANCER

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# CHARACTERIZATION OF F-BOX PROTEINS AND THEIR TARGET SUBSTRATES IN CANCER THESIS FOR DOCTORAL DEGREE (Ph.D.)

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*To my loved ones in Singapore*



## ABSTRACT

Protein degradation, by means of ubiquitylation tagging for subsequent degradation by the ubiquitin-proteasome system (UPS), has opened up a newfound way of protein degradation some three to four decades back. Termed the ‘kiss of death’, this field of study has since sparked off the quest for substrates for the main enzymes executing ubiquitylation, the E3 ligases. Ubiquitylation of proteins have been implicated in a wide variety of biological processes, many of which whose dysregulation lead to tumorigenesis. One major subgroup, the SCF-type of E3 ligases, utilizes a variable component, an F-box protein, for substrate recognition. However, with more than 70 F-box proteins in our genome, most of them poorly characterized, it remains a challenge to unravel the biological significance of each of these proteins. In this thesis, we seek to expand the understanding of two of such SCF-type E3 ligases, namely, Fbw7 and FBXO28 and their substrates in processes such as cyclin E regulation by Fbw7, MYC-mediated transcription and tumorigenesis by FBXO28 and cell motility with the focus on  $\beta$ PIX as a substrate of FBXO28.

Previous work has demonstrated that the SCF(Fbw7/Cdc4) complex is responsible for the ubiquitin-dependent degradation of cyclin E1. In the first study (Paper I), we show that a cooperation between Fbw7 $\alpha$  and Fbw7 $\gamma$  is required for driving ubiquitylation and degradation of cyclin E1 in the nucleolus. Specifically, we show that Fbw7 $\alpha$  acts as a cofactor for Pin1 and aids in isomerization of the cyclin E1 phosphodegron and subsequent translocation and targeting of cyclin E1 for degradation in the nucleolus by Fbw7 $\gamma$ .

In the two other studies, we investigate the function of FBXO28. In **Paper II**, we identify a previously uncharacterized cell cycle-regulated F-box protein, FBXO28, and explore its role in cancer. We show that the CDK1/2 phosphorylated FBXO28 protein assembles a SCF<sup>FBXO28</sup> ubiquitin ligase that targets MYC for non-proteolytic ubiquitylation and demonstrate that this is important for MYC-driven transcriptional activity. Furthermore, expression of a non-functional FBXO28 mutant or silencing FBXO28 leads to impairment in MYC-driven transcriptional activity, transformation and tumorigenesis. Lastly, we show that high FBXO28 expression and phosphorylation are indicators for poor prognosis in breast cancer. In **Paper III** we find that FBXO28 is able to interact with a group of proteins, the PAK1- $\beta$ PIX-GIT1 complex, that are key players in cell migration. FBXO28 is found to localize to the cell-matrix complex upon treatment with EGF and ubiquitylates  $\beta$ PIX in a non-proteolytic but phosphorylation-dependent manner. Additionally, we show that FBXO28 positively regulates the formation of PAK1- $\beta$ PIX-GIT1 complexes, and a depletion of FBXO28 leads to an impairment in cell migration and invasion of metastatic cancer cells. Furthermore, we demonstrate a poor prognosis for breast cancer patients with membranous staining of FBXO28.

## LIST OF SCIENTIFIC PAPERS

I. Nimesh Bhaskaran, Frank van Drogen, **Hwee-Fang Ng**, Raman Kumar, Susanna Ekholm-Reed, Matthias Peter, Olle Sangfelt, Steven I. Reed. Fbw7 $\alpha$  and Fbw7 $\gamma$  Collaborate to shuttle Cyclin E1 into the Nucleolus for Multiubiquitylation. Mol. Cell. Biol, 2013, Jan;33(1), 85-97.

II. Diana Cepeda, **Hwee-Fang Ng\***, Hamid Reza Sharifi\*, Salah Mahmoudi\*, Vanessa Soto Cerrato, Erik Fredlund\*\*, Kristina Magnusson\*\*, Hele'n Nilsson\*\*, Alena Malyukova, Juha Rantala, Daniel Klevebring, Francesc Vin~als, Nimesh Bhaskaran, Siti Mariam Zakaria, Aldwin Suryo Rahmanto, Stefan Grotegut, Michael Lund Nielsen, Cristina Al-Khalili Szigyarto, Dahui Sun, Mikael Lerner, Sanjay Navani, Martin Widschwendter, Mathias Uhle'n, Karin Jirstr~om, Fredrik Ponte'n, James Wohlschlegel, Dan Grande'r, Charles Spruck, Lars-Gunnar Larsson, Olle Sangfelt. CDK-mediated activation of the SCF<sup>FBXO28</sup> ubiquitin ligase promotes MYC-driven transcription and tumourigenesis and predicts poor survival in breast cancer. EMBO Mol Med, 2013,5, 1067-1086.

\*Equal contribution

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III. **Hwee-Fang Ng**, Malyukova A, Bhaskaran N, Magnusson K, Hamdah Shafgat Abbasi, Al-Khalili Szigyarto C, Wohlschlegel J, Str~omblad S, Grand~er D, Manser E, Sangfelt O. FBXO28 is an SCF-type ubiquitin ligase that regulates cell motility by targeting  $\beta$ PIX for ubiquitylation. 2014. Manuscript.



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## LIST OF ABBREVIATIONS

APC/C	Anaphase-promoting complex or cyclosome
$\beta$ PIX	PAK-interacting exchange factor
bHLH-LZ	Basic helix-loop-helix leucine zipper
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
CHX	Cycloheximide
CK1 $\alpha$	Casein kinase-1 $\alpha$
CP	Core particle
CPD	Cdc4 phosphodegron
CSMA	Cell Spot Microarray
DUBs	Deubiquitylation enzymes
EGF	Epidermal growth factor
GIT1	G protein-coupled receptor kinase interacting ArfGAP 1
GSK3	Glycogen synthase kinase 3
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GTP	Guanosine-5'-triphosphate
HAT	Histone acetyltransferase
HGF	Hepatocyte growth factor
I $\kappa$ B kinase	IkappaB kinase
<i>In situ</i> PLA	<i>In situ</i> proximity ligation assay
MBI	MYC Box I
MBII	MYC Box II
MCL-1	Myeloid cell leukemia 1
MEF	Mouse embryonic fibroblasts
MudPIT	Multidimensional protein identification technology
NF- $\kappa$ B	Nuclear factor- $\kappa$ B

NPM	Nucleophosmin
PAK1	p21 protein (Cdc42/Rac)-activated kinase 1
PGC-1	Peroxisome proliferator-activated receptor $\gamma$ coactivator
Q-ChIP	Quantitative chromatin immunoprecipitation
RAC1	Ras-related C3 botulinum toxin substrate 1
RAC3	Ras-related C3 botulinum toxin substrate 3
RACGEF	Rac- guanine nucleotide exchange factor
RhoA	Ras homolog family member A
RING	Really Interesting New Gene
RP	Regulatory particle
SCF	Skp1/Cul1/F-box
TAD	Transcription activation domain
TMA	Tissue microarray
TRPC4AP	Transient receptor potential cation channel, subfamily C, member 4 associated protein
UPS	Ubiquitin Proteasome System
WT	Wild-type
$\Delta$ F	F-box deleted mutant



# 1 INTRODUCTION

## 1.1 DEATH OF A PROTEIN BY UBIQUITYLATION, OR *MAYBE NOT?*

### 1.1.1 Ubiquitin-Proteasomal System (UPS) and its biological significance

Before considering the Ubiquitin-Proteasome System (UPS) and its biological significance, we shall look at the composition of this system; Ubiquitin, the E1, E2 and E3 enzymes and the 26S proteasome.

#### **Ubiquitin**

Ubiquitin is a ~8.5-kDa globular protein of 76 amino acids and was discovered in the 1970's by Goldstein [1]. Ubiquitin is extremely well conserved and exists ubiquitously in the cells signifying its important biological function [2]. Ubiquitin was later found to be an essential component of the proteolytic machinery also known as ubiquitin proteasome system (UPS) [3]. Ubiquitin can be conjugated through an isopeptide linkage to other proteins, including ubiquitin itself [4].

#### **E1, E2 and E3s**

The UPS can be viewed as an ATP driven enzymatic cascade involving three types of enzymes; E1, E2s, and E3s [5, 6]. In the first step, the E1 enzyme forms a thioester bond with ubiquitin (Ub) in an ATP-dependent manner [7]. In the second step, the activated Ub is transferred to an E2-conjugating enzyme. In the final step, the ubiquitin-charged E2 enzyme catalyzes the covalent attachment of Ub onto specific lysine residues in the target proteins, which are primarily recruited by the E3 ubiquitin ligases [4]. A polyubiquitylated protein is eventually degraded once recognized by the 26S proteasome [8] (**Figure 1**).

#### **The Proteasome**

The 26S proteasome is a large ~2.5 MDa, multisubunit proteolytic complex responsible for degradation of polyubiquitylated proteins into short peptides [9, 10]. It is composed of a barrel-shaped 20S catalytic core particle (CP) and two 19S regulatory particles (RPs), facing each end of the CP. Polyubiquitylated proteins are first recognized by ubiquitin-binding proteins in the 19S RP and subsequently unfolded by associated ATPases followed by translocation into the central cavity of the catalytic CP where the protein substrate is destroyed through cleavage of its peptide bonds [4, 8, 11, 12]. Ubiquitin is released and recycled for additional rounds of ubiquitylation by the UPS.

## **Ubiquitylation of proteins for degradation (*or maybe not*)**

The discovery of the UPS by Ciechanover, Hershko and Rose was awarded the Nobel prize in chemistry 2004 [3]. For a long time, degradation of proteins was thought to be predominantly a lysosomal-dependent process [4]. It was only many years later that a cell-free system (that allowed researchers to address non-lysosomal protein degradation) was found to recapitulate protein degradation [13, 14]. This discovery led to a new era of investigations of protein degradation in a non-lysosomal manner known today as ubiquitin dependent degradation. Ubiquitins can be cleaved from the substrates via its C-terminal glycine-76 by a group of enzymes termed deubiquitylation enzymes (DUBs) [15], thus making ubiquitylation a reversible process.

Ubiquitylation as a non-proteolytic posttranslational modification was later found to regulate many other biological processes, as studied in **Paper II** and **Paper III**. For example, non-proteolytic ubiquitin modifications regulate protein-protein interaction and therefore various biological processes such as trafficking, transcription, DNA repair, cell survival and migration, among others (**Figure 1**).

### **Variable fates of a protein *chained* to the ubiquitin depending on chain type**

With seven lysines (K6, K11, K27, K29, K33, K48, and K63) in a ubiquitin polypeptide [16], a diversity of ubiquitin chain types can be formed depending on which lysine is conjugated to the C-terminal glycine (Gly76) residue of ubiquitin. This gives the ubiquitin versatility in its role as a post-translational modification [17-19]. A number of studies suggest that whereas Lys48-linked polyubiquitin chains target proteins for proteasomal degradation, modifications with single ubiquitin (mono-Ub) or with polyubiquitin chains linked through other lysines in ubiquitin (e.g. Lys63) exert non-proteolytic functions. In addition, proteins can be modified on multiple lysines residues in the substrate resulting in multiubiquitylation [20-22].

Thus, whereas polyubiquitylation through K48 of ubiquitin will likely give rise to proteasomal degradation events, K63-linked ubiquitin chains often results in signaling and endocytosis [23]. Monoubiquitylation, on the other hand, has been described to be used in chromatin remodelling, DNA repair, viral budding, or gene expression [23, 24].

## **Cancer: When E3 ligases miscue**

Cancer is a group of diseases developing in a multistep progression manner, ultimately leading to dysregulation of several processes, thus causing cancer cells to have novel capabilities termed ‘hallmarks of cancers’; sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, tumor promoting inflammation, resisting cell death, enabling replicative immortality, inducing angiogenesis, genome instability and mutation, dysregulating cellular energetics, and activating invasion and metastasis [25]. With a delicate balance of protein synthesis and degradation governed by the UPS, it is of little wonder that the very same biological machinery, when dysregulated, has been implicated to play a significant role for tumor development and progression [22, 26]. Two major groups of genes, the so-called tumor suppressor genes (TSGs) and oncogenes [27], are particularly important for cancerogenesis. In this thesis, I have studied one SCF-type ubiquitin ligase with a well-established oncoprotein suppressor function, namely SCF<sup>Fbw7</sup>. I have also identified and functionally characterized a novel SCF ubiquitin ligase, SCF<sup>FBXO28</sup>, with a potential role in supporting oncogenesis.

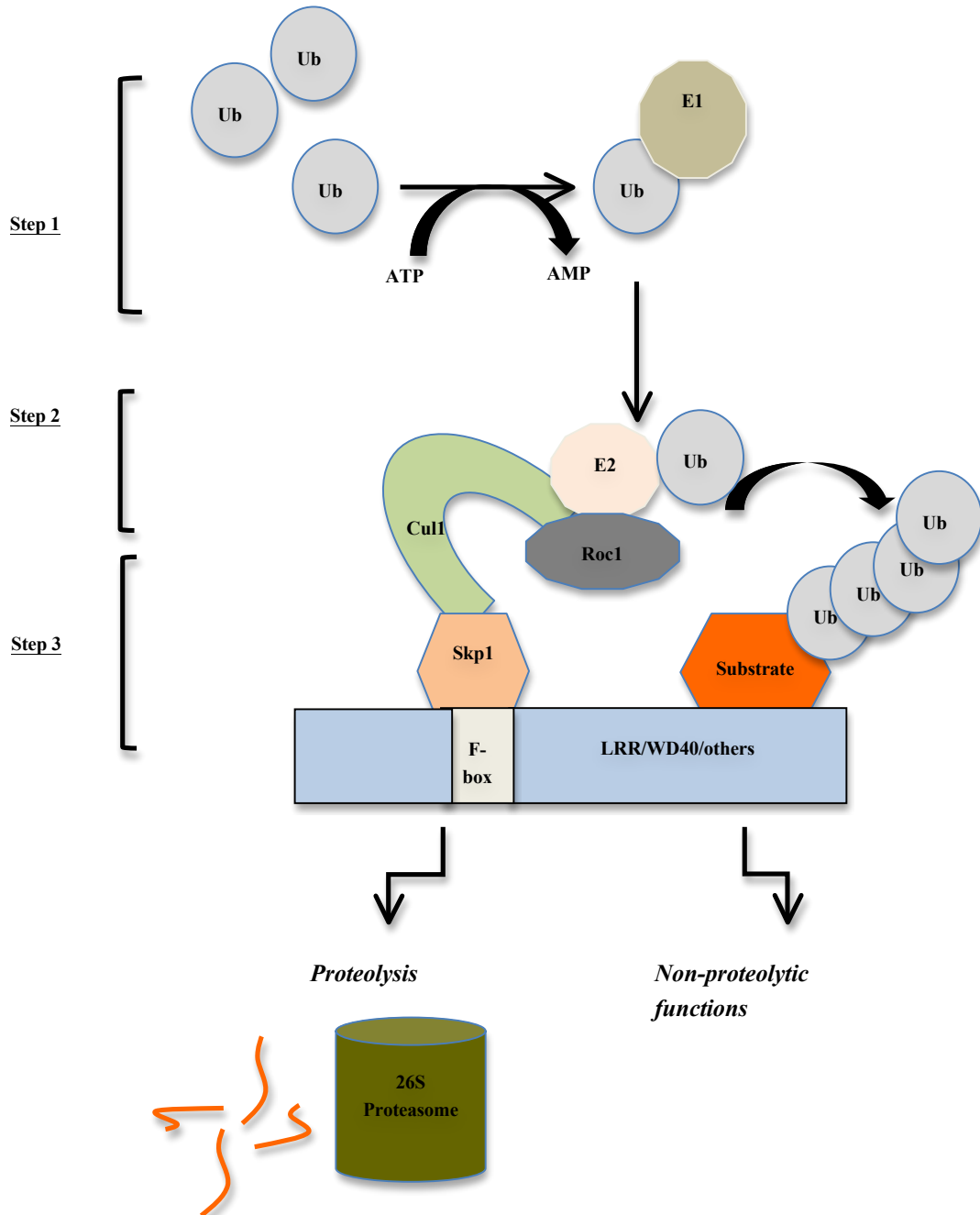
### **1.1.2 Skp1/Cul1/F-box (SCF)-type E3 ligases**

#### **SCF E3 ligases**

Among the 600 identified ubiquitin ligases, a major class is the Skp1-Cullin-F-box (SCF) complex [28-30]. SCF ligases are multi-subunit E3s belonging to the RING-finger-type (**R**eally **I**nteresting **N**ew **G**ene) family that binds the RING-domain-containing protein Roc1, through its scaffold protein Cullin1 [31]. The F-box protein is the variable component of the SCF complex and acts as an adaptor by linking the target substrate to the SCF core ligase via Skp1 (Skp1-Cul1-Roc1). More than 70 different F-box proteins have been identified in humans, but only a few SCF complexes and their specific target substrates have been well characterized to date such as SCF<sup>SKP2</sup>, SCF<sup>FBXW7</sup>, SCF<sup>BTRCP</sup> [32-39]. F-box proteins contain additional protein-protein interacting motifs, including leucine-rich repeats (LRRs) or WD40 repeats, and are named accordingly, FBXL and FBXW, respectively. There are also F-box proteins which contain other domains, including zinc fingers, cyclin domains, leucine zippers, ring fingers, tetratricopeptide (TPR) repeats, and proline-rich regions, or F-box proteins without any known domains. These latter F-box proteins are named FBXO’s [40]. F-box proteins are best known for

their function as key regulators of the cell cycle and for their role in tumor development.

In this thesis, we continue to further our understanding of the role of Fbw7 and in ubiquitylation of its substrate cyclin E (**Paper I**) and also unravel new functions of another SCF-type E3 ligase; FBXO28 in the regulation of MYC and  $\beta$ PIX (**Paper II and Paper III**).



**Figure 1. The Ubiquitin-Proteasome System and SCF-type E3 ligases.** The ubiquitin cycle of protein degradation is a three-step enzymatic cascade involving ubiquitin activating-enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-ligase (E3). Substrate recognition and attachment of poly-ubiquitin chains onto the substrate by the E3 ligases can lead to degradation by the 26S proteasome. Ubiquitylation of proteins has also been shown to have non-proteolytic consequences.



## 1.2 THERE IS A TIME FOR EVERYTHING: SCF-TYPE E3 LIGASES AND THEIR BIOLOGICAL REGULATORY ROLES

### 1.2.1 Regulation of the cell cycle

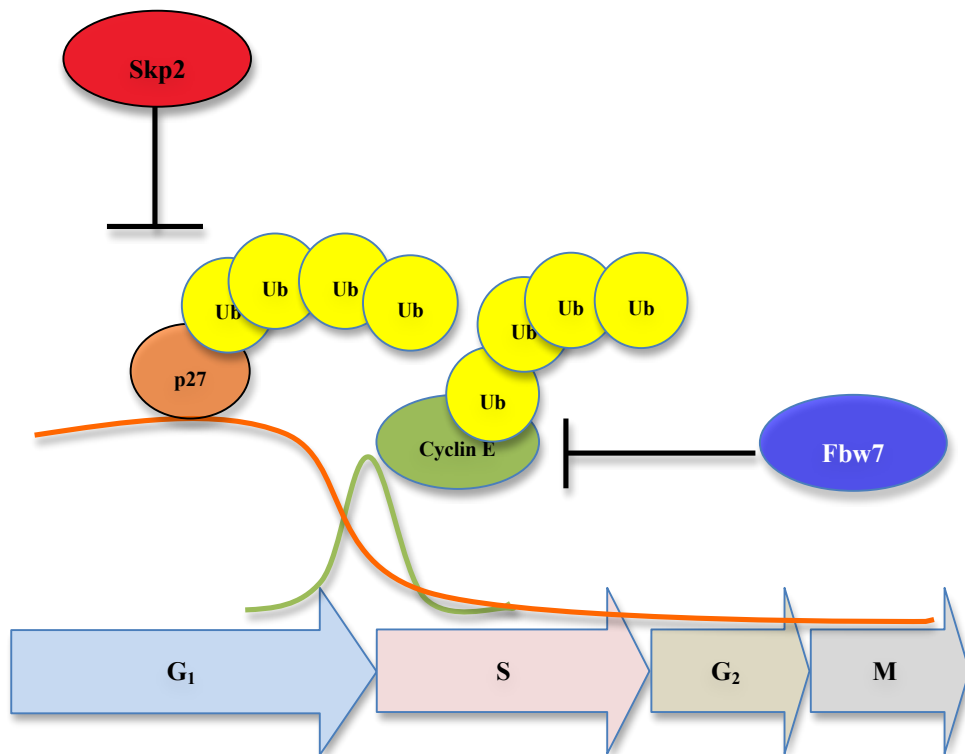
The cell division cycle is a tightly regulated process, with activation and deactivation of proteins in a timely fashion [41]. Cyclin-dependent kinases (CDKs) are responsible for driving the cell cycle forward by phosphorylation of regulatory proteins at different stages of the cell cycle. Notably, protein phosphorylation and ubiquitylation are tightly interconnected processes employed by the cells to govern the intricate balances of activities of cell cycle-regulated proteins. The Cullin-dependent ubiquitin ligases, including the SCF and the anaphase-promoting complex or cyclosome (APC/C), are master regulators of the cell cycle, enforcing the irreversible movement through the cycle by targeting a multitude of phosphorylated proteins (e.g. cyclins) for degradation [41-44].

CDK activity is negatively regulated by cyclin-dependent kinases inhibitors (CKIs) [45, 46]. SCF ubiquitin ligases have been demonstrated to eliminate CKIs through ubiquitin-dependent proteolysis [42]. In particular, three SCF-type E3 ligases have been described for their crucial function in regulating the cell cycle, including Skp2,  $\beta$ -TrCP and Fbw7 [32, 39, 42, 47]. Skp2 promotes the cell cycle by targeting several CKIs for proteasomal degradation, including p21, p27 and p57 [32, 42] (**Figure 2**).  $\beta$ -TrCP targets both positive and negative cell cycle regulatory proteins, for example Emi1/2, Wee1 and Cdc25A/B among others [42].

Fbw7 exists as 3 different splice variants encoding different protein isoforms, each with different subcellular compartmentalization, with Fbw7 $\alpha$  being nucleoplasmic, Fbw7 $\gamma$  nucleolar [48, 49], while Fbw7 $\beta$  resides in the cytoplasm and has been described to localize to membranes [50]. The three different isoforms share a common C-terminal region and only differ in their N-terminal 5'-exons [48]. The common C-terminal region contains the different functional domains, including the F-box domain (Skp1 interacting motif) [31], dimerization domain (D-domain) [51], and substrate binding domain (WD40 repeats) [52]. FBW7 substrate recognition occurs through the interaction of key residues on the  $\beta$ -propeller surface formed by the eight WD40 repeats of FBW7 and a phosphodegron motif in the substrate called Cdc4 PhosphoDegron (CPD). Fbw7 has been shown to target > 20 different proteins for degradation [37] and the vast majority of targets described to date contain a CPD sequence [52, 53] as defined by;  $\phi$ -X- $\phi$ - $\phi$ - $\phi$ -pT/pS-P-P-X-pS/pT/E, where  $\phi$  corresponds to a hydrophobic residue, and X as any amino acid residue [54]. The number of

phosphodegrons present in the substrate, as well as the sequence composition seem to be a determining factor for their recognition by Fbw7 [41]. In line with its function as a master oncoprotein repressor, inactivation of FBW7 through mutations in the substrate-binding pocket of FBW7 leads to accumulation of oncoproteins and tumorigenesis [55]. Well characterized substrates of Fbw7 include critical oncoproteins such as cyclin E [33-35, 55, 56], c-Myc [49, 57, 58] and Notch1 [59, 60]. More recently identified substrates of Fbw7 include SREBP [61], PGC-1 [62], Mcl-1 [63, 64], and NF- $\kappa$ B [65-67].

Cyclin E protein, the regulatory subunit of the cyclin E-CDK2 complex, peaks at the G<sub>1</sub>-S phase and declines rapidly during early S-phase through transcriptional and proteolytic events [68]. Fbw7 has been well studied for its role in tagging cyclin E for degradation at the G<sub>1</sub>-S boundary [33, 35] (**Figure 2**). Cyclin E contains two Cdc4 phosphodegrons; one at its N-terminus (pThr62) and the other at its C-terminus (pThr380) [69-72]. The C-terminal degron motif (Thr380), perfectly conform to the consensus CPD sequence and is phosphorylated on Thr380 and Ser384 by Cdk2 and glycogen synthase kinase (GSK3), generating a high-affinity phosphodegron [70]. Interaction between Fbw7 and cyclin E predominantly occur via pThr380, which can then form hydrogen bonds with several Arg residues (Arg465, Arg479, and Arg505) embedded within the binding pocket of the  $\beta$ -propeller structure in Fbw7 [33, 52]. These arginines in Fbw7 represent mutational hotspots in human cancers [48] but other cancer-related mutations have also been identified, including an N-terminal Fbw7 $\alpha$  specific mutation (D124Y) [48, 73] associated with defective turnover of cyclin E. The N-terminus of Fbw7 $\alpha$  in association with Pin1 presumably aids in the isomerization of phosphorylated cyclin E, priming it for subsequent recognition and ubiquitylation by Fbw7 $\gamma$  [73, 74]. Interestingly, the D124Y-Fbw7 $\alpha$  mutation is incapable of interacting with cyclin E-Pin1, thus supporting a function for Pin1-Fbw7 $\alpha$  mediated degradation of cyclin E in human cancer [73, 74].



**Figure 2.** Schematic model depicting an oncogene, *Skp2* degrades a CDK inhibitor (CKI), *p27*, while tumor suppressor, *Fbw7* targets *Cyclin E* for degradation at the *G<sub>1</sub>-S* phase of the cell cycle. *G<sub>1</sub>*, *S*, *G<sub>2</sub>*, *M* represents the different phases of the cell cycle; *G<sub>1</sub>*-phase, *S*-phase, *G<sub>2</sub>*-phase, and Mitotic phase.

In **Paper I**, we add on to the growing knowledge of the role *Fbw7* with regard to how cyclin E degradation is mediated by the cooperation between its two isoforms *Fbw7 $\alpha$*  and *Fbw7 $\gamma$* . Studies from our group further discovered *FBXO28* to be a cell cycle regulated SCF E3 ligase whose phosphorylation by the cyclin-CDK complexes peaked towards the late S-G<sub>2</sub>/M phase [75]. As outlined in **Paper II**, phosphorylation of *FBXO28* regulates SCF ligase function and promotes non-proteolytic polyubiquitylation of *MYC* to enhance its transcription activity and oncogenic capability [75]. In **Paper III**, we identify a new *FBXO28* target substrate,  $\beta$ *PIX*, and describe a role for *FBXO28* in regulation of cell motility.

## 1.2.2 Regulation of transcription

The activity and/or levels of transcription factors needs to be tightly regulated in response to extracellular cues and intracellular signaling pathways. Many oncogenes and TSGs encode transcription factors. The p53 gene is the most frequently mutated gene in human cancer and has been described as the ‘guardian of the genome’ [76]. The p53 protein binds specific DNA sequences and act as an important transcription factor in response to DNA damage and other stress signals [76]. Ubiquitylation mediated degradation of this transcription factor is one way by which the cells keep p53 activity and level in check [77]. In a response to cellular stress such as DNA damage, the p53 is activated and polyubiquitylation by E3 ligases such as Mdm2 is inhibited, thus increasing p53 levels [77-81]. In an unstressed condition, the p53 protein is instead continuously polyubiquitylated and thus maintained at a low level in cells. p53 is ubiquitylated by several other E3s [82], for instance by the SCF-type E3 ligase  $\beta$ TrCP (also known as FBXW1) which is able to degrade p53 in response to phosphorylation by I $\kappa$ B kinase 2 (I $\kappa$ B kinase) [83].

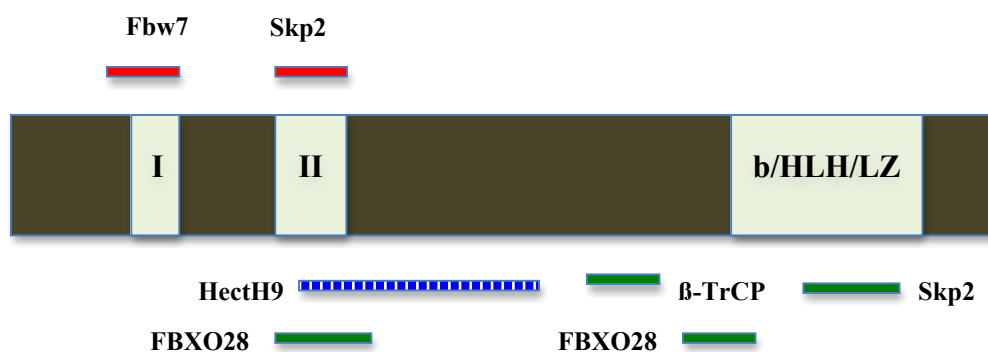
Another important transcription factor is encoded by the proto-oncogene, *c-MYC*, first identified as a homologue to the *v-myc* of the avian myelocytomatosis retrovirus [84]. MYC regulates many different biological processes and function as a master regulator of gene expression in cells and can both activate and repress transcription [85]. MYC contains a transcription activation domain (TAD) as well as a DNA binding domain. In the amino-terminal of MYC, the TAD encompasses conserved ‘MYC’ boxes (MB), MBI and MBII, which are essential for transactivation of genes [86]. The carboxy-terminus of MYC contain the basic-helix-loop-helix-zipper (bHLHZ) domain which is critical for binding to its partner, the MAX protein, creating MYC-MAX heterodimers responsible for binding DNA sequences such as the E-box sequence CACGTG [87].

Over the years, several E3 ligases have been identified to have a role in activating or inhibiting MYC function [77, 88] (**Figure 3**). For example, Skp2 can interact with MYC through its MBII region and the HLH/LZ region to regulate its stability via degradation, but also promote MYC transcriptional activity [77, 88-90]. The HectH9 (Huwe1/Mule) E3 ubiquitin ligase, triggering K63 polyubiquitylating of MYC, was reported to promote activation of MYC target genes without stimulating its degradation [91]. However, HectH9/Huwe1 has also been shown to promote MYC degradation (both N-MYC and c-

MYC) and induce neural differentiation and proliferation arrest in other studies [92] (**Figure 3**).

Other E3 ligases including Fbw7 and TRUSS (TRPC4AP), negatively regulates MYC protein stability through K48-type polyubiquitylation and proteasomal degradation [49, 57, 58, 77, 88, 93]. Fbw7 polyubiquitylates MYC in response to sequential phosphorylation of MYC's CPD. ERK or CDKs first phosphorylate MYC on serine 62 (S62), which primes for GSK3 $\beta$ -mediated phosphorylation on threonine 58 (T58). However, S62 has also been reported to be dephosphorylated through the combined actions of the PIN1 prolyl isomerase and the PP2A phosphatase before it can be targeted by Fbw7 [94]. In addition,  $\beta$ TrCP was recently reported to positively regulate MYC protein stability by antagonizing Fbw7-mediated induction of proteasomal degradation [95].

In addition to the aforementioned E3 ligases identified for their roles in ubiquitylation of MYC, we recently identified a new F-box protein, FBXO28, that mediates non-proteolytic ubiquitylation of MYC [75] (reported in **Paper II**) (**Figure 3**).



**Figure 3. E3 ligases and their regulation of MYC.** Schematic diagram of MYC with its MYC box I (MBI), MYC box II (MBII), and basic-helix-loop-helix-leucine zipper (b/HLH/LZ) domains. Red bar indicates a downregulation of MYC stability; green bars indicate that MYC activity is positively regulated; dashed bar dictates where MYC activity can be either up-regulated or down-regulated.

### 1.2.3 Regulation of cell motility

As mentioned above, the ubiquitin system has well established functions in biological processes such as cell cycle regulation and transcription. In the recent years, ubiquitylation has also emerged as an important posttranslational modification in yet another realm of biology; namely cell adhesion, cell migration and cytoskeletal remodelling [96, 97].

RhoA, Rac and Cdc42 are small GTPases that are important molecular switches (cycling between an active GTP-bound form, and an inactive GDP-bound form) in the cellular system known primarily for cell migration purposes [98]. These Rho GTPases were also found to influence other biological processes such as cell cycle regulation, cytoskeletal remodeling as well as transcriptional factor activity [99, 100]. The activity of the active GTP-bound or inactive GDP-bound forms of the Rho GTPases is regulated by a concerted effort of GTPase activating proteins (GAPs), guanine nucleotide exchange factors (GEFs), and guanine nucleotide dissociation inhibitors (GDIs) [98, 101].

Recently, an SCF-type E3 ligase, FBXL19, has been reported for its role in ubiquitylating Rho GTPases family members, such as Rac1 [102], Rac3 [103], and RhoA [104]. F-box proteins are known to associate with phosphorylated substrates [105] and FBXL19 was also shown to polyubiquitylate and degrade AKT phosphorylated Rac1. Overexpression of FBXL19 in mouse lung epithelial negatively impeded cell migration, possibly by down-regulating Rac1 [102]. FBXL19 was further shown to target phosphorylated RhoA (mediated by Erk2) [104]. Interestingly, FBXL19 displayed roles in both negatively affecting cell proliferation as well as cytoskeletal rearrangement (reduced stress fiber formation) by targeting RhoA for polyubiquitylation and degradation [104]. More recently, FBXL19 was demonstrated to interact with and degrade Rac3 [103].

The list of E3 ligases regulating cell migration has been expanding in recent years. For instance, the HECT-type E3 ligase, Smurf1 (SMAD specific type E3 ligase), is capable of degrading active GTP-bound RhoA [106] and BACURD (a cullin3-type E3 ligase) targets the inactive GDP-bound RhoA for degradation [107]. The SCF-type E3 ligases and their involvement in cell migration has not been extensively explored and we know little regarding the ubiquitylation status of the GEFs or GAPs proteins and how ubiquitylation of these proteins regulate Rho GTPase activity.

The SCF-type E3 ligase,  $\beta$ TrCP, regulates cell migration and invasion by targeting the Rap guanine exchange factor, RAPGEF2, for degradation [108]. Upon stimulation by a potent metastatic factor such as the hepatocyte growth factor (HGF) and phosphorylation mediated by the I-Kappa-B-Kinase- $\beta$  (I $\kappa$ B kinase) and casein kinase-1 $\alpha$  (CK1 $\alpha$ ), RAPGEF2 (phosphorylated on serine 1254) is recognized by  $\beta$ TrCP and subsequently ubiquitylated and degraded [108]. Importantly, abrogated degradation of RAPGEF2 leads to inhibition of epithelial cell migration and metastasis in breast cancer cells [108].

The guanine exchange factor,  $\beta$ PIX (Pak-interacting exchange factor), has been described as a binding partner of both PAK1 and GIT1 and is involved in regulation of cell shape maintenance as well as cell migration.  $\beta$ PIX acts as a GEF for the Rho GTPases; Rac1 and Cdc42 [109-111] and has shown to regulate cell motility by effects on focal adhesion maturation and disassembly [112, 113]. The role of other post-translational modifications, including protein ubiquitylation, in regulation of  $\beta$ PIX activity and cell motility is poorly understood. In **Paper III**, we identify a new FBXO28 target substrate,  $\beta$ PIX, and describe a role for FBXO28 in regulation of cell motility.

### 1.3 FINE-TUNING TAILORED CANCER TREATMENT WITH E3 LIGASES

Although E3 ligases are highly selective substrate recognition factors, they can also give rise to wide-ranging biological responses since each E3 can potentially target many different substrates involved in diverse processes. In addition, with the combination of different E2-E3 enzymes and different types of ubiquitin chains formed, one can only imagine the almost endless possibilities and resultant biological responses when this system is targeted. For instance,  $\beta$ TrCP is an E3 ligase that can target both I $\kappa$ B $\alpha$  and  $\beta$ -catenin for degradation [32]. It may seem a promising strategy to target  $\beta$ TrCP to prevent degradation of I $\kappa$ B $\alpha$  in cancer cells, thereby negatively regulating the NF- $\kappa$ B signaling pathway. On the other hand, since  $\beta$ -catenin is also a substrate for  $\beta$ TrCP, inhibiting the action of  $\beta$ TrCP in tumor cells could potentially increase the level of  $\beta$ -catenin, which will in turn be advantageous for the progress of tumorigenesis [114, 115].

Some E3 ligases such Fbw7, comes in different isoforms, thus adding yet another level to the complexity of tailored treatment. Perhaps having different isoforms could allow targeting a distinct protein isoform that is critical for disease progression. As mentioned, Fbw7 is mutated in many types of cancers and targets several potent oncoproteins such as cyclin E, c-Myc and Notch [33-35, 49, 55-57, 59, 60]. It would seem ideal then to reactive Fbw7 in cancer patients with Fbw7 inactivation. However, restoring TSG function is not a simple task. In addition, Fbw7 also targets antiapoptotic proteins including MCL1 and loss of Fbw7 contributes to drug resistance to compounds such as taxol and ABT-737 as reported in [63, 64]. On the other hand, loss of Fbw7 in the cancer cells of certain cancer patients can also potentially increase response to specific anticancer drug [65, 116, 117].

Some F-box proteins have been considered as particularly attractive targets for cancer therapy, for example, Skp2, which targets multiple tumor suppressor proteins for degradation [118, 119]. With the many uncharacterized F-boxes out there, what we know of is, however, rather the tip of the iceberg. Considering the many unknown substrates that could be potentially affected, more knowledge is clearly needed to utilize this group of proteins as targets for novel treatments. Is targeting a particular E3 ligase that is found to be dysregulated in cancer really a lifesaver? This is the question for now at least, that remains to be answered until we know more about this group of proteins and their substrates. It is therefore of imminent importance to characterize the complete set of substrates of F-box proteins so that one can have a better understanding of their multifaceted activities and role(s) in cancer development. Such knowledge may also enable the clinicians to make a better prediction on the success of survival rate for a certain group of patients with the availability of biomarkers.



## 2 AIMS

Overall, this thesis seeks to improve our understanding of F-box proteins and their substrates. The primary objective of the work has been to explore the roles that specific F-box proteins have in cancer with regard to the types of substrates they target. Specifically, the aims were to:

- Elucidate how FBW7 $\alpha$  and FBW7 $\gamma$  collaborate to shuttle cyclin E1 into the nucleolus for ubiquitylation.
- Functionally characterize FBXO28 and its role in cancer by targeting MYC for non-proteolytic ubiquitylation.
- Explore a new role of FBXO28 in cancer by analyzing its influence on cell motility by studies of a new target substrate,  $\beta$ PIX that is targeted by FBXO28 for non-proteolytic ubiquitylation.



## 3 RESULTS

### 3.1 PAPER I

#### **FBW7-ALPHA AND FBW7-GAMMA COLLABORATE TO SHUTTLE CYCLIN E1 INTO THE NUCLEOLUS FOR MULTIUBIQUITYLATION.**

Since most known targets of Fbw7 are nucleoplasmic proteins, it is reasonable to assume that these proteins are targeted by the Fbw7 $\alpha$  isoform, SCF<sup>Fbw7 $\alpha$</sup>  [120]. Likewise, the Fbw7 $\gamma$  isoform would be expected to target nucleolar substrates. However, we previously made the surprising observation that efficient polyubiquitylation of cyclin E requires both Fbw7 isoforms [73, 121]. We found that whereas Fbw7 $\alpha$  binds phosphorylated cyclin E, the SCF<sup>Fbw7 $\alpha$</sup>  ligase does not polyubiquitylate it [73]. Instead, the results suggested a role for SCF<sup>Fbw7 $\alpha$</sup> , in conjunction with the prolyl cis-trans isomerase Pin1, to carry out non-canonical isomerization of the proline-proline bond in the primary cyclin E phosphodegron (Thr380), thereby creating a high-affinity interaction with SCF<sup>Fbw7 $\gamma$</sup> , which then triggers cyclin E polyubiquitylation [73]. Accordingly, knockdown of specific Fbw7 isoforms in different tumor-derived cell lines using siRNAs targeting either Fbw7 $\alpha$  or Fbw7 $\gamma$  increased cyclin E levels (and stability), whereas the Fbw7 $\beta$ -specific and control siRNAs had no effect [121]. Together, these results suggest that Fbw7 $\alpha$  and Fbw7 $\gamma$  cooperate in ubiquitin-mediated proteolysis of cyclin E. However, we found that in cell lines overexpressing cyclin E, the requirement for Fbw7 $\gamma$  is relieved and only Fbw7 $\alpha$  was required for cyclin E degradation [121]. This suggests that an alternative pathway for cyclin E ubiquitylation and turnover prevails in cells that overexpress cyclin E. One explanation for this mechanistic discrepancy might be the usage of the second low affinity phosphodegron in cells expressing high cyclin E levels. This low affinity phosphodegron is centered around Thr62, which does not contain a proline-proline bond and therefore would not require isomerization. As Fbw7 $\alpha$  has been shown to target phosphodegrons that do not have proline-proline bonds, this could bypass the requirement for isomerization and allow ubiquitylation of cyclin E by Fbw7 $\alpha$  in the nucleoplasm (possibly through Fbw7 $\alpha$  homodimers which provides another level of substrate regulation [120]).

In paper I, we characterized the cooperative function of Fbw7 isoforms in mediating cyclin E ubiquitylation and degradation in greater detail. Initially, we investigated the levels and subcellular localization of cyclin E in cell lines where cyclin E is not overexpressed, including Saos2, HEK293A, and hTERT-immortalized human mammary

epithelial cells (IME). When such cells were subjected to proteasomal inhibitors, we found that cyclin E accumulates in the nucleolus. The fact that Fbw7 $\gamma$  is required for cyclin E degradation and cyclin E accumulates in the nucleolus when cells are incubated with proteasome inhibitors indicated that one step in the cyclin E degradation pathway may occur in the nucleolus. To address this issue, we depleted either Fbw7 $\alpha$  or Fbw7 $\gamma$  in Saos2 and HEK293A cells and found reduced levels of cyclin E in the nucleolus. This result imply that besides targeting cyclin E for ubiquitylation and proteolysis, both Fbw7 $\alpha$  and Fbw7 $\gamma$  have roles in cyclin E nucleolar localization.

In order to further elucidate the function of Fbw7 $\alpha$  and Fbw7 $\gamma$  in translocating cyclin E to the nucleolus, we expressed F-box deleted ( $\Delta$ F) versions of these proteins in Saos2 cells using a conditional tetracycline-inducible promoter and carried out immunofluorescence microscopy analysis (F-box deleted alleles can bind substrates but not the SCF core and therefore have dominant negative properties). Importantly, we found that whereas expression of  $\Delta$ F-Fbw7 $\alpha$  caused accumulation cyclin E in the nucleoplasm and eliminated most of the nucleolar cyclin E, expression of  $\Delta$ F-Fbw7 $\gamma$  led to accumulation of cyclin E in the nucleolus. Taken together with the results of isoform-specific Fbw7 silencing, these data suggest that SCF<sup>Fbw7 $\alpha$</sup> -dependent cyclin E phosphodegron isomerization and subsequent binding of the isomerized protein to Fbw7 $\gamma$  is required for localization of cyclin E into the nucleolus.

Since SCF<sup>Fbw7 $\gamma$</sup>  ubiquitylates cyclin E (when its not overexpressed), we hypothesized that phosphorylated cyclin E is translocated into the nucleolus and ubiquitylated by Fbw7 $\gamma$ . To test this, we purified nucleolar extracts with or without prior treatment with proteasome inhibitors and analyzed cyclin E ubiquitylation in different subcellular fractions. When compared to nucleolus-depleted extract and whole cell extract, we found that the nucleolar fraction was enriched for high mobility cyclin E species, indicative of polyubiquitylation. We confirmed that polyubiquitylated cyclin E primarily accumulates in the nucleolar fraction in cells transfected with HA-ubiquitin plasmids. Furthermore, immunoblotting with antibodies detecting the phosphorylated residues in the cyclin E phosphodegron verified that the nucleolar pool of cyclin E was indeed phosphorylated. Thus, cyclin E with an activated phosphodegron is nucleolar and much of it is polyubiquitylated. Since depletion of Fbw7 $\alpha$  prevents accumulation of cyclin E in the nucleolus and we previously showed that SCF<sup>Fbw7 $\alpha$</sup>  is a cofactor for Pin1-mediated isomerization of the cyclin E phosphodegron [73], we next tested if isomerization is also required for nucleolar localization. We silenced Pin1 and analyzed the level and localization of cyclin E.

Strikingly, depletion of Pin1 prevented nucleolar accumulation but generally stabilized cyclin E as previously described [73, 74]. As Pin1 isomerization affects the functions of many different proteins, we also analyzed the localization of a phosphodegron mutant of cyclin E (P382I) that can be phosphorylated (on Thr380), but not isomerized by Pin1. Cyclin E (wt and P382I) was tagged with the fluorophore mCherry (to distinguish it from endogenous cyclin E) and analyzed by immunofluorescence microscopy. Interestingly, we found that nucleolar localization of the isomerization-deficient P382I allele was greatly reduced as compared to wild-type cyclin E. This is similar to the T380A phosphodegron-deficient mutant of cyclin E (with a completely inactivated phosphodegron), however, unlike the cyclin E T380A mutant, the P382I mutant can be degraded, most likely through proteasomal degradation in the nucleoplasm by SCF<sup>Fbw7 $\alpha$</sup>  [73]. Indeed, depletion of Fbw7 $\alpha$  restored nucleolar accumulation of P382I-cyclin E, suggesting that this mutant is able to interact with Fbw7 $\gamma$  and localize to the nucleolus if its not intercepted by Fbw7 $\alpha$ . This is in sharp contrast to the T380A mutant, which is unable to localize to the nucleolus with or without silencing of Fbw7 $\alpha$ , further supporting the role of Fbw7 $\gamma$  for nucleolar translocation of cyclin E. We also studied the requirement of NPM in the nucleolar accumulation of cyclin E since NPM has been proposed to be responsible for localization of Fbw7 $\gamma$  to the nucleolar compartment [122]. Using NPM<sup>-/-</sup> mouse embryonic fibroblasts (MEFs), we found that cyclin E nucleolar localization was eliminated as compared to control MEFs. When NPM was restored in the NPM<sup>-/-</sup> MEFs, nucleolar localization of cyclin E was re-established as expected.

Functional sequestration of proteins in the nucleolus has been reported [123-125]. Why is cyclin E translocated to the nucleolus for degradation? One possible reason could be that nucleolar translocation might be a more rapid way to functionally inactivate cyclin E by separating it from CDK2-cyclin E substrates in the nucleoplasm. In an attempt to further characterize the function of cyclin E nucleolar sequestration, we analyzed accumulation of  $\gamma$ H2AX foci and replication rate (as one of the hallmarks of cyclin E overexpression is replication stress) in Saos2 cells following expression of dominant-negative  $\Delta$ F-Fbw7 $\alpha$  or  $\Delta$ F-Fbw7 $\gamma$  alleles, respectively. As these two mutants sequester cyclin E in different nuclear compartments, we assumed that by translocating phosphorylated cyclin E into the nucleolus (with expression of  $\Delta$ F-Fbw7 $\gamma$ ) this should reduce the replicative stress induced by the otherwise elevated levels of cyclin E in the nucleoplasm. Indeed, we found that forced expression of  $\Delta$ F-Fbw7 $\alpha$ , but not  $\Delta$ F-Fbw7 $\gamma$ , resulted in high levels of  $\gamma$ H2AX. Similarly,  $\Delta$ F-Fbw7 $\alpha$ , but not  $\Delta$ F-Fbw7 $\gamma$  expressing cells, exhibited lower rates of

replication. As cells depleted of either Fbw7 $\alpha$  or Fbw7 $\gamma$  exhibited nuclear accumulation of cyclin E, we also examined replicative stress and DNA replication rate following knockdown of each isoform. Depletion of either Fbw7 isoform both led to increased  $\gamma$ H2AX levels and a decrease in DNA replication, indicative of replication stress. Taken together, these results show that Fbw7 $\gamma$ -mediated sequestration of cyclin E into the nucleolus has biological consequences and indicate that separation of cyclin E from its targets in the nucleoplasm abrogates the effects of cyclin E overexpression, at least in terms of markers of replicative stress. Finally, if functional inactivation of cyclin E by means of translocation into the nucleolus should be justified, translocation is expected to occur on a more rapid time scale than ubiquitin-mediated proteolysis. Although we have not directly tested this, we observed that cyclin E localization varies as a function of progression through S phase. We found that as cells progressed from S-phase, cyclin E which had appeared to be evenly distributed in the nucleus initially, began to get depleted from the nucleoplasm and accumulate in the nucleoli instead, along with an overall reduction in cyclin E level [126-128].

Taken together, the results presented in this study support the requirement of both Fbw7 $\alpha$  and Fbw7 $\gamma$  for inactivation of cyclin E by its translocation and subsequent degradation in the nucleolus. Specifically, we have found that cyclin E phosphodegron isomerization by SCF<sup>Fbw7 $\alpha$</sup> -Pin1 potentiates binding to SCF<sup>Fbw7 $\gamma$</sup> , which then causes cyclin E to translocate or localize into the nucleolus where it is ubiquitylated prior to degradation.

## 3.2 PAPER II

### **CDK-MEDIATED ACTIVATION OF THE SCF<sup>FBXO28</sup> UBIQUITIN LIGASE PROMOTES MYC-DRIVEN TRANSCRIPTION AND TUMOURIGENESIS AND PREDICTS POOR SURVIVAL IN BREAST CANCER.**

As mentioned, only a handful of F-box proteins have been well-characterized to date [38]. As uncontrolled cell proliferation is a major hallmark of cancer, we initially attempted to identify F-box genes that could potentially have a role in tumor cell proliferation. Using a high-throughput image-based siRNA screen that entails a complete library of F-box specific siRNAs, each respective F-box gene was silenced and effects on cell proliferation was analyzed based on EdU incorporation using the Cell Spot Microarray platform (CSMA) [129]. Knockdown of the F-box gene, *FBXO28*, significantly reduced cell proliferation in multiple tumor-derived cell lines, and was therefore chosen for further functional characterization.

Using mass spectrometry, we identified a serine phosphorylated FBXO28 peptide (*LREVMESAVGNSSGSGQNEEpSPR*). Bioinformatic analysis of the *FBXO28* protein reveals that the phosphorylated serine (S344) is within a conserved CDK consensus motif (S/T)PX(K/R) in the C-terminal end of FBXO28. We generated an antibody that specifically recognizes phosphorylated serine 344 (pS344-FBXO28) and profiled the expression and phosphorylation of FBXO28 during cell cycle progression. Interestingly, FBXO28 phosphorylation peaked during the S-G2/M phase while appeared to be minimal at early G1 phase. Using an *in vitro* kinase assay with purified recombinant cyclin/CDK complexes, we confirmed that FBXO28 is phosphorylated on S344 by cyclin A-CDK2 and cyclin B-CDK1, but not cyclin E-CDK2. Further analysis demonstrated that phosphorylation of FBXO28 affects FBXO28 stability, with the pool of unphosphorylated protein being more unstable. In line with these data, a phospho-deficient mutant of FBXO28 (S344A-FBXO28) was degraded more rapidly compared to a phosphomimetic mutant of FBXO28 (S344E-FBXO28). Together, these results demonstrate that FBXO28 is a CDK substrate and tightly regulated during cell cycle progression. We also found that FBXO28 assembled an SCF complex independently of phosphorylation status. When the effect of FBXO28 knockdown was assessed on global gene expression by microarray analysis, we found a significant downregulation of genes that are involved in rRNA

processing, ribosome biogenesis, cell cycle and metabolism, an expression profile that mirrors transcriptional processes regulated by the master transcription factor and oncoprotein, MYC [85, 130-132]. Interestingly, we found that the downregulation of most of these genes occurred already after 16 hours knockdown, well before any loss of proliferation. To delve into the possibility that depletion of FBXO28 regulates MYC output as a transcription factor, we deployed Gene Set Enrichment Analysis (GSEA). Indeed, MYC-activated genes were downregulated in response to FBXO28 depletion [133, 134] and downregulation of MYC target genes was confirmed by means of qRT-PCR. We also investigated whether loss of proliferation following FBXO28 depletion depends on MYC by silencing FBXO28 and MYC separately, or together. Importantly, co-depletion of FBXO28 and MYC did not further reduce proliferation, suggesting that MYC and FBXO28 act in the same pathway. We next studied the interaction between these proteins and found that FBXO28 co-immunoprecipitated with MYC. By means of interaction mapping analysis, we were able to conclude that the highly conserved MYC Box II (MBII) and possibly the helix-loop-helix leucine zipper (HLH-LZ) domain of MYC are important regions for the interaction between FBXO28 and MYC. Since FBXO28 interacts with MYC and FBXO28 depletion reduces MYC target gene expression, we performed several experiments to investigate whether FBXO28 ubiquitylated MYC. WT-FBXO28, but not  $\Delta$ F-FBXO28, was found to ubiquitylate MYC both *in vivo* and *in vitro*. In fact, expression of  $\Delta$ F-FBXO28 severely attenuated MYC polyubiquitylation suggesting that the F-box deleted mutant potentially act in a dominant-negative manner (binds without ubiquitylating MYC). Supporting these data, we showed that expression of  $\Delta$ F-FBXO28 specifically impeded ubiquitylation of MYC during S-phase (when FBXO28 is phosphorylated at S344). Strikingly, the phospho-mimetic form of FBXO28, S344E-FBXO28, but not the phospho-deficient S344A-FBXO28, promoted ubiquitylation of MYC. Since ubiquitylation has been studied primarily as a mode of protein degradation in cells, [26, 135, 136], we next examined if MYC ubiquitylation by FBXO28 resulted in MYC degradation. Cycloheximide chase experiments showed that FBXO28 does not alter the MYC protein turnover, indicating a non-proteolytic function of FBXO28. As MYC functions as a master transcription factor [85, 91, 131, 132], we wanted to determine whether ubiquitylation by FBXO28 affected the transcriptional activity of MYC. Indeed, overexpression of  $\Delta$ F-FBXO28 or depletion of FBXO28 was sufficient to reduce a MYC-dependent luciferase reporter activity. Specifically, we demonstrated that WT-FBXO28 enhanced expression of several MYC target genes



whereas  $\Delta$ F-FBXO28 led to a reduction in the activation of these genes in S-phase cells. In particular, we also verified enrichment of FBXO28 and pS344-FBXO28 within E-box regions of several MYC target gene promoters.

The activity of the MYC/MAX transcriptional complex is influenced by the local chromatin structure at target promoters [85, 130-132]. The histone acetyltransferase (HAT) p300 protein has been shown to act as a coactivator of MYC-driven transcription [91, 137, 138]. Interestingly, we found that overexpression of  $\Delta$ F-FBXO28 led to reduced p300 and acetylated histone H4 at MYC target gene promoters. We also found that MYC-MAX binding was not significantly affected by overexpression of  $\Delta$ F-FBXO28, but the interaction between p300 and MYC at target promoters was strongly attenuated. In line with these results, we linked ubiquitylation of MYC by FBXO28 to a region in MYC that has previously been shown to be important for MYC-p300 interaction.

Since FBXO28 regulates MYC activity, we decided to explore the role of FBXO28 in tumorigenesis. Inactivation of FBXO28, either by expression of  $\Delta$ F-FBXO28 or siRNA depletion, reduced colony growth on plastics. We also engineered *P53*<sup>-/-</sup> immortalized mouse embryonic fibroblasts (MEFs) (that have been previously shown to be transformed by MYC [139]) with retroviruses encoding MYC and  $\Delta$ F-FBXO28. The results from these experiments showed that overexpression of  $\Delta$ F-FBXO28 was able to suppress MYC-induced transformation in *in vitro* soft agar assay as well as in *in vivo* using an immunodeficient mouse model system. To further explore a potential role of FBXO28 in cancer, we utilized the *in silico* transcriptomics database of the GeneSapiens System ([www.genesapiens.org](http://www.genesapiens.org)) and the Oncomine database [140] to examine FBXO28 expression in human tumors. We found that FBXO28 expression is elevated in several different tumor types, including breast cancer. When gene expression data representing 327 primary breast tumor specimens was analyzed for FBXO28 expression, we identified over 100 genes whose expression was highly related to FBXO28 expression. Importantly, when analyzed at ENCODE (<http://genome.ucsc.edu/ENCODE/> analyses) we found that most of the genes that were positively correlated to FBXO28 expression in primary tumors also had a highly significant overrepresentation of MYC binding at the promoters and a strong trend towards coassociation of p300.

Based on these results, we addressed the potential clinical significance of FBXO28 in human breast cancer. FBXO28 protein and phosphorylation was analyzed in several independent breast cancer cohorts by western blot and immunohistochemistry on tissue

microarray (TMA). Strikingly, we found a statistically significant association between a high nuclear fraction of FBXO28 and survival. Most importantly, in multivariate analysis, expression and phosphorylation of FBXO28 were independent predictors of poor survival.

Overall, this work identified a new F-box protein, FBXO28, phosphorylated by CDK1/2. SCF<sup>FBXO28</sup> targets MYC for non-proteolytic ubiquitylation, a modification we showed is important for MYC-driven target gene expression and tumor growth. This work also underscores the importance of FBXO28 as a new potential biomarker in particular patient subgroups of human breast cancer, possibly in tumors with hyperactivation of MYC.

### 3.3 PAPER III

#### **FBXO28 IS A SCF-TYPE UBIQUITIN LIGASE THAT REGULATES CELL MOTILITY BY TARGETING $\beta$ PIX FOR UBIQUITYLATION.**

In the previous study (paper II), we performed an initial characterization of the function of nuclear FBXO28 protein [75]. In paper III, we continued to explore the biological functions of FBXO28.

Initially, a proteomic mass spectrometry approach was used to search for new interactors and potential FBXO28 substrates. FBXO28 was affinity purified and interacting proteins identified by multidimensional protein identification technology (MudPIT) [141]. The MudPIT results revealed that FBXO28 is able to interact with a group of proteins with key functions in cell motility, specifically the PAK1- $\beta$ PIX-GIT1 protein complex. Next, we confirmed FBXO28 association with this group of proteins by means of biochemical immunoprecipitation experiments and *in situ* proximity ligation assay (*in situ* PLA) [142].

PAK1- $\beta$ PIX-GIT1 proteins and their effectors have been found to be important biological players in, for instance cell motility, which is a crucial step in migration and cancer metastasis [143].  $\beta$ PIX, (Pak-interacting exchange factor), has been described as a binding partner of both PAK1 and GIT1 and acts as a guanine nucleotide exchange factor (GEF) for the Rho GTPases Rac1 and Cdc42 [109-111].  $\beta$ PIX is involved in cell motility by means of regulating maturation and disassembly of cell-matrix adhesions [112, 113]. Whereas phosphorylation is a key posttranslational modification for the regulation of Rho GTPases and their effector proteins, ubiquitylation is another mode of regulation for the functions of these proteins in adhesion dynamics and cell migration [96, 97]. Based on these findings we decided to characterize the potential role of FBXO28 in cell motility.

Since FBXO28 forms a functional SCF complex and ubiquitylates the nuclear protein MYC (Paper II) [75], we sought to examine whether FBXO28 is also able to ubiquitylate  $\beta$ PIX which is predominantly a cytoplasmic protein. As Cullin-1 is the scaffold protein for a functional SCF ligase [144] we first tested whether  $\beta$ PIX associates with Cullin 1 and the dependence of FBXO28 for this interaction. Indeed, the association between  $\beta$ PIX and Cullin-1 was markedly decreased upon depletion of FBXO28. Furthermore, we found

that WT-FBXO28 (but not  $\Delta$ F-FBXO28) ubiquitylates  $\beta$ PIX both *in vivo* and *in vitro*. Knockdown of FBXO28 further demonstrated reduced polyubiquitylation of  $\beta$ PIX *in vivo*. Together, these data strongly suggest that FBXO28 acts as an SCF ubiquitin ligase for  $\beta$ PIX.

Since the PIX–GIT complex has been widely studied in the context of integrin-mediated cell spreading and focal adhesion turnover [113, 145], we decided to examine if FBXO28 possibly localizes to cell-matrix adhesion complexes. Using paxillin as a focal adhesion marker, we showed that FBXO28 was able to colocalize with paxillin using immunofluorescence. Our results thus support an additional, membrane localized function of FBXO28, possibly as a regulator of adhesion complex dynamics and cellular motility.

As  $\beta$ PIX and GIT1 act downstream of the EGFR-SRC-FAK phosphorylation signaling pathway [146, 147], we hypothesized that ubiquitylation of  $\beta$ PIX by FBXO28 might be linked to activation of this signaling cascade. To test this, we stimulated HeLa cells with epidermal growth factor (EGF), previously shown to phosphorylate  $\beta$ PIX at the cell periphery [112]. Strikingly, we found that not only does FBXO28 protein re-distribute to cell-matrix complexes upon treatment with EGF, but also more importantly, the interaction between FBXO28 and  $\beta$ PIX was significantly enhanced upon EGF stimulation. We also found that FBXO28 is able to ubiquitylate  $\beta$ PIX in a non-proteolytic and phosphorylation-dependent manner following EGF stimulation. As EGF stimulation has previously been shown to trigger phosphorylation of  $\beta$ PIX at amino acid residue Y442 [112], we also tested if FBXO28 is capable of ubiquitylating  $\beta$ PIX when this tyrosine 442 is mutated to alanine (Y442A- $\beta$ PIX). Interestingly, the Y442A  $\beta$ PIX phospho-mutant displayed resistance to ubiquitylation by FBXO28 suggesting that FBXO28 promotes ubiquitylation of  $\beta$ PIX in response to phosphorylation under EGF stimulatory condition.

Next, we sought to examine whether ubiquitylation of  $\beta$ PIX by FBXO28 could have a role in the PAK1- $\beta$ PIX-GIT1 protein complex formation. Our results showed that whilst WT-FBXO28 (but not  $\Delta$ F-FBXO28) positively promotes the formation of PAK1- $\beta$ PIX-GIT1 complexes, depletion of FBXO28 leads to a slight, but reproducible decreased interaction between PAK1-  $\beta$ PIX with endogenous GIT1 protein. Taken together, these results support a function of FBXO28 in promoting PAK1- $\beta$ PIX-GIT1 complex assembly in response to extracellular signals stimulating cell spreading and motility.

For cancer cells to migrate and invade the extracellular matrix, they need to adhere and

spread to facilitate the process. The GTPase Rac1 is well-studied for its role membrane ruffling, formation of lamellipodia and cell adhesion [148]. In particular, Rac1 translocates to the cell periphery in its GTP-bound active form [148]. Since  $\beta$ PIX binds and regulates Rac1 activity [146, 148], we next asked if knockdown of FBXO28 interfered with the translocation of Rac1 to the cell periphery. Using RNAi and immunofluorescence microscopy analysis, we showed that under conditions of FBXO28 depletion, the distribution of GTP-bound Rac1 was dramatically changed and the majority of the cells lacked membrane-bound Rac1 under EGF stimulated conditions. When cells were depleted of FBXO28 and plated on fibronectin-coated plates, we also found that cells displayed defective cell spreading. To follow on these findings, we next examined if depletion of FBXO28 could impede migration and invasiveness of cancer cells. Interestingly, overexpression of  $\Delta$ F-FBXO28 in U2OS cells and knockdown of FBXO28 in MDA-MB-231 cells (data not shown) significantly attenuated migration as measured by wound-healing assays. In addition, we found that invasiveness of metastatic MDA-MB-231 cancer cells was significantly reduced with depletion of FBXO28.

Finally, we explored whether FBXO28 protein is detected at the membrane also in primary human breast cancer cells. Using TMA analysis, we found that a high fraction of FBXO28 membranous-positive tumor cells was not associated with other adverse clinicopathological characteristics (e.g. tumor size, Ki-67 expression and grade), although a statistically significant correlation was found between high membranous fraction of FBXO28 and poor breast cancer specific survival. Importantly, using Cox modelling, we found a significant association between high membranous fraction (MF) of FBXO28 and decreased BCSS, and when analyzed by multivariate analysis, the MF of FBXO28 retained its prognostic significance as an independent predictor of poor BCSS (HR=3.0,  $p < 0.05$ ).

In summary, the results presented in this study reveal a novel function of the SCF ubiquitin ligase FBXO28 in ubiquitylation of  $\beta$ PIX in response to growth factor stimulation. The exact mechanism how ubiquitylation of  $\beta$ PIX regulates PAK- $\beta$ PIX-GIT complex dynamics and recruitment of active Rac1 to the membrane remains to be determined.



## 4 GENERAL CONCLUSIONS

This work has contributed to the understanding of SCF-type F-box proteins, FBW7 and FBXO28, in targeting proteins for ubiquitylation and their roles in cancer.

- We have shown that collaboration between Fbw7 $\alpha$  and Fbw7 $\gamma$  is required for driving the degradation of cyclin E1 in the nucleolus. The results expand upon previous findings demonstrating that Fbw7 $\alpha$  acts as a cofactor for Pin1 and aids in isomerization of the cyclin E1 phosphodegron, which is important for subsequent translocation and ubiquitylation of cyclin E1 by Fbw7 $\gamma$  in the nucleolus.
- We have identified a new F-box protein that targets MYC for non-proteolytic ubiquitylation. FBXO28 is phosphorylated by CDKs and this is important for MYC-driven transcriptional activity, transformation and tumorigenesis. Importantly, high levels of FBXO28 expression and phosphorylation are indicators for poor prognosis in breast cancer.
- We have discovered a new function of FBXO28 in cell motility, possibly by targeting  $\beta$ PIX for non-proteolytic ubiquitylation in response to growth factor stimulation. This work also demonstrates a significant association between a high fraction of FBXO28 at the membrane and decreased breast cancer specific survival.

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