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F-BOX PROTEINS AS REGULATORS OF ONCOGENIC PATHWAYS BY UBIQUITYLATION

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To my beautiful growing family

ABSTRACT

F-box proteins are the substrate-recognition components of the SCF E3 ubiquitin ligases that catalyze the ubiquitylation of many key cell cycle regulators. Functional studies indicate that the ubiquitin-proteasome system participates in the control of nearly all cellular processes through the timely degradation of short-lived regulatory proteins. Accordingly, altered protein degradation due to defective E3 ligases has been shown to underlie many human diseases, such as cancer. The studies in this thesis have focused on the functional characterization of two F-box proteins, FBXW7/hCDC4 and FBXO28, in ubiquitylation and degradation of the cell cycle regulatory proteins, cyclin E and Myc, and their potential deregulation in cancer.

The tumor suppressor protein FBXW7/hCDC4 has been linked to human tumorigenesis through the targeted degradation of several important oncoproteins, including cyclin E. The ubiquitin-dependent turnover of cyclin E1 is regulated by phosphorylation and isomerization of cyclin E1, and executed by the concerted actions of the FBXW7/hCDC4-α and -γ isoforms. Our results demonstrate that this two-isoform dependence is not employed in conditions where cyclin E1 levels are elevated. Under these circumstances, cyclin E1 can be ubiquitylated by FBXW7/hCDC4-α alone, perhaps through an alternative pathway that does not require isomerization. In the second study, we report that cyclin E2 is targeted for ubiquitin-dependent proteolysis by $SCF^{FBXW7/hCDC4}$. Interestingly, we found that cyclin E1 enhances the ubiquitin-dependent proteolysis of cyclin E2, suggesting a mechanism by which cyclin E1 regulates the abundance of cyclin E2, allowing it to possibly perform non-redundant functions in cell cycle control.

In the last two studies we characterized the novel F-box protein, FBXO28, initially identified in an RNAi screen for F-box genes that regulate cell proliferation. We show that SCF^{FBXO28} targets Myc for ubiquitylation, without altering Myc protein turnover. Instead, FBXO28 was found to be an important regulator of Myc-driven transcription through the ubiquitin-dependent recruitment of a transcriptional cofactor to Myc target gene promoters. In addition, we found that FBXO28 is a nuclear substrate for cyclin-CDK phosphorylation and that phosphorylation of FBXO28 is significantly associated with poor prognosis in patients with primary breast cancer. FBXO28 may thus constitute an important player in cell proliferation and Myc pathways during tumorigenesis.

In summary, in this thesis we present different mechanisms by which SCF-mediated ubiquitylation can regulate proliferation, thus linking ubiquitin-mediated processes to proliferative pathways often altered in human cancer.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to in the text by their roman numerals:

I. Sangfelt O., **Cepeda D.**, Malyukova A., van Drogen F., and Reed S.I.
Both $SCF^{Cdc4\alpha}$ and $SCF^{Cdc4\gamma}$ are required for cyclin E turnover in cell lines that do not overexpress cyclin E.
Cell Cycle, 2008 Apr;7(8):1075-82.

II. Klotz K., **Cepeda D.**, Tan Y., Sun D., Sangfelt O., and Spruck C.
$SCF^{Fbxw7/hCdc4}$ targets cyclin E2 for ubiquitin-dependent proteolysis.
Exp Cell Res., 2009 Jul;315(11):1832-9.

III. **Cepeda D.**, Sharifi H., Ng H.F., Mahmoudi S., Nilsson H., Fredlund E., Rantala J., Klevebring D., Grotegut S., Viñals F., Al-Khalili Szigyarto C., Pontén F., Uhlén M., Widschwendter M., Wohlschlegel J., Grandér D., Spruck C., Larsson L.G., and Sangfelt O.
The ubiquitin ligase SCF^{FBXO28} regulates Myc-driven transcription through non-proteolytic ubiquitylation and is required for cell proliferation.
Manuscript, 2011.

IV. **Cepeda D.**, Ng H.F., Magnusson K., Navani S., Mahmoudi S., Dun D., Lerner M., Spruck C., Grandér D., Jirström K., Pontén F., and Sangfelt O.
Phosphorylation of the F-box protein FBXO28 is associated with poor prognosis in patients with primary breast cancer.
Manuscript, 2011.

RELATED PUBLICATIONS

Lerner M., Corcoran M.M., **Cepeda D.**, Nielsen M.L., Zubarev R., Pontén F., Uhlén M., Hober S., Grandér D., and Sangfelt O. The RBCC gene RFP2 (*Leu5*) encodes a novel transmembrane E3 ubiquitin ligase involved in ERAD. *Mol Biol Cell*. 2007 May;18(5):1670-82.

Akhoondi S., Sun D., von der Lehr N., Apostolidou S., Klotz K., Maljukova A., **Cepeda D.**, Fiegl H., Dafou D., Marth C., Mueller-Holzner E., Corcoran M., Dagnell M., Nejad S.Z., Nayer B.N., Zali M.R., Hansson J., Egyhazi S., Petersson F., Sangfelt P., Nordgren H., Grandér D., Reed S.I., Widschwendter M., Sangfelt O., and Spruck C. *FBXW7/hCDC4* is a general tumor suppressor in human cancer. *Cancer Res.* 2007 Oct;67(19):9006-12.

CONTENTS

1. LIST OF ABBREVIATIONS

APC/C	anaphase-promoting complex/Cyclosome
ATP	adenosine triphosphate
BCSS	breast cancer-specific survival
bHLH/LZ	basic helix-loop-helix leucine zipper
B-ALL	B-cell acute lymphoblastic leukemia/lymphoma
BIM	BCL2 interacting mediator of cell death
BRCA	breast cancer susceptibility gene
CBP	CREB-binding protein
CDK	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
Cip/Kip	cdk interacting protein/cdk inhibitory protein
CKI	CDK inhibitor
CPD	CDC4 phosphodegron
D-box	destruction box
DNA	deoxyribonucleic acid
DNMT3a	DNA-methyltransferase 3a
DUB	deubiquitylating enzyme
ER	estrogen receptor
GSK3	glycogen synthase kinase 3
HAT	histone acetyltransferase
hCDC4	human cell division cycle 4
HDAC	histone deacetylase
HECT	homologous to the E6-AP carboxyl terminus
HER2	human epidermal growth factor receptor 2
HUWE1	HECT, UBA and WWE domain-containing protein 1
INK4	inhibitor of CDK4
K	lysine
Mad	Max dimerizer
Max	Myc-associated protein X
MBI/II	myc box I or myc box II
MCM	minichromosome maintenance
Mdm2	mouse double minute 2
MEFs	mouse embryonic fibroblasts
Mga	Max gene-associated protein
Miz1	Myc-interacting zinc finger protein 1
Mnt	Max-interacting protein
mRNA	messenger ribonucleic acid
Mule	Mcl-1 ubiquitination ligase E2
Mxi1	max interactor 1
NF	nuclear fraction
NF-κB	nuclear factor-kappa B
NLS	nuclear localization signal
OS	overall survival

Pin1	peptidyl prolyl *cis-trans* isomerase
PP2A	protein phosphatase 2A
Ras	rat sarcoma viral oncogene homolog
Rb	retinoblastoma protein
RBX1	RING-box protein 1
rDNA	ribosomal DNA
RFS	recurrence-free survival
RING	really interesting new gene
RNA	ribonucleic acid
RNAi	RNA interference
SCF	Skp1-Cullin-F-box
Ser	Serine
siRNA	small interfering RNA
SKP2	S-phase kinase-associated protein 2
SWI/SNF	switch/sucrose nonfermentable
TAD	transactivation domain
Thr	threonine
TMA	tissue microarray
β-TrCP	beta-transducin repeat containing protein
TSG	tumour suppressor gene
TRRAP	transactivation/transformation associated protein
TRUSS	tumor necrosis factor receptor-associated ubiquitous scaffolding and signalling protein
Ub	ubiquitin
Ubc	ubiquitin-conjugating enzyme
UPS	ubiquitin-proteasome system
USP28	ubiquitin-specific protease 28

2. LITERATURE REVIEW

The work presented in this thesis focuses on the roles of protein ubiquitylation in the control of proliferative pathways often operating at an increased rate to promote cancer. I hope that the topics that I have chosen to cover in this literature review will reflect this. Since understanding the biology of cancer has ultimately been the endpoint of these studies, let us begin there.

2.1. CANCER: THE PRICE OF MULTICELLULARITY

Most of us have a very clear reaction to the term *cancer*: fear. We consider cancer as a terrible and painful disease that greatly shortens the lifespan of the affected person. However, for the most part, we have a less clear idea of what cancer really is. Cancer can be broadly defined as a genetic disease that arises from the unrestrained and destructive proliferation of cells that is irresponsive to external signals. But how do normal cells become cancer cells? Normal physiology of multicellular organisms requires the continuous renewal of cell populations, largely accomplished by cell growth (defined as an increase in cell mass), cell division, and the programmed elimination of cells (termed *apoptosis*) in response to environmental cues. The tight control of these processes is thus essential to the proper functioning of the organism and perturbation of the cellular balance between life and death can have devastating consequences to the organism as a whole. Occasionally, a cell acquires a set of advantageous changes that allows it to escape normal regulation, and to grow and proliferate autonomously, invading other tissues and organs. These changes, called *mutations* and/or *epigenetic changes*, simultaneously affect two groups of genes: those that promote cell growth and proliferation, evading cell death, known as *oncogenes*, and those that restrain proliferative events and induce death by apoptosis, called *tumor suppressor genes* (TSGs). Through a combination of sufficiently advantageous mutations, cells ultimately acquire an array of functional capabilities, commonly referred to as *the hallmarks of cancer*. These include: (1) sustaining proliferative signalling, (2) evading growth suppressors, (3) resisting cell death, (4) enabling replicative immortality, (5) inducing angiogenesis, (6) activating tissue invasion and metastasis, (7) reprogramming of energy metabolism, and (8) evading immune destruction [1]. The genes that are altered and result in disturbance of these processes differ between tumors from different tissue types, but the acquired traits are remarkably consistent across cancers. Therefore, rather than being a single disease, the term cancer encompasses hundreds of different diseases, each with its own set of risk factors and epidemiology, which can arise from most cell types and organs. Hence, the requisite to the preservation of a long life with tissues that regenerate comes

with the ever-present risk of initiating processes that might result in fatal malignancy.

Given that in Paper III and, in particular, in Paper IV, we examine the expression of the F-box protein, FBXO28, in breast cancers, I owe the reader a short description of this type of cancer.

2.1.1. On breast cancer

Breast carcinogenesis arises from a multi-step process in which normal breast epithelium evolves into an invasive cancer that can eventually spread via the lymphatic and vascular systems to give rise to distant metastases [2]. The steps in the development of breast cancer seem to correlate to genetic alterations in either oncogenes or tumor suppressor genes.

Breast cancer can be divided into two major groups: sporadic and hereditary breast cancer. In sporadic breast cancer, mutations are acquired during the individual's lifetime, and occur in somatic cells without underlying changes in germ cells. Early mutations in sporadic breast cancer include mutational activation of oncogenes, with concomitant overexpression of the oncoprotein, such as c-Myc, cyclin D1, and HER2/neu [2-5]. In hereditary breast cancer, the individual is born with a mutation (*germline mutation*) in one copy of a tumor suppressor gene (TSG) that, in combination with inactivation of the second copy, or *allele*, can support cancer development. This is called Knudson's "two-hit-hypothesis" of carcinogenesis [6]. The most prominent predisposing mutations in hereditary breast cancer occur in the BRCA1 and BRCA2 genes (**br**east **ca**ncer susceptibility gene **1** and **br**east **ca**ncer susceptibility gene **2**, respectively). The products of these TSGs play critical roles in maintaining the integrity of the cellular genome through DNA repair. Women carrying germline BRCA1 or BRCA2 mutations have an increased lifetime risk for breast (50-90% and 40-80%, respectively) and ovarian (20-50% and 10-20%, respectively) cancers [7].

Breast cancer classification assigns breast cancers into several different categories based on multiple parameters. The purpose of the classification is to aid in the selection of the best treatment approach for each individual patient. A typical classification usually considers the following features: the histolopathological type, the grade of the tumor, the stage of the tumor, and the expression of proteins and genes. The general architecture of breast tumors determines their ***histological type***. In general, breast cancers are derived from glandular tissue, and can be divided into ductal and lobular carcinomas, with invasive ductal carcinomas being the most common form of invasive breast cancer. Tumor ***histological grade*** defines cellular differentiation, reflecting how much the tumor cells differ from the cells in the normal surrounding tissue. Grading is generally performed using the guidelines proposed by Bloom and Richardson, and later modified by Elston and Ellis [8, 9]. The histological grade is based on three features: tubule formation, nuclear pleomorphism (abnormal nuclei), and mitotic count (as a measure of proliferation rate), and divides breast tumors into three categories: grade I, II and III. Grade score increases with lack of

cellular differentiation, such that well-differentiated cells are found in grade I tumors (low-grade), moderately differentiated cells in grade II tumors (moderate grade), and poorly differentiated cells in grade III tumors (high-grade). Moreover, breast cancer ***staging*** is performed using the TNM classification that describes the size of the tumor (T), the involvement of local lymph nodes (N), and the existence of distant metastases (M). Finally, routine pathological classification of breast cancer also defines the receptor status (estrogen and progesterone receptors), gene amplifications and expression of the HER2/neu gene, and occasionally the status of the p53 tumor suppressor gene.

Overexpression of the estrogen receptor (ER) is also a frequent event in breast cancer [10]. An increased risk for breast cancer is associated with high levels of estrogen in the blood plasma, either endogenous estrogen or from exogenous sources such as hormone-replacement therapy [11]. Recent advances in gene expression profiling have further stratified breast cancers into "molecular subtypes" based on patterns of gene expression. These include two main subtypes of ER-positive tumors, termed luminal A and luminal B. As a rule, luminal A tumors have low expression of proliferation-related genes, thus carrying the best prognosis of all breast cancer subtypes, whereas the less common luminal B tumors are highly proliferative, and have therefore poor prognosis.

It is worth noting that these classifications are constantly being updated as our knowledge of breast cancer biology and prognosis develops.

I hope that, as the thesis proceeds, it becomes apparent to the reader that genetic aberrations resulting in deregulated proteolytic pathways contribute to the aetiology of cancer of the breast and other tissues.

2.2. THE CELL CYCLE AT A GLANCE

2.2.1. Cycling in the lane

To divide is a challenging task for a cell. It entails the progression through a complex sequence of events that will ultimately culminate in cell growth and the production of two daughter cells. In order for the cell to maintain its functional integrity, these events need to be tightly controlled every step of the way. Failure to do so can have devastating consequences, leading to mutations in critical genes, excessive proliferation and cellular transformation. In order to understand how cancer cells acquire the ability to proliferate uncontrollably, we need to appreciate how a normal cell divides.

Two main events characterize the cell cycle: DNA replication and nuclear division. However, for the cell to accomplish these tasks, it must pass through a series of unidirectionally steps, or phases, that make up the cell division cycle, or simply *the cell cycle*. The cell cycle is divided into four major phases: G1, S, G2, and M. Duplication of DNA, or chromosomal replication, occurs during the S phase (synthesis phase), preceded by a gap phase called G1. In G1, the cell receives and translates signals from its surrounding and makes the critical decision to progress through cell cycle and initiate DNA synthesis. S phase is followed by a second gap phase, G2, in preparation for cell division, also known as mitosis (M). Cells in G1 that are not committed to cell division can withdraw from the cell cycle and enter a resting state termed G0. However, resting cells can be stimulated to re-enter the cell cycle in response to external growth promoting signals. Once committed to divide, the cell must proceed through all the phases of the cell cycle without additional growth stimulatory signals. This "point of no return" has been referred to as the *restriction point* [12, 13]. Deregulation of factors that control the restriction point are thought to be of major importance for cancer development.

The driving force promoting the transition from one cell cycle phase to the next is governed by the activation of key regulatory protein kinases, known as cyclin-dependent kinases (CDKs). CDKs are a family of serine/threonine protein kinases that, when activated, induce downstream processes by phosphorylating proteins essential for the forward movement of the cell cycle. Activation of CDKs is dependent on binding to specific cyclin proteins. While CDK protein levels are stable throughout the cell cycle, cyclin levels rise and fall periodically during the cell cycle. Cyclins are short-lived proteins regulated at the level of transcription and protein stability [14, 15]. In this way, the abundance of cyclins determines the timing of CDK activation. Different cyclins are expressed at different stages of the cell cycle; the D-type cyclins (Cyclins D1, D2, and D3) associate with CDK4 and CDK6 and are essential for entry into G1. Unlike the other cyclins, D-type cyclins are not synthesized periodically, but are expressed as long as growth stimulatory signals are present [16]. E-cyclins are another type of G1 cyclins, which associate with CDK2 to regulate the transition from G1 to S-phase [17]. Cyclin A-CDK2 association is required during S-phase, while in late

G2 cyclin A binds to CDK1 to promote M-phase entry [18, 19]. Finally, cyclin B-CDK1 complexes are responsible for the regulation of mitosis [20].

Cyclin-CDK activity can be antagonized by a group of proteins called CDK inhibitors (CKIs). There are two major families of CKIs: the INK4 (named as inhibitors of CDK4) and the Cip/Kip (cdk interacting protein/cdk inhibitory protein) families [21]. The INK4 proteins ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$) specifically inactivate CDK4 and CDK6 by preventing cyclin association and inhibiting their catalytic activities [22, 23], whereas the Cip/Kip family of inhibitors ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) have the potential to inhibit all cyclin-CDK complexes. The expression and activities of CKIs are tightly regulated at the level of transcription, phosphorylation by other kinases, and by degradation by the ubiquitin proteasome system.

One of the major targets of cyclin D-CDK4/6 is the Retinoblastoma tumor suppressor protein, Rb. The Rb family includes three members, Rb1/p105, p107, and Rb2/p130, collectively referred to as "pocket proteins". The Rb proteins repress transition from G1 to S phase by inhibiting transcription factors of the E2F family whose activity is required for S-phase progression [24]. Thus, in its hypophosphorylated, active state, Rb inhibits cell cycle progression; however, upon growth factor stimulation, Rb is inactivated by G1 CDK-dependent phosphorylation, leading to the dissociation of E2F/DP transcription factors from Rb and transcriptional induction of a large set of genes required for S-phase progression, including cyclin E, cyclin A, CDK1, CDK2 and thymidine synthase [24, 25]. Rb remains inactive for the remainder of the cell cycle by virtue of phosphorylation by cyclin E-CDK2 [26]. During G1/S transition, the cyclin E-CDK2 complex phosphorylates its inhibitor $p27^{Kip1}$, inducing its proteasomal degradation by the SCF^{SKP2} ubiquitin ligase [27, 28]. Both cyclin E-CDK2 and cyclin A-CDK2 complexes play essential roles in DNA replication. As S-phase progression continues, cyclin E is destroyed by the ubiquitin ligase SCF^{Fbxw7} (discussed further in Section 2.4.3.), and the level of mitotic cyclins begin to rise. The activity of cyclin A-CDK1 is of particular importance in G2/M transition, while its degradation is necessary for M-phase completion [29, 30]. Finally, cyclin B-CDK1 complexes initiate essential processes including condensation of the chromosomes, mitotic spindle assembly and breakdown of the nuclear envelope, ultimately leading to cell division. At this point, the anaphase-promoting complex (APC/C) degrades cyclin B, which is necessary for mitotic exit and entry into the next G1 phase (reviewed in [31, 32]).

"Cycling in the lane" is analogous to the regulated progression of a cell through the cell cycle, with "stop" and "go ahead" signals in place at each cell cycle phase. However, when, for example the signals are misplaced, the cell starts cycling non-stop. The next section gives a few examples of regulators that, when altered, can promote uncontrolled proliferation.

2.2.2 Cycling round and round: When the cell cycle is out of control

In cancer, alterations in the molecular machinery that regulates cell growth and cell division cooperate to promote unrestrained cell proliferation that is

irresponsive to regulatory cues. Mutations have been described in genes acting at multiple levels of cell proliferative pathways, including CDKs, cyclins, CKIs, and CDK substrates. The *RB1* gene is frequently mutated in human retinoblastoma and many other tumors [33], and alterations of at least one CKI are found in nearly all human cancers [34]. For instance, decreased expression of $p27^{Kip1}$, through increased proteasome-dependent proteolysis, is a frequent event in various human cancers, and is a strong indicator of poor prognosis [35, 36]. Indeed, overexpression of SKP2, the E3 ligase responsible for $p27^{Kip1}$ degradation, is a major contributor to human malignancy [37]. Moreover, whereas cyclin E has been found to be amplified and overexpressed in a wide range of cancers [38], its overexpression has also been attributed to inactivation of the FBXW7/hCDC4 protein that is responsible for its ubiquitin-dependent proteasomal degradation [39, 40]. These examples highlight the importance of regulated protein abundance in normal cell cycle control. *Targeting of cyclin E for ubiquitin-mediated degradation by FBXW7/hCDC4 the basis of Papers I and II, and will be discussed in greater detail throughout section 2.4.*

Abnormal activation of the Myc proto-oncogene is also a frequent event leading to dysregulation of cellular homeostasis. Briefly, Myc drives proliferation and inhibits cell differentiation by stimulating the expression of several positive regulators of the cell cycle, including cyclin D2, CDK4 and E2F2 [41-43]. Accordingly, the *MYC* oncogene is deregulated in multiple human cancers by different mechanisms including amplification, point mutation, chromosomal translocation, or increased protein stability [44]. *The functions of Myc will be explored in section 2.5., and Paper III will describe a novel regulator of Myc activity.*

All of the above-described alterations have the same functional outcome: to increase cell cycle progression, often by shutting down terminal differentiation and cell death pathways. This unchecked cell division will ultimately result in the accumulation of malignant cells with the acquired ability to proliferate autonomously, thus promoting tumor formation.

2.3. UBIQUITIN-MEDIATED PROTEOLYSIS

2.3.1. One-way cycling: a role for the ubiquitin-proteasome system

As we learned in the previous section, the cell cycle consists of an orderly sequence of events characterized by the periodic synthesis and destruction of key regulatory proteins within a defined window of time. The ubiquitin-proteasome system (UPS) is responsible for the targeted degradation of numerous cell cycle proteins, including cyclins and CDK inhibitors (CKIs) [45].

The tasks of many of the proteins required for cell cycle progression are often limited to a precise period of time. Cyclins are great examples of how cell-cycle transitions are regulated by the sharp activation of specific CDKs programmed to phosphorylate a defined set of substrates. Understandably, chronic activation of CDKs leading to phosphorylation of the wrong substrates at the wrong time can have deleterious consequences for the cell. Proteolysis of cyclins via the UPS is thus important for preventing continuous CDK activation and ensuring the proper execution of cell-cycle functions. Degradation of CKIs, negative regulators of the CDKs, by the UPS is also instrumental in the rapid and irreversible transition from one cell-cycle phase to the next. Undoubtedly, cell-cycle control and ubiquitin-mediated proteolysis are intricately dependent processes. The importance of this system in the pathogenesis of many human tumors is underpinned by growing evidence of direct alterations found in the components that regulate the UPS.

2.3.2. The process of tagging

Besides its crucial role in cell cycle regulation, major roles of the UPS also include clearance of misfolded proteins, immune response, cell survival, inflammatory responses, protein trafficking, signaling and gene transcription [46-48].

Ubiquitin is a highly conserved protein of 76 amino acids that is ubiquitously expressed, thus giving rise to its name. It is translated as a fusion product, either fused to a ribosomal protein or as a linear repeat comprised of a chain of ubiquitin molecules linked together. Single ubiquitin monomers are produced through cleavage of the fusion proteins by ubiquitin C-terminal hydrolases [49]. While ubiquitin protein is highly abundant due to constitutive expression, stability and recycling, the pool of free ubiquitin molecules is rather limited [50]. This is because the majority of ubiquitin is found conjugated to target proteins, reflecting the extensive usage of ubiquitin in diverse cellular processes. Consequently, there is a dynamic equilibrium between assembly and disassembly of ubiquitin molecules through the opposing actions of ubiquitylation and deubiquitylation enzymes.

Ubiquitin regulates protein turnover in the cell by “tagging” specific proteins for degradation. Whereas the majority of the proteins in the cell are long-lived, short-lived proteins are typically regulatory proteins or abnormal proteins that need to be removed. Thus, by eliminating such proteins, cells can

rapidly turn on or off specific biological process. This is a very effective way to irreversibly regulate protein activity, but is also energetically expensive, as the protein needs to be re-synthesized if needed again. Furthermore, the processes of ubiquitin conjugation and proteasomal degradation require energy in the form of ATP. But why is this, since no energy is needed for hydrolysis of proteins? The reason for this is that specialized machinery needs to be engaged in the specific tagging of proteins for ubiquitin-mediated proteolysis. It was this discovery, that regulated protein degradation was an energy-dependent process catalyzed by the covalent attachment of multiple ubiquitin molecules and degradation in the proteasome, that awarded Aaron Ciechanover, Avram Hershko, and Irwin Rose the Nobel Prize in chemistry in 2004 [51, 52].

The biological system responsible for conjugation of ubiquitin to specific target proteins (also known as ubiquitylation) is regulated by the sequential action of three key enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). The E1 enzyme generates a high-energy thioester bond between E1 and ubiquitin, activating ubiquitin in an ATP-dependent reaction. In the second reaction, ubiquitin is transferred from E1 to the active site cysteine of an E2 ubiquitin-conjugating enzyme. Finally, ubiquitin is ¨tagged¨ to a specific lysine residue in the protein substrate with the help of an E3 ubiquitin ligase. In some cases, the activated ubiquitin can be alternatively conjugated to the N-terminal residue of the protein, conforming to the so-called “N-end rule” [53]. E3 ubiquitin ligases generally function in concert with E2s, and they are particularly important in the recognition of a specific substrate. Successive rounds of ubiquitylation ligation, linking additional ubiquitin molecules together in chains of different lengths, result in the formation of polyubiquitin chains on the substrate. While there are two E1s and approximately forty E2 enzymes [54], it is estimated that hundreds of E3 ligases exist in the human proteome. Targeting of substrates by E3 ligases is both specific and versatile: one given E3 ligase can ubiquitylate several different substrates, and the same substrate can be recognized by various E3 ligases [55].

E3 ligases can be divided into two discrete groups on the basis of structural motifs and biochemical function: The HECT-type and the RING-finger-type [56]. The HECT (homologous to E6AP C-Terminus) domain E3s are structurally similar to E6-AP, which degrades the p53 tumor suppressor by HPV E6. During the transfer of ubiquitin to the target substrate, HECT ligases themselves form a transient linkage with ubiquitin through a conserved cysteine residue, before catalyzing the subsequent ubiquitin conjugation. In contrast, RING (Really Interesting New Gene) E3 ligases do not directly participate in the ubiquitylation reaction; by interacting with the E2 via the RING domain, they act as scaffolds to facilitate the transfer of ubiquitin to substrates [57].

The RING E3s constitute the largest family of ubiquitin ligases and can be further divided into two groups: single subunit ligases, where individual proteins interact with both the E2 and the substrate, or multi-subunit complexes composed of distinct RING and substrate adaptors to recruit the E2 and substrate, respectively. Mdm2, the ubiquitin ligase responsible for destruction of p53, is an example of single-subunit RING enzymes [58]. Two canonical examples of multisubunit RING E3 ligases are the SCF (SKP1/Cullin/F-box) and the APC/C (Anaphase Promoting Complex/Cyclosome), each of which utilizes multiple substrate-binding subunits and the same core ligase to target specific substrates.

The SCF and APC/C complexes have been demonstrated to be the major E3 ligase complexes implicated in the targeted destruction of key cell cycle regulatory proteins [59], although they are also thought to play additional roles in other aspects of cell biology.

Despite the similarities, both in structure and function, between the SCF and the APC/C complexes, they differ in their timing of activity: the APC/C is active from mid-mitosis until the end of the subsequent G1 phase, while the SCF complexes function throughout the cell cycle, predominantly from late G1 to early M-phase (Figure 1). They also use different strategies for targeting substrates for ubiquitylation. The APC/C is active in two alternative forms, depending on the availability of substrate-binding factors, Cdc20/Fizzy and Cdh1/Hct1/Fizzy-related. APC/C substrates typically carry either of two recognition motifs, (the D-box or the KEN-box), known as degrons, that can be specifically targeted by Cdc20 and/or Cdh1[60]. Hence, a single substrate recognition adapter can recognize multiple substrates, allowing the APC/C to target many substrates with only two such adapters. The strategy used by the SCF complexes allows for fine-tuned targeting of a multiplicity of substrates and a great flexibility with respect to timing. This flexibility lies partly in the ability of SCF E3 ligases to recognize only substrates that are phosphorylated at specific motifs called phosphodegrons. It is therefore the timing of phosphorylation of target proteins that largely controls their degradation. Furthermore, in contrast to the two forms of APC/C, SCF complexes rely on one of many interchangeable substrate-recognition factors called F-box proteins, thus adding another layer of flexibility to this system (reviewed in [45]).

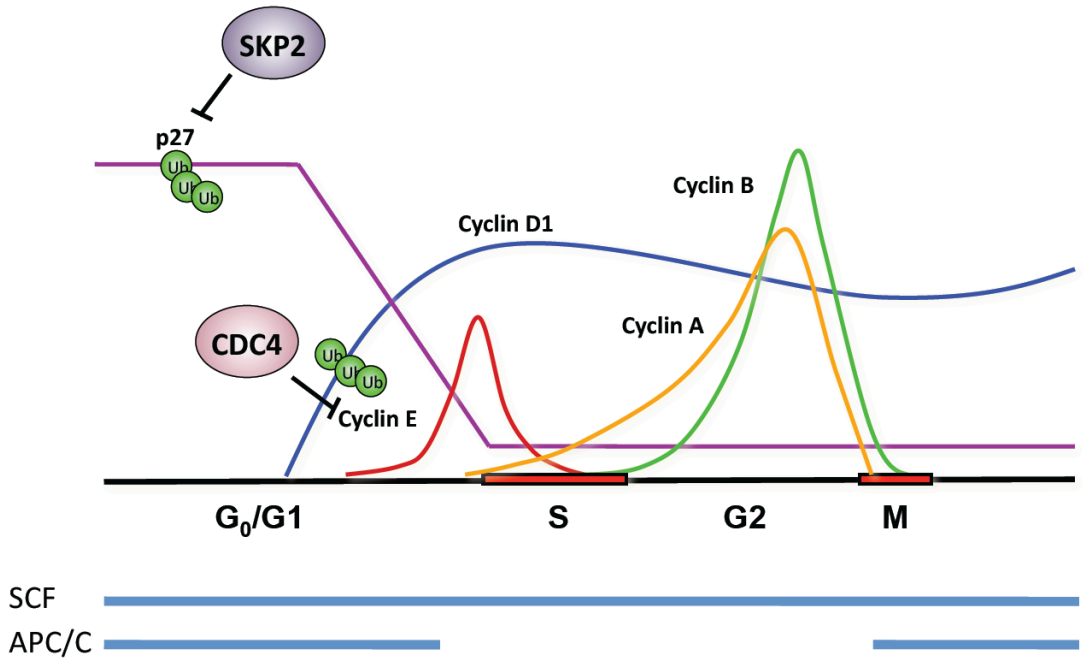

Figure 1. A simplified overview of the interplay between SCF and APC/C E3 ubiquitin ligases in cell cycle regulation. Shown are examples of two SCF ligases, SKP2 and CDC4, in the ubiquitin-mediated degradation of p27 and cyclin E, respectively.

Despite their differences, it is clear that there is a tight interplay between the APC/C and SCF E3 ligase complexes in the control of cell-cycle regulators [61-63], as well as in regulating the activities of one another [64]. However, and as mentioned, genetic alterations in these multisubunit E3 ligases are frequently associated with cancer development, though they have been found to be significantly higher for the SCF complex than for the APC/C [59], likely as a result of the flexibility discussed above.

We will soon explore the SCF-type E3 ligases in greater detail. After all, they are the central players in this thesis. But first, let us look into the fate of ubiquitin and the tagged substrates.

2.3.3. Chained by Ub

The functional outcome of protein ubiquitylation depends primarily on two factors: the length of the ubiquitin chain, and the type of ubiquitin linkage. Ubiquitin can modify substrate proteins as a single moiety (monoubiquitylation) or can be conjugated to other ubiquitin molecules to form a chain (polyubiquitylation or multiubiquitylation). Monoubiquitylation is not normally implicated in degradation, but has been shown to control numerous cellular processes such as receptor transport, histone regulation and DNA repair [65, 66]. Furthermore, monoubiquitin modifications can serve as protein interaction surfaces, as several proteins contain domains (e.g UBM, UIM) that allow them to interact with ubiquitin molecules. Ubiquitin itself contains seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) all of which can function as receptor sites for another ubiquitin molecule to form polyubiquitin chains of different linkages. Attachment of four or more ubiquitin chains through K48 linkages typically targets a protein for proteasomal degradation, whereas polyubiquitin chains linked through other lysines often perform nonproteolytic functions ([67, 68] and reviewed in [69]). K63-linked chains, for instance, have been implicated in signal transduction through the NF-κB pathway, receptor endocytosis and DNA repair (Reviewed in [70, 71]).

Protein ubiquitylation can be reversed by the action of isopeptidases called deubiquitylation enzymes, or DUBs. These proteins are responsible for processing of monomeric ubiquitin molecules from precursor ubiquitin fusion proteins, proofreading ubiquitin-protein conjugates and recycling ubiquitin. Moreover, DUBs can regulate the UPS by rescuing substrates from degradation (Reviewed in [72]).

2.3.4. Execution by the 26S proteasome

Proteins that are modified by polyubiquitin chains of an appropriate type of linkage are ultimately presented to the 26S proteasome. Proteasomes are large multisubunit complexes of about 2000 kDa in molecular mass, composed of one 20S core particle carrying the proteolytic activity, and two 19S regulatory caps [55]. In order for a protein to access the core particle, it must first be recognized

by the 19S regulatory subunit. Recognition is mediated by binding to the polyubiquitin chain on the target protein, and the substrate is then unfolded in an ATP-dependent fashion. The pores that lead to the central proteolytic chamber of the 20S core particle are so narrow, that only unfolded polypeptides are able to pass through [73]. The 20S core particle consists of four heptameric ring structures stacked on top of each other forming a barrel-shaped structure. Within this barrel a set of catalytic subunits with chymotrypsin, trypsin and peptidyl-glutamyl peptide hydrolysing specificities break down proteins into small peptides. Ubiquitin chains are not degraded in the process, but are detached from ubiquitin-protein conjugates by proteasome-associated DUBs, to be re-used by the UPS [72].

Now that we know how a protein is targeted and degraded by the ubiquitin-proteasome system, let us take a step back to explore how the SCF E3 ligases recognize their substrates.

2.3.5. SCF complex: CULling substrates via the F-box protein

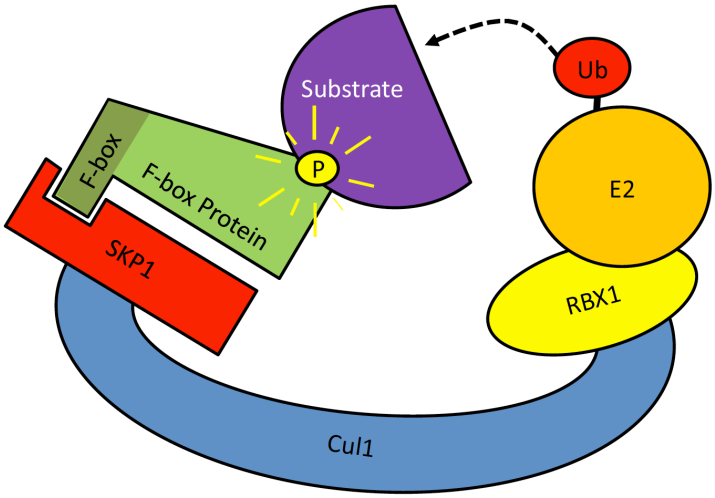

Figure 2. Illustration of the components of the SCF E3 ligase, showing the interaction with the substrate protein through the F-box protein, as well as with the E2 ubiquitin-conjugating enzyme via the RING protein RBX1.

The RING-finger type SCF ubiquitin ligase is composed of core components CUL1, SKP1 and RBX1, and a variable F-box protein. The cullin subunit CUL1 functions as a molecular scaffold that simultaneously interacts with the adaptor subunit SKP1 (S-phase-kinase-associated protein-1), and with the RING-finger protein RBX1 (also known as Roc1), which in turn recruits a specific E2 enzyme, such as

Ubc3, Ubc4 or Ubc5. SKP1 mediates the interaction between CUL1 and one of many different F-box proteins, which are responsible for substrate recognition. A graphical representation of the SCF complex is presented in Figure 2.

F-box proteins are defined by the presence of a conserved F-box domain through which they associate with SKP1 [74]. The F-box motif, so named after its discovery in the first identified F-box protein cyclin F, is generally found towards the amino-terminus of the protein. F-box proteins often recruit their specific substrates through a protein-interaction domain that lies towards the carboxy-terminus of the F-box domain in the sequence. It is based on their substrate-interaction domains that the F-box proteins have been classified into three groups: those containing WD40 repeats (FBXW), leucine-rich repeats (FBXL) or other domains (FBXO) [75-77]. Typically, F-box proteins specifically recognize phosphorylated substrates [78]. For example the WD40 substrate-binding domain in the FBXW group is a β-propeller structure that presumably recognizes specific Ser/Thr phosphorylation at consensus sequences, which have been dubbed phosphodegrons. Such consensus sequences have been identified for FBXW1 (also known as β-TrCP1) and FBXW7 (CDC4) [79]. To date, approximately 70 F-box proteins have been identified in humans, but the functions of only a few have been described. Three well-characterized F-box proteins, in particular, target important substrates that implicate them in human malignancy. As mentioned, SKP2 (FBXL1) targets key negative regulators of the cell cycle, including $p27^{Kip1}$ [28, 80, 81], $p21^{Cip1}$ [82, 83], and $p57^{Kip2}$ [84] and p130 [85] for degradation, thus promoting cell cycle progression (Figure 1). SKP2 has been demonstrated to be an oncogene in mouse models and is found overexpressed in many human cancers [86]. β-TrCP (FBXW1/11) functions in diverse pathways, targeting several cell cycle proteins such as Emi1/2 [61, 62, 87], Wee1A [88] and CDC25A/B [89-91], as well as the signalling proteins β-catenin and IκB [37] for ubiquitin-dependent proteolysis. Both mutation and overexpression of β-TrCP have been reported in cancers [86]. Finally, FBXW7/hCdc4 promotes the degradation of positive regulators of the cell cycle such as Myc, Jun, Notch and cyclin E, and is often mutated in various tumor types [59] (shown in Figure 1 and discussed in greater detail in section 2.4). Thus, by recruiting different substrate-recognition factors (i.e., the F-box proteins) using a common core, SCF ubiquitin ligases are able to "cull", or sort, a broad array of substrates involved in a variety of cellular functions.

Given that Papers I and II in this thesis will deal with the regulation of cyclin E by the FBXW7/hCDC4 E3 ligase, we will now learn more about these proteins and their involvement in cancer.

2.4. FBXW7/hCDC4: MULTI-TASKING IN TUMOR SUPPRESSION

2.4.1. The FBXW7/hCDC4 protein

The *FBXW7* gene (also known as *FBW7* or *hCDC4* in humans, *Cdc4* in *S. cerevisiae*, *Sel-10* in *C. elegans*, or *Ago* in *Drosophila melanogaster*) encodes three protein isoforms in humans, designated α, β, and γ, each containing a unique amino-terminus, and a common carboxy-terminal region. All three isoforms share motifs critical for recognition and ubiquitylation of substrates, namely the F-box motif for recruitment of the SCF core proteins, the WD40 repeats that form the typical β-propeller structure for substrate binding, and a D-domain that allows for dimerization [92]. Each FBXW7/hCDC4 isoform localizes to different subcellular compartments, mediated by signals in their unique N-terminus. The α isoform is found in the nucleus, β in the cytosol and membrane of the endoplasmic reticulum [93], and γ is enriched in the nucleolus. Given that all three isoforms share the same substrate-recognition domain, differential localization might be important for the compartmentalized targeting of substrates to specific locations in the cell. Substrate recognition by FBXW7/hCDC4 is dependent on phosphorylation of the substrate within a consensus motif called the CDC4-phosphodegron (CPD) [94]. Comparison between known FBXW7/hCDC4 substrates has allowed the identification of a defined CPD sequence: Φ-X-Φ-Φ-Φ-pT/pS-P-P-X-pS/pT/E, where Φ corresponds to a hydrophobic amino acid and X to any amino acid [95]. Many of the substrates contain clusters of phosphorylation sequences that deviate from the defined "ideal" CDP, but which still mediate binding to FBXW7/hCDC4. Therefore, efficiency of substrate recognition is determined both by the number of CPDs in the substrate, as well as their sequence similarity to the consensus CPD [45].

2.4.2. $SCF^{FBXW7/hCDC4}$: One ligase, a multitude of substrates

To date, the FBXW7/hCDC4-containing SCF ubiquitin ligase, $SCF^{FBXW7/hCDC4}$, has been reported to target more than 20 different proteins for ubiquitylation in different species. Substrates range from CDK inhibitors, such as the yeast proteins Sic1 [96] and Far1 [97], to transcription factors, like c-Jun [98] and Myc [99, 100]; from signalling factors, such as Notch [101, 102], to positive cell-cycle regulatory proteins, such as cyclin E [39, 40, 103] an yeast Cdc6 [104]. Although the targets in mammalian cells regulate various biological processes, they seem to have one property in common: nearly all have been categorized as oncoproteins.

Two of the substrates – cyclin E and Myc– are critically involved in cell cycle regulation and are often deregulated in cancer. Both cyclin E and Myc are of particular interest to the topics of this thesis, and thus are described more thoroughly in subsequent sections.

2.4.3. The E-cyclins: easy prey for SCF$^{FBXW7/hCDC4}$

E-type cyclins (cyclin E1 and cyclin E2) are expressed from late G1-phase until the end of S-phase of the mammalian cell cycle and, together with their kinase subunit CDK2, regulate passage from the G1 restriction point into S-phase. The cyclin E2 gene was so named due to the presence of a characteristic cyclin box motif and a 47% similarity to the original cyclin E (also known as cyclin E1) [105]. Just like cyclin E1, cyclin E2 binds and activates CDK2 [105-107]. While most of what we know about cyclin E comes largely from extensive studies of cyclin E1, cyclin E1 and cyclin E2 have been described to have, for the most part, overlapping functions; hence, in this text I will refer to the E-type cyclins simply as "cyclin E", unless otherwise specified.

Cyclin E-CDK2 complexes have direct roles in S-phase entry and initiation of DNA replication by the stimulation of S-phase genes, partly via the E2F transcription factors (explained below). Other S-phase events controlled by cyclin E-CDK2 include modulation of DNA synthesis, recruitment of DNA polymerase α, loading of MCM proteins onto replication origins, centrosome duplication, among others (reviewed in [108]).

Since most of the functions of cyclin E relate to DNA, cyclin E is mainly found to reside in the nucleus. Nuclear localization is partly dependent on the presence of a nuclear localization signal (NLS) in the cyclin E sequence, and nuclear shuttling by the proteins importin α and importin β [109]. Moreover, CDK2, the catalytic partner of cyclin E, depends on interaction with cyclin E for nuclear import [108]. Cyclin E is normally expressed periodically in the cell cycle, peaking at the G1 to S transition. This periodicity is achieved by E2F-dependent transcription in late G1 and ubiquitin-mediated proteolysis of active cyclin E-CDK2 complexes in early S-phase [110]. Complementary mechanisms, such as inhibition by CKIs $p21^{Cip1}$ and $p27^{Kip1}$, are critical factors limiting cyclin E-CDK2 activities at the G1 phase [21].

As described in section 2.2. of this thesis, the transcriptional activation of the cyclin E gene at the end of the G1-phase is dependent on phosphorylation of the Rb transcriptional repressors by the cyclin D-CDK4/6 complexes. Phosphorylation of Rb activates E2F transcription factors, which can subsequently induce transcription of cyclin E. Since cyclin E-CDK2 can also phosphorylate the Rb family of proteins, increased cyclin E expression results in increased free E2F, further promoting expression of cyclin E [26, 111-113]. This constitutes a positive feedback loop that allows cyclin E to stimulate its own expression. In contrast, transcription silencing of the cyclin E gene occurs by the assembly of repressive complexes containing hypophosphorylated Rb, histone deacetylase (HDAC), and the SWI/SNF chromatin remodelers together with E2F transcription factors bound to the cyclin E gene promoter [114].

Not surprisingly, the proper regulation of cyclin E is important for maintaining genomic stability and its deregulation has been associated with a broad spectrum of human malignancies. As mentioned, the $SCF^{FBXW7/CDC4}$ ubiquitin ligase mediates the degradation of cyclin E1 in a phosphorylation-dependent fashion [39, 40, 103]. Cyclin E1 has two CDC4 phosphodegrons (CPDs): one "ideal" high-affinity CPD at the C-terminus centered around residues Thr380 and Ser384, and one N-terminal low-affinity CPD at Thr62 [115-118]. Phosphorylation at Thr380 and Ser384 depends on autophosphorylation of

cyclin E1 by active cyclin E-CDK2 complexes, and by the GSK3 kinase at Thr380 [116, 117]. Since cyclin E1 has an optimal CPD, a single phosphorylation event at Thr380 within the high affinity CPD may be sufficient to induce ubiquitylation of cyclin E1 by FBXW7/hCDC4 [45]. Nonetheless, phosphorylation of Ser384 has been demonstrated to increase the efficiency of the binding between cyclin E1 and FBXW7/hCDC4, as pSer384 is capable of interacting with a basic surface on the interaction region of FBXW7/hCDC4 [94]. Interestingly, ubiquitylation of cyclin E1 protein by $SCF^{FBXW7/CDC4}$ has been shown to involve the sequential action of the FBXW7/hCDC4-α and -γ isoforms, both *in vitro* and *in vivo* [119]. *In vitro* reconstitution experiments showed that the role of the FBXW7/hCDC4-α isoform is to serve as a co-factor for the prolyl *cis-trans* isomerase Pin1 in the isomerization of a non-cannonical proline-proline bond in the cyclin E1 high-affinity degron [119]. This isomerization event constitutes a signal for subsequent binding and translocation of cyclin E1 by the FBXW7/hCDC4-γ isoform to the nucleolus where cyclin E1 is subsequently multiubiquitylated ([120] and Bhaskaran *et al.* unpublished). The FBXW7/hCDC4-β isoform does not seem to directly contribute to cyclin E1 degradation *in vivo*, although it could theoretically regulate cytosolic cyclin E, which has been suggested [118].

Paper I explores the interplay between FBXW7/hCDC4-α and –γ isoforms in ubiquitylation of cyclin E1. The regulation of ubiquitin-dependent proteolysis of cyclin E2 by FBXW7/hCDC4 will be described in Paper II.

2.4.4. Cyclin E in cancer

Cyclin E is overexpressed in many human tumors and the cyclin E1 gene (*CCNE1*) is sometimes amplified in cancers [121]. However, more often, tumor cells display increased cyclin E1 protein stability and loss of periodic expression. Unlike cyclin E1, which is expressed both in normal and tumor cells, cyclin E2 expression has been reported to be very low to undetectable in non-transformed cells, and to be elevated particularly in tumor cells, indicating an important role for cyclin E2 in tumorigenesis [105]. Overexpression of cyclin E has been shown to cause premature initiation of DNA synthesis, increased centrosomal duplication and genomic instability [122-125]. In breast carcinoma, increased levels of cyclin E have been correlated to poor prognosis and worse survival [126-128]. Evidence for an oncogenic function of cyclin E is also evident from mouse models [129]. Finally, cyclin E has been demonstrated to transform rat embryonic fibroblasts *in vitro*, from the simultaneous expression of cyclin E and the constitutively form of the Ha-Ras oncoprotein [130], supporting its role in tumorigenesis.

As compared to many other oncoproteins, the major mechanism for deregulation of cyclin E in cancer is through decreased turnover by interfering with the cyclin E proteolytic pathway. Indeed, the main ligase for cyclin E, FBXW7/hCDC4 is frequently inactivated in a broad spectrum of human tumors, with concominant cyclin E deregulation [40, 131, 132]. This will the topic of section 2.4.6.

2.4.5. Regulators of FBXW7/hCDC4 activity

Given its critical function as a master regulator of various cell cycle regulatory proteins, it is not surprising that $SCF^{FBXW7/CDC4}$ activity is controlled by both positive and negative regulators. As mentioned, the prolyl isomerase Pin1 in complex with FBXW7/hCDC4-α promotes ubiquitylation of cyclin E1 by the $SCF^{FBXW7/hCDC4}$-γ ligase [119, 133]. Importantly, a mutation in FBXW7/hCDC4-α that abrogates binding to Pin1 was found to increase cyclin E1 stability, illustrating the requirement of Pin1 for cyclin E1 turnover [119]. As will be described in section 2.5.4., ubiquitylation of the Myc oncoprotein is also regulated by Pin1, as well as by the protein phosphatase PP2A [134]. A negative regulator of Myc and cyclin E1 ubiquitylation by the $SCF^{FBXW7/CDC4}$ ligase is the deubiquitylating enzyme USP28 (ubiquitin-specific protease 28) (also described in section 2.5.4.) [135]. It is not known whether USP28 regulates the ubiquitylation of other FBXW7/hCDC4 substrates, though it has been suggested.

2.4.6. FBXW7/hCDC4 in cancer

The fact FBXW7/hCDC4 is implicated in the degradation of several oncoproteins and is frequently inactivated by mutations and chromosomal rearrangements in many cancers supports its function as a general TSG. An extensive screen of primary human tumors recently reported mutations in tumors of diverse origin, including those of the stomach, breast, blood, colon, endometrium, lung, ovary, pancreas, bile duct, and prostate, with an overall mutation frequency of ~6% [136]. While mutations are found to occur throughout the coding region, more than half of the mutations are localized to six mutation hotspots corresponding to residues in the β-propeller structure of the WD40 repeats critical for substrate binding [40, 94, 95, 136]. Moreover, mutation of a single *FBXW7/CDC4* allele in mouse has been found to cooperate with inactivation of p53, suggesting that *FBXW7/CDC4* is a haploinsufficient tumor suppressor (i.e., loss of one copy is enough to cause disease) [137]. However, recent data rather support a function for dominant-negative mutations in the substrate-binding domain of *FBW7/hCDC4*. Dimerization of FBXW7/hCDC4 proteins through their conserved D-domains has also been suggested to promote ubiquitination activity, presumably by enhancing accessibility of the ligase to the different lysine residues on the substrate, resulting in more efficient polyubiquitylation [138-141]. Thus, an FBXW7/hCDC4 mutant that forms a complex with wild-type FBXW7/hCDC4 may act in a dominant-negative manner by limiting the ubiquitylation activity of the wild-type protein. Indeed, expression of a mutant form of FBXW7/hCDC4 in cells without *FBXW7/hCDC4* genetic alterations has been shown to repress substrate ubiquitylation [136, 142, 143].

FBXW7/hCDC4 inactivation by mechanisms other than mutations have also been reported. Methylation of the *FBXW7/hCDC4-β* gene promoter, which results in decreased expression, was found to be a frequent event in both breast cancer cell lines and primary tumors, demonstrating that promoter methylation is an alternative mechanism for the suppression of FBXW7/hCDC4 activity [144]. Finally, FBXW7/hCDC4 is negatively regulated by microRNAs. Two microRNAs, miR223 and miR27a, were recently implicated in cyclin E regulation through the

post-transcriptional repression of FBXW7/hCDC4 [145, 146]. Overexpression of these microRNAs results in decreased FBXW7/hCDC4 mRNA levels, with concomitant increases in cyclin E levels and stability, and DNA replication stress. Importantly, increased levels of miR27a expression, which correlated with low FBXW7/hCDC4 levels, were found in pediatric B-ALL [146].

To conclude, the plethora of mutations and other types of alterations of the *FBXW7/hCDC4* gene found in cancers highlights the importance of this tumor suppressor in human malignancy.

2.5. THE MYC ONCOPROTEIN

In the final part of this literature review, we will turn our attention to the Myc protein. Although, as we learned, Myc is also an important substrate for ubiquitin-mediated degradation by FBXW7/hCDC4, this will not be the main focus of the present section. Rather, we will concentrate on aspects of Myc function that are of relevance to Paper III in this thesis.

2.5.1. Can't do without Myc

The Myc family of proto-oncogenes (comprising c-Myc, N-Myc, and L-Myc) play important roles in the regulation of normal cellular processes such as cell proliferation, growth, metabolism, survival, and differentiation. First identified as transforming factors transduced by avian retroviruses, Myc genes encode transcription factors that regulate the expression of an estimated 10-15% of all mammalian genes. Since Myc controls many pathways with key roles in the hallmarks of cancer, it is hardly surprising to find that Myc is deregulated in most tumors. Indeed, alterations in expression or activity of the Myc oncogenes contribute to the genesis of a wide range of human cancers including hematopoietic malignancies as well as various solid tumors ([44] and reviewed in [147, 148]).

2.5.2. MAXimizing transcription by Myc

The Myc genes encode proteins of the basic region/helix-loop-helix/leucine zipper (bHLH/LZ) family that, upon heterodimerization with their obligate partner Max (Myc-associated protein X), bind DNA and activate gene expression. Myc:Max complexes specifically recognize a so-called E-box sequence (5'-CACGTG-3') in the promoters of target genes. Two main regions of the Myc protein are particularly important for Myc function. These include the amino-terminal transactivation domain (TAD) and the carboxy-terminal bHLH/LZ domain (Figure 3). The TAD region contains the conserved Myc boxes I and II (MBI and MBII), which are critical for cofactor binding, transactivation activity, cellular transformation, and virtually all Myc-dependent biological functions. Myc utilizes its helix-loop-helix domain to dimerize with Max, which results in the formation of a complex capable of binding DNA via the basic region. The bHLH/LZ is also essential for full transformation of primary and immortalized cells (reviewed in [147-149]).

Transcriptional activation by Myc is believed to occur through recruitment of a number of transcriptional cofactors involved in the modulation of chromatin structure including histone acetyl transferases (HATs), chromatin remodelling complexes, demethylases, and ubiquitin ligases. One such protein, TRRAP (transactivation/transformation associated protein), is recruited to Myc-regulated gene promoters through binding to the MBII domain of Myc. TRRAP is

the core subunit of the TIP60 and GCN5 histone acetyl transferase complexes, which are also recruited to promoters through association with TRRAP. Found in the TIP60 complex are two ATPases, TIP48 and TIP49, which are required for the function of several chromatin remodelling complexes. They bind the MBII region independently of TRRAP (reviewed in [150]). Furthermore, recruitment of the E3 ubiquitin ligase SCF^{SKP2} and proteasomal subunits have been shown to be required for transactivation of several Myc target genes [151]. Not all Myc cofactors, however, require MBII for binding to Myc. Examples of this are the histone acetyltransferases CREB-binding protein (CBP) and p300, which interact with the C-terminal region of Myc. While GCN5 and TIP60 preferentially acetylate histones, it is believed that CBP/p300 can also acetylate other substrates, including transcription factors, such as Myc itself [152, 153] and subunits of the RNA polymerase [154, 155]. The action of Myc-associated cofactors can therefore activate gene transcription by enhancing accessibility of chromatin for subsequent binding and activation by other transcription factors.

While Myc has been predominantly associated with gene activation, there is also evidence for its involvement in gene repression. Although the mechanisms of repression are not as well studied as those resulting in gene activation, the present model suggests that Myc:Max complexes are recruited to non-E-box binding sites through the interaction with other transcription factors, including Miz-1. Myc:Max dimers block transcriptional activation by Miz-1 both by blocking association between Miz-1 and p300, and by recruiting the histone methyltransferase DNMT3a (reviewed in [150, 156]).

The function of Myc as a transcription factor can be antagonized by competition with the Mad/Mnt family of transcriptional repressors. These families, composed of Mad1, Mxi1, Mad3, and Mad4; Mnt and Mga, are bHLH/LZ proteins that behave much like Myc in that they can readily heterodimerize with Max and bind DNA at E-box sequences. The Mad:Max or Mnt:Max complexes repress transcription by recruiting histone deacetylases via the adaptor protein Sin3. Given that Max is a stable and constitutively expressed protein suggests that regulation of transcription is dependent on the abundance of Max-associated transcription factors [156].

2.5.3. Myc's many targets

The number of genes that are regulated by Myc is unusually large compared to other transcription factors. Myc activities regulate the expression of genes that cover a broad range of biological functions, although some gene pathways seem to be overrepresented. Myc-regulated genes, including protein coding genes as well as functional RNAs, are transcribed by all three RNA polymerases. Myc regulates *cell growth* by supplying the cell with a variety of building blocks. It activates the production of components important in protein biosynthesis, including ribosomal RNAs and proteins, as well as RNA processing and translation factors. Myc has also been shown to stimulate transcription of genes involved in metabolism and mitochondrial biogenesis, leading to increased energy production. Further, Myc regulates *cell cycle* progression by transactivating several genes, including cyclins and CDKs, while inhibiting the expression of genes that attenuate cell cycle progression, including cyclin-

dependent kinase inhibitors (CKIs), such as $p21^{CIP1}$. Other genes that are commonly down-regulated by Myc include those that promote differentiation, inhibit signal transduction pathways and reduce cell adhesion and cell-cell communication (Reviewed in [147]). Thus, the ability of Myc to stimulate cell growth and proliferation, as well as to inhibit cell cycle arrest, makes Myc a very powerful gene that, when deregulated, contributes to the limitless proliferative potential characteristic of cancer cells.

Paradoxically, Myc's ability to induce cell proliferation is limited by the potential of Myc to induce apoptosis. Although the mechanisms of Myc-induced cell death are not exactly known, two major pathways have been suggested (reviewed in [157, 158]). The first involves the induction of $p14^{ARF}$, which stabilizes p53 by sequestering the E3 ligase Mdm2 which is responsible for p53 degradation [159, 160]. When stabilized, p53 can activate proapoptotic signals and induce cell death. The second mechanism involves the induction of the pro-apoptotic protein Bim and simultaneous repression of anti-apoptotic signals, contributing to the release of cytochrome *c* from the mitochondria [161, 162]. This implies that apoptosis is a major tumor barrier that needs to be overcome for Myc-induced transformation. Indeed, loss of pro-apoptotic genes including $p14^{ARF}$, p53, or BIM, or overexpression of anti-apoptotic genes facilitates myc-induced tumorigenesis [160, 163, 164]. However, it has been recently shown that, in normal cells, significantly higher levels of Myc are required for inducing apoptosis than are needed for driving proliferation, thus allowing Myc's normal activities to proceed without resulting in cell death [165].

Finally, Myc has also been reported to both suppress and promote cellular senescence, an irreversible arrest of proliferation [148]. Myc is able to repress senescence and contribute to immortalization of primary cells by, for instance, promoting expression of *hTERT*, and inhibiting expression of important senescence-promoting CKIs *p21* and *p16* [166, 167]. On the other hand, Myc can also promote senescence under conditions where protective factors such as WRN and CDK2 are defective [168].

2.5.4. Regulation of the Myc protein: living a short sweet life

The widespread binding of Myc to DNA and its involvement in many important cellular functions would give the impression that Myc is a very abundant protein. However, this is not the case. Myc is turned over at a very high rate via the ubiquitin-proteasome system, involving at least four ubiquitin ligases, to date: SCF^{SKP2}, $SCF^{FBXW7/hCDC4}$, HectH9/Huwe1/Mule, and TRUSS. Moreover, the $SCF^{\beta\text{-}TrCP}$ complex and the ubiquitin-specific protease USP28, have been shown to antagonize SCF^{Fbxw7}–mediated Myc turnover. Considerable effort has been made towards understanding whether an extended half-life of Myc protein, stemming from inefficient protein turnover, lies behind the increased Myc protein commonly observed in cancers. Let us take a closer look at each of these factors, in particular how they alter Myc protein stability and their involvement in cancer.

SCF^{SKP2}: SKP2 is a proto-oncogene overexpressed in different cancers [169]. It promotes the degradation of several negative regulators of the cell cycle,

including the cyclin dependent kinase inhibitor $p27^{Kip1}$, a tumor suppressor which is frequently inactivated in cancer cells [28] (for a review see [170]). SKP2 was the first identified E3 ubiquitin ligase to target Myc for ubiquitin-mediated degradation. However, SKP2 also promotes transactivation of Myc target genes, resulting in S-phase transition; SKP2 binds to Myc through the MBII and HLH/LZ regions and is considered to be a coactivator of Myc:Max transcriptional complexes [151, 171]. Interestingly, it has been demonstrated that subunits of the proteasome are recruited, along with SKP2, to Myc target gene promoters, implying an important relationship between Myc activation and the UPS [151, 171].

$SCF^{FBXW7/hCDC4}$: The $SCF^{FBXW7/hCDC4}$ is considered to be a major regulator of Myc protein turnover, and has so far only been associated with the negative regulation of Myc function. Targeted destruction of Myc by FBXW7/hCDC4 is a complex process that depends on GSK3-β-mediated phosphorylation at Thr58 in the MBI region. GSK3-β phosphorylation of Myc is primed by ERK-mediated Ser62 phosphorylation and also requires the activity of the *cis-trans* prolyl isomerase Pin1. Interestingly, the action of Pin1 has been reported to be required for recruitment of the protein phosphatase 2A (PP2A) and subsequent dephosphorylation of the stabilizing Ser62 phosphate in Myc. Remarkably, it is the nucleolar FBXW7/hCDC4-γ isoform that finally targets Myc for ubiquitylation and proteasomal degradation [99, 100, 134, 172, 173]. Several mechanisms can render Myc resistant to degradation by $SCF^{FBXW7/hCDC4}$ in human cancers; for instance, point mutations at Thr58 of Myc have been found in lymphomas [174], and mutational inactivation of FBXW7/hCDC4 in several cancers [175] (discussed in section 2.4.6.).

HectH9/Huwe1/Mule: The Hect-domain ubiquitin ligase HectH9 has been shown to promote transcriptional activation of c-Myc through lysine 63 (K63)-linked ubiquitylation. Adhikary *et al.* demonstrated that HectH9-mediated ubiquitylation did not result in proteasomal degradation of Myc protein, but instead promoted transactivation of Myc target genes through recruitment of the coactivator p300 [176]. It is worth noting that a truncated version of HectH9, lacking the first 2472 amino acids, was used in this study.

Interestingly, an independent study showed that HectH9 (referred to as Huwe1) induced neural differentiation and proliferation arrest by the proteasomal-mediated destruction of N-Myc through K48-linked polyubiquitylation [177]. In this study, HectH9 was able to bind and ubiquitylate both N-Myc and c-Myc in a K48-linked fashion, albeit to different extents, suggesting that c-Myc is also a substrate for degradation by HectH9.

Whether HectH9 is a potent activator [176], or even a repressor of Myc [177], remains to be determined. HECT-domain containing E3 ligases are thought to form only homogeneous ubiquitin chains on their substrates (i.e., either K48- or K63-linked chains only) [178]. In fact, HectH9 has been shown to catalyze ubiquitylation of a number of substrates, including p53 [179], Cdc6 [180], Histone H2A [181], Mcl-1 [182], Miz-1 [183], as well as N-Myc and c-Myc described above, through the formation of K48-linked ubiquitin chains. This is an important issue that yet needs to be further clarified.

TRUSS/TRPC2AF: The tumor necrosis factor receptor-associated ubiquitous scaffolding and signalling protein (TRUSS) is a receptor of the DDB1-CUL4A E3 ubiquitin ligase complex , which belongs to the family of the cullin-RING finger ligase complexes [184]. TRUSS was recently shown to mediate ubiquitylation of both c-Myc and N-Myc and repress Myc-dependent transcription and cellular transformation. Expression analyses in cancer cell lines demonstrated that TRUSS is downregulated in certain cell lines, suggesting that TRUSS-mediated Myc degradation may be impaired in some cancer cells [185].

SCF$^{\beta\text{-}TrCP}$: The SCF$^{\beta\text{-}TrCP}$ ubiquitin ligase is one of the most well-studied SCF E3 ubiquitin ligases. β-TrCP (FBXW1) plays important roles in diverse cellular pathways and a wealth of evidence indicates that β-TrCP is mainly oncogenic (Review [86]). Its numerous targets can be mainly divided into two groups: cell cycle regulators and pro-apoptotic regulators. Interestingly, a recent report showed that β-TrCP stabilizes Myc during the S and G2 phases of the cell cycle, by assembling heterotypic ubiquitin chains that carry different lysine linkages in the amino-terminus of Myc, possibly around the same region important for FBXW7/hCDC4 targeting of Myc. In this manner, β-TrCP may antagonize ubiquitylation and degradation of Myc by the SCF$^{FBXW7/hCDC4}$ ligase [186].

USP28: As mentioned, ubiquitin-specific proteases counteract the activity of ubiquitin ligases. USP28 was first identified in an shRNA library screen for genes that regulate Myc function. USP28 is an interacting partner of FBXW7/hCDC4 and was shown to prevent FBXW7/hCDC4-mediated Myc ubiquitylation, thereby stabilizing Myc protein in the nucleus [135]. This mechanism was further proven important in the cellular response to DNA damage where USP28 was shown to dissociate from FBXW7α upon UV irradiation, thus allowing degradation of Myc [187].

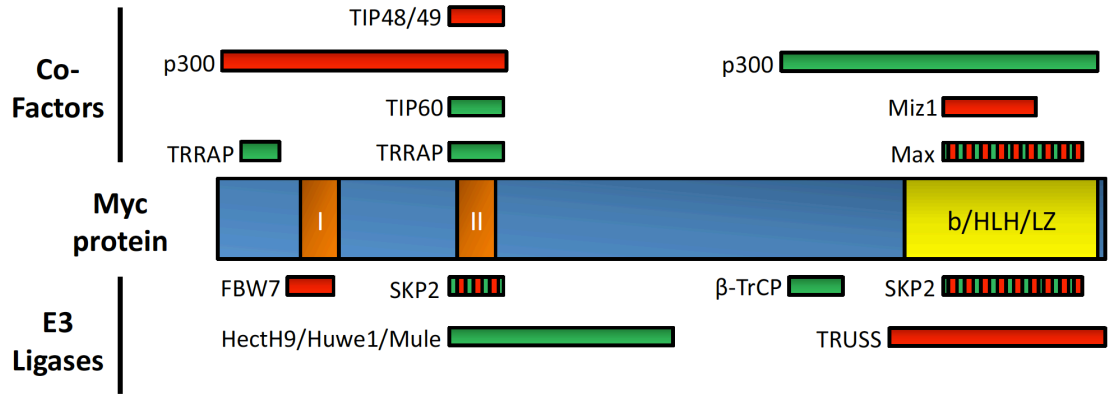

Figure 3. Schematic representation of the functional domains of the Myc protein, and regions that mediate binding of the different Myc co-factors and E3 ligases. If the interaction results in Myc transcriptional repression, the domain is shown as a red bar, if it results in Myc activation, it is shown in green, and if it mediates both activation and repression, the domain is indicated by a dashed bar. I=MBI, II=MBII.

The fact that Myc is tightly regulated by ubiquitin-dependent pathways reflects the importance of these processes in the control of Myc function. *Paper III deals with the discovery of SCF^{FBXO28}, a novel E3 ubiquitin ligase that regulates cellular proliferation and Myc transactivation.*

2.5.5. Myc-induced transformation: too much of a good thing

As outlined above, Myc regulates a myriad of pathways that contribute to neoplasia. However, it should be noted that, like other oncogenes, Myc alone fails to transform normal cells and additional mutations in other oncogenic pathways, for example the Ras pathway, cooperate with Myc in cellular transformation (reviewed in [150]). As mentioned, deregulation of Myc activities can have devastating consequences to the cell, resulting in an enlarged population of highly proliferating, self-renewing cells that are prone to acquiring further oncogenic changes to ultimately promote tumor formation. By modifying the expression of a large number of target genes, Myc activation increases the cell's capacity to grow and proliferate, promoting migration and angiogenesis, while inhibiting terminal differentiation and senescence. Accordingly, increased Myc expression and/or activity is most likely a requirement for tumor development, and from this we could speculate that many other genes involved in regulation of Myc activity must also be deregulated in cancer cells.

Given the crucial function of Myc in multiple biological pathways, it is clear that various fail-safe mechanisms must keep Myc activities in check to prevent Myc from inducing cellular transformation. As mentioned, Myc has the ability to induce apoptosis under conditions of cellular stress or limited survival factors [188, 189]. However, in Myc-driven tumors, certain mutations abrogate Myc induced apoptosis. Common mutations affect the function of the tumor suppressor genes p53 and $p14^{ARF}$. However, mutations in Myc itself have also been shown to be directly associated with decreased apoptotic potential of Myc. One example is the mutation of Thr58 in the transactivation domain (MBI) of Myc found in Burkitt's lymphomas. Since phosphorylation at Thr58 is essential for proteasomal degradation of Myc, mutation at this site could result in Myc accumulation, which can contribute to tumorigenesis [174, 190]. However, a recent study demonstrated that Thr58 mutation also abolished the ability of Myc to induce the pro-apoptotic gene Bim in the absence of p53 or $p14^{ARF}$ mutations [191]. Therefore, either mutations of p53 and $p14^{ARF}$ in combination with wild-type Myc, or mutations of Myc that fail to transactivate Bim, are seemingly sufficient for escaping apoptosis and inducing tumorigenesis.

3. AIMS OF THE THESIS

The overall purpose of this thesis was to explore the function of two F-box proteins, FBW7 and FBXO28, in ubiquitylation and degradation of the cell cycle regulatory proteins, cyclin E and Myc, and their potential deregulation in cancer.

More specifically, the aims of the studies presented here were:

I. To investigate the requirement for the FBXW7/hCDC4α and FBXW7/hCDC4γ isoforms in the *in vivo* regulation of ubiquitin-mediated degradation of cyclin E1.

II. To characterize the involvement of the $SCF^{FBXW7/hCDC4}$ ligase in the ubiquitin-dependent turnover of the cyclin E2 protein.

III. To identify and elucidate the roles of the novel ubiquitin ligase, SCF^{FBXO28}, in cell proliferation and myc-dependent transcriptional activity.

IV. To study the phosphorylation of the FBXO28 protein and its potential prognostic significance in breast cancer survival.

4. RESULTS AND DISCUSSION

PAPER I.

Both $SCF^{Cdc4\alpha}$ and $SCF^{Cdc4\gamma}$ are required for cyclin E turnover in cell lines that do not overexpress cyclin E

In this study, we have followed up on a previous study from our group and collaborators. The original observation that both the nuclear isoforms of FBXW7/hCDC4, hCDC4α and hCDC4γ, are required for cyclin E1 turnover indicated that the different isoforms collaborate in cyclin E1 ubiquitylation [119]. *In vitro* ubiquitylation analysis showed that the role of hCDC4α in cyclin E1 degradation, rather then ubiquitylation, is to serve as a cofactor for isomerization catalyzed by the prolyl isomerase Pin1 of a non-cannonical proline-proline bond in the cyclin E1 phosphodegron. This step was also demonstrated to be essential for the subsequent multiubiquitylation of cyclin E1 by the hCDC4γ isoform.

In this study, we asked whether this two-step mechanism and requirement for two hCDC4 isoforms is general in cells. To test this, we carried out RNAi-mediated silencing experiments targeting each individual hCDC4 isoform in a panel of human cell lines: 2 immortalized normal cell lines, five tumor-derived cell lines, as well as the transformed 293A cell line. Using a combination of Western blotting and quantitative immuofluorescence analyses, we observed accumulation of cyclin E1 protein upon hCDC4α-specific siRNA depletion in all cell lines analyzed. However, when hCDC4γ was siRNA depleted, cyclin E1 was elevated in only a subset of these cell lines, including normal and non-transformed cells. Increased cyclin E1 levels were shown to be due to decreased turnover of cyclin E1 protein, rather than to differences in isoform-specific mRNA expression.

Since cyclin E1 levels are abnormally elevated in many cancer cell lines, we hypothesized that the differences in isoform requirement could be explained by the relative expression cyclin E1. Indeed, when comparing cyclin E1 protein levels in different cell lines, it became apparent that the cell lines where depletion of either hCDC4 isoform (hCDC4α or hCDC4γ) stabilized cyclin E1 protein, expressed relatively low levels of cyclin E1. In contrast, cell lines with high levels of cyclin E1 were only responsive to hCDC4α siRNA depletion. To test if high levels of cyclin E1 can bypass the requirement for hCDC4γ, we overexpressed cyclin E1, while simultaneously silencing each hCDC4 isoform, in cells that require both hCDC4α and hCDC4γ for cyclin E1 turnover. Indeed, cyclin E1 levels were no longer responsive to silencing of hCDC4γ, suggesting that the normal cyclin E1 degradation pathway is not employed when cyclin E1 levels are abnormally high. Because overexpression of cyclin E1 abrogates the requirement

for hCDC4γ, we propose that hCDC4α targets cyclin E1 for ubiquitylation via a low affinity phosphodegron (pThr62) that does not require prolyl isomerization, thereby bypassing the requirement for Pin 1 activity and hCDC4γ.

In summary, based on the proposed mechanism for the ubiquitylation of cyclin E1 by the sequential actions of the $SCF^{hCDC4\alpha}$ and $SCF^{hCDC4\gamma}$ complexes, in this paper we show that this model is general in cells where cyclin E1 protein levels are not elevated. However, under conditions where cyclin E1 is overexpressed, $SCF^{hCDC4\alpha}$ is partly sufficient for turnover of cyclin E1 protein.

PAPER II.

$SCF^{Fbxw7/hCdc4}$ targets cyclin E2 for ubiquitin-dependent proteolysis

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Cyclin E2 is, like cyclin E1, often over-expressed in human tumors, and this alteration is an independent prognostic factor for poor patient prognosis [128, 192]. Both E-type cyclins display similar expression, peaking during late G1 and progressively decreasing during S-phase, due to a combination of transcriptional and, in the case of cyclin E1, targeted degradation by $SCF^{FBXW7/hCDC4}$ [39, 40, 103]. Given the similarity between cyclin E1 and cyclin E2, in this study we aimed to investigate whether cyclin E2 is a novel $SCF^{FBXW7/hCDC4}$ substrate.

To determine if FBXW7/hCDC4 regulates the turnover of endogenous cyclin E2 protein, we performed *in vivo* cycloheximide chase analysis and ubiquitylation experiments using RNAi-mediated depletion or ectopic expression of FBXW7/hCDC4, respectively. Whereas cyclin E2 protein was stabilized in cells where FBXW7/hCDC4 was depleted, overexpression of FBXW7/hCDC4 significantly increased ubiquitin-conjugated cyclin E2. These results were confirmed *in vitro* using recombinant cyclin E2 and $SCF^{FBXW7/hCDC4}$ complexes, indicating that cyclin E2 is directly targeted for ubiquitylation by the $SCF^{FBXW7/hCDC4}$ ubiquitin ligase. To explore this in more detail, we investigated whether cyclin E2 possesses a CDC4-phosphodegron (CPD). Analogous to cyclin E1, we found two putative CPDs, one high-affinity degron and one low-affinity degron in cyclin E2, which correspond to the consensus CPDs found in cyclin E1. Mutation of these putative phosphorylated residues increased the stability of cyclin E2 *in vivo*, likely abrogating ubiquitylation by $SCF^{FBXW7/hCDC4}$.

A surprising finding in this study was the apparent involvement of cyclin E1 in the ubiquitin-dependent degradation of cyclin E2. Whereas knockdown of cyclin E1 caused an increase in the steady-state levels of cyclin E2, ectopic expression of cyclin E1 stimulated cyclin E2 degradation and ubiquitylation by $SCF^{FBXW7/hCDC4}$ *in vivo*. This would imply that accumulation of cyclin E2 would require that cyclin E1 levels are low. Indeed, analysis of the expression profile of cyclin E2 protein during the cell cycle demonstrated that expression of cyclin E2 peaked slightly later than cyclin E1, and persisted into mitosis, suggesting that cyclin E2 displays different windows of expression and degradation as compared to cyclin E1.

The putative role of cyclin E1 in the regulation of cyclin E2 degradation could be mediated through phosphorylation of cyclin E2 at CPDs by the cyclin E1-CDK2 complex, stimulating binding and ubiquitylation by FBXW7/hCDC4. Our studies also indicate that cyclin E1 may compete with cyclin E2 for binding to FBXW7/hCDC4 during G1 and early S-phase. Thus, we propose that by regulating the abundance of cyclin E2, cyclin E1 ensures prolonged cyclin E2-CDK2 activities, allowing it to perform non-redundant functions in cell cycle control. However, further studies will be required to address the validity of these molecular mechanisms.

To summarize, in this paper we report that $SCF^{FBXW7/hCDC4}$ targets cyclin E2 for ubiquitin-dependent proteolysis. Since FBXW7/hCDC4 is often inactivated in several human cancers, alteration of this pathway could contribute to the deregulation of both E-type cyclins, further promoting tumorigenesis.

PAPER III.

The ubiquitin ligase SCF^{FBXO28} regulates Myc-driven transcription through non-proteolytic ubiquitylation and is required for cell proliferation

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Given that, to date, the majority of F-box proteins have remained uncharacterized, at the beginning of this study we aimed to identify new F-box genes with potential roles in regulation of cell proliferation. Initially, we employed a functional RNAi screen using an siRNA library targeting the entire family of human F-box genes, and assessed for effects on cell proliferation in four different tumor-derived cell lines by immunofluorescence microscopy. From these analyses, several candidate F-box genes were identified. These results were subsequently validated in a genome-wide screen that included 53 F-box genes. Interestingly, in both screens FBXO28 was one of the top candidates affecting cell proliferation and was thus chosen for further in-depth functional analysis.

Examination of the amino acid sequence revealed that FBXO28 is highly conserved in various species, supporting a critical biological function of this F-box protein. FBXO28 contains an F-box motif capable of co-precipitating with SCF core components SKP1 and CUL1, demonstrating that FBXO28 is part of an SCF E3 ubiquitin ligase. To identify pathways that might be regulated by FBXO28, we carried out microarray expression analyses of HCT116 cells transfected with FBXO28 siRNAs. FBXO28 depletion resulted in changes in the expression of multiple genes involved in important biological processes including cell cycle control and metabolism. Interestingly, detailed analyses of the gene expression data revealed that, among others, a considerable number of Myc target genes were significantly affected.

To further investigate a potential relationship between FBXO28 and Myc, we assessed for the ability of these two proteins to interact *in vivo* using

immunoprecipitation assays. We could establish that both wild-type FBXO28 and an F-box deletion mutant, ΔF-FBXO28 (which can not associate with SCF core components) associate with Myc, implying a role for FBXO28 in the regulation of Myc function. Ectopic expression of FBXO28 was found to promote conjugation of ubiquitin chains on Myc, whereas expression of ΔF-FBXO28 or silencing of FBXO28 abolished Myc ubiquitylation. Interestingly, examination of Myc protein stability using cycloheximide chases showed that FBXO28 does not affect Myc turnover. Since ubiquitin modification of proteins involved in transcription has been demonstrated to regulate their activities, we then speculated that ubiquitylation of Myc by FBXO28 may control Myc-dependent gene transactivation.

To this end, we performed chromatin immunoprecipitation assays (ChIP) to investigate whether FBXO28 was associated with Myc target promoters. Endogenous binding of FBXO28 was detected, together with the Myc:Max complex at the E-box regions of several known Myc target gene promoters, indicating that FBXO28 regulates Myc activity at these promoters. In line with these data, aberrant expression of FBXO28 affected the association of Myc cofactors at these promoters. Together, our results indicate that SCF^{FBXO28}-mediated ubiquitylation of Myc is important for the binding of cofactors to Myc target gene promoters.

This is the first functional study of the previously uncharacterized F-box protein, FBXO28. In summary, we demonstrate that FBXO28 is a regulator of the Myc:Max transcriptional complex, controlling Myc transactivation by regulating the availability of cofactors at Myc target genes in an ubiquitin-dependent but proteolysis-independent manner.

PAPER IV.

Phosphorylation of the F-box protein FBXO28 is associated with poor prognosis in patients with primary breast cancer

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In Paper III we showed that FBXO28 regulates cell proliferation and Myc transcriptional activation. To gain further insight into the biological functions of FBXO28, we set out to investigate whether FBXO28 itself is regulated, for instance by post-translational modifications. Immunopurification of FBXO28, followed by mass spectrometric analysis, identified specific phosphorylation of FBXO28 at serine 344 (S344). Additional experiments using phosphospecific FBXO28 antibodies showed that pS344-FBXO28 resides in the nucleus and is phosphorylated in a cell-cycle regulated fashion by the CDKs. Efficient phosphorylation of FBXO28 *in vitro* was observed with cyclin A-CDK2 and cyclin B-CDK1, but not cyclin E-CDK2, arguing that FBXO28 is a novel CDK1/2 substrate.

Given the previous findings that FBXO28 is critical for cellular proliferation, we next sought to investigate the role of FBXO28 phosphorylation

on proliferation. Ectopic expression of a phosphorylation-deficient mutant of FBXO28 (S344A-FBXO28) in tumor cell lines attenuated cell proliferation and, importantly, reduced the transforming potential of Myc in p53-/- MEFs. These results indicated that CDK phosphorylation of FBXO28 at Ser344 has an important role in cellular proliferation and possibly in Myc-induced transformation.

Encouraged by these observations, we set out to examine if FBXO28 phosphorylation has clinical significance using immunohistochemistry on tissue microarrays (TMA). A panel of 144 primary breast cancers was analyzed for FBXO28 phosphorylation using the pS344-FBXO28 antibody. The vast majority of the tumors displayed strong nuclear intensity. However, the nuclear fraction (NF) of pS344-FBXO28 was found to vary significantly between the different tumors. Indeed, a high NF of pS344-FBXO28 correlated with clinicopathological parameters associated with several established markers of poor patient outcome, including tumor size, high grade and ER-negative status. Importantly, a strong association between high NF of pS344-FBXO28 and overall survival (OS), breast cancer specific survival (BCSS) and recurrence-free survival (RFS) were found, and multivariate analyses further established that a high NF of pS344-FBXO28 as an independent predictor of poor OS in breast cancer.

Additional analysis assessing pS344-FBXO28 in specific breast cancer subtypes revealed that a high NF of pS344-FBXO28 was significantly associated with decreased BCSS in the low proliferative luminal A tumor subtype, suggesting that FBXO28 phosphorylation is not just a marker for highly proliferative luminal B tumors. Importantly, the prognostic impact of phosphorylated FBXO28 was also revealed when FBXO28 phosphorylation was analyzed in patients with moderate-grade tumors, where a high NF of pS344-FBXO28 predicted poor OS.

From this study we conclude that the nuclear fraction of FBXO28 phosphorylation is an independent prognostic factor for poor survival in breast cancer. It will be important to confirm these findings in an independent patient cohort and to investigate whether FBXO28 phosphorylation can be a valuable clinical tool as a prognostic biomarker in breast cancer, as well as other types of cancer. In the future, it will also be important to define the role of FBXO28 phosphorylation in Myc-induced transcription and cellular transformation.

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7. ORIGINAL PUBLICATIONS AND MANUSCRIPTS