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The role of the tumor suppressor gene, FBW7, and mechanisms of its inactivation in cancer

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To my beloved Shirin and Atrin
To my parents

ABSTRACT

The F-box protein FBW7 is a tumor suppressor and SCF ubiquitin ligase targeting several key oncoproteins for proteasomal degradation. In this thesis we addressed whether the *FBW7* gene is inactivated by mutations in various human tumor types and explored alternative mechanism(s) for the inactivation of *FBW7*. We also explored how inactivation of FBW7 relates to substrate degradation (including cyclin E, Notch1 and c-Myc) and its potential prognostic significance. Furthermore, we have investigated novel regulatory mechanism(s) for *FBW7* expression and activity.

Our results demonstrate that *FBW7* is a general TSG, which is frequently inactivated by mutations (with an average mutation frequency of 6%) in various malignancies. Heterozygous missense mutations altering specific arginines residues required for efficient substrate interaction are the most frequent mutations in FBW7. Our functional analysis indicates that heterozygous mutations might act in a dominant-negative manner. The highest mutation frequency was observed in cholangiocarcinomas and pediatric T-cell acute lymphocytic leukemias (T-ALL) (35 and 31 %, respectively). We also found that *FBW7* gene mutation is an infrequent event in several malignancies, including breast cancer and pediatric B-ALL. This finding prompted us to investigate whether alternative mechanism for regulation and inactivation of *FBW7* occurs in cancer, including promoter hypermethylation and miRNA induced repression. Our results demonstrate that both the 5'-UTR and the 3'-UTR of *FBW7* is epigenetically regulated. The promoter of *FBW7-beta* is frequently hypermethylated in primary breast tumors and its inactivation is associated with improved survival in certain patient subgroups. Similarly, mutational inactivation of *FBW7* and/or *NOTCH1* in T-ALL is also associated with increased overall survival. Analyses focusing on the 3'UTR of *FBW7* revealed that FBW7 expression is regulated by miR-27a, a putative oncogenic miRNA. miR-27a was identified as a critical repressor of FBW7 expression during cell cycle progression with potential consequences for FBW7-mediated turnover of cyclin E. These results indicate that miR-27a serves an important cell cycle regulatory function by repressing FBW7 and at specific cell cycle stages, but releasing it from repression during the G1 to S-phase transition.

In summary, our findings demonstrate that *FBW7* is inactivated by several different

mechanisms, including mutation, deletion, promoter methylation and possibly miRNA-induced repression of gene expression. Our pre-clinical analysis further suggests that inactivation of *FBW7* in certain malignancies might be associated with improved survival, thus implicating *FBW7* as a potential prognostic predictor in the some cancers.

LIST OF PUBLICATIONS

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

Paper I:
Malyukova A, Dohda T, von der Lehr N, **Akhoondi S**, Corcoran M, Heyman M, Spruck C, Grandér D, Lendahl U, Sangfelt O., The Tumor Suppressor Gene hCDC4 Is Frequently Mutated in Human T-Cell Acute Lymphoblastic Leukemia with Functional Consequences for Notch Signaling, Cancer Res. 2007 Jun 15;67(12):5611-6.

Paper II:
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 SZ, Nayer BN, Zali MR, Hansson J, Egyhazi S, Petersson F, Sangfelt P, Nordgren H, Grander D, Reed SI, Widschwendter M, Sangfelt O, Spruck C., FBW7is a general tumor suppressor in human cancer. Cancer Res. 2007 Oct 1;67(19):9006-12.

Paper III:
Akhoondi S, Lindström L, Widschwendter M, Corcoran M, Bergh J, Spruck C, Grandér D, Sangfelt O., Inactivation of FBXW7/hCDC4-beta expression by promoter hypermethylation is associated with favorable prognosis in primary breast cancer. Breast Cancer Res. 2010;12(6):R105.

Paper IV:
Lerner M[1st], Lundgren J[1st], **Akhoondi S**[2nd], Jahn A, Ng H. F., 2, Vrielink J, Agami R, Grandér D and Sangfelt O., miRNA-27a controls FBW7-dependent Cyclin E degradation and cell cycle progression. Cell cycle 2011.

Table of Contents

1 LIST OF ABBREVIATIONS

Ago	argonaute AMFR
APC	anaphase-promoting complex
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3 related
B-ALL	B-cell Acute Lymphoblastic Leukemia
BRCA	breast cancer
bTRCP	Beta Transducin Repeat Containing Protein
Cdk	Cyclin-dependent kinase
CPD	Cdc4 phosphodegron
CRL	Cullin-RING E3 ubiquitin-Ligase
CSL	CBF1 Suppressor of Hairless-Lag1
CSN	COP9 signalosome
Cul1	cullin1
DD	dimerization domain
DNA	deoxyribonucleic acid
DUB	deubiquitylating enzyme
E6AP	E6-associated protein
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
Far1	fatty acyl CoA reductase 1
FBXW7	F-box and WD-40 domain protein 7
GSK	glycogen synthase kinase
hCdc4	human cell division cycle 4
HECT	homologous to the E6-AP carboxyl terminus
ICD	intracellular domain
LOH	loss of heterozygosity
LRR	Leucine rich repeat
MAM	mastermind-like
MCL1	myeloid cell leukemia sequence 1
Mdm2	mouse double minute 2
miRNA	microRNA
mRNA	messenger RNA
Myc	myelocytomatosis
NICD	Notch intracellular domain
ORC	origin of replication complex
PARP	Poly ADP-ribose polymerase

PEST	proline (P), glutamic acid (E), serine (S), and threonine (T)
PI3K	Phosphatidylinositol 3-kinases
Pop1, 2	processing of precursor 1, 2
PP2A	protein phosphatase 2 A
PTEN	phosphatase and tensin homolog
RAS	rat sarcoma viral oncogene homolog
RING	really interesting new gene
RNA	ribonucleic acid
RNAi	RNA interference
SCF	Skp1/Cul1/F-box
Ser	serine
Sic1	stoichiometric inhibitor of Cdk1-Clb
Skp1	S-phase kinase-associated protein 1
Skp2	S-phase kinase-associated protein 2
T-ALL	T-cell Acute Lymphoblastic Leukemia
TGF	transforming growth factor
Thr	threonine
TM	transmembrane
TP53	tumor protein p53
TSG	Tumor suppressor gene
Ub	ubiquitin
Ubc	ubiquitin conjugating enzyme
UBD	ubiquitin binding domain
UPS	ubiquitin proteasome system
USP28	ubiquitin specific peptidase 28
UTR	untranslated region
WNT	wingless

2 INTRODUCTION

This thesis touches upon various concepts in cell biology including tumor suppressor genes, oncogenes, gene regulatory mechanisms such as DNA methylation and microRNA expression, phosphorylation, ubiquitylation and some other aspects of processes that are dysregulated in cancer. The reason is that *FBW7*, a gene originally shown to be a tumor suppressor through its negative effect on proteins promoting proliferation, is now known to be a multifunctional gene involved in many different biological processes. Since it became apparent that FBW7 is a master regulator of many well-known oncoproteins, it was important to determine how it may be regulated and how it is functionally inactivated in tumors. The studies in this thesis show that the FBW7gene is in fact inactivated via various mechanisms in different human tumors. Although is not fully understood how *FBW7* gene inactivation contributes to cancer development and progression, it is the beginning of the way that will eventually lead to better understanding of the role of *FBW7* in tumorigenesis and treatment of cancer.

2.1 CANCER

2.1.1 History of cancer

The oldest documented case of cancer was recorded on a papyrus from ancient Egypt, in 1500 b.c. However, Hippocrates, the great Greek physician (460-370 B.C), who is referred to as the father of medicine is thought to be the first person who named ¨cancer¨. Hippocrates used the name karkinos (the Greek name for crab, in English carcinos or carcinomas) to describe a skin wound that he thought of as a particular disease. His writings contain detailed descriptions of different types of cancers involving various sites in the body [1].

2.1.2 Development of cancer

In ancient Egypt, it was believed that cancer was caused by the Gods. Hippocrates believed that an excess of black bile in any given site in the body caused cancer and it was also the general thought for the cause of cancer in the next 1400 years. Despite stunning progress in our understanding of the cells that build up the human body

during the last four decades, it is not yet possible to exactly define cancer in a way that covers all its characteristics. In the simplest version, cancer has been defined as increased proliferation and survival of cells. A normal cell knows when and to what extent to grow and divide or maybe die. Normal cells are also responsive to the negative regulatory signals that they receive from the external environment. A cancer cell however, is insensitive to these signals. Cancer cells have achieved the capacity to escape from many different kinds of controls, which have elegantly been summarized by Hanahan and Weinberg in " The Hallmarks of Cancer" [2, 3]. Today we know that the development of cancer is a multistep process, and as recently described, there are 10 hallmarks of cancer [3] that collectively describe a malignancy (Figure 1). These capabilities namely, self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis are shared by most, and perhaps all, types of human cancers. Hopefully, knowing these underlying mechanisms of tumor development will facilitate the discovery of novel treatment strategies for the clinic. Many of the anti-cancer drugs that have been generated during the last decades target one or multiple of these characteristic hallmarks.

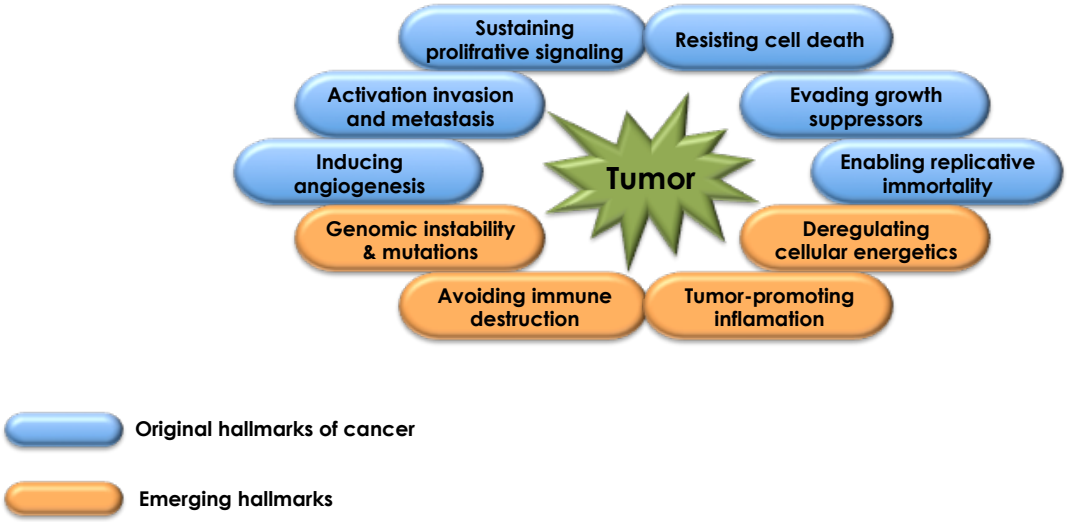


Figure 1. Hallmarks of cancer

Globally, there are approximately 7.4 million cancer deaths annually. Cancer is a leading cause of death worldwide and it accounts for approximately 13% of deaths from all causes. Encouragingly, during recent years, cancer mortality has stabilized in

many countries. Part of this success may be attributed to the development of new cancer agents, collectively called 'targeted therapies' [4, 5].

2.1.3 Oncogenes

The discovery of the double helical DNA structure in 1953 by James Watson and Francis Crick was a revolution in biology since it explained DNA's potential for replication and information encoding [6]. Simultaneous development of techniques such as DNA hybridization suddenly enabled scientists to address many key issues in cancer biology. Although foreboded by earlier studies such as those of chromosome abnormalities, hereditary cancer and the involvement of mutagens in tumorigenesis, modern molecular biology enabled scientists to define the involvement of specific mutations in cancer development. This led to the breakthroughs in the 1970s and early 80s, when oncogenes and tumor suppressor genes were discovered. An oncogene is a gene that when mutated into an activated form has the potential to cause cancer. Oncogenes encode proteins that are involved in processes such as cell proliferation and apoptosis [7]. The products of oncogenes act at multiple levels in the cell and can be transcription factors [8], chromatin remodelers [9-11], growth factors [12], growth factor receptors [13], signal transducers [14] and apoptosis regulators [15, 16].

2.1.3.1 Mechanisms of activation

A proto-oncogene is a normal gene converted into an oncogene if altered and activated. The resultant protein is called oncoprotein [17, 18]. Activation mechanisms of oncogenes can be categorized into three main classes: 1) Chromosomal rearrangements, 2) Mutations and 3) Gene amplification, all of which confer a growth advantage or increased survival of cells carrying such alterations [19]. All three mechanisms cause either structural alterations or an increased, deregulated expression of the oncoprotein [7, 20]. Examples of proto-oncogenes frequently dysregulated in cancer development are RAS, WNT and MYC. The expression of oncogenes can also be dysregulated by other processes such as epi-genetic alterations as well as microRNAs (miRNAs). The latter are a newly discovered group of small regulatory RNAs, that control gene expression through either translational repression or mRNA degradation [21]. Dysregulation of such miRNAs can thus lead to the subsequent activation of the oncogene [22].

2.1.4 Tumor suppressor genes

The discovery of proto-oncogenes and oncogenes provided a powerful explanation of how the growth and proliferation of cells is positively regulated. Accepting the assumption that biological systems are balanced indicated that there could be genes that counteract proto-oncogenes-and prevent cells from uncontrolled growth. It was in the 1970s and early 1980s a second, and fundamentally different type of growth regulating genes began to accumulate [23]. These genes were named ”Tumor suppressor genes“ (TSGs) and possess anti-proliferative properties [23]. TSGs are defined as recessive genes that must sustain mutations or deletions of both alleles in order to contribute to cancer formation. This definition was described by Knudson and it known as Knudson’s 'two-hit' rule [24]. It was later shown that some TSGs are exceptions to the Knudson’s 'two-hit' rule, referred to as haploinsufficient TSGs. Those genes contribute to tumor development even in the presence of one functional allele [25]. The functions of tumor-suppressor proteins are diverse but can be classified into different categories, including cell cycle regulation (e.g pRb, p27, p53), DNA damage (e.g ATM, ATR) and repair (i.e BRCA1, BRCA2), apoptosis (e.g p53, BAX), migration and invasion (e.g DLC1, PTEN), senescence (e.g p16, p19) [26-33].

2.1.4.1 Mechanisms of inactivation

Haploinsufficient TSGs may become inactivated following deletion or mutation of one single allele. However, since TSGs are often recessive, cells that contain one functional and one mutated gene (heterozygous) still behave normally. There are several mechanisms by which the normal allele may then become inactivated, which in turn can predispose a cell to cancer development. Among these mechanisms are large homozygous deletions or duplication of the mutated chromosome and complete loss of the normal counterpart, leading to so called Loss of Heterozygozity (LOH), as well as small deletions, point mutations, insertions, promoter hypermethylation and dominant negative effects [34, 35].

2.2 UBIQUITIN PROTEASOME SYSTEM (UPS)

2.2.1 Background/history

A requirement for normal cellular homeostasis [36, 37] is that proteins are continuously synthesized and degraded inside our cells. This was first postulated by Schönheimer in 1942 in his book ¨The dynamic State of Body Constituents [38]. Proteins that have served their functions and are no longer needed have to be removed. Additionally, misfolded or damaged proteins also need to be rapidly eliminated which could otherwise cause various kinds of problems to the cell [39]. For a long time, proteins were thought to be degraded in lysosomes [40], and it took several decades before the pioneering work by Ciechanover, Hershko and Rose resulted in the discovery of a regulated energy-dependent protein degradation process which is catalyzed by specific enzymatic activities and a small ubiquitous protein named ubiquitin (Ub) [40]. This discovery was awarded the Nobel Prize in chemistry in 2004 [41]. Ubiquitylation is the covalent attachment of ubiquitin to one or more lysine residues on a substrate protein and involves a cascade of key enzymatic activities: *1*) a ubiquitin-activating enzyme (E1), that activates ubiquitin in an ATP dependent manner; *2*) a ubiquitin-conjugating enzyme (E2); and *3*) a ubiquitin-ligase (E3), that transfers the ubiquitin to specific lysine residues on target substrates (Figure 2). Ubiquitylated substrates are then recognized by a large multi-subunit protease complex, which is named the 26S proteasome complex [42, 43]. Thus, the UPS can be divided into two distinct steps: ubiquitylation and proteasomal degradation (Figure 2). Importantly, ubiquitin contain seven lysines, which all serve as potential acceptor sites for another ubiquitin molecule and can therefore result in different ubiquitin chains on substrates [40]. In fact, the attachment of different types of ubiquitin chains on substrates can take several forms, each with potentially different biological outcomes. For example, attachment of a single ubiquitin (monoubiquitylation) often activates transcription factors, regulates protein trafficking, or targets proteins to the secretory/endocytic pathway [44]. Attachment of a chain of lysine-48 linked ubiquitins (also called poly-ubiquitylation) usually targets the substrate to the 26S proteasome for ubiquitin-mediated proteolysis [43] (Figure 2). However, other types of polyubiquitin chains (e.g lysine-63) can exert nonproteolytic functions, including kinase and transcription factor activation and chromatin remodelling [45]. In addition, many proteins contain so-called ubiquitin-binding domains (UBDs) [46, 47] enabling an

additional level of regulation and translation of ubiquitin modifications into diverse cellular outputs, similar to protein phosphorylation. Finally, ubiquitylation is counteracted by deubiquitylating enzymes (DUBs) highlighting the reversibility of this enzymatic system [40]. Given the critical function of an intact UPS, altered degradation of cellular regulators directly contributes to the development of hallmarks of cancer.

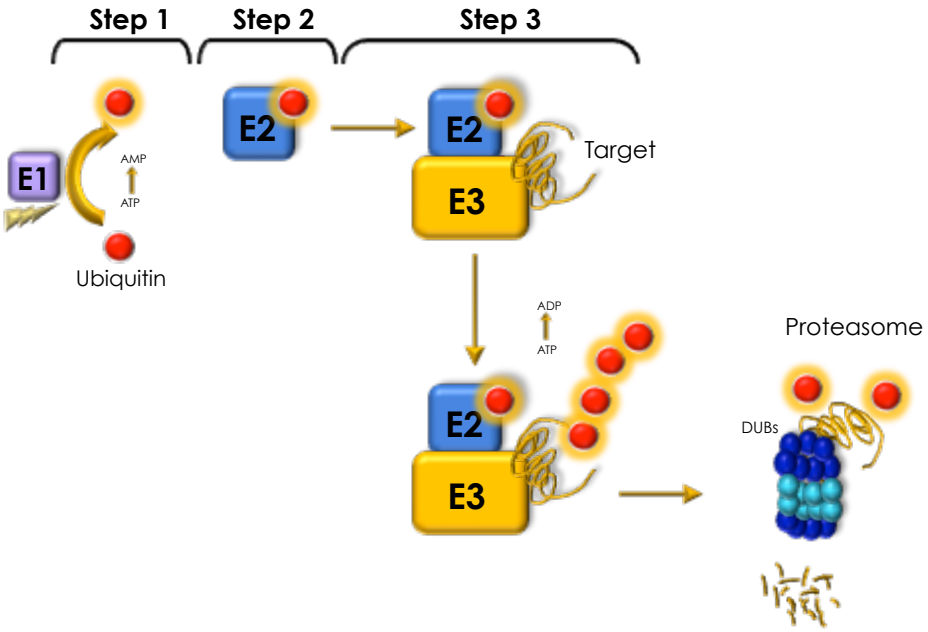


Figure 2. Ubiquitin Proteasome System (UPS)

2.2.2 E3 Ubiquitin ligases

E3 ubiquitin ligases are responsible for facilitating the attachment of the ubiquitin molecule to a lysine residue on the target protein via isopeptide bonds and therefore act as substrate specificity factors [48]. There is a huge number of E3 ligases in the cell and can be characterized by the presence of either a HECT (Homologues to E6AP C-Terminus) domain or a RING (Really Interesting New Gene) domain [49, 50]. The HECT domain containing E3s directly transfer ubiquitin from the E3 onto the substrate, whereas the RING domain provides a docking site for the E2 enzyme, which mediate the transfer of ubiquitin to the substrate [49]. E3 ligases can also be classified into single subunit E3s (e.g. Mdm2, Cbl) and multi-subunit complexes. The multisubunit RING E3s (e.g APC and SCF) includes several hundred members and can be further subdivided into different families based on the complex composition [50]. E3 enzymes bind their cognate target substrates through various protein-protein interaction

domains (e.g WD40 repeats). However, for a substrate to be recognized by an E3 ligase it generally first has to be post-translationally modified, for example by phosphorylation or proteolytic cleavage [48]. The modified motif in the substrate has been termed degron [51]. There are many different types of degrons (e.g phosphodegron, PEST). Once modified, a degron in a substrate might be recognized by a specific E3 ligase, which forms the basis for its subsequent ubiquitylation.

2.2.3 SCF ubiquitin ligases and Cancer

An important family of multimeric E3s is known as SCF (Figure 3). SCF ligases are composed of an invariant core ligase comprised of the adaptor protein Skp1, the scaffold protein Cullin1 and the RING domain protein Rbx1 (also called Roc1 or Hrt1), which recruits the E2 enzyme Cdc34 (also known as UBCH3) and one variable component called the F-box protein. It is the F-box protein that provides substrate specificity, usually through its C-terminal protein-protein interacting domains (often containing WD 40 and LRR repeats). The F-box protein associates with the SCF core through binding to the adaptor protein Skp1, via its conserved F-box domain (Figure 3) [52, 53]. The F-box is a protein domain of approximately 40-50 amino acids in length. It was first identified in cyclin F, as its name implies [52]. In the human genome, there are about 70 genes with F-box domains, each likely targeting a unique set of substrates for degradation [54]. Notably, a majority of the F-box proteins are still completely uncharactierzed [55].

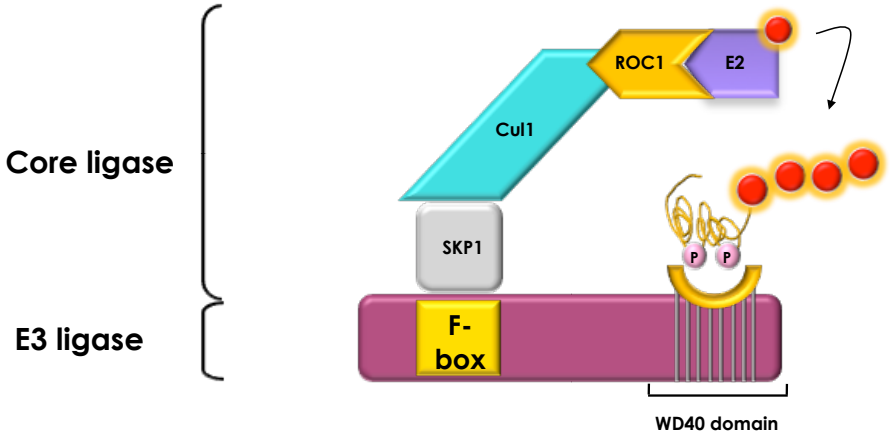


Figure 3. SCF ligase

A wealth of evidence suggests that SCF ligases play importnant roles in the control of cell division, which is almost a hallmark itself for this category of genes [Pagano]. Not surprisingly, several F-box proteins have been directly linked to tumorigenesis [56]

through their role in the regulation of critical substrates frequently dysregulated in cancer. Skp2 (FBL1) is an oncoprotein that catalyzes the ubiquitylation of p27, a cyclin-dependent kinase (CDK) inhibitor and tumor suppressor that negatively regulates cell cycle progression. Interestingly, Skp2 also catalyzes ubiquitylation and degradation of multiple other proteins regulating cell cycle progression and tumor development (eg, p130, p21) [56, 57]. Indeed, many studies clearly show that E3 ligases and F-box proteins themselves act as critical oncoproteins or tumor suppressor proteins consistent with their role in ubiquitinating an array of substrates directly linked to the development of cancer.

2.3 FBW7

2.3.1 History/function

FBW7 (also known as *FBXW7, FBW7* or *hCDC4* in humans, *Cdc4* in Saccharomyces cerevisiae, *Sel-10* in Caenorhabditis elegans, or *Ago* in Drosophila melanogaster), is a substrate specificity factor for the SCF ubiquitin ligase SCF^{FBW7}[58]. *FBW7* was first identified in a genetic screen for cell division cycle (Cdc) mutants in S. cerevisiae [59] and was later implicated in the ubiquitin-dependent proteolysis of the cyclin-dependent kinase (Cdk) inhibitors Sic1 and Far1 [60], among others. In mammals, FBW7 also regulates cell cycle progression and development through the degradation of specific transcription factors and cyclins [48, 61] (see below). Thus, FBW7 is a master regulator of cellular proliferation with important implications for several pathways regulating differentiation and development.

2.3.2 Gene/transcript/protein

In humans, the *FBW7* gene spans over 200 kb on the long arm of chromosome 4 (4q31). It contains 13 exons and is alternatively spliced into three transcripts with unique 5'-exons termed alpha, beta and gamma [61] [62]. The 5'-exon of each transcript is linked directly linked to exon 2 and each mRNA thus share all C-terminal exons (exon 2-11) (Figure 4). Importantly, the individual 5' exons are coding and therefore generate three distinct protein isoforms that differ only at their N termini [63]. This genomic organization is highly conserved in mammals and also allows for

isoform-specific transcriptional regulation, as each *FBW7* isoform is expressed from its own promoter [63]. The FBW7 protein contains several protein-protein interaction domains (Figure 4). The F-box domain that recruits the SCF core complex through interaction with SKP1 protein [52] is localized in the N-terminal portion of the protein. The C-terminal exons in FBW7 encode eight WD40 repeats that form a barrel-shaped beta-propeller structure that is responsible for protein-protein interaction [64]. An additional domain in the N-terminus of the protein, termed the dimerization domain (DD), has been shown to mediate FBW7 dimerization [65-67]. While all FBW7protein isoforms share the DD, F-box and WD40 domains, the alpha and gamma exons lack obvious domain structures, whereas the beta isoform encode a putative transmembrane domain (TMD) [68-70]. The physiological significance for having three protein isoforms with exactly the same substrate-binding domain is still unclear, although the different FBW7 isoforms are differentially expressed among different tissues. Importantly, the FBW7 isoforms occupy different subcellular compartments in the cell [68]. A cis-acting signal in the 5' specific exons directs the alpha and beta isoforms to the nucleoplasm and cytoplasm, respectively [71]. In contrast, the nuclear gamma isoform also localizes to the nucleolus [68]. The beta isoform was recently reported to be localized to in the membrane of the Endoplasmatic Reticulum (ER) [72], but may also localize to mitochondria (Steve Reed, personal communication).

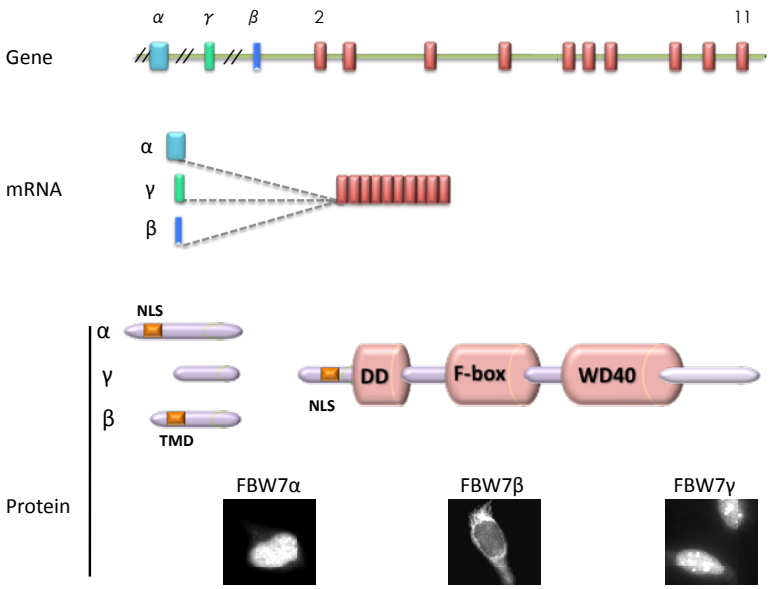


Figure 4. The FBW7 gene structure

2.3.3 Substrates/degrons

The interaction between FBW7 and its target substrates is dependent on a specific motif common in all substrates [73, 74]. This motif was identified through structural studies of Cdc4 in S. cerevisiae and a human phosphopeptide from cyclin E [74, 75] and was named the Cdc4-Phospho-Degron (CPD). Importantly, data from several substrates defined a CPD consensus sequence; Φ-X-Φ-Φ-Φ-pT/PS-P-P-X-pS/pT/E (Φ corresponding to a hydrophobic residue and X any amino acid) [76], which is found in all target proteins. Structural and mutational studies demonstrated that the major contacts between the FBW7 and its substrate(s) occurs through hydrogen bonds between a phosphorylated threonine/serine in the CPD and several arginine residues located in a binding pocket on the inner rim of the beta-propeller surface of FBW7 [69, 75]. However, the affinity for a specific substrate also seem to depend on the number of CPDs, and on the actual amino acid sequence in each respective substrate [76]. To date, FBW7 has been reported to target 20 different proteins for degradation (Table 1) and as such, FBW7is involved in the regulation of multiple biological processes (Figure 5, 6). Importantly, the common denominator for these substrates is that they all act as critical oncoproteins. Some of these substrates are described in greater detail below.

Table 1. FBW7 substrates in mammals

Name	Degron	Protein category	Function
	θXθθθ(**S/T**)PXX **(S/T/E)**		
Cyclin E	LL**T**PPQ**S**GK IP**T**PDK**E**DD	Regulatory	Proliferation*
c-Myc	LP**T**PPL**S**PS	Transcription factor	Proliferation*
c-Jun	GE**T**PPL**S**PI	Transcription factor	Proliferation*
SREBP 1	TL**T**PPP**S**DA	Transcription factor	Metabolism
SV40 large T antigen	PP**T**PPP**E**PE	Oncoprotein	Transformation
Notch 1	FL**T**PSP**E**SP	Transcription factor	Differentiation*
Presenilin 1	IY**T**PFT**E**DT	Regulatory	signaling
Cyclin E2	LL**T**PPQ**S**GK IP**T**PDK**E**DD	Regulatory	Cell cycle*
MCL1	GSAGASPPST ADAIMSPEEE	regulatory	Apoptosis
C/EBPalpha	HP**T**PPP**T**P	Transcription factor	Differentiation
KLF5	LN**T**PDLDM PP**S**PPS**S**E NL**T**PPP**S**Y	transcription factor	Proliferation
Aurora A	?	Kinase	Proliferation*
mTOR	LL**T**PSIHL	Kinase	Proliferation*
c-Myb	?	Transcription factor	Proliferation
SRC	**S**PMA**S**	Transcription factor	Proliferation
PGC1	PL**T**PES**P**N GL**T**PPT**T**P	Transcription factor	Metabolism
n-Myc	?	transcription factor	Proliferation
TGIF1	FN**T**PPP**T**P	regulatory	signaling
Ebp2 (pseudosubstrate)	?	Regulatory	Ribosome biogenesis
PKC (atypical substrate-no degradation)	?	Kinase	Signaling

θ, hydrophobic amino acid; X, any amino acid, * additional functions

2.3.3.1 Cyclin E1

Cyclin E, an activator of Cdk2, is involved in the initiation of DNA replication and other cell cycle functions [77]. Cyclin E/Cdk2 catalyzes the phosphorylation of many substrates that are involved in diverse S phase processes such as histone biosynthesis, centrosome duplication, and the licensing of origins of replication (ORC), among others [78]. In normal cells, the level of cyclin E peaks at the G1/S phase boundary and rapidly declines as cells progress into S phase. However, in many tumors cyclin E protein levels are elevated and/or dysregulated relative to cell cycle progression and these

alterations have a strong prognostic impact for poor outcome in patients with breast cancer [79, 80]. Overexpression of cyclin E has been shown cause a premature initiation of DNA synthesis [77, 81] and induce chromosome instability in cultured cells [78]. Furthermore, deregulated cyclin E expression in mice leads to tumor formation [82]. Finally, cyclin E can transform rat embryo fibroblasts together with oncogenic Ras [83], in line with its oncogenic function. The first indication for ubiquitin ligase mediated proteolysis of cyclin E was provided by reports of the Cul1 knockout mouse [84, 85]. Cul1 deficiency in mice resulted in embryonic lethality with arresting cells containing elevated levels of cyclin E [84]. In 2001, three groups including our own, independently identified the F-box protein FBW7 as a novel SCF ligase responsible for cyclin E ubiquitylation and degradation [69, 86, 87]. FBW7 was found to bind specifically to cyclin E that is phosphorylated on two CPDs, one surrounding Thr62 and one at Thr380 [88-91]. Phosphorylation of cyclin E1 is mediated by Cdk2 on residues Thr380 and Ser384 through autophosphorylation and also by glycogen synthase kinase 3 (GSK3) [89]. Interestingly, whereas FBW7-beta does not appear to be involved in cyclin E turnover, both FBW7-alpha and gamma are required for cyclin E degradation in cultured cells [91, 92]. Using in vitro and in vivo ubiquitylation assays, we found that ubiquitylation of cyclin E requires sequential function both the alpha and the gamma isoforms [91].

2.3.3.2 Notch 1

The *Notch1* gene is a member of the Notch family of transmembrane receptors [93]. Notch proteins have an extracellular domain consisting of multiple epidermal growth factor-like (EGF) repeats, and an intracellular domain. Notch proteins play a key role in a variety of developmental processes and regulate differentiation, survival, and proliferation through an evolutionarily conserved intercellular signaling pathway. Canonical Notch signaling is initiated and activated following ligand (Jagged) interaction and sequential proteolytic cleavages by ADAM metalloproteases and the gamma-secretase complex [93]. Proteolytic release of the intracellular domain of Notch (NICD) from the cell membrane results in translocation of the NICD to the nucleus where it promotes the transcriptional activation of DNA-binding transcription factor including CSL and MAM. NICD forms a trimeric complex with CSL and the co-activator MAM, which is essential for NICD-dependent transcription *in vitro* and *in vivo* [94]. The first Notch receptor gene to be identified in humans,

Notch1, was discovered through the analysis of DNA flanking the breakpoints of a recurrent t(7;9)(q34;q34.3) chromosomal translocation seen in a small subset (< 1%) of human pre–T-cell acute lymphoblastic leukemias [95]. *Notch*1 is frequently mutated in T-ALL but is also deregulated in variety of other human tumors, including breast cancer and various subsets of human lymphomas [96, 97]. The first link between Notch and FBW7 came from studies demonstrating that LIN-12 (the NOTCH1 orthologue in C. elegans) is regulated by SEL-10 (the Cdc4 orthologue in C. elegans) [98, 99]. In 2001, the mouse and human homologs of FBW7 was shown to target Notch1 for ubiquitylation [100]. Interestingly, Notch1 is also controlled by several other ubiquitin ligases [99, 101-103]. The PEST domain of NICD is known to regulate protein stability and is often mutated in primary human and mouse T-ALLs [104]. Indeed, the Notch1 PEST domain contains putative CPD motifs [105]. The important function of FBW7 in Notch regulation is underscored by the fact that *FBW7* knockout mice die early during embryonic development mainly due to abnormalities in vascular development which is attributed to failure in the degradation of Notch [103, 106].

2.3.3.3 c-Myc

The *MYC* proto-oncogene encodes a nuclear transcription factor and regulates up to 15% of all genes [107]. Because of this, MYC regulates a broad spectrum of cellular functions, including cell growth, proliferation and apoptosis among others [108-110]. Loss of Myc results in embryonic lethality in mice and depletion of Myc forces cells to exit the cell cycle. Conversely, Myc expression stimulates progression through the cell cycle and its overexpression in cells or animals induces transformation [111, 112]. Indeed, the MYC gene is one of the most frequently overexpressed oncogenes and is estimated to contribute to as many as 70% of all human cancers [113-116]. Thus, a tight control of Myc expression and activity is essential for normal cellular homeostasis. Like cyclin E, MYC expression is regulated by upstream kinases such as the PI3K-AKT-GSK3b pathways, which is responsible for the rapid degradation of Myc by the ubiquitin-proteasome pathway. Increased stability of Myc is frequently observed in various human tumors and this has mainly been attributed to point mutations in the N-terminus of Myc, a region, which also encodes a CPD. Indeed, subsequent studies showed that FBW7 targets Myc for ubiquitin-mediated proteolysis in a phosphorylation dependent manner [117-119]. The exact mechanism for Myc

ubiquitylation is not known. Like cyclin E, Myc degradation appears to depend on additional cofactors (see below) and both the nuclear isoforms of FBW7 [68]. Interestingly, at least five ubiquitin ligases have been implicated in Myc ubiquitylation and turnover, including three F-box proteins (Cdc4, SKP2 and bTRCP) [120].

1.3.3.4 Other substrates

As mentioned above, the list of novel FBW7specific target substrates is rapidly expanding, as is the number of biological pathways regulated by FBW7. Figure 5 and Table 1 summarize the extensive number of substrates we know today. From this list, it is evident that FBW7is not only responsible for the tight regulation of the cell division cycle, but also control growth, signal transduction, differentiation, survival and metabolism. Although most established substrates are nuclear proteins degraded by the nuclear isoforms of FBW7, alpha and gamma [68], it is intriguing that there are yet no defined substrates for the cytoplasmic FBW7-beta isoform [61]. Whereas knockout mice lacking FBW7 are not viable, a recent study shows that mice specifically deleted for the FBW7-beta isoform do not exhibit any apparent development defects. However, primary cultures of neurons prepared from the mutant mice are more sensitive to oxidative stress than those prepared from the wild-type mice. Conversely, overexpression of FBW7-beta renders cells resistant to the oxidative stress. These results thus suggest that FBW7-beta might contribute to the protection of cells from oxidative stress [72].

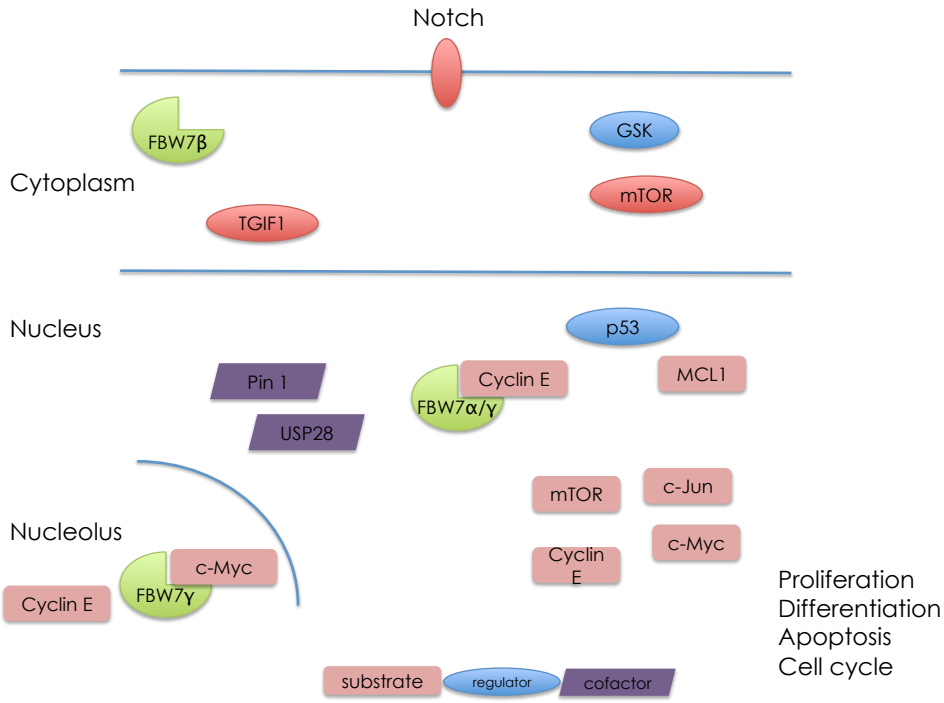


Figure 5. FBW7substrates and regulators

2.3.4 Mechanism of regulation

2.3.4.1 Expression

As described above, FBW7 is a critical suppressor of mammalian cancerogenesis through its negative regulation of various oncoproteins (Figure 5). However, we still have very limited knowledge about the regulatory mechanisms controlling FBW7 expression and activity in cells. The fact that different isoforms posses distinct promoters suggest that each isoform is differentially regulated [58]. Indeed, each isoform specific transcript is differentially expressed in different tissues [62, 87]. FBW7-alpha is ubiquituously expressed compared to [63] FBW7-beta which is highly expressed in brain but absent in skeletal muscle and liver [121]. FBW7-gamma expression is low in most tissues but high in muscle [62]. Very little is known how isoform specific expression is regulated. The alpha promoter resides within a 2 kb CpG island, whereas the gamma and beta promoters lack obvious CpG islands. The FBW7-alpha and beta transcripts are transcriptionally induced by the p53 tumor suppressor [70, 122] in response to DNA damage. However, only the beta promoter contains a consensus p53 binding site [70]. However, we have shown by ChiP-seq analysis that p53 binds a region downstream of exon 11 (Selinova G., unpublished observations) which could promote p53-induced transcriptional activation of the

alpha isoform. Interestingly, the inflammatory response gene CCAAT/enhancer binding protein-δ, C/EBPδ, has been shown to directly inhibit expression of FBW7during hypoxia through targeting the promoter region of FBX7-alpha [123]. Interestingly, we recently discovered that the gamma transcript is induced upon serum-withdrawal mediated myoblast differentiation of C2C12 cells (unpublished data). Furthermore, gene expression data indicate that FBW7 expression may be transcriptionally induced under low oxygene conditions. A potent epigenetic mechanism for the regulation of gene expression during normal development and cellular differentiation in higher organisms is DNA methylation [124]. We recently demonstrated that the beta promoter is hypermethylated in multiple different tumor-derived cell lines and primary breast tumors (paper III) and it is thus possible that methylation of the different FBW7promoters determines isoform specific expression in different normal tissues. Another potential mechanism for regulation of FBW7 expression is microRNAs (miRNAs). miRNAs are short non-coding RNAs of about 21-23 nucleotides in length that regulate gene expression at the post-transcriptional level [125, 126]. Despite their recent discovery, miRNAs are now widely accepted as critical gene expression regulatory factors [125, 126]. miRNAs are expressed by RNA polymerase II as parts of longer primary transcripts or miRNA precursors termed pri-microRNAs [127, 128]. Maturation of miRNAs involves the sequential processing by protein complexes containing an RNAse III enzyme Drosha and the Dicer-TRBP complex respectively [129], followed by incorporation into a complex termed RISC (RNA-induced silencing complex) [130, 131]. The degree of complementarity between microRNA and mRNA will generally dictate the outcome; perfect or near-perfect complementarity will result in cleavage of the mRNA, while imperfect complementarity will trigger translational repression [132]. The human genome contain between 400 to 1000 different miRNAs, as estimated by computational methods [133]. Approximately 50% of all genes are predicted targets of miRNAs [134]. Apparently, each miRNA targets many different genes and each 3'UTR often contains binding sites for many different miRNAs [135-137]. Because of their widespread role in regulation of gene expression, it is easy to understand that miRNAs have profound effects on tumor development. In fact, many miRNAs have been shown to act as tumor suppressors and oncogenes [22]. With this in mind, we set out to identify miRNAs that might regulate FBW7 expression. Using a combination of different screening procedures, we found that the 3'UTR of FBW7 is regulated by at

least 5 different miRNAs. Interestingly, one of these miRNAs, miR-27a, was found to regulate FBW7 expression during cell cycle progression (paper IV). In fact, this is the first demonstration that FBW7 expression is regulated during specific cell cycle phases under physiological conditions (paper IV). Interestingly, miR-223 was recently reported to regulate FBW7 expression as well [138]. Interestingly, miRNAs often act in concert with specific RNA binding proteins. It is becoming increasingly clear that miRNA function often depends on RNA binding proteins [137]. There are still no reports on the role of RNA-binding proteins regulating FBW7 expression. However, our results indicate that miR-27a-mediated repression of FBW7 is minimal at the G1/S border, without any obvious change in miR-27a expression levels. This suggests that the interaction between miR-27a and the FBW7-3'UTR is regulated by additional factors, as has been demonstrated for the cyclin-dependent kinase inhibitor p27 mRNA and the miRNAs miR-221/miR-222 which is dynamically regulated by the RNA-binding protein Pumilio-1 [139]. Future studies will reveal in greater detail how FBW7 is regulated by miR-27a during the cell cycle and whether it's regulated by specific RNA binding proteins.

1.4.1.2 Activity

FBW7function and activity is also regulated at the post-translational level. As described above, FBW7 function as a SCF ubiquitin ligase when bound the Skp1-Cul1-Roc1 complex. Thus, the general regulation of components in the core ligase is also of importance for FBW7-specific substrate ubiquitylation. Indeed, it has been shown that SCF activity requires an active neddylation system [140]. For example, recent observations indicate that conformational changes induced by Cullin-RING E3 ubiquitin-Ligases (CRL) dimerization and by conjugation of the ubiquitin-like protein NEDD8 on the cullin subunit stimulates substrate polyubiquitination [141]. Conversely, Deneddylation, in which the COP9 signalosome (CSN) removes NEDD8 from cullins, inactivates CRLs [142]. Interestingly, it is very difficult to detect endogenous FBW7 protein expression in cells although FBW7 mRNA levels are easily detected. The reason for this is not completely understood, but could be due to miRNA-mediated translational repression, or possibly, decreased protein stability. Indeed, we, (unpublished data) and others [143] have shown that FBW7 protein is unstable and auto-ubiquitylated in vivo. Notably, the WD40 beta-propeller structure was recently shown to function as an ubiquitin-binding domain and ubiquitin

interaction by Cdc4 was demonstrated to promote its autoubiquitylation and turnover [144].

We have very limited knowledge of direct post-translational modifications regulating FBW7 activity. However, FBW7 is phosphorylated through ATM/ATR pathway upon induction of DNA damage [145]. Additionally, FBW7 was recently reported to be a novel substrate for PKC and mutational analysis revealed that PKC-mediated phosphorylation of the FBW7-alpha isoform at serine 10 regulate its nuclear localization [146]. There are as yet no reports for post-translational modifications of the beta and gamma isoforms.

In addition to the F-box and WD40 domains, FBW7 contains a dimerization domain (DD)(Figure 4) located immediately N-terminal to the F-box domain. The D-domain is highly conserved among different species [66, 147] and is also found in other F-box proteins. For example, human beta-TrCP1 and beta-TrCP2, form hetero- and homodimers in vivo [66, 147] and the two FBW7 homologs in fission yeast, Pop1 and Pop2, also form hetero- and homo-complexes [148]. Human FBW7also forms homodimers and possibly heterodimers [67]. The role of dimerization of F-box proteins is not exactly understood, but has been proposed to increase the ubiquitylation efficiency of substrates with low-affinity phosphodegrons [65, 66], possibly through reducing the distance between the substrate and the SCF catalytic site. Juxtaposition of two E2 sites in proximity to the substrate may also increase reaction kinetics [66]. However, FBW7dimerization is not an absolute requirement for cyclin E degradation [65]. Additional studies need to firmly establish how dimerization regulates substrate ubiquitylation. Moreover, SCF^{FBW7} localization and activity is also regulated by various cofactors. We, and others have previously shown that SCF^{FBW7} mediated cyclin E and Myc ubiquitylation depends on the peptidyl-prolyl cis/trans isomerase Pin1 [91, 149]. Pin1 is a multifunctional enzyme that isomerizes many proteins with S/TP motifs [150]. We showed that ubiquitylation of cyclin E requires the sequential function of FBW7-alpha and gamma isoforms [91]. Whereas FBW7-gamma polyubiquitylates cyclin E, FBW7-alpha forms a ternary complex with cyclin E and Pin1 and isomerizes the cyclin E phosphodegron. Pin1 is also required for FBW7 mediated Myc degradation, in conjunction with the protein phosphatase 2A (PP2A) [118]. Thus, PP2A is another factor regulating FBW7 activity towards specific

target substrates [118]. The functional effects of these cofactors during tumor development are complex but may be of therapeutic potential in cancer treatment [151-155]. The deubiquitylating enzyme, ubiquitin-specific protease 28 (USP28), is an example of a negative regulator of SCF^{FBW7}activity. USP28 also forms a complex with FBW7-alpha and cyclin E or c-Myc [156] in the nucleus, possibly counteracting ubiquitylation by the FBW7-alpha isoform in the nucleoplasm [156]. Interestingly, upon DNA damage induced by UV irradiation, the USP28-associated ternary complex is disassembled [157] which result in declined steady-state levels of Myc protein rapidly after exposure [156]. Degradation of Myc and cyclin E (unpublished data) by FBW7 may be compartmentalized, as both Myc and cyclin E have been shown to accumulate in the nucleolus upon proteasomal inhibition ([91], Bhaskaran et al, unpublished). Together, these data suggest that ubiquitylation of Myc and cyclin E is catalyzed by the FBW7-gamma isoform in the nucleolus [91]. At present, it is unclear whether ubiquitylation of other FBW7substrates are also regulated in a similar compartmentalized manner. Nucelophosmin (NPM), also termed B23, is a dynamic, multifunctional protein that is tightly regulated during proliferation and it was recently shown to regulate FBW7-mediated Myc ubiquitination and transcriptional activity [158, 159]. Our own unpublished data also support a function for NPM in the regulation of cyclin E stability. Thus, it is possible that NPM may be involved in shuttling these substrates to the nucleolus. Ebp2 is another potential factor regulating the translocation of FBW7 to nucleoli by functioning as a pseudosubstrate for FBW7 [71]. Finally, FBW7-dependent substrate ubiquitylation is also dependent on upstream signaling pathways, including the PI3K/Akt/GSK3b pathway [160] and possibly Ras signaling [161].

2.3.5 Mechanisms of inactivation

2.3.5.1 Phenotypes

While FBW7 null mice die around 10.5 days post coitus due to a combination of deficiencies in hematopoietic and vascular development attributed to dysregulation of Notch1 [103], tumors with inactivation of FBW7 exhibit various functional alterations related to other FBW7 substrates. Considering the fact that FBW7 orchestrates the destruction of a number of key onco-proteins it is maybe not surprising that FBW7 function has been linked to several important processes such as proliferation, apoptosis and differentiation. Thus, FBW7 inactivation causes the undermined regulation of several specific cellular programmes associated with the major hallmarks of cancer (Figure 6).

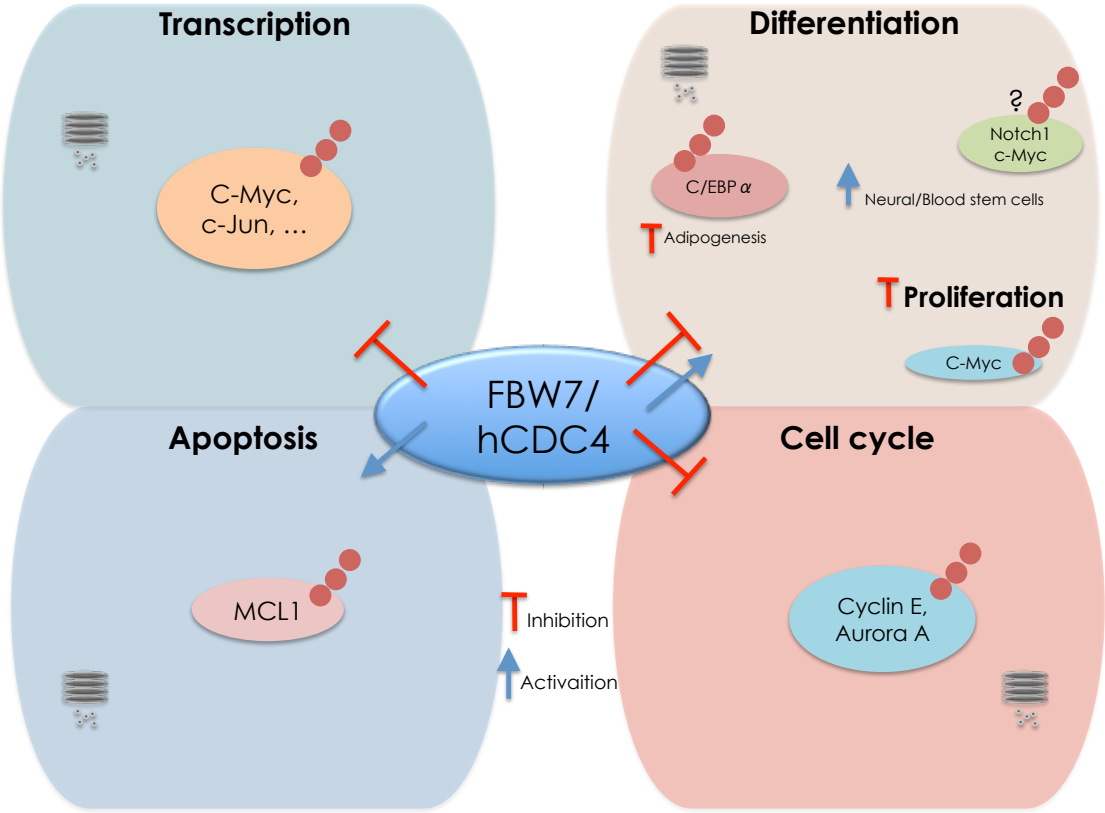


Figure 6. A simplified overview of cellular processes regulated by FBW7

Recently *FBW7-beta* deficient mice have been generated. Interestingly, no cancer development was detected in these mice up to 1 year. Also these animals did not exhibit any apparent abnormalities in development, however longer observation will be necessary to determine whether Fbxw7b-deficient mice are predisposed to cancer development [72].

One obvious phenotype in cells with FBW7 inactivation is genetic instability [162, 163]. The genetic instability phenotype might be mediated due to increased levels and dysregulated expression of a several key oncogenic FBW7 target substrates, including c-Myc, cyclin E1 and STK15/AURKA protein [164]. During recent years there has been an effort to show which of these substrate(s) that is more likely to be responsible for the genomic instability caused by FBW7 ablation, with ample data supporting the relevance of dysregulated cyclin E1. Overexpression of cyclin E can also recapitulate phenotypes observed in cells with inactivated or suppressed FBW7, and conversely, knockdown of cyclin E expression in cancer cell lines with a genomic instability phenotype reduces percentage of micronuclei formation [162]. Taken together, cyclin E1 dysregulation phenocopies several phenotypes observed upon ablation of *FBW7*[78]. The genomic instability of *FBW7*-deficient cells is at the microscopic level manifested as nuclear atypia characterized by micronuclei, lobulated or elongated nuclei, aberrant chromosomal structures at metaphase, abnormal number of centromeres and multipolar spindles [162]. Furthermore, FBW7-deficient tumor cells undergo extensive mitotic slippage and endoreduplication when exposed to spindle toxins such as vinblastine or taxol, which in turn render them polyploid [165].

2.3.5.2 Mutation

Early reports analyzing the *FBW7* gene sequence identified chromosomal rearrangements and mutations in the *FBW7* gene in human tumor-derived cell lines [86, 87]. Shortly thereafter, it was shown that primary endometrial tumors contain *FBW7* mutations [63]. Interestingly, *FBW7* mutation was found to correlate with cyclin E dysregulation in these tumors [63]. Mutations were subsequently identified in colon carcinomas and pancreatic cancer [63, 166, 167]. Our original discovery of *FBW7* mutations in primary endometrial adenocarcinomas was the basis for continued analysis of *FBW7* mutations in primary tumors. Mutational analysis of

FBW7 in pediatric T-ALL showed that this malignancy has a particularly high frequency of mutations (paper I). These studies were followed up with a comprehensive screen for *FBW7* mutations in 1556 primary human tumor samples from twelve different malignancies (paper II). The results from these analysis established that FBW7 represent a general TSG in human cancers [168]. The overall mutation frequency of FBW7 in different tumors is 6% and the highest mutation frequencies were found in tumors of the bile duct (cholangiocarcinomas, 35%), blood (T-cell acute lymphocytic leukemia, 31%), endometrium (9%), colon (9%), and stomach (6%) [168]. The most common mutations in FBW7 are missense mutations of three arginine residues (Arg^{465} and Arg^{479}) in the substrate binding pocket of FBW7 [168, 169]. Interestingly, these mutations presumably function in a dominant-negative manner, possibly through dimerization with the functional wild-type allele. The reason why mutations frequently occur in these specific nucleotides of FBW7 (exon 8 and exon 9) is not known, but could be due to spontaneous deamination of 5'-methylcytosine to thymine in DNA. Indeed, the cytosine corresponding to the mutation hotspot at nucleotides 1,435–1,436 (Arg479) is methylated in vivo [168]. Some discrepancies in the mutation frequencies have been reported. For example in T-ALL samples, FBW7 has been shown to be mutated between 10 to 30 % [170-172], but all laboratories report clustering of mutations in FBW7 binding pocket. One plausible explanation for such differences is the ethnical differences between the patients enrolled in different studies. Other factors could be differences in sample size or other types of selection biases.

Various types of single nucleotide polymorphisms (SNPs)(Synonyme, nonsynonyme and frameshift) possibly affecting FBW7 function have been reported to occur in all three isoforms (ensembl.org).

2.3.5.3 Deletion

Deletion of the FBW7 gene, which localizes chromosome 4q31 is a frequent event in human tumors and occurs in more than 30% of all neoplasms [173]. Several studies have reported deletion of one allele at 4q31 [173, 174]. We have also demonstrated small deletions of FBW7 occurring in a breast cancer cell line [87]. A previous report showed that FBW7-/+ mice crossed with p53 heterozygous mice have a greater susceptibility to radiation-induced tumors [122]. Interestingly, most of these tumors retain and express the wild-type *FBW7* allele, indicating that *FBW7* might be a *p53*-

dependent haploinsufficient tumor suppressor gene [122]. In another study it was shown that FBW7 exhibited intestinal tumors carry heterozygous FBW7 mutations, again indicative of FBW7 being a haploinsufficient tumor suppressor gene [175]. However, in these studies, detailed analysis of FBW7 isoform specific expression was not performed.

1.5.1.4 Methylation:

Several recent reports have shown that FBW7expression is downregulated in some tumor types, including gliobastomas [176], gastric [177] and colorectal cancer [178]. However, the mechanism for loss of expression was not clarified in these studies [179]. DNA methylation is generally defined as the addition of a methyl group to the 5 position of a cytosine base, but it can occur on adenine base as well. Methylation is epigenetically inherited from parental cells to the daughter cells upon cell division. [180]. DNA methylation is an important mechanism for the regulation of gene expression during normal development and cellular differentiation in higher organisms [124, 180]. Alterations in the methylation pattern, particularly at TSG gene loci, is also a frequent event in cancer [124]. In paper III, we report that *FBW7-beta* promoter is hypermethylated in human breast cancer and various tumor derived cell lines [121, 181]. Comparison of *FBW7-beta* promoter methylation and FBW7-beta expression demonstrated that methylation correlates with loss of expression, suggesting that promoter hypermethylation is an alternative mechanism for FBW7 gene inactivation (paper III). Taken together, these data indicate that methylation might be an important mechanism of *FBW7-beta* gene inactivation in different tumor types [121]. A recent report showed that the *FBW7-beta* promoter is also methylated in thymoma [181]. Whether the other FBW7 promoters are methylated in cancer remains to be shown, but our methylation analysis in breast cancer specimens show no evidence for hypermethylation of the alpha promoter (paper III).

1.5.1.5 microRNAs

miRNAs are now widely accepted as critical factors involved in the regulation of gene expression [125, 126]. In most instances, the microRNA will bind to sites within 3' untranslated regions (3'-UTRs) of the targets. Today, the link between microRNAs and tumorigenesis is well-established [125] and microRNAs have been found to be deleted, downregulated or overexpressed in many different tumor types and have

been demonstrated to function as both tumor suppressors or oncoproteins [22]. *FBW7* has a long and extremely well-conserved 3'-UTR with multiple putative microRNA binding sites. Thus, FBW7 is likely to be regulated by miRNAs. The first report that FBW7 is indeed regulated by miRNAs was just recently published [138]. The granulocyte specific miR-223 was identified as a regulator of FBW7 using a screen for miRNAs downregulated in cyclin $E^{T74A, T393A}$ knock-in erythroblasts (Xu *et al,* 2010). Interestingly, miR-223 is responsive to acute alterations in cyclin E regulation by FBW7 and it was suggested that miR-223 is part of a feedback loop connecting cyclin E activity to the regulation of FBW7 expression [138]. miR-223 is also overexpressed in several solid tumors [182] and increased expression has also been reported in T-ALL [183]. The relationship between miR-223 and FBW7 in tumors is however not known. We recently identified miR-223 and five additional miRNAs, miR-27a/b, miR-182 and miR-363/92 as potential regulators of FBW7 expression (paper IV). However, our data suggest that miR-27a is the most general regulatory miRNA for FBW7. Indeed, our data indicate that miR-27a is a physiological regulator of FBW7 expression during cell cycle progression and that is required for tight regulation of cyclin E degradation at the G1/S border. Thus, it is possible that overexpression of miR27 may contribute to dysregulated cyclin E expression and tumor development. Our findings were also confirmed by a recent study demonstrating miR27 as critical suppressor of FBW7 expression [184]. Interestingly, in this study, upregulation of miR-27a was shown to contribute to the malignant transformation of human bronchial epithelial cells induced by the SV40 small T antigen [184].

2.3.5.4 Viruses

Many major TSGs have been shown to be inactivated by specific tumor viruses. Simian virus 40 (SV40) is a well-characterized member of the polyomavirus family. The SV40 encodes three proteins based on alternative splicing, the large T (LT), small T (ST) and 17 kT antigens [185-187].

Interestingly, SV40 Large T Antigen can also inhibit FBW7-driven cyclin E turnover in vivo resulting in increased cyclin E associated kinase activity. LT contains a CPD, which is recognized by FBW7 and thus alters the normal capacity of FBW7 in targeting other substrates [187]. Thus, the binding of LT to FBW7 occurs via a decoy phospho-epitope within the C-terminus of LT that closely mimics the consensus Cdc4 phospho-degron (CPD) found within FBW7 substrates [187].

Human adenoviruses are a group of small DNA viruses that are capable of transforming rodent cells in culture and also induce tumors in hamsters or rats. Two viral oncogenes, termed E1A and E1B, have been identified as critical factors for adenoviral induced transformation through inactivation of the TSGs RB and p53 [188]. A recent report suggests that E1A can interfere with FBW7 function by targeting the SCF^{FBW7}complex [189]. E1A was found to directly bind to Roc1/Rbx1 and CUL1 and inhibit the ubiquitin ligase activity of core ligase, resulting in impaired degradation of several proto-oncogene products that are normally degraded by the SCF^{FBW7}ubiquitin ligase [189].

2.3.5.5 Other factors

As mentioned above, SCF^{FBW7} activity is regulated by several cofactors and most likely various post-translation modifications that could be of major importance for inactivation of FBW7 in cancer. There are contradictory results regarding the role of Pin1 in tumorigenesis [152, 154, 155] but it could potentially interfer with FBW7 function [91]. The deubiquitylating enzyme USP28 has been shown to be overexpressed in colon and breast carcinoma [156] and could possibly prevent FBW7-mediated degradation cyclin E and c-Myc [157]. Other cofactors, including NPM and Ebp2 could also in principle also interfere with FBW7 substrate degradation. Indeed, NPM is frequently mutated in AML and NPM mutations have been demonstrated to mislocalize FBW7 protein [190]. Upstream signaling cascades stimulating FBW7 substrate degradation, including the GSK3b [191] and the Ras [192] pathway, are frequently dysregulated in human tumors and may therefore be of major importance preventing FBW7 from degrading specific oncoproteins.

2.4 TARGETING THE UPS IN CANCER

During recent years there have been major efforts to develop drugs targeting the UPS system in cancer cells [193]. The fact that the UPS regulates important tumor suppressors first raised the possibility that inhibition of the 26S proteasome might be beneficial in the treatment of cancer patients. However, targeting the 26S proteasome will also stabilize critical oncoproteins and it is not possible to predict whether the inhibition of the 26S proteasome will necessarily be beneficial. Nevertheless, Bortezomib (Velcade), a reversible inhibitor of the chymotrypsin-like activity of the 26S proteasome, was approved by the US Food and Drug Administration (FDA) for the treatment of relapsed or refractory multiple myeloma in 2004. Today, Bortezomib is used for the treatment of hematologic malignancies as well as solid tumors such as breast cancer and several clinical trials, utlizing Bortezomib in combination with other drugs are ongoing [190, 194-198]. However, more specific drugs are warranted. The E3 ligases are particularly attractive drug targets since they are responsible for the recognition and ubiquitylation of specific substrates. In addition, it is now clear that one single E3 ligase often targets multiple proteins for degradation. For example, the F-box protein oncoprotein Skp2, targets several important tumor suppressor proteins, including the CDK inhibitor p27 [199], which is frequently downregulated in many different tumors [200]. As outlined in this thesis, FBW7 also targets multiple proteins for degradation, all being critical oncoproteins. Furthermore, we recently showed that the small-molecule inhibitor, RITA, reactivates p53-induced FBW7 expression resulting in proteasomal degradation of several FBW7 target substrates [201]. Interestingly, our results indicate that FBW7 inactivation is associated with a favorable prognosis and increased survival (paper I, III, IV). The reason for this is presently unclear. However, exciting results demonstrating synthetic lethal interaction for certain genes, including ATM and p53 [202], and PARP and BRCA1 [203], may hint that FBW7 could also be synthetic lethal with specific genes. A recent study identified a compound that act as an inhibitor of SCF^{Cdc4} in yeast [204], demonstrating that this type of E3 ligase can in principle be inactivated by small-molecules. The future will clarify whether targeting FBW7 or its cofactors with drugs can be used in the treatment of patients with cancer.

3 AIMS OF THIS THESIS

The overall aim of this thesis was to explore the mechanisms by which FBW7is inactivated in human cancers.

Specific aims:

- To investigate if FBW7is mutated in pediatric B- and T-ALLs (paper I)

- To elucidate if FBW7is a general tumor suppressor gene (Paper II)

- To explore if methylation is an alternative mechanism for inactivation of FBW7expression in cancer (Paper III)

- To investigate if FBW7is regulated by microRNAs (Paper IV)

4 RESULTS AND DISCUSSIONS

4.1 PAPER I

The Tumor Suppressor Gene hCDC4 Is Frequently Mutated in Human T-Cell Acute Lymphoblastic Leukemia with Functional Consequences for Notch Signaling.

At the beginning of this project, very little was known about whether FBW7 is mutationally inactivated in specific tumors. The findings that Notch1 is a novel FBW7 target substrate that is frequently targeted by gain of function mutations in T-ALL prompted us to investigate the possibility that FBW7 inactivation contributes to the development of pediatric leukemia.
We used SSCP (Single Strand Conformation Polymorphism assay) and sequence analysis to screen the entire coding region of *FBW7* and *Notch1* in 26 pediatric leukemic T- and 20 B-lineage ALL samples.

Interestingly, *FBW7* mutations were found in 8 of the 26 T-ALL specimens (31%) while no mutations were observed in leukemic cells from B-ALL patients. All mutations were missense mutations and the majority (88%) led to aminoacid changes of Arginine 465 and Arginine 479, which are located within the substrate recognition site of FBW7. FBW7 mutations were absent in cells from matching nonmalignant leukocytes obtained from the same patients, indicating that these mutations were of somatic origin. Additionally, similar mutations were also found in several cell lines derived from various hematological malignancies, particularly of T-cell origin. No hCDC4 mutations were identified in any other leukemia or lymphoma cell lines analyzed, with the exception of the multiple myeloma cell line U266-1984, which harbored an Arginine 505 missense mutation. Notably, one leukemic sample was found to harbor a Notch1 missense mutation resulting in a threonine to methionine substitution at position 2,484 in the PEST domain of Notch1. The amino acids surrounding threonine 2484 in Notch1 resembles a consensus CPD motif, suggesting that this mutation might prevent its recognition by FBW7. However, several truncating PEST domain mutations occur downstream of this motif, which suggests that multiple CPD motifs may be present in Notch1 ICD and regulate its degradation [74].

In line with Notch1 being a FBW7 substrate, Notch1 protein analysis in T-ALL cell lines showed that cell lines with hCDC4 mutations had elevated levels and increased stability of Notch1 ICD, as compared with T-ALL cell lines where hCDC4 was not mutated. Furthermore, we found that wild-type (wt), but not mutant *FBW7-alpha/gamma* isoforms, could restore ubiquitylation of Notch1 in vivo, and that expression of wt-FBW7 (but not mutant FBW7) decreased Notch1 signaling.

Importantly, our data indicated that mutations in *Notch1 and/or FBW7* associates with a favorable long-term survival in children with T-ALL. This was the first report showing that FBW7is mutated in pediatric T-ALL with functional consequences for Notch signaling. Previous studies from our group and others reported *FBW7* mutations in endometrial adenomcarcinomas [63] and colon [167]. However, the mutation frequency in those solid tumors were lower (16% and 6%).

In summary, previous work had shown that Notch1 signaling is commonly upregulated in T-ALL. In this paper, we show that the negative regulator of Notch1, *FBW7*, is frequently mutated in T-ALL. This data also extends previous findings, and suggests that mutation in hCDC4 and/or NOTCH1 is associated with improved overall survival of children with T-ALL. The finding that two genes in the same pathway, NOTCH1 and FBW7, are both frequently mutated in children with T-ALL is remarkable and emphasizes the importance of this pathway in T-ALL development and possibly therapeutic intervention and refined risk stratification.

4.2 PAPER II

FBXW7/hCDC4 Is a General Tumor Suppressor in Human Cancer.

Inspired by the frequent mutations of FBW7 found in T-ALL, we wondered whether FBW7 is inactivated in other tumors as well, and if it could represent a general tumor suppressor gene in human malignancy.

A total of 534 primary tumor specimens from 12 different tumor types were included in this study and analyzed by SSCP (Single Strand Conformation Polymorphism assay)

and sequence analysis for mutations in all 13 exons, including sequences unique to the three isoforms, alpha, beta and gamma. We next compiled these results with pre-existing *FBW7* mutation data (a total of 1556 samples). Table 2 summarizes of the mutational analysis.

Table 2. Summary of FBW7 mutational analysis

Mutation type	**Percentage (%)**	**Additional information**
Single amino acid change	96	(missense 74%, nonsense 26%)
Deletion and insertion	4	
Isoform specific mutations	6	
Average mutation frequency	***6***	***87 mutations out of 1556***
Tumor types		
Breast	0.8	
Bladder	0	
Cholangiocarcinoma	35	
Colon	9.5	
Endometrium	9	
esophagus	0	
Leukemia	31	T-ALL (31%), B-ALL (0), B-CLL (0), HCL (0), AML (0)
Liver	0	
Lung	3	NSCLC
Melanoma	0	
Bone	0	
Ovarian	0	
Prostate	1	A proline insertion in Alpha isoform
Pancreas	9	Only one study (1 mutation in 11 patients)
Stomach	15	

Abbreviations: B-CLL, B-cell chronic lymphocytic leukemia; B-ALL, B-cell acute lymphocytic leukemia; T-ALL, T-cell acute lymphocytic leukemia; HCL, hairy cell leukemia; NSCLC, non–small cell lung carcinoma.

Analysis of this large set of tumors confimed and extended previous analysis. The majority of the mutations cluster in the critical arginines previously shown to be responsible for the interaction between FBW7 and the phosphodegron of specific

substrates. Thus, mutations clustered at hotspot codons, encoding several arginine residues in the FBW7 WD40 repeats.

Investigation of the mutational hotspots showed that all are centered on CG dinucleotides, which are potential sites of DNA methylation and a source for mutations due to deamination of 5'methylcytosine to thymidine in DNA.

Indeed, we could show that the cytosine at the hotspot codon 479 was methylated in all tumors analyzed. Thus, these mutations seem to correspond to methylated CG sites, which could explain the high frequency of such missense mutations.

To examine the consequence on substrate binding of the hotspot mutations, we analyzed their ability to bind one of the FBW7 key substrates, cyclin E. None of the mutants could bind cyclin E (in contrast to wt-FBW7) and this was not due to mislocalization of the FBW7 mutants.

Regarding the fact that majority of the mutations were heterozygous raised the possibility that these mutations might interfere with the protein expressed from the normal wild-type allele of *FBW7*. Indeed, when co-expressed, mutant-FBW7 resulted in a marked accumulation of cyclin E1 despite expression of wt-FBW7. These results are well in agreement with findings that FBW7 forms dimers in vivo and it is therefore possible that mutations in only one allele of *FBW7* is enough to compromise substrate degradation through a dominant-negative function.

Interestingly, our analysis also demonstrates that mutations occur in the unique isoform specific 5'-exons. The functional consequences of mutations in specific isoforms are not fully understood, but could for example to mislocalization of a specific isoform. Indeed, we could show that a proline insertion in a prostate cancer specimen that resided at the amino acid 16 of the FBW7-alpha isoform mislocalized FBW7-alpha to the cytoplasm and thus preventing it from interacting with cyclin E1. Additionally, one FBW7-alpha specific mutation has been shown to disrupt the interaction between FBW7 and its cofactor Pin1 [91]. Together, these data suggest that FBW7 isoforms are non-redundant.

In summary, this paper is the first comprehensive screen for mutations in *FBW7* in diverse human tumor types. The results demonstrate an overall average mutation frequency of 6% and potential dominant negative hotspot mutations clustering in exon8 and exon9 of FBW7.

4.3 PAPER III

Inactivation of FBXW7/hCDC4-b expression by promoter hypermethylation is associated with favorable prognosis in primary breast cancer.

During the work with paper II, it became obvious that many tumors lack mutations in *FBW7*, although several of these tumors are known to have increased levels of specific FBW7 target substrates. For example, elevated cyclin E levels are frequently observed in breast tumors, but FBW7 is not found to be mutated in this malignancy. In addition, our preliminary analysis of FBW7 expression in both primary tumors and derived cell lines indicated that the expression of FBW7 varied significantly between and within different tumor types.

In the present study, we therefore examined the possibility that FBW7 expression is downregulated through promoter specific hypermethylation, as an alternative epigenetic mechanism for inactivation of FBW7 in cancer.

The FBW7- beta isoform was chosen for further analysis; since it showed the most variable expression pattern among a panel of different tumor-derived and immortalized cell lines. Interestingly, using bisulphate sequenceing and methylation-sensitive restriction digestion analysis, we found that the *FBW7-beta* promotor is heavily methylated in multiple tumor cell lines. Expression analysis showed that hypermethylation of the beta- promoter associates with a decreased expression of the FBW7-beta mRNA. In order to establish a direct link between methylation and expression we treated cells with a methylated beta- promoter with the demethylating agent, 5-AZA cytidine (AZA). Bisulphite sequencing and expression analysis revealed that expression of FBW7-beta was restored and its promotor was demethylated upon 5-AZA treatment. Additional investigations, including luciferase reporter assays with

an in vitro methylated promoter, also support the conclusion that *FBW7-beta* expression is regulated by promotor specific methylation.

We next asked whether *FBW7-beta* methylation also occurs in primary tumors. A total of 161 breast cancer specimens, from two independent cohorts (Austria and Sweden), were subject to methylation and expression analysis. The methylation status in each tumor sample was defined by restriction digestion test using McrBc enzyme. This method uses methylation-sensitive restriction McrBc enzyme, to cleave DNA at specific methylated-cytosine residues followed by amplification of the resultant products. The PCR amplification bands were then subject to quantification using Image J software. FBW7 isoform specific mRNA expression levels were analyzed by real time PCR (RT-PCR). Importantly promoter methylation was absent in normal breast tissue (and in other noncancerous tissue DNA extracted from paraffin-embedded breast cancer specimens).

A total of 71 out of 139 (51%) patient samples showed significant methylation of the beta promoter as defined by McrBc digestion, compared to its undigested control. Thus, as in tumor cell lines, there was a significant inverse correlation between promoter methylation and FBXW7/hCDC4beta expression in primary breast cancer specimens.

Interestingly, comparison of the *FBW7-beta* promoter methylation status of the individual samples with various clinicopathological factors, demonstrated that methylation associates with high-grade tumors (p= 0.017) and possibly estrogen receptor-negative tumors (p=0.08), thus tumors usually associated with an adverse prognosis in breast cancer. Remarkably, methylation of the FBW7beta promoter was anyhow found to be associated with a decreased risk of death ((cohort 1 hazard ratio 0.53 (0.23 to 1.23) and cohort 2 (HR) 0.50 (95% CI 0.23 to 1.08)) in both cohorts despite the correlation between methylation and high-grade tumors. When methylation was compared to overall survival in defined subgroups of the breast cancer, including patients with p53 mutation or lymph node negative tumors, patients with a methylated beta promotor had an improved overall survival.

In summary, this study is the first report of *FBW7* promoter hypermethylation and loss of expression in primary tumors. Our data support promoter methylation as an

alternative mechanism for FBW7 inactivation in cancer. The frequency of methylation is high (>50%) and links loss of FBW7-beta expression to high-grade tumors. However, patients with a methylated beta promotor still appear to have a decreased risk of death. The reason why methylation is linked to improved survival is presently unclear, but could possibly relate to increased sensitivity of methylated tumors to certain therapeutic drugs. This possibility, and its potential as a novel biomarker are areas that need further investigation.

4.4 PAPER IV

miRNA-27a controls FBW7/hCDC4-dependent Cyclin E degradation and cell cycle progression.

In paper III, we showed that FBW7 expression is regulated by methylation of specific CpG dinucleotides in the 5' UTR of FBW7. Another mechanism for regulation of gene expression is through miRNA binding to specific seed sequences in the 3'UTR of genes [205]. Interestingly, the FBW7 3'UTR is long and extremely conserved and contains a large number of putative miRNA binding sites. The aim of this study was therefore to investigate if FBW7expression is regulated by miRNAs.

An initial observation that FBW7 protein expression is elevated in cells with a targeted deletion of the miRNA-processing enzyme, Dicer, suggested that FBW7 3'UTR is indeed controlled by miRNAs .

In order to identify relevant candidate microRNAs that potentially repress FBW7expression, we employed a combination of computational predictions, luciferase reporter assays, miRNA library screens and expression profiling analysis. Luciferase reporter assays was initially utilized to identify cell lines where FBW7 is repressed by endogenous miRNA expression. miRNA expression profiling in combination with computer-based prediction of candidate miRNAs and a miRNA library screen identified miR-27a as a strong candidate for the repressive effect in the model cell lines utilized.

Further validation analysis confirmed that miR-27a is a negative regulator of FBW7expression. Mutation of two putative miR-27a binding sites located in the 3'UTR

region of FBW7completely abolished the suppressive effect of miR-27a and inhibition of miR-27a by antagomirs increased FBW7 expression. In addition, miR-27a overexpression reduced endogenous FBW7 protein levels in a dose-dependent manner.Together, these results confirm a direct function for miR-27a in the negative regulation of FBW7.

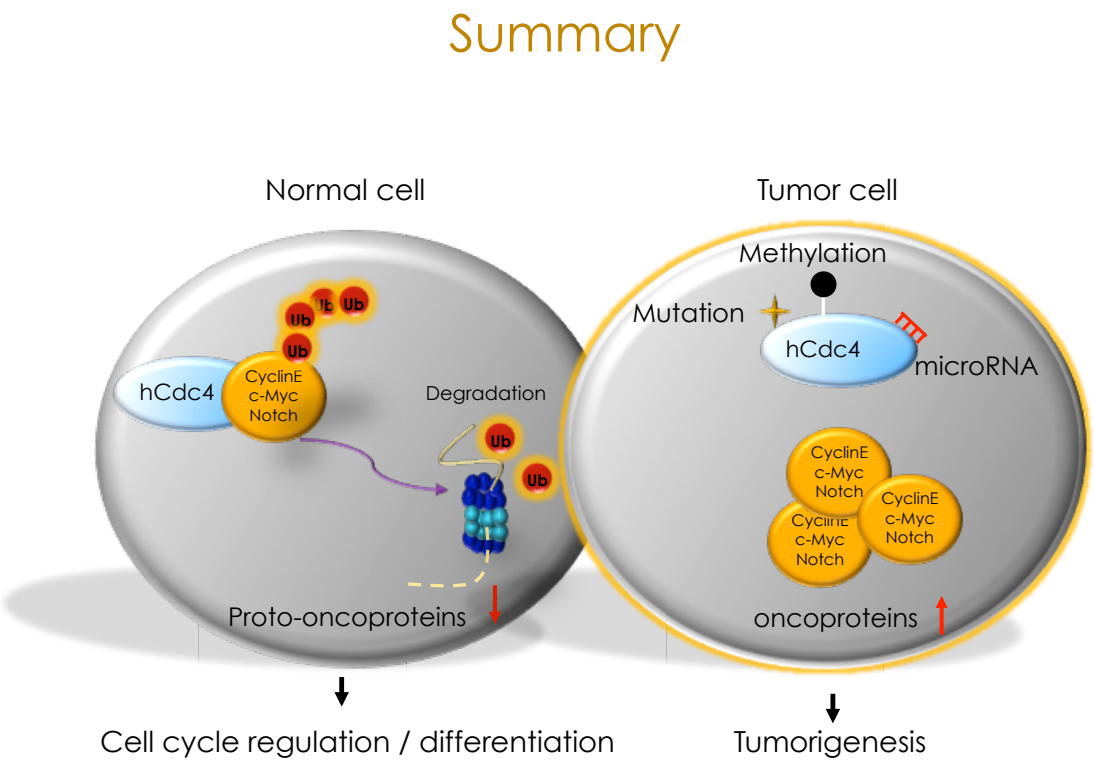
To evaluate the functional effect of miR-27a on FBW7, we performed several experiments to test if miR-27a specifically regulates the stability and ubiquitylation of FBW7target substrates. miR-27a overexpression resulted increased steady-state levels of cyclin E and myc which was due to an increased stability and decreased ubiquitylation of these proteins.
To date, we have very limited knowledge on if and how FBW7 is regulated during the cell cycle. To directly test if miR-27a regulates FBW7 during the cell cycle, we analyzed *FBW7*-3'UTR reporter activity (by comparing the wild-type 3'UTR with the miR-27a-mutated 3'UTR) in U2OS cells progressing from mitosis towards S-phase. Importantly, miR-27a was found to potently repress FBW7 at the G2/M and early G1 phases, but not at the G1/S transition. First of all, these results provide evidence that miR-27a regulates FBW7 in a physiological setting, during cell cycle progression. Second, the lack of repression at the G1 to S-border, indicate that miR-27a may release FBW7 from repression at this critical cell cycle stage, just prior to FBW7-mediated cyclin E degradation at early S-phase. Third, overexpression of miR-27a significantly elevated cyclin E levels in later cell cycle stages and increased the number of cells S-phase. In line with these results, miR27 overxoression induced DNA double-strand breaks (DSBs), as analyzed by gamma-H2AX foci formation, a phenotype directly related to dysregulation of cyclin E protein. Importantly, our results demonstrate that these effects are likely to be FBW7-dependent, as cyclin E degradation and gamma-H2AX foci formation was rescued by expression of FBW7 lacking the 3'UTR.

The fact that miR-27a is a negative regulator of FBW7 and is overexpressed in tumors, suggest that it may act as a novel oncogene. Interestingly, analysis of miR27a expression in pediatric B-ALL, a tumor type in *FBW7* is not mutated, showed that miR27 is significantly overexpressed in several B-ALL subtypes compared to normal bone marrow cells and $CD34_+$ B cells, Additionally, in hyperdiploid pre-B-ALL, a

significant inverse correlation between miR-27a expression and FBW7 mRNA expression was evident.

In summary, this study has unraveled a novel role for miR-27a, a ubiquitously expressed and putatively oncogenic miRNA, in the fine-tuned regulation of FBW7 function during the cell cycle. Our results also demonstrate that miR-27a is overexpressed in pediatric B-ALL, and show that in hyperdiploid B-ALL cases, increased levels of miR-27a are generally correlated with low levels of FBW7.

4.5 SUMMARY



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