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On 13q14 deletions in chronic lymphocytic leukemia

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ABSTRACT

Malignant tumors arise as a consequence of a multistep process where an accumulation of genetic and epigenetic changes drives the transformation of normal cells into tumor cells. The identification and characterization of genes targeted by these alterations is of crucial importance for an increased understanding of the biology of cancer.

The studies in this thesis have focused on genetic aberrations in chronic lymphocytic leukemia (CLL), and specifically those affecting chromosome band 13q14.3. Deletions involving 13q14 occur in more than 50% of all CLL cases and loss of this locus is also observed in several other malignancies. Results from numerous previous studies have indicated that a segment containing the *DLEU2* and *RFP2/LEU5* genes is targeted by these deletions. We characterized these genes with the purpose of elucidating the driving force behind these changes.

The *RFP2/LEU5* gene is the only protein-coding gene in the region. We demonstrated that Rfp2 is a novel RING E3 ubiquitin ligase. A series of experiments furthermore established Rfp2 as a membrane-bound protein specifically involved in endoplasmic reticulum-associated degradation (ERAD), a process responsible for the clearance of misfolded and improperly assembled proteins from the endoplasmic reticulum.

Characterization of *DLEU2* allowed us to demonstrate that it functions as a regulatory host gene for the microRNAs *miR-15a* and *miR-16-1*. These microRNAs were shown to target the G1 cyclins D1 and E1 for translational repression. In line with this, ectopic expression of *DLEU2*, and hence also *miR-15a/miR-16-1*, inhibited the colony-forming ability of tumor cell lines. Finally, we demonstrated that *DLEU2* is transcriptionally regulated by the oncoprotein Myc that associates with and represses the two alternative *DLEU2* promoters. Together, our data strongly support an important function for *DLEU2* in regulation of G1 cyclin protein levels and further suggest a novel mechanism for Myc-induced proliferation. In this way, inactivation of *DLEU2* could promote G1 cyclin deregulation and tumor progression.

In another study, we investigated the underlying molecular mechanism behind genomic aberrations in CLL. By analyzing genetic breakpoints from CLL patients, we could identify a CLL-specific signature. Our studies revealed that CLL breakpoints are characterized by an overrepresentation of short direct repeats, a feature not found in other malignancies analyzed. This indicates that repeats of this kind are specifically involved in genetic recombination leading to deletions and translocations in CLL.

The results presented in this thesis provide insight into the nature of genetic alterations in CLL, and specifically help define the molecular basis of 13q14.3 aberrations in malignancy.

LIST OF PUBLICATIONS

I. Martin M. Corcoran, Marianne Hammarsund, Chaoyong Zhu, **Mikael Lerner**, Bagrat Kapanadze, Bill Wilson, Catharina Larsson, Lars Forsberg, Rachel E. Ibbotson, Stefan Einhorn, David G. Oscier, Dan Grandér, and Olle Sangfelt.
DLEU2 encodes an antisense RNA for the putative bicistronic *RFP2/LEU5* gene in humans and mouse.
Genes Chromosomes Cancer. 2004 Aug;40(4):285-97.

II. **Mikael Lerner**, Martin M. Corcoran, Diana Cepeda, Michael L. Nielsen, Roman Zubarev, Fredrik Pontén, Mathias Uhlén, Sophia Hober, Dan Grandér, and Olle Sangfelt.
The RBCC gene *RFP2 (Leu5)* encodes a novel transmembrane E3 ubiquitin ligase involved in ERAD.
Mol Biol Cell. 2007 May;18(5):1670-82.

III. **Mikael Lerner**, Masako Harada, Jakob Lovén, Juan Castro, Marie Henriksson, Olle Sangfelt, Dan Grandér, and Martin M. Corcoran.
DLEU2 downregulates the G1 Cyclins D1 and E1 through microRNA-mediated repression.
Submitted.

IV. **Mikael Lerner***, Martin Enge*, Marianne Farnebo, Rachel E. Ibbotson, Anne Gardiner, Mats Merup, David G. Oscier, Galina Selivanova, Dan Grandér, and Martin M. Corcoran.
Analysis of chronic lymphocytic leukemia breakpoints implicates short direct repeats as a disease-specific cause of double strand break formation.
Submitted.

* Both authors contributed equally.

RELATED PUBLICATIONS

1. Marianne Hammarsund, **Mikael Lerner**, Chaoyong Zhu, Mats Merup, Monika Jansson, Gösta Gahrton, Hanneke Kluin-Nelemans, Stefan Einhorn, Dan Grandér, Olle Sangfelt, and Martin M. Corcoran.
   Disruption of a novel ectodermal neural cortex 1 antisense gene, *ENC-1AS* and identification of *ENC-1* overexpression in hairy cell leukemia.
   Hum Mol Genet. 2004 Dec 1;13(23):2925-36.

2. Wen-Hui Weng, **Mikael Lerner**, Dan Grandér, Jan Åhlén, See-Tong Pang, Johan Wejde, Weng-Onn Lui, and Catharina Larsson.
   Loss of chromosome 13q is a frequently acquired event in genetic progression of soft tissue sarcomas in the abdominal cavity.
   Int J Oncol. 2005 Jan;26(1):5-16.

LIST OF ABBREVIATIONS

Ago	argonaute
AMFR	autocrine motility factor receptor
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
BCL2	B-cell lymphoma 2
bHLH/LZ	basic helix-loop-helix leucine zipper
BiP	immunoglobulin heavy chain binding protein
Cbl	casitas B-lineage lymphoma
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
CDK	cyclin-dependent kinase
CFTR	cystic fibrosis transmembrane conductance regulator
ChIP	chromatin immunoprecipitation
Cip/Kip	cdk interacting protein/cdk inhibitory protein
CKI	CDK inhibitor
CLL	chronic lymphocytic leukemia
CP	core particle
CPY	carboxypeptidase Y
DDOST	dolichol-diphosphooligosaccharide-protein glycosyltransferase
DGCR8	DiGeorge syndrome critical region 8
DLEU	deleted in lymphocytic leukemia
Dltet	K+-channel tetramerization domain homologous ORF deleted in leukemia
DNA	deoxyribonucleic acid
Dnd1	dead end 1
dsRNA	double-stranded RNA
DUB	deubiquitinating enzyme
EDEM	ER-degradation enhancing α-mannosidase-like protein
eIF	eukaryotic translation initiation factor
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FISH	fluorescence in situ hybridization
GRaBD	Gross Rearrangement Breakpoint Database
GTP	guanosine triphosphate
hCdc4	human cell division cycle 4
HECT	homologous to the E6-AP carboxyl terminus
Hrd1	HMG-CoA reductase degradation 1
IFN	interferon
Ig	immunoglobulin
INK4	inhibitor of CDK4
JDR	junctional direct repeat
K	lysine
KCNRG	K+ channel regulator
lin	abnormal cell lineage

Mad	Max dimerizer
MARCH VI	membrane-associated ring finger (C3HC4) 6
Max	Myc-associated factor X
MBL	monoclonal B-cell lymphocytosis
Mcl-1	myeloid cell leukemia sequence 1
MCM	minichromosome maintenance
Mdm2	mouse double minute 2
MDR	minimally deleted region
MHC	major histocompatibility complex
Miz1	Myc-interacting zinc finger protein 1
Mnt	Max-interacting protein
Myc	myelocytomatosis
NAT	natural antisense transcript
NF-kB	nuclear factor-kappa B
Npl4	nuclear protein localization 4
NZB	New Zealand black
PABP1	polyadenylate-binding protein 1
Pael	Parkin-associated endothelin receptor-like
PDGFB	platelet-derived growth factor beta
piRNAs	Piwi-interacting RNAs
PML	promyelocytic leukemia
PTEN	phosphatase and tensin homolog
RAG	recombination-activating gene
Ran	Ras-related nuclear protein
Ras	rat sarcoma viral oncogene homolog
Rb	retinoblastoma protein
RBCC	RING finger B-box coiled-coil
RFP2	Ret finger protein 2
RFP2OS	*RFP2* opposite strand
RING	really interesting new gene
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RP	regulatory particle
RT-PCR	reverse transcriptase polymerase chain reaction
SCF	Skp1-Cullin-F-box
SERCA2A	sarcoplasmic/endoplasmic reticulum calcium ATPase 2
shRNA	short-hairpin RNA
Skp2	S-phase kinase-associated protein 2
SNRPN	small nuclear ribonucleoprotein polypeptide N
SNURF	SNRPN upstream reading frame
TCR	T-cell receptor
TGF	transforming growth factor
TM	transmembrane
TP53	tumor protein p53
TRBP	human immunodeficiency virus transactivating response RNA-binding protein
TRIM	tripartite motif

TSG	tumor-suppressor gene
TTP	tristetraprolin
Ub	ubiquitin
Ubc	ubiquitin conjugating enzyme
Ufd1	ubiquitin fusion degradation 1
UPS	ubiquitin-proteasome system
UTR	untranslated region
V(D)J	variable (diversity) joining
VCP	valosin containing protein
v-src	viral sarcoma
YFP	yellow fluorescent protein
ZAP-70	zeta-chain-associated protein kinase 70

TABLE OF CONTENTS

1 CANCER

1.1 CANCER AS A MULTISTEP GENETIC AND EPIGENETIC DISEASE

General descriptions of the nature of cancer are problematic since the term "cancer" in reality represents hundreds of different diseases each with its own tissue of origin, etiology and optimal treatment. This notwithstanding, some general features have been described that apply to most, if not all, human malignancies.

It is now commonly accepted that cancers form as a consequence of a multistep process that involves the evolution of normal cells into neoplastic counterparts [1]. Normally, cells in multicellular organisms are restricted to grow, divide and die in a way that is of benefit to the organism as a whole. To this end, most cells in the human body are only granted permission to proliferate a limited number of times and are furthermore required to undergo programmed cell death (apoptosis) when no longer necessary or when they become damaged. While small subsets of undifferentiated stem cells exist that are responsible for the replenishment of tissue compartments and that have unlimited self-renewal capacity, the vast majority of cells are differentiated with a reduced capability to divide. This tight regulation of cell division and death is crucial for the proper functioning of the organism and a perturbation of this balance is a critical underlying factor in the transformation of normal cells into tumor cells.

The endpoint of tumor progression is the formation of a malignant tumor. Fortunately, this stage is rarely reached since a set of basic defense mechanisms have been developed during the course of evolution to thwart the emergence of tumorigenic outgrowths. During tumor progression the cancer cell acquires capabilities through various genetic and epigenetic alterations that enable it to escape these control measures. Genetic changes include point mutations, deletions, amplifications and translocations [2]. Epigenetic events, i.e. heritable changes in gene expression that do not involve alterations of the primary DNA sequence, can be exemplified with the recurrent repression of certain genes through promoter methylation [3]. A single somatic cell will acquire a random genetic (or epigenetic) change that will give it a proliferative advantage over the neighboring cells. This cell will expand more readily than the surrounding cells and eventually produce a clone. Once this clone has reached a sufficient size, another advantageous mutation will make one of the cells more proliferative than the rest of the population. This process is then reiterated until a fully malignant phenotype is evident. The emerging tumor cell is thus under selective pressure to develop more and more advantageous changes in order to compete out its neighboring cells [4].

However, recently doubts have been raised whether all cells within an emerging clone have the same capability to evolve further. From studies on acute myeloid leukemias, breast cancers and brain tumors it appears that certain subpopulations of malignant cells produce tumors when introduced into mice in small quantities, while much larger quantities of other subpopulations do not [5-7]. Analysis of the individual subpopulations indicated that the minor populations capable of forming tumors are composed of less differentiated stem-cell like cells. This has led to the notion of cancer stem cells and implies that cancerous tissues have a similar organization as normal

tissues, with a small population of cells capable of self-renewal and a larger population of daughter cells with limited renewal capacity.

Due to the breaching of cellular control circuits, tumor cells sooner or later become genetically unstable. Deregulated proliferation leads to untimely cellular division allowing for the increased risk of transmitting defective genes to daughter cells. Furthermore, mutations in genes controlling DNA repair also accelerate the accumulation of genetic changes. Because of this the evolution of clones is not strictly linear. Instead, large numbers of genetically different subclones will exist within one single tumor [8].

1.2 THE NATURE OF THE GENETIC CHANGES

While having briefly discussed the sequence of changes that lead to cancer, we have not yet described the exact nature of these changes. What ultimately distinguishes a tumor cell from a normal cell is that it has acquired a set of capabilities making it more autonomous. The genes that are deregulated in the process of obtaining these characteristics differ between tumor types, but the capabilities themselves are remarkably consistent. Fundamentally, cancer is characterized by the abnormal accumulation of a group of cells at the expense of the multicellular organism. This accumulation is due to excessive proliferation, defective apoptosis or a combination of the two. In most cases a set of other traits must also be acquired in order for a sustainable tumor mass to develop, such as the ability to proliferate indefinitely (i.e. become immortalized), the ability to induce the formation of new blood vessels (angiogenesis), and the ability to metastasize (i.e. to be able to invade neighboring tissues and build colonies at distant sites in the body). As mentioned above, the acquisition of all these individual traits is facilitated by the relative genetic instability of tumor cells [9]. In the end, for tumor development to take place changes must occur in specific genes that in their normal capacity regulate the above-mentioned processes. These genes can be crudely divided into two major classes: oncogenes and tumor-suppressor genes.

1.3 ONCOGENES

Oncogenes are genes that have acquired gain-of-function mutations that give them the ability to promote the development of cancer. Their discovery dates back to the early 20th century when Peyton Rous identified an infectious agent that causes tumor formation in birds [10]. Later it was found that this retrovirus, Rous sarcoma virus, carries a gene called *v-src* that is responsible for its tumorigenic potential. Work by Michael Bishop and Harold Varmus in the 1970s demonstrated that a homologue of *v-src* is present in humans and this led to the notion that oncogenes represent inappropriately activated normal genes [11].

In their normal state oncogenes are termed proto-oncogenes and generally function to regulate cell growth and differentiation [12]. Upon activation, the proto-oncogene becomes an oncogene and can promote cancer. Proto-oncogenes encode diverse proteins such as growth factors (e.g. *PDGFB*), growth factor receptors (e.g.

ERBB2), intracellular signaling intermediates (e.g. *KRAS*) and transcription factors (e.g. *MYC*). They can be inappropriately activated in many different ways; by amplifications, point mutations and translocations. Mutations affecting proto-oncogenes are usually dominant and only one allele of the gene needs to be affected.

As a result these changes help the cancer cell to attain an autonomous growth state, which in turn is instrumental in malignant transformation.

1.4 TUMOR-SUPPRESSOR GENES

When a tumor-suppressor gene (TSG) is functioning normally it acts to prevent cancer formation [13]. The concept of tumor-suppression originated from experiments by Harris and coworkers that found that malignant cells initially become non-tumorigenic when fused to normal cells. However, continued cultivation of these fusion cells leads to progressive loss of chromosomes and reversion to malignancy [14]. Additional support for the existence of TSGs came from studies on familial cancer, where affected individuals have a predisposition to develop cancer since they inherit one damaged allele of a TSG. In 1971 Alfred Knudson formulated his "two-hit-hypothesis", based on statistical analysis of the incidence of inherited and sporadic cases of retinoblastoma [15]. Knudson suggested that several "hits" affecting the DNA were required for cancer to develop. In children with familial retinoblastoma, the first hit was inherited in the DNA and any subsequent hit would rapidly lead to cancer. In sporadic cases, on the other hand, two independent hits would have to take place during the individual's lifetime before cancer could develop. This would explain why familial retinoblastoma occurs much earlier in life than sporadic retinoblastoma and why multiple tumors develop in the inherited form. TSGs can thus be considered recessive in that both alleles of a TSG generally need to be inactivated for the tumor-suppressive function to be lost. This is not always true however and for several TSGs the presence of one functional copy yields a mutant phenotype. This can be due to haploinsuffiency, where the amount of gene product generated by one allele is not sufficient or to dominant negative mutations that block the function of the protein produced from the wild-type allele. Yet another alternative is that expression from the remaining allele is lacking due to mechanisms such as genomic imprinting [16].

TSGs are generally inactivated by deletions, point mutations and epigenetic silencing [2, 3]. The consistent loss of specific chromosomal regions can therefore be an indication of the presence of a TSG. This notion has been the basis of the current thesis, where the recurrent deletion of a locus on chromosome 13q14 has led to the functional characterization of the genes in that region in the search for putative TSGs (Papers I-III).

TSGs are involved in several critical cellular pathways such as apoptosis regulation, cell cycle control, signal transduction and DNA repair. According to Kinzler and Vogelstein, two major groups of TSGs can be discerned: "gatekeepers" and "caretakers" [17]. Genes of the former group are directly involved in the suppression of a tumorigenic phenotype by affecting cell proliferation, cell death and differentiation. The latter group is represented by genes that indirectly suppress tumor development by ensuring the integrity of the DNA. These genes are involved in DNA repair and the prevention of genomic instability. One should be mindful that this subdivision is not

absolute. Since some genes have the ability to both prevent abnormal growth and assist in maintaining genomic stability it is perhaps more relevant to talk about "gatekeeper" and "caretaker" *functions*. One example of this is *TP53*. This widely known TSG acts as a gatekeeper by inducing cell cycle arrest and apoptosis and as a caretaker by being involved in the cellular response to DNA damage [18]. The importance of *TP53* is underlined by the fact that it is the single gene most frequently found mutated in cancer. It is estimated to be inactivated in more than 50% of all human cancers [19].

2 THE CELL CYCLE

In order to understand how excessive proliferation leads to cancer it is first important to be aware of how cell division is regulated in normal cells. The division of cells entails replication of the DNA followed by division of the nucleus and partitioning of the cytoplasm to yield two daughter cells. To accomplish this, the cell proceeds through a series of steps that are collectively referred to as the cell division cycle, or simply the cell cycle.

For simplicity, the cell cycle has been divided into the following major phases: G0, G1, S, G2 and M (Figure 1). The cell's DNA is replicated during S and the cell physically divides during the mitotic (M) phase. In between these phases two gap stages exist (G1 and G2 respectively) that are necessary to give the cell opportunity to respond to signals from its extracellular environment and to check that everything proceeds without error. Additionally, sometimes cells reversibly enter a quiescent state termed G0 in which they can remain for long periods of time until induced to divide again [20].

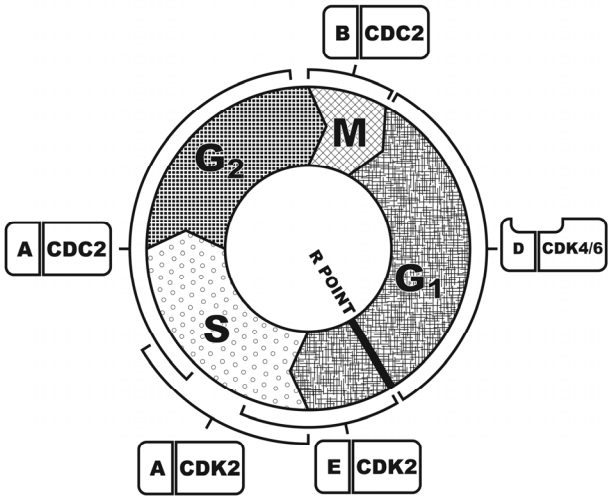

Figure 1. *The cell cycle.*

The actual part of the cell cycle that has been studied most extensively in tumor biology is the transition between G1 and S phase. During G1, the cell makes the critical decision whether to divide again or enter quiescence. This decision is mainly based on the presence of mitogenic and growth inhibitory factors in its surrounding [21]. Once a cell makes the decision to divide it proceeds autonomously throughout the rest of the cell cycle, not requiring further signals from the outside. This critical point in G1 has been referred to as the restriction point [22, 23]. Ultimately, it is the molecular machinery that governs transition through the restriction point that is deregulated in most cancers.

What applies to all eukaryotic cells is that they need to activate a group of proteins called cyclin-dependent kinases (CDKs) in order to proceed through the cell

cycle [21]. CDKs are serine/threonine protein kinases that phosphorylate a set of substrates essential for normal cell cycle progression. Different members of the CDK family have different target specificities. Of importance is that CDKs are dependent on binding to cyclin proteins for enzymatic activity [24]. CDK proteins are expressed throughout the cell cycle, whereas cyclins oscillate dramatically in levels [25]. They are short-lived proteins that are mainly regulated at the level of transcription and protein stability [24, 26]. Thus, the temporal specificity of the cyclin-CDK complexes is in major part dictated by the abundance and availability of cyclins during different cell cycle stages.

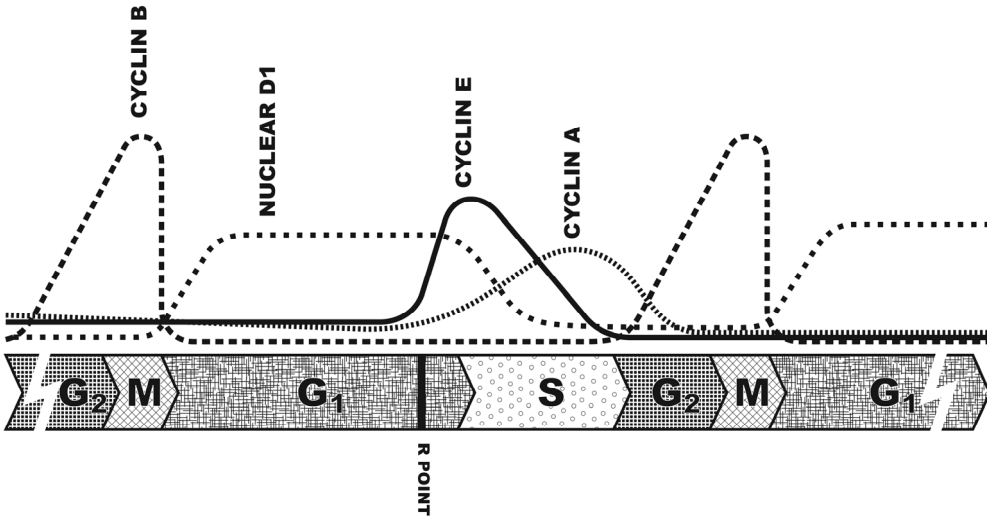

Figure 2. *The cyclins.*

Most cyclins fluctuate consistently during the cell cycle. E-cyclins are for example expressed after the restriction point at the G1/S border, while A-cyclins are expressed during S and G2 phases (Figure 2) [27, 28]. A major exception is the D-type cyclins (D1, D2 and D3), the expression of which is controlled by extracellular signals during G1 [29, 30]. The abundance of these specific cyclins influences whether the cell will divide or not. Their importance has been illustrated in experiments where overexpression of D-cyclins in mouse fibroblasts shortens G1 phase [31]. Cyclins D1, D2 and D3 associate with CDK4 and CDK6 and these complexes can drive the cell past the restriction point, thereafter being superseded by the other cyclin-CDK complexes that make sure that the cell division cycle is completed. However, it has become clear that these players are not mandatory for G1/S progression to take place. Actually, mice lacking all D-type cyclins develop normally until mid-gestation, a stage when most tissues and organs are already formed. Furthermore, fibroblasts prepared from triple knockout embryos proliferate at nearly the same rate as wild-type cells [32]. These results clearly indicate that there exist alternative mechanisms that allow progression through the restriction point.

The cell cycle is additionally regulated by a group of proteins called CDK inhibitors (CKIs). Two major groups of CKIs exist: the INK4 (originally named as inhibitors of CDK4) and Cip/Kip (cdk interacting protein/cdk inhibitory protein)

proteins [33]. The INK4 proteins ($p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$) inhibit the cyclin D-CDK4/CDK6 complexes by distorting the cyclin-binding site of the CDK. Additionally, they bind the ATP-binding site and in that way inhibit catalytic activity [34]. The Cip/Kip proteins ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) on the other hand are able to inhibit all the remaining cyclin-CDK complexes (E-CDK2, A-CDK2, A-CDC2, and B-CDC2) presumably by blocking the ATP-binding site in the catalytic site of the CDK [35]. Like cyclins, all the CKIs are regulated by intrinsic and extrinsic signals. For example, anti-mitogenic factors such as TGF-β and IFN-α induce $p15^{INK4B}$ which helps to bring the cell cycle to a halt [36, 37].

The CKIs additionally exert their effects by a process termed CKI exchange [26]. While inhibiting the above-stated cyclin-CDK complexes, $p21^{Cip1}$ and $p27^{Kip1}$ can actually have a stimulatory effect on cyclin D-CDK4/6 complexes. This leads to the following scenario: as D-type cyclins accumulate in G1, they bind the Cip/Kip inhibitors and sequester them away from cyclin E-CDK2 complexes. These complexes are thereby liberated to activate their downstream targets. This demonstrates how different waves of signaling ensure that the cell cycle transition is finalized once the decision to divide has been made.

So how does cyclin D-CDK4/6 activity actually promote cell cycle progression? One principal target of cyclin D-CDK4/6 is the Rb tumor suppressor protein [38]. In order for the cell to progress into S phase, the Rb protein must be extensively phosphorylated and thereby inactivated. This process is initiated by cyclin D-CDK4/6 and completed by Cyclin E-CDK2 [39]. Unphosphorylated or hypophosphorylated Rb is associated with a set of E2F transcription factors, the activity of which is essential for G1/S progression. As long as E2Fs are bound to Rb, they cannot function as activators of transcription [21]. Upon hyperphosphorylation of Rb, heterodimeric complexes containing E2Fs and DP subunits (DP1 or DP2) are liberated and can proceed to regulate the transcription of a large set of genes. Their targets include different mediators of DNA replication such as thymidine synthase, ribonucleotide reductase and DNA polymerase α. Also, E2Fs activate genes that ensure that the remaining, "autonomous", part of the cell cycle is completed, such as cyclin B and different DNA quality control components [40]. Furthermore, cyclin E transcription is also induced by E2Fs. This "feed-forward loop" makes sure that the cell committed to cell division sticks to its task. Cyclin E function is not restricted to hyperphosphorylating Rb however, but is crucial for several aspects of S-phase progression. For example, cyclin E has an important role in the assembly of pre-replication complexes by promoting the loading of MCM proteins onto chromatin [41]. In this fashion, the cell proceeds to duplicate its DNA content and later complete cell division.

2.1 CELL CYCLE DEREGULATION IN CANCER

Deregulation of the molecular machinery controlling G1/S transition is a common theme in most, if not all, human cancers. The G1 cyclins are inappropriately expressed in a wide variety of tumors. Overexpression of cyclin D1, due to amplification or other mechanisms, is for example evident in over 50% of breast cancers [42]. While not normally expressed in hematopoietic cells, cyclin D1 is activated in mantle cell lymphomas due to the consistently found t(11;14)(q13;q32) translocation [43]. Cyclin

D2 deregulation, on the other hand, is frequently found in testicular and ovarian cancers [44, 45]. Furthermore, a considerable amount of human cancers show excessive levels of cyclin E [46]. This is frequently a result of amplification of the Cyclin E gene or functional inactivation of the hCdc4 protein that normally is responsible for ubiquitin-dependent proteasomal degradation of this cyclin [47]. In this thesis, a novel way of deregulating G1 cyclins by way of microRNA inactivation is discussed in Paper III.

The *RB1* gene was actually the first TSG to be identified and it is found deleted or mutated in various tumors, such as retinoblastomas, bladder cancers and small cell lung cancers [48]. Additionally, CDK4 amplifications are observed in glioblastomas and sarcomas [49, 50]. Furthermore, the relevance of CKIs as negative regulators of the cell cycle is underpinned by their frequent inactivation in a wide variety of cancers [21, 48].

Another common way for cancer cells to promote unlicensed cell cycle progression is through the oncogene *MYC* that is deregulated in many different human cancers due to amplification, point mutation, enhanced translation, or chromosomal translocation [51]. Myc is part of a family of basic helix-loop-helix leucine zipper (bHLH/LZ) transcription factors that also includes proteins such as Max, Mad and Mnt [52]. The encoded protein product forms heterodimeric complexes with other members of this transcription factor family. These bind specific DNA motifs designated E-boxes and regulate the expression of a large number of downstream genes. The exact functional outcome of Myc activation depends on the magnitude of its expression as well as on what partner proteins it is associated with. Myc-Max heterodimers bound to DNA promote transcription. In contrast, Myc represses transcription of target genes when it is additionally associated with Miz1. A crucial regulatory feature of the Myc signaling network is that Max can form complexes with other bHLH/LZ proteins than Myc. Proliferating, non-differentiated cells have predominant expression of the Myc-Max complex. During differentiation, proliferation is halted as increased expression of Mad leads to an abundance of Mad-Max complexes that conversely act to repress transcription [53]. Simply put, Myc drives proliferation and counteracts differentiation by enforcing the expression of several positive regulators of the cell cycle, such as cyclin D2, CDK4 and E2F2 [54-56]. In concert with Miz1, is can assist in cell cycle progression by repressing $p15^{INK4B}$ and $p21^{Cip1}$ [57, 58]. In Paper III a novel pathway integrating Myc and cyclin signaling is described.

All the described alterations have one functional consequence in common; they push the cell to enter S phase instead of differentiating or dying. The unwarranted and sometimes premature cell division results in the accumulation of malignant cells and additionally leads to further mutations of benefit to the cancer cell.

3 CHRONIC LYMPHOCYTIC LEUKEMIA

3.1 EPIDEMIOLOGY

Chronic lymphocytic leukemia (CLL) is the most common hematological malignancy in the Western world accounting for approximately 30% of all leukemias [59]. The disease mainly affects middle-aged and elderly patients and the median age at diagnosis is around 65 years. Men are affected at twice the rate as women [60]. While exposure to pesticides and other chemicals has been proposed to lead to increased risk of acquiring CLL, strong evidence supporting the involvement of environmental risk factors is still lacking [59]. A familial component has been suggested and several studies have demonstrated that first-degree relatives of patients with the disease are more than two times more likely to be diagnosed with CLL than the general population [61].

3.2 CLINICAL FEATURES

CLL is a disease characterized by the accumulation of mature-looking malignant B cells in peripheral blood, bone marrow and lymph nodes. Common signs include persistent lymphocytosis, lymphadenopathy, splenomegaly and B symptoms such as fever and night sweats. Patients at an early stage are often asymptomatic despite a heavy tumor burden. The median survival for all patients ranges from 9 to 12 years [60, 62]. It is often an indolent disease and some patients live for many years without treatment. In other cases, the disease is more aggressive and requires immediate treatment. This differential outcome underlines the importance of a careful diagnosis based on several clinical and laboratory parameters. These include lymphocyte doubling time, hemoglobin count, extent of organ infiltration, cytogenetic changes, expression status of the protein kinase ZAP-70 and IgV_H mutation status (see below) [63-66]. While CLL can be successfully treated using alkylating agents (e.g. chlorambucil) or purine analogues (e.g. fludarabine), complete remissions are rarely seen and it can therefore be considered a chronic, incurable disease. In addition to chemotherapy, other treatment modalities are being used with increased frequency, such as monoclonal antibodies (e.g. alemtuzumab) and hematological stem cell transplantation for younger patients [63, 67].

3.3 THE CLL CELL

The dominating cell in CLL is a mature B lymphocyte that is slightly bigger than normal B cells in the peripheral blood. It mostly has a round nucleus with tightly packed chromatin and a scanty, agranular cytoplasm. CLL cells express normal B-cell markers such as CD19 and CD20 and have faint expression of surface immunoglobulins, typically of the IgM or IgD class. A feature distinctive of CLL cells is surface expression of the CD5 marker. This transmembrane protein is usually found on thymocytes and mature T cells. It is also present in a small subpopulation of B cells in

the mantle zone of the lymph node that is expanded in several autoimmune disorders. This latter observation is particularly interesting considering the propensity for autoimmune complications in CLL [63]. T-cell type CLL (T-CLL) has been described but the designation is debated as this rare condition, although similar in morphology, differs from the B-cell counterpart both clinically and biologically [68].

Generally, insight into the origin of individual B-cell malignancies can be gained by analyzing the mutational status of the genes encoding immunoglobulin variable regions (IgV_H). In order to reach maturation normal B cells undergo a series of programmed genetic recombination steps first in the bone marrow and later in secondary lymphoid organs such as lymph nodes. Upon encounter with antigen the B cell enters the germinal center of the lymph node and the IgV_H undergo somatic hypermutation in order for the cell to be able to produce high-affinity antibodies. Therefore, leukemias originating from pre-germinal center cells have unmutated immunoglobulin genes, while leukemias originating from post-germinal center cells have mutated counterparts. This scenario is more complex in CLL since it represents one of the few leukemias where both cases with or without somatically hypermutated IgV_H are seen. The mutational status is furthermore of clinical interest since it is a highly relevant prognostic factor. CLL patients with mutated IgV_H genes survive significantly longer than the ones without [66]. With respect to both the immunophenotype and the mutational status of the immunoglobulin genes it is consequently difficult to identify the normal counterpart to the CLL cells. Intriguingly, gene expression profiling of CLL cells has demonstrated that they are related to normal memory B cells [69]. The following model regarding the putative origin of the CLL cell can be posited based on the above-stated data. Either the cell experiences a primary genetic lesion in the bone marrow and consequently encounters antigen within the germinal center in the context of antigen-presenting cells restricted by T cells. This would lead to leukemic memory cells with hypermutated immunoglobulin genes. In contrast, the cell might also be unconventionally stimulated outside of the germinal center by a T-cell independent antigen or by superantigen which would result in an "experienced" B cell without somatic IgV_H mutations [70].

Traditionally, the accumulation of malignant cells in CLL has been considered mainly a consequence of an inherent apoptosis defect, rather than of excessive proliferation in the growing clone. This notion has been supported by how CLL cells appear in a light microscope having several morphological features associated with quiescence (small cells with condensed chromatin, high nuclear-to-cytoplasmic ratio, lacking prominent nucleoli) [71]. Additionally, flow cytometric analysis of CLL cells has demonstrated that a large proportion of peripheral lymphocytes in CLL patients are in the G0 phase of the cell cycle [72]. CLL cells generally overexpress the antiapoptotic protein Bcl-2 which is in line with their proposed increased life span. This is normally due to hypomethylation of the *BCL2* gene [73], but other mechanisms have been suggested, such as decreased expression of miR-15a/miR-16-1 leading to diminished translational repression of the Bcl-2 transcript [74]. However, the concept of CLL as a non-proliferative disease has been convincingly challenged during recent years and several independent observations have led to a reconsideration regarding this matter. For example, the telomeres of CLL cells are significantly shorter than those of age-matched normal B cells, indicating that the leukemic cells have completed more cell division cycles than their normal counterparts [75]. Furthermore, the birth and death rate of CLL cells has been determined *in vivo* in patients and this led to the conclusion

that between 10^9 and 10^{12} leukemic cells are produced each day. Importantly, the extent of proliferation of CLL cells correlates with the overall change in white blood cell counts in patients and also with the clinical course observed [71, 76]. These findings are specifically relevant to the findings presented in paper III of this thesis.

3.4 GENETIC CHANGES IN CLL

Many hematological malignancies are characterized by defining recurrent chromosomal translocations, such as the t(9;22) translocation seen in nearly all chronic myelogenous leukemia cases [77]. Such a feature is not observed in CLL, where instead a set of different recurring chromosomal alterations, mostly deletions, are seen. Today, genomic aberrations are detected in over 80% of all cases using such methods as fluorescence in situ hybridization (FISH) [78]. The by far most frequent genetic alteration is deletion of the long arm of chromosome 13 (see below). Other relevant genetic abnormalities include trisomy 12 and deletions at chromosome arms 11q, 17p and 6q [78]. The frequency of these recurring aberrations is listed in Table 1.

Aberration	Minimal region defined*	Frequency in CLL patients (%)**
13q deletion	13q14.3	55
11q deletion	11q22.3-q23.1	18
12q trisomy	12q13-q15	16
17p deletion	17p13.3	7
6q deletion	6q21-q23, 6q25-q27	6

* As reviewed in [78].
** As presented in a FISH study of 325 CLL patients [65].

Table 1. *Genetic abnormalities in CLL.*

In many instances, the molecular basis for these abnormalities is unknown. The minimally deleted region (MDR) on chromosome 11q harbors the *ATM* gene, known to be important for the cell to adequately sense and respond to DNA damage [79]. While established as a tumor-suppressor gene, the exact role of *ATM* in the pathogenesis of CLL remains to be determined. 17p alterations are believed to target *TP53* that resides in the MDR and that is also mutated in 10-15% of all CLL cases [78].

There is ample data supporting the prognostic relevance of these individual genetic aberrations (Figure 3). For example, both 11q and 17p alterations are associated with resistance to chemoptherapy and poor survival [65, 78].

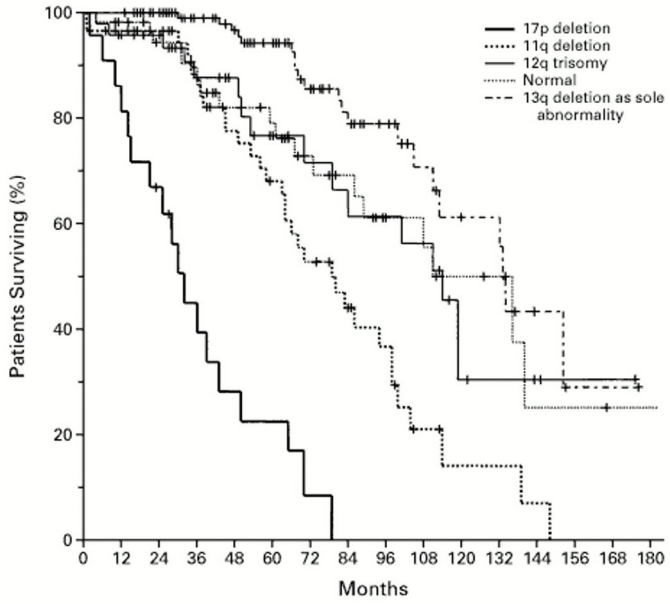

Figure 3. *The median survival times from the date of diagnosis among CLL patients with different cytogenetic abnormalities [65].*

3.4.1 13q14 deletions in CLL

Deletion of a segment of chromosome band 13q14 is the most frequent genetic abnormality in CLL and approximately 50% of all patients show heterozygous loss at this locus. In some 10-20% of these, a biallelic loss of 13q14 is observed [65, 80-86]. The consistent occurrence of these deletions in CLL and also other malignancies (see below) argues for the existence of a TSG at this locus. Initially, it was thought that these deletions target the *RB1* gene present on chromosome band 13q14.2 [87]. However, detailed deletion mapping has demonstrated that the critical region of loss lies approximately 2 megabases telomeric to *RB1*. Additionally, no inactivating mutations have been found in patients with monoallelic loss of *RB1* [85].

In a large proportion of cases, 13q14 deletions are observed as the sole genetic abnormality indicating that it may represent one of the initiating events in the transformation of CLL [65, 81]. Interestingly, 13q14 deletions are also detected in more than 50% of cases of monoclonal B-cell lymphocytosis (MBL), a condition that has been suggested to be a precursor state to CLL [88]. Abnormalities on chromosome 13 are manifested as both interstitial deletions of varying sizes as well as translocations. However, in the latter cases no recurrent fusion transcripts are observed and translocations consistently occur with concomitant loss of the 13q14 MDR [89]. This suggests that deletion is the driving force behind genetic aberrations at this locus in CLL.

With regard to prognosis, patients with 13q14 aberrations as sole cytogenetic abnormality fare reasonably well. This patient group usually has long median survival times and long treatment-free intervals. Close to one third of patients do not require therapy at all (Figure 3) [65].

A large set of studies has dealt with the extent of 13q14 deletions in CLL. A consensus MDR comprising approximately 600 kb has been described [90]. Additionally, considerably smaller MDRs have been reported by our and other groups [82, 84, 91] (Figure 4). Various candidate genes have been proposed to be of importance to CLL development such as *DLEU1*, *DLEU2*, *DLEU7*, *TRIM13*, *miR-15a* and *miR-16-1* [74, 92-95]. None of the genes have however been demonstrated to be mutated in the remaining allele of patients with hemizygous 13q14 loss which would argue against a gene inactivation mechanism along the lines of Knudson's two-hit hypothesis [15]. When only taking the smallest described MDR into consideration, two genes are present in the critical region of loss, namely *DLEU1* and *DLEU2* [91](Paper IV). The nature of 13q14 deletions, their functional consequences and the underlying mechanisms generating them, constitute a major part of this thesis and are extensively discussed in the Results and Discussion parts (Papers I-IV).

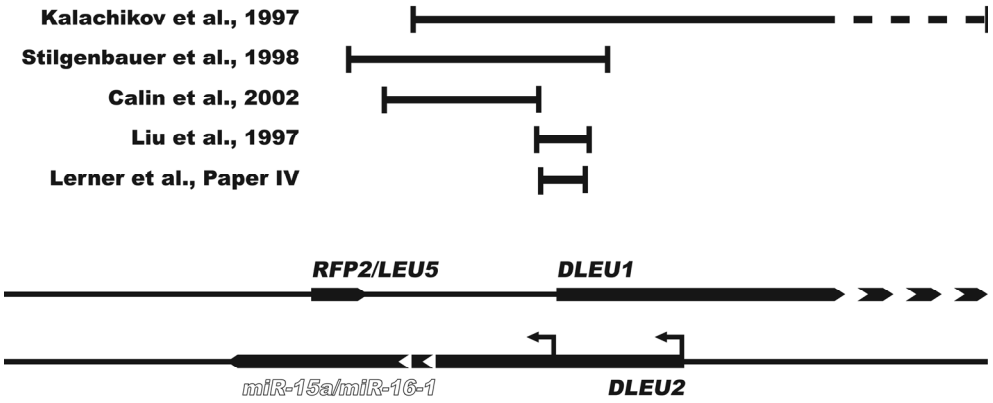

Figure 4. *The CLL 13q14 MDR. Genes are depicted as arrows. Brackets denote extent of deletion as mapped in different studies [82, 84, 91, 96]. Dashed line indicates that the deletion extends further than shown in the figure.*

3.4.2 13q14 deletions in other malignancies

The notion that a *bona fide* tumor-suppressor gene is located at the 13q14 MDR described in CLL is supported by the fact that the same region is targeted for deletion in numerous other malignancies. It is the region most frequently lost in mantle cell lymphoma and multiple myeloma occurring in 55-70% and in 30-70% of all cases, respectively [82, 97-101]. Additionally, recurring deletions targeting 13q14 distal to *RB1* have also been reported in head and neck tumors [102] and prostate cancer [103]. While the 13q14 deletions in these tumor types have not been as finely mapped as those in CLL, the respective MDRs in all these diseases overlap, raising the possibility that the same tumor-suppressive elements(s) is targeted. The functional characterization of the genes in the MDR in CLL (Papers I-III) might therefore be of relevance to the understanding of the pathogenesis of these other malignancies as well.

4 REGULATION OF GENE EXPRESSION

Regulation of gene expression serves to control the amount and activity of gene products according to cellular needs. The generation of a functional gene product involves several steps and essentially any step may be modulated, from the transcription of a gene to the post-translational modification of a protein.

In Papers I-III of this thesis, two regulatory systems and their putative involvement in cancer are discussed; functional RNAs and proteasomal degradation. Therefore, an introduction to these control systems is given below. If not otherwise indicated, gene and proteins in this section will be denoted by their human names.

4.1 FUNCTIONAL RNAS

The importance of so-called functional RNAs that exert their action without giving rise to proteins has become increasingly appreciated in recent years. Specifically those involved in regulation of gene expression, such as antisense RNAs, microRNAs, and Piwi-interacting RNAs (piRNAs), have attracted immense interest.

In Papers I and III, the *DLEU2* gene is described. It does not seem to encode a protein but instead functions as both a natural antisense RNA and a microRNA host gene. A brief description of these types of functional RNAs is presented.

4.1.1 Natural antisense RNAs

Natural antisense RNAs are transcripts that are complementary in sequence to other ("sense") transcripts [104]. Two major classes of natural antisense transcripts (NATs) exist: *cis*-acting and *trans*-acting. Pairs of *cis*-NATs are transcribed from opposing DNA strands at the same genomic locus and hence have perfect complementarity, whereas *trans*-NAT pairs are transcribed from separate loci and generally display imperfect complementarity. In this respect, microRNAs (see below) can be considered a subtype of *trans*-NATs.

Cis-NATs have been discovered in the genomes of viruses, prokaryotes and eukaryotes [105-107]. It is estimated that over 20% of all human transcripts have antisense partners [108]. The notion that many of these are functionally relevant is supported by their evolutionary conservation. By comparing human, mouse and *Fugu* genomes, Dahary and coworkers found that orthologous gene pairs with an antisense relationship were twice as likely to preserve their genomic organization throughout evolution as compared to nonantisense pairs [109].

The mechanism by which NATs regulate gene expression is in most instances not known. Results obtained from studies on individual sense-antisense pairs indicate that the regulation is context-dependent and consequently several different modes of action have been proposed [104]. One model suggests that sense-antisense RNAs can transcriptionally interfere with each other [110, 111]. RNA polymerase II complexes moving along opposite DNA strands can potentially collide in the overlapping region

which would hinder transcriptional progression and result in silencing of one or both of the transcripts. Competition for transcription factors at the promoter level might also lead to similar changes in gene expression. Another model suggests that NATs exert their function by hybridizing to their sense transcripts and thereby give rise to a double-stranded RNA (dsRNA) duplex. This could interfere with protein-RNA interactions and consequently change the splicing, polyadenylation, transport, translation or stability of the mRNA. For example, the thyroid hormone receptor gene *erbAα* is regulated by its antisense transcript in this fashion. Expression of the antisense RNA results in a shift in the expression ratio of two antagonistic splice variants of *erbAα* due to selective interference with the splicing of the downregulated isoform [112, 113]. Another possibility is that formed dsRNA duplexes act as substrates for processes such as RNA editing or RNA interference (RNAi). Finally, a third model implicates NATs in epigenetic alterations, such as promoter methylation and chromatin remodeling. This is especially relevant for the establishment of monoallelic gene expression, where antisense transcription has been found important in processes such as X-chromosome inactivation and genomic imprinting [114, 115]. The notion that NATs are involved in epigenetic silencing is supported by a study by Tufarelli and colleagues, in which an aberrant antisense transcript identified in a patient with α-thalassemia was demonstrated to induce methylation of its associated CpG island [116]. A recent report has provided the first experimental evidence of NAT-mediated silencing of a TSG. An antisense transcript to the CKI $p15^{INK4B}$ was identified and was shown to inhibit the expression of its sense partner by triggering heterochromatin formation. Consistent with this, an inverse relation between the expression of $p15^{INK4B}$ and the antisense transcript was found in both normal lymphocytes and primary leukemic cells [117].

Sense-antisense transcripts are a common feature of the human genome according to computational predictions. However, only a minor fraction of the putative cis-NATs have been experimentally verified. In paper I, a novel cis-NAT is described and its possible role in tumor development is discussed.

4.1.2 MicroRNAs

4.1.2.1 Biogenesis and function

MicroRNAs are a group of short non-coding RNAs of about 21-23 nucleotides in length that regulate gene expression at the post-transcriptional level. With few exceptions this is achieved by negatively controlling mRNA translation or stability in the cytoplasm [118]. MicroRNAs were initially described in *Caenorhabditis elegans*. There, the *lin-4* gene was demonstrated to produce microRNAs that suppress the lin-14 mRNA [119]. This regulation in turn is essential for the proper developmental timing of larval stages [120]. Later, a combination of computer-based predictions and experimental approaches established that microRNAs are present in diverse organisms ranging from plants to mammals [121]. The human genome is believed to contain between 400-1000 different microRNAs, as estimated by computational methods [122].

MicroRNAs are expressed as parts of longer primary transcripts termed pri-microRNAs. They can either be located in intergenic regions as independent transcription units with their own promoters or they may be located within genes [121].

In these cases they are often, but not exclusively, residing within introns of non-coding or protein-coding genes [123]. Just like mRNAs, most pri-microRNAs are transcribed by RNA polymerase II and are capped and polyadenylated [124, 125]. Production of mature microRNAs involves sequential processing by protein complexes containing an RNAse III enzyme (Drosha or Dicer) and a double-strand RNA-binding protein (DGCR8 or TRBP) (Figure 5).

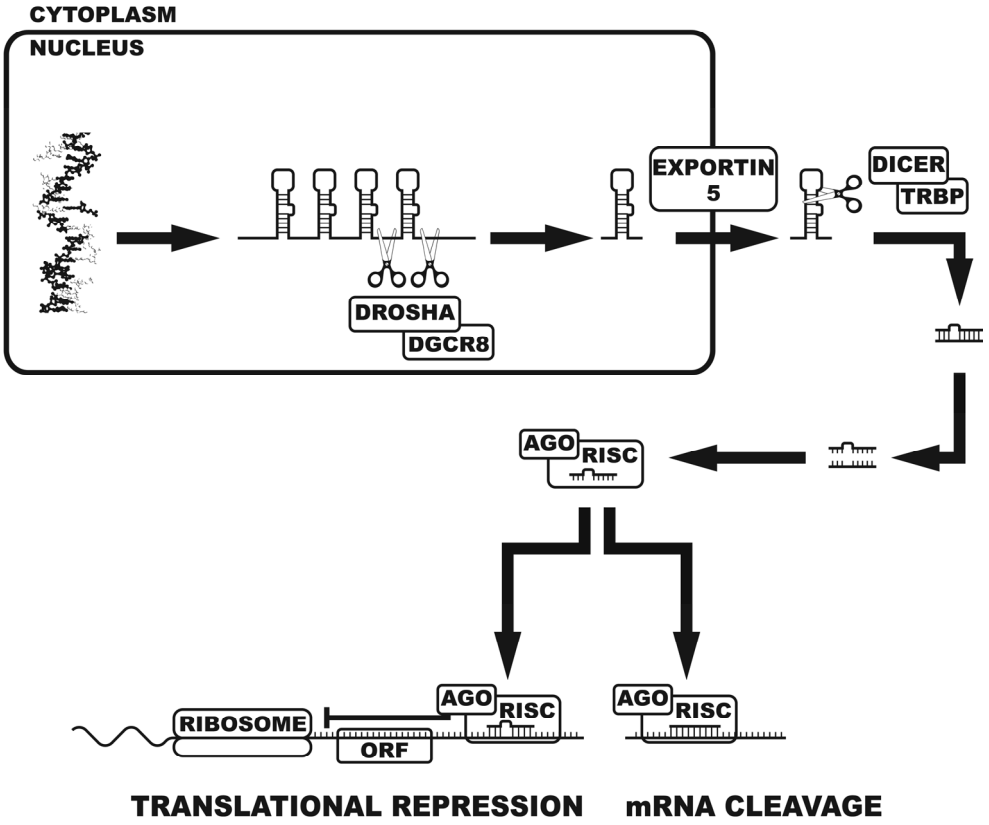

Figure 5. *MicroRNA processing.*

Initially, the pri-microRNA is recognized by a complex termed the Microprocessor consisting of Drosha and DGCR8 [126]. The Microprocessor complex cleaves the stem-loop structure within the pri-microRNA yielding an approximately 70 nucleotide long precursor microRNA (pre-microRNA)(Figure 5). This pre-microRNA is subsequently transported out of the nucleus by the Ran-GTP-dependent Exportin 5 protein [127] and is cleaved in the cytoplasm by the Dicer-TRBP complex [128, 129]. The resulting final product is a ~22 nucleotide double-stranded microRNA duplex. One strand of the mature microRNA is thereafter incorporated into a complex termed RISC (RNA-induced silencing complex) while the other one is degraded [130, 131]. A defining feature of RISC is the presence of Argonaute (Ago) proteins. The TRBP protein probably has an important role in bringing microRNAs into the RISC as it has been demonstrated to bind Ago2 [129]. Once within the RISC, the microRNA guides the complex to target transcripts that are supposed to be silenced. This is mediated by base-pairing interactions between the microRNA and the target mRNA and in most instances, the RISC will bind to sites within 3' untranslated regions (3'UTRs) of target

mRNAs [118]. 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]. However, it has become increasingly clear that microRNAs can bring about gene silencing by yet other mechanisms such as destabilizing mRNAs by promoting deadenylation [118]. For the sake of simplicity, a description of these mechanisms will be omitted from the following discussion.

Recent investigations have shed light on the mechanisms by which translational repression by microRNAs is accomplished. The process of mRNA translation can be divided into three phases: initiation, elongation and termination. While some contradictory results have been presented, it is now generally believed that microRNA-mediated repression mainly occurs at the initiation of translation [118]. The initiation step is subject to extensive regulation and involves a set of multisubunit complexes containing eukaryotic translation initiation factors (eIFs). The process starts with the recognition of the 5'-terminal cap structure of the mRNA by the eIF4E subunit. Another initiation factor subunit, eIF4G, has an important role by acting as a scaffold for different interactions necessary for the assembly of the ribosome initiation complex. It interacts with the polyadenylate-binding protein 1 (PABP1) bound to the 3' end of the mRNA and with eIF4E at the 5' end. The simultaneous binding of eIF4G to eIF4E and PABP1 brings the two ends of the transcript in proximity and leads to the "circularization" of the mRNA. This conformation of the mRNA is believed to stimulate translation initiation [133]. Several studies have demonstrated that microRNA-dependent translational repression is dependent on the presence of a 5' cap and poly(A) tail in the mRNA. Work by Kiriakidou and coworkers demonstrated that Ago proteins contain a region that is partly homologous to the cap-binding region of eIF4E [134]. By virtue of this domain, the Ago proteins can compete with eIF4E for cap binding and thereby suppress translation initiation. Such a model could also explain why several target sites need to be present in the 3'UTR of the target transcript for potent microRNA repression to occur. Since Ago proteins have a lower affinity for the 5' cap than eIF4E, then the presence of several copies of Ago proteins might be necessary to compete out this initiation factor. The proposed model has been corroborated by experiments where addition of purified eukaryotic initiation factor complex rescued microRNA-mediated gene silencing [135]. It is noteworthy that other mechanisms of repression have been proposed where microRNAs suppress translation after initiation and it is therefore possible that microRNAs affect protein production at several different levels [136].

Associated mRNAs, microRNAs and Ago proteins have been found to accumulate in specific compartments of the cytoplasm termed P (processing) bodies [137]. These cytoplasmic foci are enriched in proteins that are implicated in mRNA degradation. While known to be generally involved in the catabolism of mRNAs, the exact role of the P bodies in the microRNA pathway remains to be determined. Interestingly, disrupting P bodies does not interfere with microRNA-mediated repression and it has been proposed that P bodies constitute sites for temporary storage of repressed mRNAs [138].

Plant microRNAs generally bind with near perfect complementarity to target mRNAs which results in cleavage. Metazoan microRNAs on the other hand typically pair with their targets imperfectly. The binding follows a set of criteria that have been established using experimental and bioinformatic approaches [118]. Of primary

importance is that there is perfect base pairing between a stretch of 6-8 nucleotides at the 5' end of the microRNA, termed the "seed sequence", and the target mRNA. Imperfect binding of the seed region can be somewhat compensated for by extensive base pairing between the 3' region of the microRNA and the target. MicroRNAs are generally divided into families with identical seed regions. Individual members of a family are believed to have at least some degree of overlap in target specificity. In this way, microRNA family members are believed to cooperate in the downregulation of specific targets.

Several computational tools for the prediction of putative microRNA targets exist that are based on the extent of sequence complementarity between microRNA and target, the free energies of the microRNA-mRNA duplex and the degree of evolutionary conservation of target sites [122]. Using these bioinformatic applications, a myriad of putative targets are predicted for each microRNA. However, the significance of these predictions remains largely unknown and therefore each microRNA-target interaction must be validated experimentally. To further complicate matters, microRNA function has been shown to be very context-dependent. Sequences other than the microRNA target site are of importance as they can affect the accessibility of both the microRNA itself and RNA-binding proteins. For example, a recent report has demonstrated that the protein Dnd1 can bind to specific uridine-rich regions in mRNAs and thereby prevent microRNAs from associating and effecting gene silencing [139].

MicroRNAs have been implicated in a wide variety of biological pathways including control of differentiation, apoptosis and proliferation [122, 140]. Since microRNAs can coordinately regulate the abundance of hundreds of targets, they might be very important for the orchestration of global changes in gene expression. The fact that *Dicer* deficiency causes embryonic lethality in both zebrafish and mice helps illustrate the profound relevance of microRNAs in general biological processes [141, 142].

4.1.2.2 MicroRNAs and cancer

A link between microRNAs and tumorigenesis was proposed soon after their discovery in humans and several microRNAs have been found to be downregulated or overexpressed in different tumor types.

The first microRNAs that were demonstrated to have altered expression in tumor cells were miR-15a and miR-16-1 [96]. These microRNAs are located on chromosome 13q14 and were shown to be downregulated in the majority of CLL cases. Paper III of this thesis focuses on miR-15a/miR-16-1 and they will be discussed thoroughly in the Results and Discussion part. These initial findings have been followed by many more studies implicating microRNAs in cancer development. Some examples of malignancy-associated microRNAs are given below.

The *miR-17-92* cluster encoding seven microRNAs is found amplified in both B-cell lymphomas and lung cancers [143-145]. Overexpression of miR-17-92 accelerated c-Myc-induced tumorigenesis in a mouse model of B-cell lymphoma presumably by reducing the apoptotic signaling that normally accompanies Myc activation [145]. The *miR-155* microRNA and its host gene *BIC* are likewise overexpressed in a variety of B-cell lymphomas [146, 147]. Mice carrying a *miR-155*

transgene whose expression is targeted to B cells developed B-cell malignancy after initially exhibiting preleukemic pre-B-cell proliferation [148]. Another set of microRNAs implicated in tumor development are miR-372 and miR-373 that can collaborate with oncogenic Ras in cellular transformation [149]. Importantly, the expression of miR-372/miR-373 allowed transformation of primary fibroblasts in the presence of functionally active p53. In line with this, mutation of p53 and expression of miR-372/miR-373 were found to be mutually exclusive events in primary testicular germ cell tumors.

Considering that several experimentally validated microRNA targets are oncogenes and TSGs such as PTEN [150], $p27^{Kip1}$ [151, 152], $p21^{Cip1}$ [153], and Mcl-1 [154], it is likely that many additional microRNAs will be found deregulated in tumors in the future.

4.2 THE UBIQUITIN-PROTEASOME SYSTEM

4.2.1 General introduction

One way for the cell to regulate protein function is by posttranslational modifications. Both the activity and the abundance of proteins can be fine-tuned by modifications such as phosphorylations, acetylations, and glycosylations. This type of regulation is however not restricted to the addition of functional groups but can also include attachment of small proteins such as ubiquitin. While we know today that ubiquitination leads to diverse downstream effects, it was originally recognized as a signal for protein degradation [155].

Before the discovery of the ubiquitin-proteasome system (UPS), proteins were believed to be degraded mainly within lysosomes. During lysosomal degradation, proteins are exposed to hydrolases and indiscriminately degraded at approximately the same rate. It eventually became evident, however, that the majority of proteins are broken down in an energy-dependent tightly regulated process. Pioneering work in the 1970s and 1980s by among others Aaron Ciechanover, Avram Hershko, and Irwin Rose, defined that one essential factor of this proteolytic system is the small protein ubiquitin [156]. A new cellular pathway was discovered where proteins are first tagged by the covalent attachment of multiple ubiquitin molecules and then degraded in the multisubunit protease complex that is known as the proteasome. While some proteins are degraded within minutes, such as the tumor-suppressor p53 and the oncoprotein c-Myc, other appear to be stable for days or even years.

The UPS is involved in most, if not all, cellular processes [157]. The timely degradation of diverse sets of short-lived proteins is essential for basic cellular functions. For example, cell division is intimately linked to the UPS-regulated turnover of cyclins and CKIs at different phases of the cell cycle [158]. An equally important function is the clearance of defective proteins from the cell. Proteins that are misfolded need to be destroyed as they can potentially participate in dominant negative protein-protein interactions or multimerize into insoluble protein aggregates. Indeed, deregulation of the UPS has been implicated in several neurodegenerative disorders, where aberrant proteins accumulate and eventually induce cell death [159, 160]. A functioning UPS is also necessary for the immune system. In the proteasome, proteins

are digested into smaller peptides. During antigen presentation some of these peptides are translocated to the endoplasmic reticulum (ER), loaded on major histocompatibility complex (MHC) class I and subsequently transported to the cell surface for display [161]. Presented peptides are representative of the total pool of proteins in the cell and can therefore indicate if a cell has been infected with an exogenous agent. Interestingly, not all proteins are completely degraded by the proteasome. Some are instead processed into truncated forms with altered activities. For example, one component of the NF-κB transcription factor, p50, is activated following ubiquitination and limited proteolysis of the p105 precursor [162]. Finally, ubiquitination can also have non-proteolytic functions and these have been implicated in such diverse processes as DNA repair and endocytosis [163, 164].

4.2.2 Ubiquitin and the process of ubiquitin conjugation

Ubiquitin is a protein of 76 amino acids that is ubiquitously expressed (hence its name). It is extremely well conserved and the yeast and human counterparts differ only by three amino acid residues. Ubiquitin is translated as a precursor protein, either consisting of ubiquitin fused to a ribosomal protein or as a chain of several ubiquitin molecules fused together in a head-to-head arrangement. Monomeric forms are produced via cleavage of these fusion proteins by ubiquitin C-terminal hydrolases [165]. Adequate levels are maintained due to high expression levels, exceptional stability of the protein and recycling of conjugated ubiquitin molecules. However, the amount of free ubiquitin is restricted to a minor fraction of the total pool as the majority is found conjugated to different target proteins [166]. Consequently, ubiquitin molecules are dynamically reshuffled between proteins through the action of ubiquitination and deubiquitination enzymes.

The process of ubiquitin conjugation to protein substrates occurs through the sequential action of three specific enzymes: the E1, E2 and E3 (Figure 6) [167, 168]. First, the ubiquitin molecule is activated by the E1 ubiquitin-activating enzyme in an ATP-dependent reaction that generates an ubiquitin-E1 high-energy thiol ester bond. The human genome, like that of most organisms, has a single E1 enzyme. The encoded protein product is thus responsible for the activation of all ubiquitin molecules needed for conjugation reactions. Second, the thiol-linked ubiquitin is transferred from E1 to the active site cysteine of an E2 ubiquitin-conjugating enzyme. E2s are characterized by a conserved core domain of approximately 150 amino acids containing the critical cysteine residue. Finally, the ubiquitin is ligated through an isopeptide bond created between the C-terminal glycince residue of ubiquitin and an ε-NH2 group of an internal lysine residue in a specific target protein. In some instances, however, the ubiquitin moiety can alternatively be conjugated to the N-terminal residue of the protein. This final ligation generally requires the activity of an E3 ubiquitin ligase that can interact with both the E2 and the substrate. The E3 ligases therefore confer specificity to the reaction by dictating which proteins that are tagged with ubiquitin molecules. Once one ubiquitin moiety is bound to the substrate, additional ones can be added by successive rounds of ubiquitination. Each ubiquitin molecule is linked to a lysine residue on the preceding ubiquitin and in this way a polyubiquitin chain can be built. While some 30 different E2 enzymes exist in mammals [169], the group of E3s is

substantially larger, comprising hundreds or maybe thousands of proteins. There is considerable flexibility at the level of the E3 ligases. The same substrate can be targeted by different E3 ligases and, conversely, one given E3 ligase can ubiquitinate several different substrates [167].

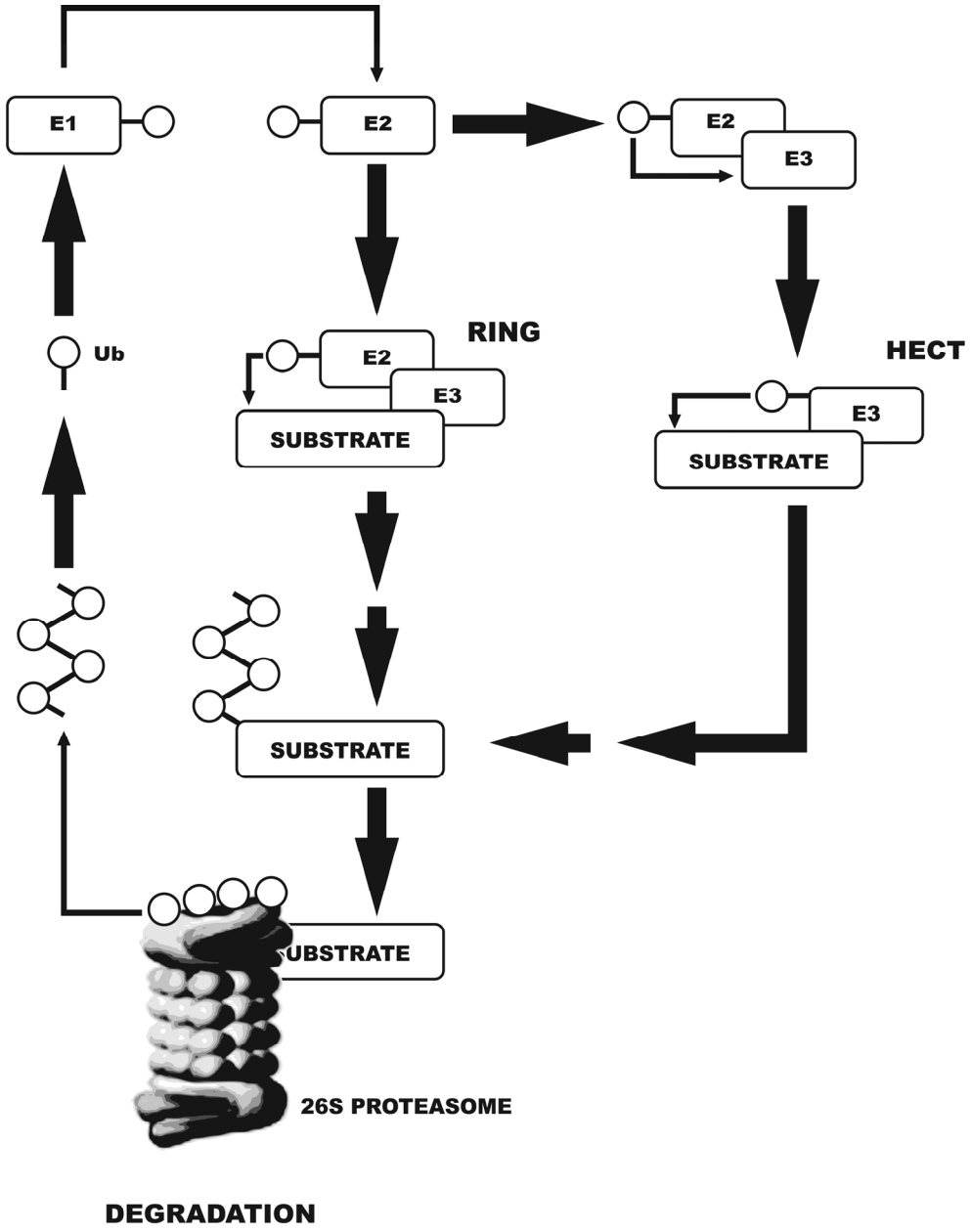

Figure 6. *The ubiquitination process.*

The vast majority of E3 ubiquitin ligases can be divided into two groups: HECT domain- and RING finger-containing E3s [168]. One major difference between these two classes is related to how they mediate ubiquitin transfer. HECT (homologous to the E6-AP carboxyl terminus) domain E3 ligases function as chemical catalysts and the ubiquitin is transferred from the E2 to the E3, forming a thiol ester intermediate, before subsequently being transferred to the substrate. The defining HECT domain is a

~350 amino acid long region containing the critical cysteine residue necessary for thiol ester formation with ubiquitin. With RING (really interesting new gene) domain E3s, the ubiquitin is transferred directly from the E2 to the substrate and the ligase is believed to function as a scaffold that places these in a conformation optimal for ubiquitin transfer [170].

RING E3s constitute the largest group of ubiquitin ligases and are characterized by the RING domain that is essential for ubiquitination activity [171]. This domain consists of a series of histidine and cysteine residues that can bind two zinc atoms in a cross-braced fashion. Of note is that several RING finger E3s have been demonstrated to undergo autoubiquitination [168, 172]. This type of modification probably serves as a mechanism to regulate the abundance or activity of the E3 itself. Depending on how RING E3 ligases recognize their substrates, they can be subdivided into two groups, single subunit or multisubunit E3 ligases. In the first class, the substrate-binding site and the RING finger domain are contained within the same molecule. Examples of single subunit E3 ligases include the p53 ubiquitinating enzyme Mdm2 and c-Cbl that is critically involved in the downregulation of activated receptor tyrosine kinases [173, 174]. In multisubunit E3 ligases, the RING finger protein is part of a complex and substrate recognition is mediated by a different protein. SCF (Skp1-Cullin-F-box) complexes are typical multisubunit E3s that are responsible for the selective degradation of various phosphorylated proteins, many of which are involved in cell cycle regulation. In these the RING finger component, Rbx1/Hrt1/Roc1, recruits the relevant E2 enzyme while a separate subunit, the F-box protein, binds the substrate [167]. Some proteins directly involved in the ubiquitination reaction are not readily classified as E1s, E2s or E3s. For instance, proteins containing a domain called the U-box have been classified as E4 enzymes [175]. U-box proteins have been demonstrated to be able to elongate existing ubiquitin chains independent of E3 ligases [176].

The functional outcome of ubiquitination depends largely on the length and linkage of the ubiquitin chain. Proteins can be modified by the attachment of ubiquitin monomers (monoubiquitination) or polyubiquitin chains. Monoubiquitination does not lead to proteasomal degradation but instead has an impact on various protein interactions as several proteins carry domains capable of binding to ubiquitin molecules. This particular posttranslational modification has been implicated in both trafficking of membrane proteins and histone regulation [163]. The ubiquitin molecule contains seven different lysines and in theory all of them can function as binding sites for the C-terminal glycine residue of another ubiquitin molecule. In this way, different polyubiquitin chains can be built. The typical chain that serves as a signal for proteolysis is one where each ubiquitin is conjugated to a preceding ubiquitin via a lysine residue at position 48 [177]. Attachment of four or more ubiquitins in such a K48-linked manner will target the protein for degradation in the proteasome [178]. A subset of the other possible polyubiquitin conformations has been detected *in vivo*, such as K63 chains. Interestingly, K63 chains do not trigger proteasomal degradation but rather appear to be involved in processes such as endocytosis, DNA repair, and translation [179-181].

The UPS is dynamic and ubiquitin chains can be decimated by the action of deubiquitinating enzymes (DUBs). These proteins are necessary for the generation of free ubiquitin monomers from translated fusion proteins and for the recycling of ubiquitin. Additionally, some substrates can be rescued from degradation by the action of DUBs [182].

Eventually, proteins that are conjugated with polyubiquitin chains of the appropriate linkages are presented to the proteasome. Proteasomes are large multisubunit complexes that generally consist of a 20S core particle (CP) carrying the proteolytic activity and two 19S regulatory particles (RPs) [167]. They are located in both the cytoplasm and in the nucleus. The CP is composed of four rings with seven subunits each that together make up the barrel-shaped structure of the proteasome. Within the CP, a set of trypsin-, chymotrypsin-, and post-glutamyl peptide hydrolytic proteases digest any protein into small peptides. However, in order to access the inner core of the CP, proteins must first be recognized and selectively bound by the RPs. Recognition is partly mediated by distinct subunits of the RP that have polyubiquitin binding activity. The pores that lead to catalytic core are less than 2 nm wide which ensures that only unfolded polypeptides can enter the proteasome [183]. This unfolding is believed to be effected by AAA-ATPases in the RPs. Ubiquitin chains are cleaved off by DUBs that are tightly associated with the proteasome and presumably also by the S13 subunit of the RP.

Given the important role of the UPS in cells, it is perhaps not surprising that deregulation of protein degradation is implicated in a large set of diseases, including cancer. Malignancies can develop as a consequence of stabilization of oncogenic proteins or destabilization of tumor-suppressor proteins [184]. For example, several growth-promoting factors such as c-Myc, c-jun, and various growth factor receptors are tightly regulated by the UPS. Proteins involved in inhibiting growth, such as p53 and $p27^{Kip1}$ are likewise targeted for proteasomal degradation. All these proteins have been demonstrated to be deregulated as a result of defective or excessive proteolysis. For example, abnormally low levels of the CKI $p27^{Kip1}$ have been detected in several malignancies including colorectal, breast and prostate cancer [185]. Often this is a result of increased levels of Skp2, the F-box protein that recognizes and targets $p27^{Kip1}$ for degradation. In line with this, overexpression of Skp2 was shown to increase the tumorigenic potential of rat embryo fibroblasts injected into nude mice [186].

4.2.3 Endoplasmic reticulum-associated degradation

The ER is an organelle dedicated to the translation, folding, modification, and transport of membrane and secretory proteins. Newly synthesized proteins are translocated into the ER via a channel formed by the Sec61 complex and are folded with the assistance of chaperones. At this stage some proteins may additionally acquire disulfide bonds or N-linked glycans. They may also oligomerize into higher-order complexes [159]. Within a short period after their synthesis, the majority of proteins leave the ER and travel to the Golgi apparatus for further modification and transport. However, subsets of proteins fail to reach their fully mature state and will be retained in the ER. There exists a stringent quality control system to prevent the release of proteins that are defective. The major reason for this is that the potential toxicity of aberrant secretory or transmembrane proteins is not restricted to the cell in which they are synthesized. As an example, defective transmembrane proteins exposed at the cell surface can profoundly affect cell-cell communication.

Sooner or later, proteins that fail to mature in an adequate fashion have to be eliminated. The degradation however does not take place in the ER lumen. Instead,

these defective proteins need to be transported back to the cytosol where they are degraded by the UPS. This stepwise process of recognition, retrotranslocation, ubiquitination and degradation of aberrant ER proteins is termed ER-associated degradation (ERAD) (Figure 7) [159, 187].

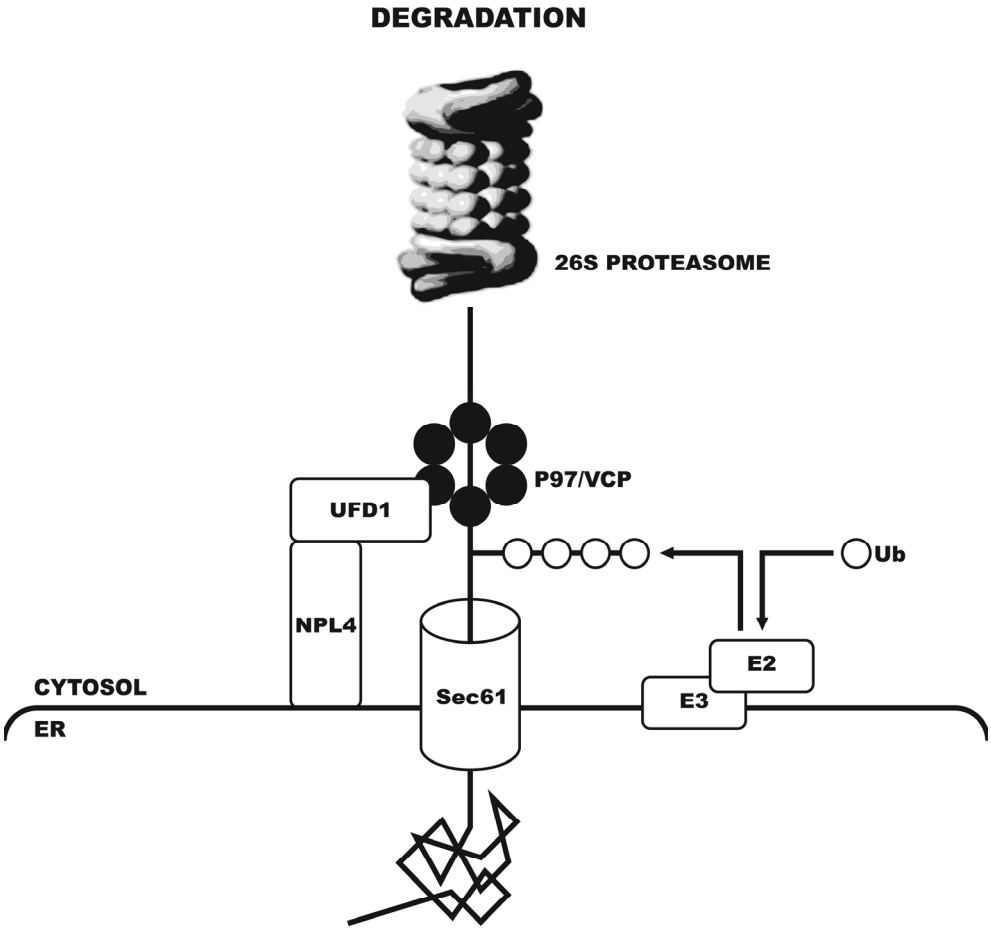

Figure 7. *The ERAD pathway.*

One major difference between the E3 ubiquitin ligases involved in ERAD and other E3s is that the former are generally spatially separated from their substrates by the ER membrane. Initial recognition of substrates is therefore mediated by components other than the ligases themselves or their direct partner proteins. An important role in this process is played by various chaperones such as BiP, calnexin, and calreticulin. These factors can recognize features that are indicative of protein immaturity or misfolding such as hydrophobic patches, unpaired cysteines and immature glycans. One crucial aspect of this quality control is that chaperones somehow have to distinguish between folding intermediates and terminally defective proteins. Some insights into the process of ERAD substrate recognition has been gained from studies on N-glycosylated proteins. Immature glycoproteins are retained in the ER bound to a pair of lectin-type chaperones, calnexin and calreticulin [188]. However, if a glycoprotein is maintained in the ER for prolonged periods of time, it is eventually deemed terminally misfolded and is targeted for degradation. This occurs when

mannose residues of N-glycans are removed. In this respect, a set of mannosidases present in the ER lumen function as molecular timers that prevent refolding from proceeding indefinitely [160].

It is generally believed that proteins exit the ER for degradation the same way they came in, namely through the Sec61 channel [159]. Nevertheless, other mechanisms for transport across the ER membrane have been proposed as the retrotranslocation of some substrates occurs independently of Sec61 [189]. The Derlin-1 protein has been demonstrated to be necessary for degradation of certain ERAD substrates and it has been suggested to be part of an export pore [190, 191]. Additionally, some ERAD E3 ligases, such as yeast Hrd1p and Doa10p, have multiple transmembrane domains that might form a possible dislocation channel [192].

Retrotranslocated proteins are ubiquitinated by specific E2 and E3 enzymes dedicated to ERAD. However, retrotranslocation and ubiquitination are not completely separate events and some substrates cannot be fully dislocated into the cytosol if polyubiquitination is prevented [193]. An important player in the translocation process is the cytosolic AAA-ATPase p97/VCP. It exists in protein complexes together with Npl4 and Ufd1, that both are able to bind ubiquitinated proteins. The p97-Npl4-Ufd1 complex is thought to physically extract proteins from the ER in an ATP-dependent manner [194]. An alternative is that the complex rather acts to make substrates available to the proteasome for degradation after they have already crossed the ER membrane [195].

The majority of the components of the ERAD system identified to date have been characterized in *Saccharomyces cerevisiae*. Two ERAD E3 ubiquitin ligases have been well-defined in yeast: Hrd1p/Der3p and Doa10p [196]. Both are multipspanning transmembrane RING finger proteins that function together with the two E2 conjugating enzymes that are specific to the ER, Ubc6p and Ubc7p. One feature that sets them apart is their respective substrate specificities and it appears that the exact position of the misfolded domain is of importance. While the Hrd1p complex mainly ubiquitinates substrates with lumenally exposed misfolded domains, Doa10p targets substrates with lesions in their cytoplasmic domains [197]. The importance of these proteins is underscored by the fact that yeast cells deficient in both Hrd1p and Doa10p exhibit a marked increase in unfolded proteins [192].

The number of putative ERAD E3 ligases is greater in mammals than in yeast. However, for most of these relatively little is known regarding their regulation and substrate specificity. The first mammalian ERAD E3 ligase to be described was gp78/AMFR [198]. Its relevance has been demonstrated in experiments where depletion of gp78 by short-hairpin RNAs (shRNAs) leads to stabilization of several prototypical ERAD substrates [199]. In addition, functional mammalian homologues of the two major yeast ERAD E3s exist. Synoviolin/Hrd1 and TEB4/MARCH VI are human homologues of Hrd1p/Der3p and Doa10p respectively [200, 201]. Apart from these ER membrane-bound E3 ligases, a set of cytosolic E3s have been described that also function in mammalian ERAD. For example, Parkin ubiquitinates the unfolded Pael receptor at the ER and a subset of SCF complexes specifically target certain glycoproteins for degradation [202, 203].

Considering the diversity of ERAD substrates, additional mammalian ER E3s will undoubtedly be identified in the future. One novel ERAD E3, Rfp2, was identified in the process of functionally characterizing the genes in the 13q14 MDR in CLL and is the topic of Paper II of this thesis.

AIMS OF THE THESIS

The overall aim of this thesis was to analyze 13q14 deletions in CLL at the molecular level.

More specifically the aims were:

- To analyze the commonly deleted chromosome 13q14 region in CLL with respect to genomic organization.
- To functionally characterize the genes in the 13q14 MDR and to examine their putative role in tumor development.
- To investigate the underlying mechanism behind 13q14 aberrations in CLL.

RESULTS AND DISCUSSION

PAPER I

DLEU2 encodes an antisense RNA for the putative bicistronic *RFP2/LEU5* gene in humans and mouse

Critical genes, including those involved in malignant transformation, tend to be highly conserved at the nucleotide level. With this in mind, our group had previously analyzed the degree of conservation of the 13q14.3 region between human and mouse [204]. These studies were extended in the present investigation and 1.3 Mb of genomic sequence surrounding the CLL 13q14 MDR was subjected to comparative sequence analysis.

Three genes have been consistently associated with 13q14 deletions in CLL; *DLEU1*, *DLEU2* and *RFP2/LEU5* [82, 84, 91]. The sequence comparison studies indicated that while *DLEU2* and *RFP2/LEU5* are conserved, *DLEU1* is not. We therefore chose to characterize the two former genes at the RNA level. Our previous studies had additionally demonstrated that the *DLEU2* gene is only conserved at the nucleotide level, and not at the amino acid level. This led to the hypothesis that it might act as a functional RNA.

Initially, we analyzed the region using different bioinformatic tools. Novel transcriptional units were identified using EST (expressed sequence tag) databases and gene prediction programs. In this way, several new potential exons for *DLEU2* and *RFP2/LEU5* were found both in humans and mice (Figure 8). These exons were experimentally verified as being part of the respective genes by sequencing of RT-PCR products and northern blot analysis. Interestingly, the sequence of a newly identified exon of *DLEU2* was found to match that of an antisense transcript to *RFP2/LEU5* (termed *RFP2OS*) previously reported by our group [205]. Using RT-PCR, the antisense transcript was shown to represent *DLEU2* exon 11. These results indicated that DLEU2 might function as a *cis*-NAT that regulates *RFP2/LEU5* through interaction at the mRNA level. The antisense exon overlaps the splice site of exon 1A of the *RFP2/LEU5* gene. We identified *RFP2/LEU5* transcripts that retain intron 1, which raises the possibility that *DLEU2* might influence *RFP2/LEU5* expression by masking this particular splice site.

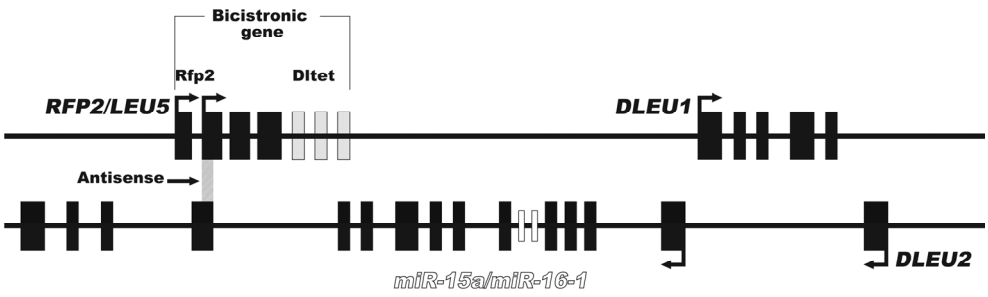

Figure 8. *Genomic organization of the CLL 13q14 MDR.*

While there was no evident direct exon overlap between *RFP2/LEU5* and *DLEU2* in mouse, a similar regulatory mechanism was identified. We found an expressed mouse *Dleu2* transcript containing a section that is imperfectly complementary to *Rfp2/Leu5* exon 3. Therefore, in contrast to the situation in humans, mouse *Dleu2* may function as a *trans*-NAT to *Rfp2/Leu5*. NAT regulation at the 13q14 locus is particularly interesting in light of the recent finding that the $p15^{INK4B}$ tumor suppressor is negatively regulated by its antisense partner [117].

The comprehensive analysis also resulted in the identification of several new *RFP2/LEU5* exons. This gene encodes an E3 ubiquitin ligase that is the focus of Paper II of this thesis. Surprisingly, one of the identified *RFP2/LEU5* exons was found to contain an open reading frame (ORF) termed Dltet (for K^+-channel tetramerization domain homologous ORF deleted in leukemia). This protein, also known as KCNRG (K^+ channel regulator) has a high degree of homology to the cytoplasmic tetramerization domain (T1) of voltage-gated K^+ channels. While largely uncharacterized, it has been demonstrated to suppress K^+ channel activity in a human prostate cell line [206]. Our first notion was that Dltet could represent a separate domain of the Rfp2 protein. However, despite several attempts to connect these ORFs by sequencing RT-PCR products from a variety of cDNA templates, we could not detect any transcript in which Dltet and Rfp2 were in the same reading frame. This suggests that *RFP2/LEU5* has the potential to actually encode two separate proteins, the previously described Rfp2 protein and the Dltet protein (Figure 8). The two ORFs can be expressed from either separate transcripts (monocistronic forms) or from the same transcript (bicistronic form). Northern blot analysis confirmed the existence of the bicistronic form as the same 7.5 kb transcript was detected using probes from the two separate ORFs. Rfp2 and Dltet were found to be highly conserved in mouse, where both monocistronic and bicistronic forms could be detected as well. Transfection of a bicistronic *RFP2/LEU5-DLTET* construct only resulted in expression of the Rfp2 protein. This indicates that translation of both proteins from the same transcript only occurs under specific circumstances. Alternatively, expression of Rfp2 from a bicistronic transcript might actually inhibit Dltet translation to prevent coexpression of these proteins.

Bicistronic genes are frequently found in viruses and bacteria but are rare in humans. There are, however, examples of bicistronic genes in the human genome, such as the *SNURF-SNRPN* and *CCL15-CCL14* genes. Similar to the *RFP2/LEU5* gene, both can be expressed as several alternative mono- and bicistronic transcripts [207, 208]. The functional significance of mammalian bicistronic expression is not clear.

In summary, this paper proposes that *DLEU2* and *RFP2/LEU5* compose one functional unit, where *DLEU2* is a putative antisense regulator of the bicistronic *RFP2/LEU5* gene. The smallest 13q14 MDR defined in CLL encompasses only one conserved element, namely *DLEU2* [91](Paper IV). One possible consequence of the sense-antisense relationship between these two genes is that deletion of *DLEU2* indirectly could lead to a deregulation of *RFP2/LEU5*.

PAPER II

The RBCC gene *RFP2 (Leu5)* encodes a novel transmembrane E3 ubiquitin ligase involved in ERAD

In this paper, we sought to functionally characterize *RFP2/LEU5* as it is the only obvious protein-coding gene in the 13q14 MDR in CLL.

The Rfp2 protein belongs to the RBCC/TRIM (RING finger, B-box, coiled-coil/tripartite motif) family of proteins. Members of this family are characterized by a conserved domain composition generally consisting of a RING finger domain, one or two B-boxes, and a coiled-coil domain [209, 210]. The order and the spacing between the domains is generally maintained and it has been suggested that this tripartite motif represents one functional structure, rather than a collection of separate modules. In line with this the entire RBCC domain is generally required for proper function and intracellular localization of proteins containing these motifs. RBCC proteins have by some researchers been described as a subclass of single subunit RING E3 ubiquitin ligases due to the presence of the RING domain [211]. However, experimental proof of E3 ligase activity *in vitro* or *in vivo* has only been presented for a fraction of the total number of RBCCs. Interestingly, several RBCC proteins have been implicated in cancer development. One notable example is PML that is consistently deregulated in acute promyelocytic leukemia [212].

In order to functionally characterize Rfp2, we created a polyclonal antibody directed against the protein. The specificity of the antibody was confirmed by transfection experiments with siRNA oligos directed against Rfp2. Our initial observation was that Rfp2 is a labile protein that is stabilized upon treatment with proteasomal, but not lysosomal, inhibitors. Consequently, we proceeded to determine if Rfp2 is ubiquitinated in cells. By mass spectrometric analysis of immunoprecipitated Rfp2, we were able to establish Rfp2 as a mono-/oligoubiquitinated protein. These results were corroborated by another set of experiments where we ectopically coexpressed Rfp2 and ubiquitin with different tags, pulled down Rfp2 and immunoblotted for ubiquitin. Using a similar approach, we proceeded to demonstrate that Rfp2 is polyubiquitinated. Importantly, this particular modification was dependent on the presence of the Rfp2 RING finger as a mutant lacking this domain, Rfp2-ΔRING, was not polyubiquitinated. This suggested that Rfp2 might be an E3 ubiquitin ligase undergoing auto-polyubiquitination. In order to directly assess whether Rfp2 has catalytic E3 ubiquitin ligase activity, we performed *in vitro* ubiquitination assays with both wild-type Rfp2 and an Rfp2 mutant with a point mutated critical cysteine residue in the RING domain, Rfp2[$C^{13}A$]. Efficient polyubiquitination could only be detected with the wild-type protein, arguing that Rfp2 functions as a proper RING finger E3 ubiquitin ligase.

While the N-terminal domain structure is similar between RBCC proteins, the C-terminal regions vary considerably. Bioinformatic analysis revealed that Rfp2 contains a single transmembrane domain in its C-terminus. Examination of all the remaining RBCC proteins showed that this particular domain composition, RBCC-TM, is only present in one additional member, namely TRIM59. Rfp2 and TRIM59 thus

constitute a novel RBCC-transmembrane subgroup. By way of fractionation of cell lysates and immunofluorescence staining we could demonstrate that Rfp2 indeed is a membrane protein located in the perinuclear compartment of the cell. Furthermore, this localization was dependent on the C-terminal part containing the transmembrane domain. Simultaneous staining for Rfp2 and different ER-resident proteins, such as the mammalian homologue of the yeast ERAD E2 enzyme Ubc6p, allowed us to define Rfp2 as an ER-located protein.

In order to obtain additional clues regarding the biological function of Rfp2, we performed a screen for putative interacting proteins by using a mass spectrometric approach. In line with the results from the localization studies, immunopurification of endogenous Rfp2 followed by tandem mass spectrometry analysis enabled the identification of several putative Rfp2-interacting proteins localized to the ER. These included sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2A), dolichol-diphosphooligosaccharide-protein glycosyltransferase (DDOST), and calmodulin. Interestingly, p97/VCP was also identified and was furthermore confirmed as an interacting partner to Rfp2 by immunoprecipitation and immunblotting experiments.

Given its apparent ubiquitin conjugating activity, its localization to the ER, and its interaction with p97/VCP, we next examined whether Rfp2 could function specifically as an ERAD E3 ligase. Several proteins have been identified that are consistently degraded in a manner that requires core ERAD components. Among these are misfolded proteins such as mutant CFTR (cystic fibrosis transmembrane conductance regulator) and mutant yeast CPY* (carboxypeptidase Y) [213, 214]. However, the ERAD machinery does not only rid the cell of misfolded proteins but also degrades native proteins that lack their proper oligomerization partners. Two such substrates are the T-cell receptor (TCR) subunits TCR-α and CDR-δ [198, 215]. These become ERAD substrates when they fail to assemble with other TCR components. We coexpressed the dominant negative Rfp2[$C^{13}A$] mutant together with CDR-δ in HEK293 cells and analyzed the turnover of the latter protein by pulse-chase metabolic labeling. Expression of Rfp2[$C^{13}A$] led to pronounced stabilization of CDR-δ. Importantly, knockdown of Rfp2 with siRNA oligos had a similar effect. In contrast, silencing of Rfp2 had no effect on a non-ERAD substrate, Ub-R-YFP. These results formally demonstrate that Rfp2 functions as an ERAD E3 ubiquitin ligase. Furthermore, the finding that knockdown of endogenous Rfp2 leads to CDR-δ stabilization implies that Rfp2 has an essential role in the turnover of this prototypical ERAD substrate.

In conclusion, this study identifies Rfp2 as a new ERAD E3 ligase. One obvious outstanding question relates to what other proteins that Rfp2 targets for degradation. It is worthwhile to keep in mind that Rfp2 ligase activity could extend to cytosolic non-ERAD substrates as well. It would furthermore be interesting to investigate how Rfp2 recognizes its targets. One possibility is that Rfp2 substrate selection is directed towards misfolded proteins with certain types of lesions or to specific aberrantly assembled multisubunit complexes. Identification of additional substrates will be instrumental in uncovering the cellular pathways that Rfp2 is involved in and determining whether Rfp2 has a role in tumor development.

PAPER III

DLEU2 downregulates the G1 Cyclins D1 and E1 through microRNA-mediated repression

MiR-15a and miR-16-1 were the first microRNAs that were demonstrated to be deregulated in cancer. A study from 2002 by Calin and colleagues showed that miR-15a/miR-16-1 are downregulated in approximately 70% of all CLL cases and that they are located within the larger consensus 13q14 MDR [96]. However, the extent of miR-15a/miR-16-1 downregulation in CLL has been debated. A recent expression profiling study using microRNA cloning and quantitative real-time-PCR demonstrated that only 11% of all CLL cases analyzed had low expression of miR-15a/miR-16-1. All of these cases except one showed biallelic loss of the 13q14 locus [216]. A further link to CLL was provided through analysis of New Zealand Black (NZB) mice that spontaneously develop a lymphoproliferative disorder highly reminiscent of CLL. In these mice, a point mutation in the 3' flanking sequence of pre-miR-16-1 was identified that is not present in other mouse strains. Furthermore, lymphoid tissue from NZB mice has decreased expression of miR-16. Overexpression of this microRNA in a NZB-derived malignant B cell line resulted in increased apoptosis and decreased proliferation, effects not seen in a non-NZB cell line control [217]. Interestingly, the location of the murine mutations is similar to that of rare germline mutations that have been found in some CLL patients [218]. Finally, a very recent study has demonstrated that miR-15a/miR-16-1 can inhibit tumor growth of leukemic cells engrafted in nude mice [219].

Two reports have associated miR-15a and miR-16-1 function with inhibition of cell cycle progression. One study demonstrated that enforced expression of miR-16 family members leads to proliferation arrest and the microRNA-mediated repression of genes specifically related to cell cycle regulation [220]. Another study attempting to identify functional relationships between microRNA families and putative mRNA targets using expression profiling data, implicated both miR-15a and miR-16-1 in cell cycle regulatory processes [221].

Thus, several investigations support the notion that miR-15a/miR-16-1 function as tumor suppressors. However, the microRNAs are located in intron 4 of *DLEU2* and are consequently outside of the smallest region of loss at 13q14 in CLL as defined by our group and others (Figure 4)[84, 86, 91].

In this study, we sought to resolve this discrepancy by studying the relationship between DLEU2 and miR-15a/miR-16-1. Specifically, we wanted to determine whether *DLEU2* could act as a microRNA host gene. Additionally, we investigated both the function and regulation of miR-15a/miR-16-1.

A substantial part of all microRNAs are located within introns of genes [121]. Generally they are in the same orientation as their host genes and in these cases microRNAs can be processed from the intronic sequences, rather than being transcribed from their own promoters. In order to determine whether DLEU2 can function as a regulatory host gene for miR-15a/miR-16-1, we knocked down the microRNA-processing enzyme Drosha. This resulted in an accumulation of DLEU2, indicating that DLEU2 transcripts normally are cleaved to produce functional

microRNAs. A recent report had demonstrated that Myc represses a large set of microRNAs including miR-15a/miR-16-1 [222]. We reasoned that if miR-15a/miR-16-1 are produced exclusively from DLEU2 transcripts then this repression should occur through downregulation of DLEU2 expression. Using a doxycycline-regulated model system where one can switch off Myc expression, we observed that repression of miR-15a/miR-16-1 was accompanied by downregulation of DLEU2. Furthermore, as shown by chromatin immunoprecipitation (ChIP) assays, this repression occurs by Myc binding directly to the two alternative *DLEU2* promoters. In contrast, Myc binding could not be detected to any loci immediately upstream of the microRNAs, arguing against that these are expressed as an individual transcription unit. These findings led us to conclude that *DLEU2* functions as a host gene for miR-15a/miR-16-1.

To gain some insight into the mechanisms by which miR-15a/miR-16-1 inhibit cell cycle progression, we searched for putative targets of these microRNAs using the Mirbase and PicTar databases. This analysis yielded Cyclin D1 and Cyclin E1 as conserved high confidence targets.

Interestingly, we managed to clone an alternatively spliced isoform of DLEU2 that retains parts of intron 4 containing the microRNAs. The transcript was differentially expressed in a wide variety of human and mouse tissues, excluding the possibility of this being a PCR artefact. We proceeded to overexpress the microRNA-containing DLEU2 transcript which resulted in the expected upregulation of miR-15a and miR-16-1. Transfection of DLEU2 in different cell lines resulted in pronounced downregulation of both Cyclin D1 and E1 protein levels. In contrast, use of a deletion construct lacking miR-15a/miR-16-1, DLEU2Δ-MIR, did not result in changes in the abundance of these cyclin proteins. We additionally transfected cells with a miR-16 inhibitor in order to determine whether Cyclin D1 and E1 are relevant physiological targets. This led to increased protein levels of both cyclins, with a specifically robust effect being evident for Cyclin E1. These results imply that endogenous miR-16 family members exert a potent inhibitory effect on Cyclin D1/E1 under normal conditions.

RT-PCR and cycloheximide chase analysis established that the DLEU2-mediated downregulation of cyclin proteins does not occur as a result of altered mRNA levels or increased cyclin protein turnover. Furthermore, downregulation was also observed in a synchronized cell population indicating that indirect cell cycle effects are not responsible for the change in cyclin abundance. Finally, a luciferase reporter assay demonstrated that the DLEU2-mediated downregulation of Cyclin D1 and E1 is dependent on the 3'UTR of these targets.

In relation to Cyclin D1 it is interesting to note that this mRNA has been shown to be negatively regulated by a complex consisting of the AU-rich-binding protein Tristetraprolin (TTP) and miR-16. MiR-16 guides the active TTP complex to specific recognition motifs in the 3'UTR of Cyclin D1. This results in the rapid deadenylation and loss of the mRNA through the exosome pathway [223, 224]. The expression of TTP is tissue-specific and it is not expressed in HEK293 cells making it possible to discount these effects in our experiments [225]. However, in TTP-positive cells Cyclin D1 may be regulated by miR-16, both at the level of mRNA stability and by the translational repression effects we demonstrate in this study.

In order to investigate the putative tumor-suppressive effects of DLEU2 and the microRNAs, we performed colony formation assays in osteosarcoma (U2OS) and breast adenocarcinoma (MCF-7) cell lines. Ectopic expression of DLEU2 led to an ~80% decrease in colony number as compared to both mock- and DLEU2-ΔMIR-

transfected cells. The reduction in colony number was primarily due to inhibited proliferation as determined by analysis of DNA content in cells. In parallel, we assessed the colony-forming ability of cells ectopically expressing the Rfp2 and Dltet ORFs. No reduction in colony number was observed for these proteins (unpublished data). Hence, with regard to the genes consistently affected by 13q14 deletions in CLL, only *DLEU2* containing *miR-15a/miR-16-1* had a growth-suppressive effect under these conditions.

This study presents a set of data that help elucidate the function and regulation of miR-15a and miR-16-1. Importantly, the identification of *DLEU2* as a regulatory host gene of these microRNAs explains how CLL deletions in the 13q14 region not encompassing *miR-15a/miR-16-1* nevertheless lead to their functional loss. The finding that miR-15a/miR-16-1 can target several cyclins for translational repression and in turn are negatively regulated by the oncoprotein Myc enables us to understand why they are deregulated in diverse cancer types. The data additionally demonstrate how, under normal circumstances, activation of Myc can lead to induction of multiple G1 cyclins in a post-transcriptional manner.

In the future, it would be interesting to investigate in greater detail how miR-15a/miR-16-1 regulate cell cycle progression. We have not observed any changes in miR-15a/miR-16-1 abundance in different cell cycle phases (unpublished data). However, it remains possible that the activity of miR-15a/miR-16-1 changes during the cell cycle due to the regulated expression of RNA-binding proteins or changes in intracellular microRNA localization. Yet another alternative is that a constitutive high expression of miR-15a/miR-16-1 serves to reduce the potential leaky expression of cyclins to insignificant levels in cell cycle phases when they are no longer needed.

PAPER IV

Analysis of chronic lymphocytic leukemia breakpoints implicates short direct repeats as a disease-specific cause of double strand break formation.

This study differs significantly from the other three presented in this thesis since it deals with the underlying mechanism behind genetic aberrations in CLL. As described in the introduction, much is known regarding the biological consequences of chromosomal aberrations in malignancy. However, the molecular mechanisms generating these structural changes remain largely unknown.

Deletions and translocations in cancer are non-randomly distributed in the human genome [226]. One obvious reason for this is the selective growth advantage gained from amplifications or losses of specific loci. Another important factor is that chromosomal aberrations often are associated with recombination "hotspots" that have an increased propensity for genomic rearrangement [227]. Certain DNA sequences appear to be causally involved in the recombination process itself. Examples of such sequences include cryptic V(D)J recombination signals and different types of repetitive sequences [228, 229]. The notion that sequence influences the chance of genetic breakage is illustrated by t(14;18) translocations in follicular lymphomas. In about 70% of these translocations, the chromosomal breaks occur within a 150 bp region in the 3' UTR of the *BCL2* gene, although double strand breakage anywhere within a region of 30 kb would result in a translocation with the same functional properties [230]. It has been demonstrated that the affected region is fragile because it adopts non-B DNA conformations that can be cleaved by the RAG (recombination-activating gene) complex.

In light of this, one way to gain insight into deletion and translocation mechanisms is by cloning genetic breakpoints from tumor DNA and analyzing the sequence context of the genomic break. This can provide clues to how the aberration came about in the first place. In this report, we cloned a set of CLL 13q14 breakpoints and analyzed them together with CLL breakpoints available from public databases. In total 17 sequences from different genetic loci were utilized.

As an added finding, the cloning of the 13q14 deletion breakpoints allowed for a refinement of the MDR at the nucleotide level. The critical region of loss described in our previous analysis was confirmed and narrowed down to 8.5 kb. MiR-15a and miR-16-1 are located approximately 31.5 kb centromeric to this MDR and are retained in four out of the eight 13q14 breakpoint cases detailed in this study.

To begin with, we assessed whether 12 different motifs previously linked to genetic recombination were overrepresented at the CLL breakpoints. Interestingly, none of the motifs were significantly associated with the CLL sequences. On the other hand, we observed a conspicuous accumulation of short direct repeats spanning the breakpoint junctions. Therefore, a computational method was developed that could automatically find such repeats and assess their overrepresentation in a given set of sequences. The region surrounding the breakpoint was analyzed in the following fashion. A set of parameters describing a repeat were optimized to maximize

overrepresentation of repeats conforming to the parameters in a "query" set compared to a larger "background" set. Our query set was the 17 CLL breakpoint sequences and the background set was a random draw of 100 sequences from a group of 1571 intronic controls. A distribution of background scores was obtained by drawing random sets of 17 from the controls and using them as query sets. In order to specifically evaluate the occurrence of repeats in the immediate vicinity of the breakpoint, only repeats bridging the breakpoint junction, i.e. those having one repeat half-site on either side of the break, were taken into account. Interestingly, we found a clear overrepresentation of these junctional direct repeats (JDRs) in our CLL sequences with only 1/500 of the random sets displaying a higher score ($p=0.002$). The JDRs found were between 4-8 bp in length with a maximum gap between half-sites of 28 bp (although the majority had smaller gap sequences). No overrepresentation was observed when analyzing repeats that were not constrained to span the breakpoint junction, indicating that the accumulation of JDRs around CLL breakpoints was not due to a general repetitiveness of the region harboring the breaks.

Using this approach, we then proceeded to investigate whether this overrepresentation of JDRs is specific to CLL. For this, we extracted different breakpoint sequences from the Gross Rearrangement Breakpoint Database (GRaBD). No accumulation of JDRs was observed in an analysis of 69 genetic breakpoints with mixed tumor origin. Next, we analyzed six tumor types (breast cancer, acute lymphoblastic leukemia, mantle cell lymphoma, follicular lymphoma, multiple myeloma, and chronic myelogenous leukemia) individually for the overrepresentation of JDRs. Importantly, none of the malignancies displayed a statistically significant overrepresentation of junctional repeats (p-values ranging from 0.23 and upwards).

The data presented in this study indicates that CLL breakpoints are characterized by a dramatic overrepresentation of short spanning direct repeats. Moreover, this feature appears to be CLL-specific as it was not found in tumor breakpoints in general or in breakpoints from any of the six tumor types that we analyzed individually.

The overrepresentation of JDRs at CLL breakpoint junctions is highly statistically significant. However, experimental data is still lacking to prove that JDRs are a causative factor in genetic recombination in CLL. Therefore, experimentally determining the recombination potential of sequences carrying JDRs will be important. Of particular interest is that the observed feature appears to be disease-specific. One possibility is that an enzyme or enzyme complex specifically present in CLL cells or their precursors can mediate recombination through JDRs. If this is the case, then identification of this factor would be a priority.

GENERAL CONCLUSIONS

Based on the findings in this thesis, we can conclude that;

- The refined 13q14 MDR in CLL encompasses only one conserved element, namely *DLEU2*.
- *DLEU2* is a noncoding RNA gene with two separate functions;
    - It acts as an antisense transcript to Rfp2/Leu5.
    - It is a regulatory host gene for the microRNAs miR-15a and miR-16-1.
- *DLEU2* is repressed by the oncoprotein Myc and can through expression of miR-15a and miR-16-1 negatively regulate Cyclin D1 and Cyclin E1 at the translational level.
- The Rfp2 protein is a new ERAD E3 ubiquitin ligase.
- Short direct repeats are implicated in the process of genomic aberration in CLL but not in other malignancies.

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