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DISSERTATION

Titel der Dissertation

„Mechanisms of target regulation by the chimeric oncogene EWS-FLI1 in Ewing´s sarcoma“

Verfasserin

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Abstract

Ewing sarcoma is a highly aggressive pediatric cancer characterized by a chromosomal translocation leading to the chimeric ETS transcription factor EWS-FLI1 in 85% of cases.

We have performed gene expression analysis to identify genes regulated at the transcriptional level by EWS-FLI1. To elucidate which of these are direct targets of EWS-FLI1 chromatin immunoprecipitation followed by sequencing (ChIP-seq) was performed. Integrative bioinformatic analysis revealed that EWS-FLI1 directly binds to 50% of E2F target genes in Ewing Sarcoma. Furthermore, several E2F factors were also found to be directly regulated by EWS-FLI1. Since E2F family members regulate cell cycle progression, regulation of E2F target genes by EWS-FLI1 might be a major pathway of cell cycle deregulation in Ewing Sarcoma. Therefore, we studied the functional interaction of EWS-FLI1 with E2F.

Ten arbitrarily chosen EWS-FLI1/E2F candidate targets including *E2F3*, *RAD51*, *GEMIN4* and *ATAD2* were chosen for an in-depth promoter activity analysis. We confirmed direct EWS-FLI1 promoter binding (ChIP-PCR) and observed decreased reporter activity for all ten studied promoters upon knockdown of EWS-FLI1 by RNAi. Furthermore, the study of promoter occupancy by different E2F family members revealed that silencing of EWS-FLI1 results in the exchange of activating E2F3 for repressive E2F4 on the promoters of their jointly regulated target genes. Importantly, testing *E2F3* promoter occupancy on wildtype and ETS motif mutated promoter constructs in Ewing sarcoma cell lines revealed that binding of E2F3 to its target promoters is dependent on an intact ETS binding site. Mutation of the ETS motif resulted in decreased binding of E2F3.

These data suggest a model in which EWS-FLI1 actively recruits an activating E2F factor thereby replacing a repressing E2F factor and in which EWS-FLI1 binding is essential for E2F binding.

Strikingly, the functional E2F/ETS transcriptional module detected in Ewing Sarcoma for the chimeric ETS factor EWS-FLI1 was found in TMPRSS2-ERG expressing prostate cancer cells. Our findings therefore suggest that this mechanism might be also relevant to other ETS fusion driven cancers.

The majority of these data will be published in:

Bilke S*, Schwentner R**, Yang F, Kauer M, Jug G., Walker RL, Davis S, Zhu YJ, Pineda M, Meltzer PS, Kovar H. (2012), A functional liaison between E2F and ETS in aberrant ETS oncogene driven tumors (submitted) * authors contributed equally

Schwentner R, Bilke S, Kauer M, Ban J, Jug G, Walker RL, Meltzer PS, Kovar H. (2012) EWS-FLI1 is the cofactor of E2Fs in Ewing Sarcoma (in preparation).

Zusammenfassung

Ewing Sarkome sind hoch aggressive pediatrische Tumore, die sich durch eine chromosomale Translokation auszeichnen, welche in einem ETS Transkriptionsfaktor resultiert.

Wir führten Genom weite Expressionsanalysen durch um transkriptionell regulierte Zielgene von EWS-FLI1 zu identifizieren. Um herauszufinden welche davon direkt reguliert werden, wurden Chromatin Immunoprezipitationen gefolgt von Sequenzierung (ChIP-seq) durchgeführt. Bioinformatische Analyen zeigten dass EWS-FLI1 an 50% der E2F Zielgene bindet. Darüber hinaus sind einige E2F Faktoren ebenfalls direkt durch EWS-FLI1 reguliert. Da E2F Faktoren den Zellzyklus regulieren, könnte die Regulierung von E2F Zielgenen durch EWS-FLI1 ein Weg sein, auf dem der Zellzyklus im Ewing Sarkom dereguliert wird. Auf Grund dessen untersuchten wir die funktionelle Interaktion zwischen EWS-FLI1 und E2F.

Zehn zufällig ausgewählte Zielgene deren Promotoren von EWS-FLI1 und E2F gebunden waren, unter anderem *E2F3, RAD51* und *ATAD2,* wurden in detaillierten Promoteranalysen durch Luziferase Reporter Assays in Kombination mit Mutationsanalysen für die EWS-FLI1 und E2F DNA Bindungsstellen studiert. Alle Promotoren der untersuchten Zielgene zeigten nach Knock-down von EWS-FLI1 verminderte Aktivität. Durch ChIPs konnten wir die direkte Bindung von EWS-FLI1 and die oben genannten Promotoren zeigen. Weiters konnten wir durch eine Analyse der Promoter Belegung demonstrieren, dass durch den Knock-down von EWS-FLI1 ein aktivierender E2F Faktor, E2F3, durch einen reprimierenden, E2F4, ausgetauscht wird. Interessant ist, dass dieser Austausch nur bei intakter ETS Bindungsstelle funktioniert, nicht aber wenn EWS-FLI1 nicht mehr an den Promoter binden kann. Die Mutation der ETS Bindungsstelle resultierte in reduzierter E2F3 Bindung.

Unsere Daten stehen mit einem Modell in Einklang, in welchem EWS-FLI1 aktiv E2F3 zu Promotoren von gemeinsamen Zielgene rekrutiert und dadurch den reprimierenden Faktor E2F4 verdrängt. Dabei ist die Bindung von EWS-FLI1 essentiell für diesen Vorgang.

Besonders hervorzuheben ist, dass dieser Mechanismus auch in einer TMPRSS2-ERG expremierenden Prostatakarzinom Zelllinie, nicht aber in einer nicht ETS-Fusion exprimierenden Zelllinie, gezeigt werden konnte.

1 Introduction

1.1 Ewing Sarcoma

Ewing Sarcoma is the second most common bone tumor after osteosarcoma in children and young adults with a peak incidence at the age of 15 [1]. It arises most frequently in bones, preferentially in the long bones of the extremities, pelvis, chest wall and spine [2]. Less than 10% of tumors originate in the soft tissue [3]. Ewing Sarcoma is slightly more common in males and Caucasians and hardly occurs in Asian and African individuals [2]. Approximately 15-25% of patients present with visible metastasis at diagnosis. It is supposed that patients without overt metastases harbor micro-metastases. This assumption is based on the fact that patients with primary tumor resection who do not receive a systemic chemotherapy relapse with distal metastases in about 90% of cases [3-5]. Primary metastasis usually occurs in lungs, bone, bone marrow but rarely in lymph nodes, the liver or the central nervous system [1]. Previously, when patients were not treated with chemotherapy, the survival rate was less than 10%. After the implementation of multimodal treatment regimens, including chemotherapy followed by surgery, if applicable, or radiotherapy, a long term survival rate >60% for patients with localized disease at diagnosis was achieved [5]. Still, patients who present with metastasis can only be cured in less than 25% of cases [6].

Ewing Sarcoma is a member of the Ewing Sarcoma family of tumors which includes Askin's tumor and peripheral neuroectodermal tumors (pPNET), all of which harbor the same chromosomal translocations which will be discussed in the following chapter [4]. pPNET and Ewing Sarcoma have high expression levels of the cell surface glycoprotein CD99. In contrast to pPNET, Ewing Sarcoma largely lacks neural features [7, 8]. Histologically, Ewing Sarcoma is a small blue round cell tumor with high nuclear to cytoplasmic ratios. The cells have scant, weakly eosinophilic cytoplasm, indefinite cytoplasmic borders and round nuclei with regularly distributed finely granular chromatin and low mitotic activity. The cytoplasm typically contains glycogen appearing in periodic-acid-Schiff-positive diastase-digestible granules. Strong expression of the glycoprotein CD99 is present in 90-100% of Ewing Sarcoma [1, 6].

James Ewing first described Ewing Sarcoma in 1921 as a “diffuse endothelioma of the bone” arising in the blood vessels of the bone tissue with a clear discrimination from myeloma or osteosarcoma, the latter mainly due to its responsiveness to radiotheraphy [9]. In the last decades, the cell of origin of Ewing Sarcoma was controversially discussed, but mesenchymal [10-13] and neural crest derived progenitor cells [14-16] are considered the most promising candidates. Studies by Riggi et al. and Castillero-Trejo showed that the introduction of EWS-FLI1 in murine mesenchymal progenitor cells (MPS) or unselected primary murine bone marrow cells resulted in Ewing Sarcoma like tumors in immunocompromised mice [10, 12]. Riggi et al. further showed that the expression of EWS-FLI1 in human mesenchymal stem cells (MSC) induces a genome expression profile highly overlapping with that of Ewing Sarcoma [17]. Vice versa Tirode et al. showed that the knock down of EWS-FLI1 in Ewing Sarcoma cell lines results in a gene expression profile similar to that of mesenchymal progenitor cells and that sustained EWS-FLI1 silencing induces the capability to differentiate into several lineages [13]. Kauer et al. recapitulated these results in a larger study comparing the gene expression signature of several EWS-FLI1 silenced Ewing Sarcoma cell lines with human MPC expression profiles [11]. On the other hand there is evidence for a neural crest cell of origin. Lipinski et al. described neuroectodermal surface antigens on Ewing Sarcoma cell lines, such as ganglioside GD2, a marker of neuroectodermal tissues and tumors [14]. In a gene expression profiling study Staege et al. revealed that genes expressed in neural tissues or during neuronal differentiation are highly expressed in Ewing Sarcoma [16]. Furthermore, von Levetzow et al. showed that the expression of EWS-FLI1 in undifferentiated human neural crest stem cells (hNCSC) and their neuro-mesenchymal stem cell progeny resulted in altered expression of known and novel EWS-FLI1 target genes. More importantly gene expression profiling studies revealed that the Ewing Sarcoma signature is more similar to the gene expression signature of hNCSC than any other normal tissue, including MSC [15].

Taken together, compelling evidence for both theories has been obtained, but a conclusive answer to the question of histogenetic origin is still missing. This may be explained by the findings of Takashima et al. who revealed that neurally-derived MSCs are present in the bone marrow of the developing mouse, and of Lee et al. who demonstrated that neural crest stem cells contain mesenchymal lineage

plasticity. This suggests that Ewing Sarcoma arises from a neurally-derived MSC or a neural crest stem cell with mesenchymal potential [3, 18, 19].

1.1.1 EWS-FLI1

Ewing Sarcoma is characterized by a chromosomal translocation leading to the fusion of EWS and an ETS transcription factor. In 85% of cases the t(11;22)(q24;q12) translocation combines *EWS* (Ewing Sarcoma breakpoint region 1) on chromosome 22 with *FLI1* (Friend leukemia virus integration site 1) on chromosome 11 [20, 21]. This results in a very potent oncogenic transcription factor EWS-FLI1 comprising the FLI-1 ETS DNA binding domain and the transactivation domain of EWS [22].

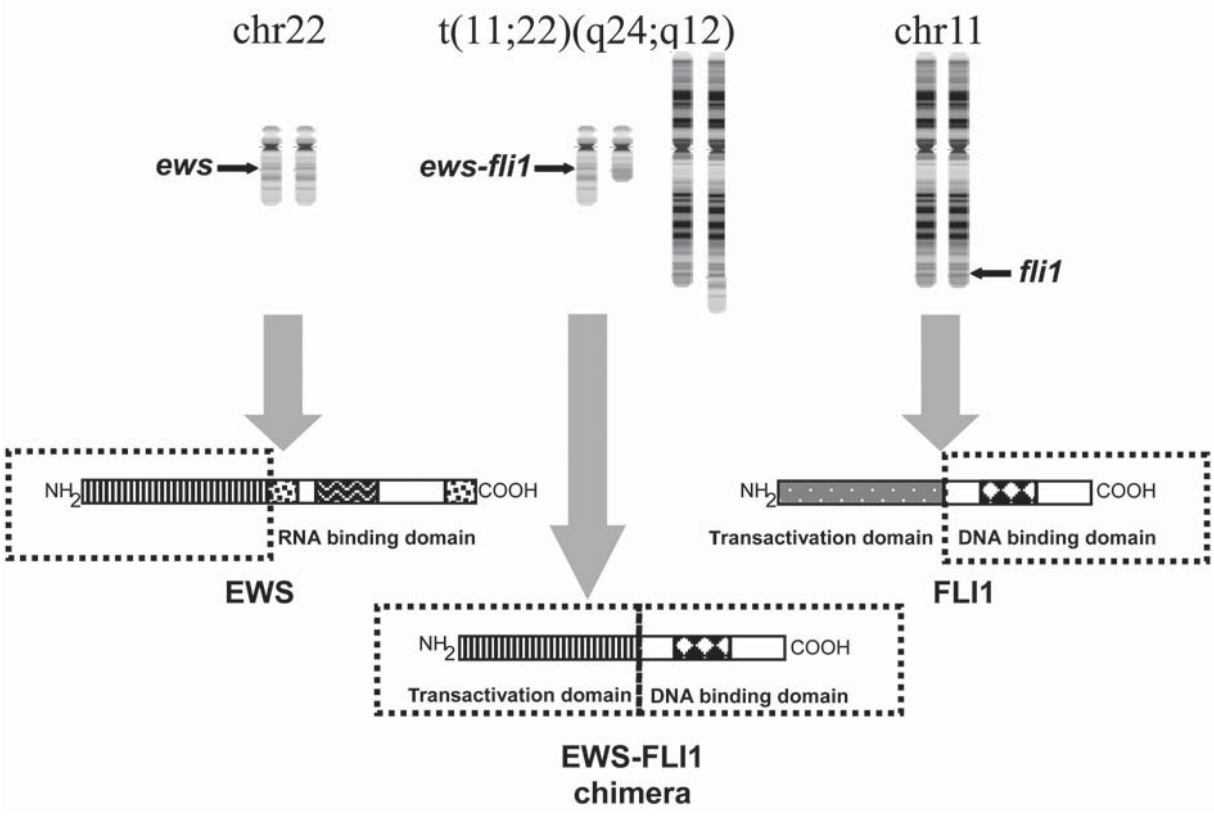

Figure 1: Translocation between Chromosome 11 and 22 leads to *EWS-FLI1* fusion gene. The reciprocal translocation between chromosomes 11 and 22 results in the formation of an *EWS-FLI1* fusion gene that consisits of the the N-terminal transcriptional regulatory domain of *EWS* and the ETS DNA-binding domain from *FLI1* [1].

1.1.2 EWS

The EWS protein belongs to the FET family of proteins, which includes EWS, FUS/TLS and TAF15 [23]. Interestingly, all members of the FET family are involved in genomic rearrangements with transcription factors in several human sarcomas and leukemias, leading to the expression of an aberrant transcription factor. Full-length EWS, like the other family members, is an RNA-binding protein, playing an important part in RNA transcription, mRNA maturation, processing of small non-coding RNAs, RNA transport and DNA repair [23, 24]. Due to the fusion with FLI1, and other members of the ETS transcription factors family (see 1.1.3), EWS loses its C-terminal RNA binding domain but maintains its N-terminal domain, containing several repeats of serine-tyrosine-glycine-glutamine rich sequences [4, 23]. This kind of repeats resembles transcriptional activation domains in transcription factors, and fusion of this domain to a DNA binding domain results in a strong transcriptional activator [4, 25].

1.1.3 FLI1

FLI1 is a member of the ETS family of transcription factors [26]. ETS proteins regulate gene expression in a variety of tissues by the binding of their DNA-binding ETS domain to promoters and enhancers and recruiting other components of the transcription machinery. The major characteristic of the ETS family members is the highly conserved ETS domain responsible for recognition of a common core binding motif 5'GGA(A/T)-3', for EWS-FLI1 in the context of a CGGAAG motif [11, 26]. This conserved domain consists of three α-helices on a small four-stranded, antiparallel β-sheet scaffold. Besides FLI1, the following proteins belong to the ETS transcription factor family: ERG, FEV, GABPA, ETV1-ETV8, ETV3L, ELK1-4, ERF, ETS1-2, SPDEF, ELF1-5, EHF, SPI1, SPIB and SPIC [26]. Several members of this family are fusion partners of EWS in Ewing Sarcoma, or are involved in oncogenic gene rearrangements in prostate cancer (as will be discussed in chapter 1.4.). Furthermore, the fusion of ETV6 with RUNX1 results in a chimeric transcription factor in acute lymphoblastic leukemia, underlining the importance of ETS transcription factors in human malignancies [21, 27, 28]. The expression of FLI1 is normally limited to hematopoietic and neural crest lineages. In Ewing Sarcoma rearrangement with EWS causes aberrant expression of chimeric FLI1 from the constitutively active EWS promoter [4].

1.1.4 Gene regulatory mechanisms of EWS-FLI1

Because EWS-FLI1 is a transcription factor with a very potent transactivation domain fused to a DNA binding domain, it is expected to act rather like a transcriptional activator. However, EWS-FLI1 activates and represses comparable numbers of target genes. Yet whereas repressed genes mainly annotate to differentiation and cell communication, EWS-FLI1 activated genes are involved in cell cycle regulation and proliferation. Thus, the fusion protein appears to combine, by different mechanisms, two key functions of oncogenic transformation in one molecule stimulated proliferation and differentiation blockade. Kauer et al. demonstrated that EWS-FLI1 activated genes are significantly enriched in ETS binding motifs in their promoter sequences, whereas the ETS binding motif was clearly under-represented in EWS-FLI1 repressed promoters. This finding suggests a different, possibly indirect, mode of target gene repression by EWS-FLI1 [11].

EWS-FLI1, for example, up-regulates NKx2.2, which is a transcription factor harboring both transcriptional activation and repression domains dependent on the cellular context. In Ewing Sarcoma gene expression profiling revealed that NKX2.2 functions only as a repressor and that its expression signature overlaps with the EWS-FLI1 down-regulated signature. Furthermore linking transcriptional profiling with ChIP-on-chip data demonstrated that a significant portion of the NKX2.2-repressed gene expression signature was directly mediated by NKX2.2 binding [29-31]. Since NKX2.2 is an EWS-FLI1 activated gene, the repressive signature of EWS-FLI1 seems to be, at least partially, mediated through NKX2.2.

While in the above described mechanism EWS-FLI1 indirectly represses its target genes via up-regulation of a repressive factor, our lab previously reported a direct mechanism of target gene repression via the master regulator FOXO1. We found that EWS-FLI1 directly represses FOXO1 on the transcriptional level and via activation of CDK2 and AKT on the post-translational level. Gene expression profiling showed a significant overlap between EWS-FLI1 repressed and FOXO1 activated genes. Activation and targeting of FOXO1 to the nucleus resulted in reduced proliferation and clonogenicity of Ewing Sarcoma cells [32].

The work described in this thesis focuses on EWS-FLI1 induced mechanisms for gene activation and hence the above mentioned examples are not discussed further.

Several mechanisms of target gene activation used by EWS-FLI1 have been described in the past. These include direct target gene activation through binding to its promoter, indirect activation via deregulation of an upstream regulator (see for example ID2 chapter 1.3, which is indirectly and directly activated), combinatorial binding with a cofactor to a target gene promoter or direct protein – protein interactions. I will discuss target gene activation through direct protein interaction in the following chapter 1.1.4.1 and will give an example of combinatorial binding in the below paragraph.

Molecular analysis revealed that a number of EWS-FLI1 target genes contain ETS and AP-1 binding motifs within near proximity. Gel shift assays were used to study binding of Fos-Jun and EWS-FLI1 to the uridine phosphorylase (UPP) promoter and showed co-operative binding to ETS and AP1 tandem elements in the UPP promoter. Furthermore DNA binding of Fos-Jun complexes enhances binding of EWS-FLI1. While complex formation of Fos-Jun, binding of the complex and recruitment of EWS-FLI1 to DNA could be shown, a direct interaction between Fos-Jun and EWS-FLI1 could not be demonstrated. Interestingly, this cooperative promoter occupancy was also observed for other ETS family members, which participate in gene fusions in Ewing Sarcoma, but not for other ETS factors [33].

1.1.4.1 EWS-FLI1 target gene activation trough interaction partners

Among the various possible mechanisms used by EWS-FLI1 to modulate target gene expression, only few examples of direct protein-protein interactions are known. One such example is NR0B1, which was identified to physically interact with EWS-FLI1 using a yeast two-hybrid screen. Mutations of NR0B1, which disrupt the association with EWS-FLI1, lead to an abrogated oncogenic transformation and also have transcriptional consequences. This suggests that the interaction between NR0B1 and EWS-FLI1 may mediate the transformed phenotype of Ewing Sarcoma [34].

Furthermore, in a study by Kinsey et al., transcriptional profiling data from three Ewing sarcoma cell lines in the presence and absence of EWS-FLI1 were analyzed and revealed NR0B1 (also known as DAX1) to be the most consistently EWS-FLI1

up-regulated gene and to be expressed in primary tumors [29]. NR0B1 is an orphan nuclear receptor that is important for adrenal gland development and was, until this study, not demonstrated to be involved in oncogenesis [35]. Comparison of the transcriptional profiles of NR0B1 and EWS-FLI1 revealed an overlap of regulated genes. Kinsey et al. further analyzed the genome-wide binding pattern of EWS-FLI1 and NR0B1 using ChIP-on-chip and revealed that they bind in a large number to the same genes implying they co-ordinately modulate gene expression [29].

Another and perhaps more general gene regulatory mechanism used by EWS-FLI1 is its association with several proteins of the basal transcription machinery. Among them are RNA polymerase II and its subunit hsRPB7 [36, 37]. While wildtype EWS binds to hyperphosphorylated RNA polymerase II and subsequently attracts splicing factors, EWS-FLI1 binds hyperphosphorylated RNA polymerase II but lacks the ability to recruit splicing factors [36]. CREB-binding protein (CBP) functions as transcriptional co-activator and tumor suppressor, binding of EWS-FLI1 to the amino-terminal region of CBP leads to loss of interaction between CBP and nuclear-receptor transcriptional activity [38]. RNA helicase A (RHA) is not only part of the basal transcription machinery, but also part of the posttranscriptional RNA metabolism [39, 40]. RHA was identified to directly interact with EWS-FLI1 and modulates EWS-FLI1 transcription and anchorage-independent cell growth. Moreover RHA and EWS-FLI1 bind to target gene promoters of EWS-FLI1 like ID2, which showed enhanced activity upon overexpression of RHA. Toretsky et al. conclude that RHA interacts with EWS-FLI1 as a transcriptional cofactor to enhance its function [41].

1.2 Cell Cycle deregulation in Ewing Sarcoma

Deregulation of the cell cycle and resulting sustained proliferation is one main hallmark of cancer and absolutely necessary for tumor growth and progression. Accordingly it is not surprising that several key regulators of the cell cycle are deregulated in Ewing Sarcoma. Kauer et al. reported that most of the EWS-FLI1 activated genes annotate to cell cycle GO terms. In addition they revealed that in EWS-FLI1 activated promoters the E2F consensus binding motif is the second most common transcription factor binding motif after ETS, implying a cooperativity between EWS-FLI1 and E2Fs, which are known to play an important role in cell cycle regulation [11].

In the following chapters I will discuss the phases of cell cycle and its regulators, with a specific focus on E2F factors and their regulators, the pocket proteins, to underline the importance and complexity of this regulatory network. In chapter 1.3 I will focus on the mechanisms used by EWS-FLI1 to enhance cell proliferation and therefore tumor growth.

1.2.1 Cell cycle

Continuous proliferation is maybe the most important trait of cancer. Normal tissue carefully controls the division and proliferation of cells by growth promoting and arresting signals, yet in cancer these signals are deregulated leading to sustained proliferation of cancerous cells [42]. Therefore, our knowledge of cell cycle regulation is essential for our understanding of cancer promotion.

The cell cycle can be divided into interphase and mitosis, which includes prophase, metaphase, anaphase and telophase. In the mitotic phase, the daughter chromosomes are separated and the cells are divided (cytokinesis). While mitosis and cytokinesis last only about an hour and cells usually divide every 24h, the cells stay most of the time in the interphase, in which the chromosomes are decondensed and distributed throughout the nucleus. The interphase consists of G1, S and G2 with G1 and G2 representing the gaps in the cell cycle between mitosis and DNA synthesis (S phase). In the G1 phase, cells are preparing for DNA synthesis while at G2 cells prepare for mitosis. Cells in the G0 phase are not actively cycling. [43, 44]

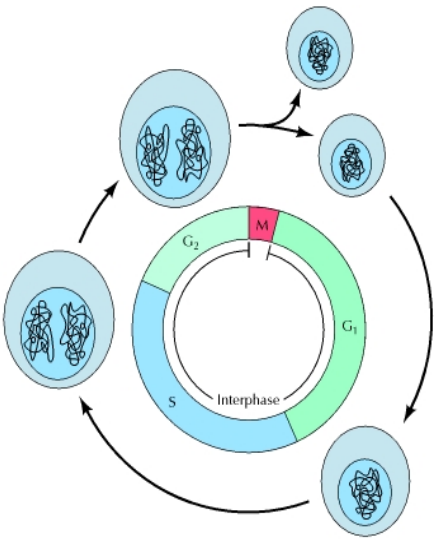

Figure 2: The stages of cell cycle. The cell cycle is divided into four phases: M (mitosis), S(DNA synthesis) and G1 and G2 [43].

1.2.2 Cell cycle regulation

Cell cycle progression is a highly complex regulatory system controlled by several mechanisms. If these mechanisms are impaired unscheduled proliferation and, as a consequence, cancer initiation occurs. In this chapter I will give an overview of the major regulators of cell cycle which are often impaired by EWS-FLI1, as will be discussed in chapter 1.3.

The key regulators of cell cycle maintenance are cyclin dependent kinases (CDK) in association with their activating subunits, cyclins. CDKs are serine and threonine protein kinases activated at specific time points in the cell cycle [45, 46]. While CDKs are expressed throughout the whole cell cycle, their activating partners, the cyclins, are synthesized and degraded at specific time points during the cell cycle and thereby periodically activating CDKs [45, 47]. Prior to progression into the next phase, the cyclin of the current phase has to be degraded and the subsequent cyclin has to be synthesized [44]. According to current knowledge in mammalian cells, 11 *CDK* and 9 *CDK*-like genes are expressed, with only a few of them being involved in cell cycle regulation [47, 48] including three interphase CDKs (*CDK2, CDK4, CDK6*,) and a mitotic CDK (*CDK1*). The ten cell cycle associated cyclins belong to four groups, the A-, B-, D- and E- cyclins [45, 49]. Cyclins D1, D2 and D3 form a complex with CDK4 and CDK6 thereby stimulating the G1 phase entry. Progression from G1 to S phase is promoted by association of cyclin E with CDK2. During S phase, cyclin A forms a complex with CDK2, which is exchanged for CDK1 in late G2 and early M to initiate M phase. CDK1 and cyclin B form the complex required for further regulation of mitosis [47]. In addition to the well established network of the above mentioned CDK/cyclin complexes, a few further complexes were suggested to play a role in cell cycle or gene regulatory mechanisms. CDK3/cyclinC was demonstrated to initiate the G0-G1 transition via phosphorylation of pRB [50]. CDK10 is supposed to be involved in G2-M transition and was demonstrated to associate with the N-terminal domain of Ets2, which in turn regulates CDK1 [51].

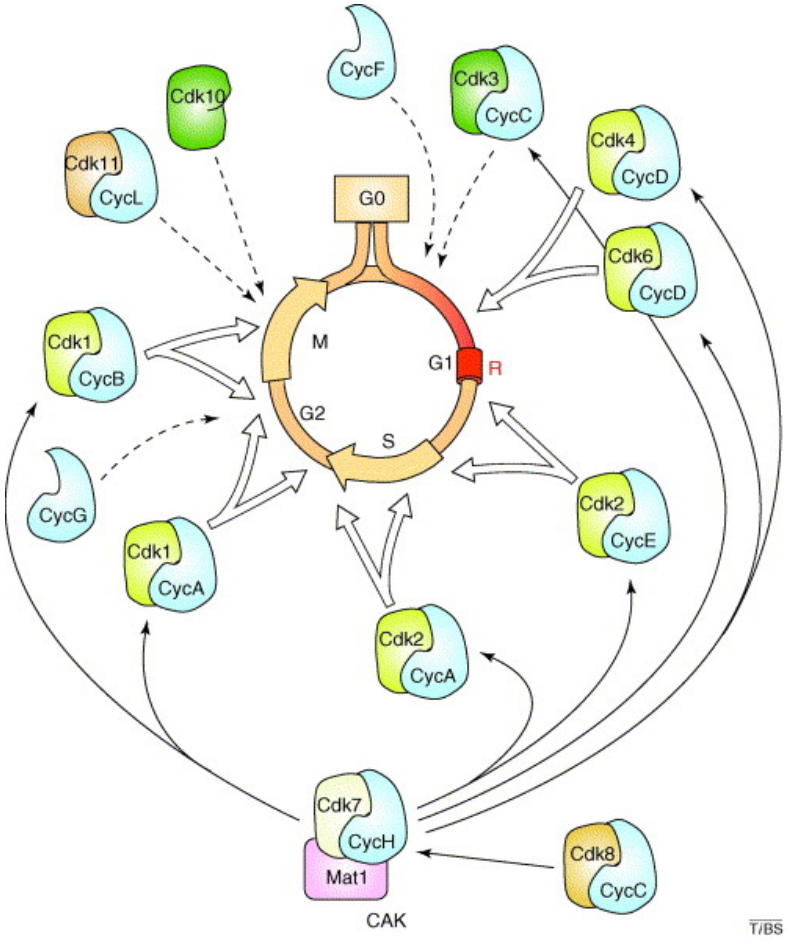

Figure 3: Overview of CDK and Cyclin complexes through the cell cycle. CDK4–cyclinD, CDK6–cyclinD and CDK3–cyclinC complexes regulate the G0–G1 transition and the early phases of G1 by phosphorylating the retinoblastoma protein (pRb). CDK2–cyclinE complexes complete phosphorylation of pRb. CDK2–cyclinE complexes are as well involved in the G1–S transition. During S phase CDK2 associates with cyclin A. CDK1 participates in the S–G2 and G2–M transitions by binding to cyclin A followed by binding to cyclin B. CDK-activating kinase (CAK) phosphorylates, and presumably activates, all cell-cycle CDKs. CAK, a protein complex formed of CDK7, CyclinH and Mat1, is a substrate for CDK8–CyclinC (filled arrows). CDK10 and CDK11 might be involved in mitosis. Cyclin F might be required for entry into G1 and Cyclin G is implicated in the DNA damage response during the G2–M transition. Well described interactions are indicated by open arrows. Dotted arrows indicate interactions based on preliminary data [48].

Beside activation of CDKs by binding of their regulatory subunits, the cyclins, CDKs are controlled by phosphorylation by CAK [44].

In addition, CDK inactivation is regulated via CDK inhibitors (CKI) which bind to CDKs alone or to the complex. Currently, two CKI families are known, the INK4 family with p15 (INK4b), p16 (INK4a), p18 (INK4c) and p19 (INK4d) and the Cip/Kip family including p21 (Waf1, Cip1), p27 (Cip2) and p57 (Kip2). CKIs themselves are

under control of internal and external signals. [47]. For example, the expression of p21 is regulated by the tumor suppressor p53, which is important in the DNA damage checkpoint [49].

By their classification as kinases, active CDK/cyclin complexes phosphorylate their substrates. CDK4 or CDK6 and D-type cyclins phosphorylate members of the pocket protein family, including Rb, p107 and p130 [48]. Phosphorylation of Rb leads to a disruption of its complex with the histone deacetylase proteins and the transcription factors of the E2F family and DP-1, which activate genes necessary for S phase progression like *CDC25*, cyclin A or cyclin E. Hyperphosphorylated pRB remains throughout the cell cycle under the control of CDK2/cyclin E [47].

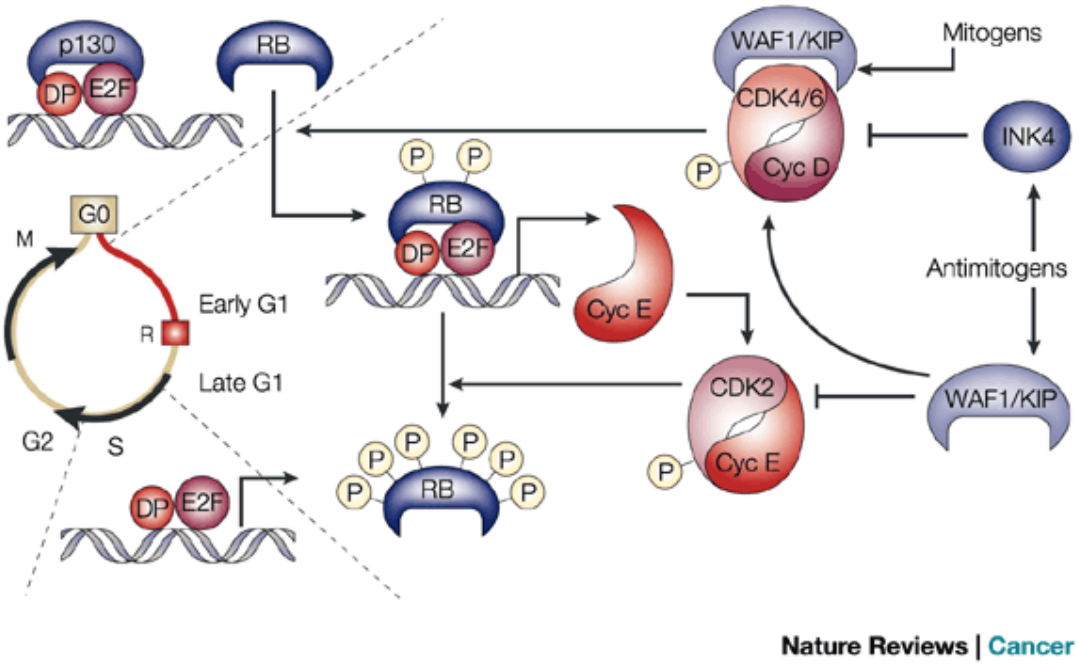

Figure 4: Regulation of the G1/S transition: In G0 cells E2F-DP is bound to p130, the main pocket protein in this phase, and thereby inactive. In G1 E2F-DP is bound to Rb. Formation of CDK4 CDK6/cylin D complexes and initial Rb phosphorylation result from mitogenic signalling and subsequent cyclin D synthesis. Partially phospohorylated Rb is still bound to E2F/DP but they can already transcribe cyclin E, which then activates CDK2. CDK2 in the following phosphorylates and completely inativates Rb. E2F is therefore released and able to induce transcription of genes involved in G1/S transition and DNA replication initiation. INK4a and waf1/kip can inhibit CDK4/6/cyclin D CDK2/cyclin E, respectively. [49]

Figure 4 summarizes the involvement of Rb into the complex network of CDKs and cyclins. In the following two chapters I will discuss the importance of the tumor suppressor Rb and the transcription factor family E2F.

1.2.3 E2F family of transcription factors

The E2F family of transcription factors consists of eight genes, which are transcribed into nine proteins and jointly regulate cell cycle progression [52-54]. Traditionally the members are divided into transcriptional activators (E2F1-3A, B) and transcriptional repressors (E2F4-8) [54-57] although recent studies reveal that this categorization is limited [58]. E2F1, E2F2 and E2F3a, E2F3b contain a transcriptional activating domain at their C-terminus and are associated exclusively with pRB [57, 59]. While E2F3b is constitutively expressed throughout the cell cycle like E2F4 and E2F5, E2F1-3a levels increase upon cell cycle entry [57]. Although E2F1-5 proteins comprise the same pocket protein binding domain, their interaction partner varies. E2F4 and E2F5 interact with p130, while p107 is exclusively bound by E2F4, which is also associated with pRB. E2F6-8 do not bind pocket proteins but act as transcriptional repressors [56, 59]. E2F1–6 require dimerization with members of the differentiation-regulated transcription factor-1 polypeptide (DP) family to form functional transcription complexes on DNA. E2F7 and 8 lack a dimerization domain but comprise tandem repeats of an E2F DNA binding domain [60, 61].

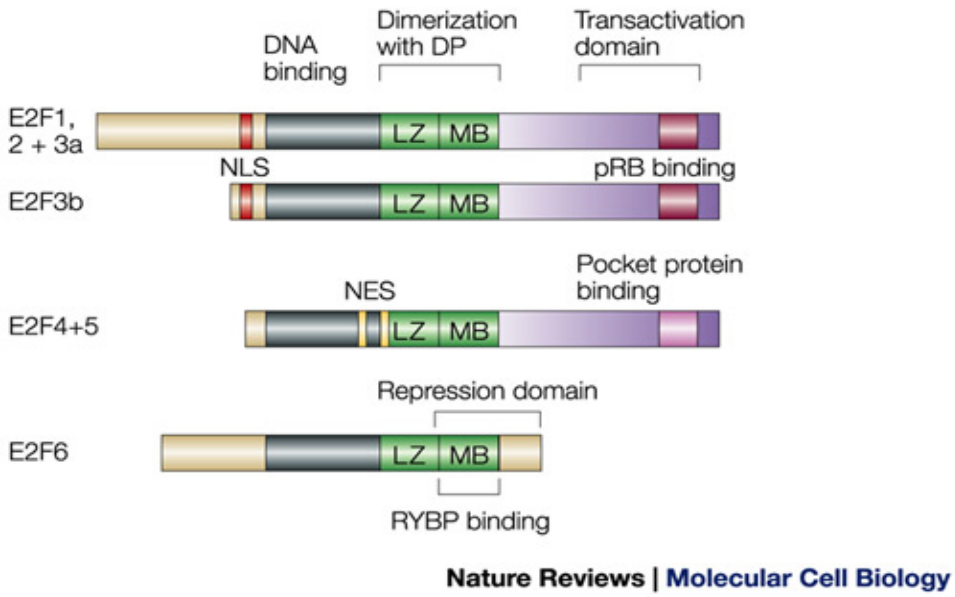

Figure 5: E2F family members. All members of the E2F family share the core domains that mediate DNA binding or dimerization with DP (encompassing both the leucine zipper (LZ) and marked Box (MB) motifs). Sequences that are required for transcriptional activation and pocket-protein binding are only present in E2F1–E2F5. E2F1, E2F2 and E2F3a+b share a canonical basic nuclear localization signal (NLS) that is absent in E2F4 and E2F5, which have nuclear export signals (NES). E2F6, like E2F7 and 8 (not shown), is lacking a pocket protein binding domain but is able to recruit Ring1 and YY1 binding protein (RYBP) - and therefore the polycomb complex [62].

E2F1–3a function as transcriptional activators when released from hyperphosphorylated pocket proteins Rb1 and RBL1/p107 and form complexes with histone acetyltransferases (HATs) p300 and CBP [63]. Alternatively, they act by recruiting the HATs Tip60 and GCN5 to their target gene loci through their essential cofactor transactivation/transformation-domain associated protein (TRRAP) [64-66]. Repressor E2Fs (E2F4 and 5) are localized in the nucleus when bound to the hypophosphorylated retinoblastoma (Rb) like protein RBL2/p130, which appear to be involved in the repression of E2F-responsive genes by assembling transcriptional repressor complexes, including histone deacetylases (HDACs) and/or mSIN3B, the chromatin remodeling complex SWI/SNF and histone methyltransferases [67, 68].

On the basis of these transcriptional properties, the transcriptional upregulation of E2F-responsive genes during the G1/S-phase transition is conferred by the replacement of repressor E2Fs with activator E2Fs at the E2F-binding element in their responsive gene promoters [59].

As already mentioned, E2F transcription factors bind to a number of genes such as cyclin A and E, DNA or polymerase subunits. Binding of activating E2Fs to the promoter regions results in transcription of genes necessary for S phase entry and enables cell cycle progression. This happens only under conditions in which cell cycle integrity is sustained. In case of for example DNA damage; Rb will stay in a complex with E2F and will not allow progression of the cell cycle until DNA is repaired. Following repair, Rb is hyperphosphorylated and releases E2F from the complex, allowing transcription of target genes [69].

1.2.4 Pocket proteins

The retinoblastoma gene Rb1 was the first member of the pocket proteins to be identified. Its discovery was based on its mutation or loss in retinoblastoma, a finding that has been also been discovered in a large number of other cancers. It was one of the first genes which provided evidence that the loss of a gene provokes tumor formation which subsequently led to the concept of tumor suppressor genes [70].

Due to sequence similarities, two further members of the pocket protein family have been identified, *RBL1* and *RBL2* which code for p107 and p130 proteins, respectively [71].

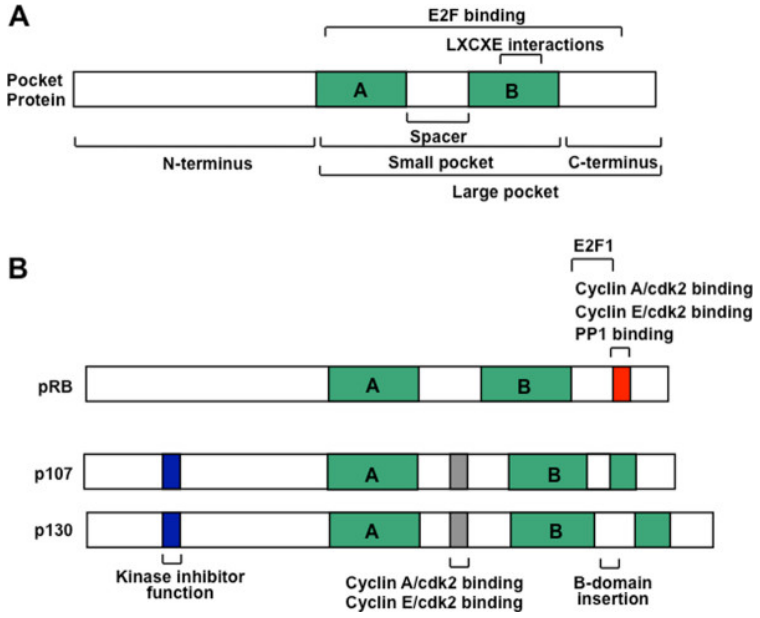

Figure 6: pRB, p107 and p130 homology. A) The small pocket domain is the central feature of RB family proteins. The large pocket is the minimal growth suppressing domain able to bind E2Fs. B) p107 and p130 harbor also a kinase inhibitory site, a cyclin binding site and a B-domain insertion [71].

The small pocket is the central feature of the pocket protein family and consists of the A and B domains that are conserved both across species and among the related proteins. The spacer region between these two domains varies in length and is not conserved. p107 and p130 contain cyclin binding sites within this linker region [71, 72]. This spacer region allows for the assembly of the two domains into a pocket like structure [69]. The pocket was previously thought to be the minimal region in Rb sufficient for binding of viral oncoproteins such as adenovirus E1A, simian virus 40 large T antigen and human papillomavirus E7 [72]. It was later discovered that those viral proteins contain a peptide motif called LXCXE, which they share with several cellular proteins often involved in chromatin regulation [71]. Proteins interacting with the pocket domain disrupt the binding of Rb to the E2F transcription factor family [72]. The large pocket consists of the small pocket and the C-terminal domain, which is not necessary for viral protein binding. The large pocket, in turn, is necessary for E2F binding which results in inactivation of E2F dependent transcriptional activity [73]. E2Fs do not harbour a LXCXE motif but interact with Rb through a distinct site from histone deacetylases (HDACs), allowing the assembly of an HDAC-Rb-E2F repressor complex. Pocket proteins interact with HDAC1-3, which remove acetyl groups from histone tails, thereby promoting the necessary nucleosome formation to form a repressing complex on E2Fs [74].

1.3 EWS-FLI1 target genes involved in cell cycle regulation

As described in the last chapters the cell cycle is regulated by a large number of factors, which are themselves tightly regulated. Deregulation of those factors and mechanisms results in sustained proliferation and, consequently, tumor growth. EWS-FLI1, like other oncogenes interferes with this regulatory system by repression of cell cycle inhibitors and activation of cell cycle promoting factors. In the following chapter I will discuss some of the known factors involved in EWS-FLI1 mediated aberration of the cell cycle (Fig. 7).

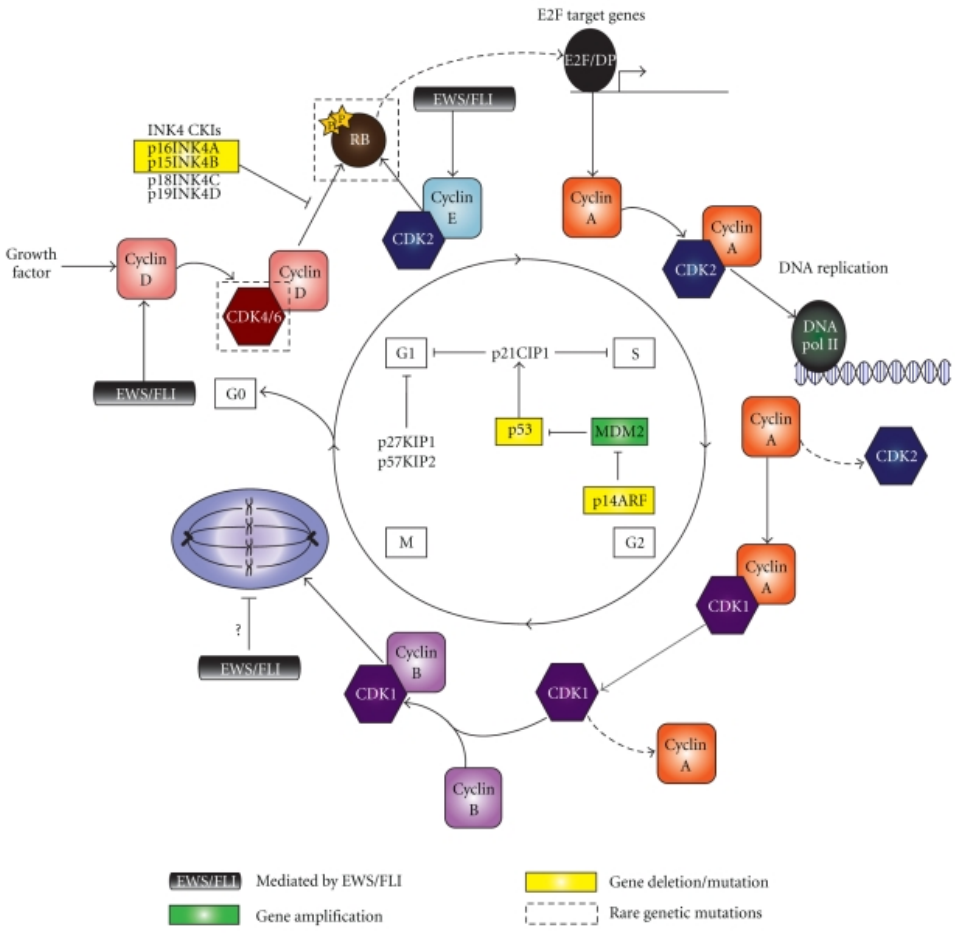

Figure 7: Overview of cell cycle deregulation in Ewing Sarcoma [75]

Depletion of EWS-FLI1 in Ewing Sarcoma cell lines leads to decreased cell proliferation, increased apoptosis and cell cycle arrest [76]. It is suggested that this is achieved through hypophosphorylation and thereby inactivation of Rb induced by EWS-FLI1 knockdown. Furthermore, it was shown that the regulatory proteins p27 and p57 were increased, while the cell cycle promoting cyclin D1 and CDK2 were

decreased upon EWS-FLI1 depletion. These aberrations, together, may result in a deregulation of the cell cycle [76, 77].

Evidence for a direct repression of p21 in Ewing Sarcoma cell lines by binding of EWS-FLI1 to a putative ETS transcription factor binding site within the p21 promoter was published by Nakatani et al. [78] - which is contrary to our findings, which strongly indicate the involvement of p53 in the regulation of p21 expression by EWS-FLI1 [79]. Nevertheless, EWS-FLI1 represses p21, either directly or via p53, in that way eliminating a cell cycle inhibitor.

The most common genetic aberration in Ewing Sarcoma, apart from the translocation, is deletion of the CDKN2A (p16) locus resulting in the loss of p16 and p14ARF expression [75, 80]. It has been reported that between 13 and 39% of Ewing Sarcoma patients harbor a deletion of this locus [80]. Notably, CDKN2B maps to the same chromosomal region as CDKN2A (p15) resulting in the simultaneous loss of p15 and p16. Deletion of this locus is associated with poor overall survival [75].

p53 is mutated or truncated in 50% of all cancers [81], but only in 10% of Ewing Sarcoma and this subset of patients has been reported to show poor outcome [82, 83]. p53 itself is under control of MDM2 and p14ARF. While MDM2 alterations are extremely rare in Ewing Sarcoma, the frequent homozygous loss of CDKN2A deletes p14ARF, a positive regulator of p53 [75].

A further regulator of cell cycle is the transcription factor c-MYC which has been shown to induce the expression of cyclins D1, D2 and CDK4 thereby stimulating G1 phase progression. In addition, c-MYC is able to increase E2F2 and cyclin A2 levels, both active in S phase. Dauphinot et al. demonstrated that besides cyclin D1, CDK4, Rb and p27, c-MYC is constantly highly expressed in Ewing Sarcoma cell lines [75, 84]. However, c-MYC is not only driving proliferation through activation of cell cycle stimulating agents, but it is also upregulating ID2, which in turn is also a directly activated target of EWS-FLI1. ID2 was shown to interact with the tumor suppressor Rb sustaining it in its inactive state and unable to inhibit cell cycle progression [85, 86].

Although the deregulation of the cell cycle in Ewing Sarcoma has been studied for years and decades and already a number of altered key players have been found, the mechanisms have not yet been resolved for all of them.

1.4 ETS fusion driven cancers – Prostate cancer

Although this thesis focuses on Ewing Sarcoma there is evidence supporting the notion that deregulated pathways and mechanisms are shared with other ETS fusion driven cancers including prostate cancer.

Prostate cancer is the second most commonly diagnosed cancer in the United States and the second cause of cancer mortality in men [87]. Although age, environmental factors such as smoking or alcohol consumption, as well as Afro-American origin are well described risk factors, genetic alterations play an important role in prostate cancer development [88]. Apart from hereditary genetic aberrations, like mutations in the androgen receptor genes, the majority of prostate cancers is caused by spontaneous mutations and gene rearrangements. Recently it has been shown that more than 50% of prostate cancers contain gene rearrangements resulting in fusion genes [27, 88, 89]. The first fusion genes described in prostate cancer were fusions of the 5' untranslated region of TMPRSS2 to the ETS transcription factors ERG and ETV1 [90]. ERG is overexpressed in 72% of cases of prostate cancer thereby making it the most prominent oncogene in this kind of cancer [89]. TMPRSS2 is a serine protease secreted in response to androgen exposure from prostate epithelial cells. Fusions of this gene to ETS or other transcription factors lead to an androgen induced oncogene [27, 89, 91]. Additional androgen responsive 5' partners of ERG are SLC45A3, HERPUD1 and NDRG1, wherein SLC45A3 also fuses with ELK4, ETV1, ETV5 and FLI1 and TMPRSS2 fuses also with ETV1, ETV4 and ETV5 [27, 92]. Additional partners for ETV1 include HERV-K22q11.23, C15orf21 and HNRPA2B1, and for ETV4 KLK2 and CANT1 were identified as fusion partners [89]. In 1-2% of prostate cancers, ETS transcription factors are not involved, but RAF kinase gene fusions were found, namely SLC45A3-BRAF, ESPRP1-RAF1 and RAF1-ESPR1 [93]. Several studies outlined that patients with a TMPRSS2-ERG fusion have a significantly worse outcome and that ETS fusion positive cancers have a significantly dissimilar transcriptional profile from ETS fusion negative prostate cancers, implying that these two subsets represent two different kinds of cancer [91]. 40 to 80% of PSA screened prostate cancers harbour an ETS-fusion, while 20-60% do not and are lacking a biomarker or prognostic factor. An analysis for top meta-outliers revealed SPINK1 to have outlier expression in ETS fusion negative prostate cancers and to be associated with clinical recurrence of prostate cancer [94]. Figure

8 summarizes the different gene rearrangements present in prostate cancer and outlines again the significant over representation of ETS gene fusions.

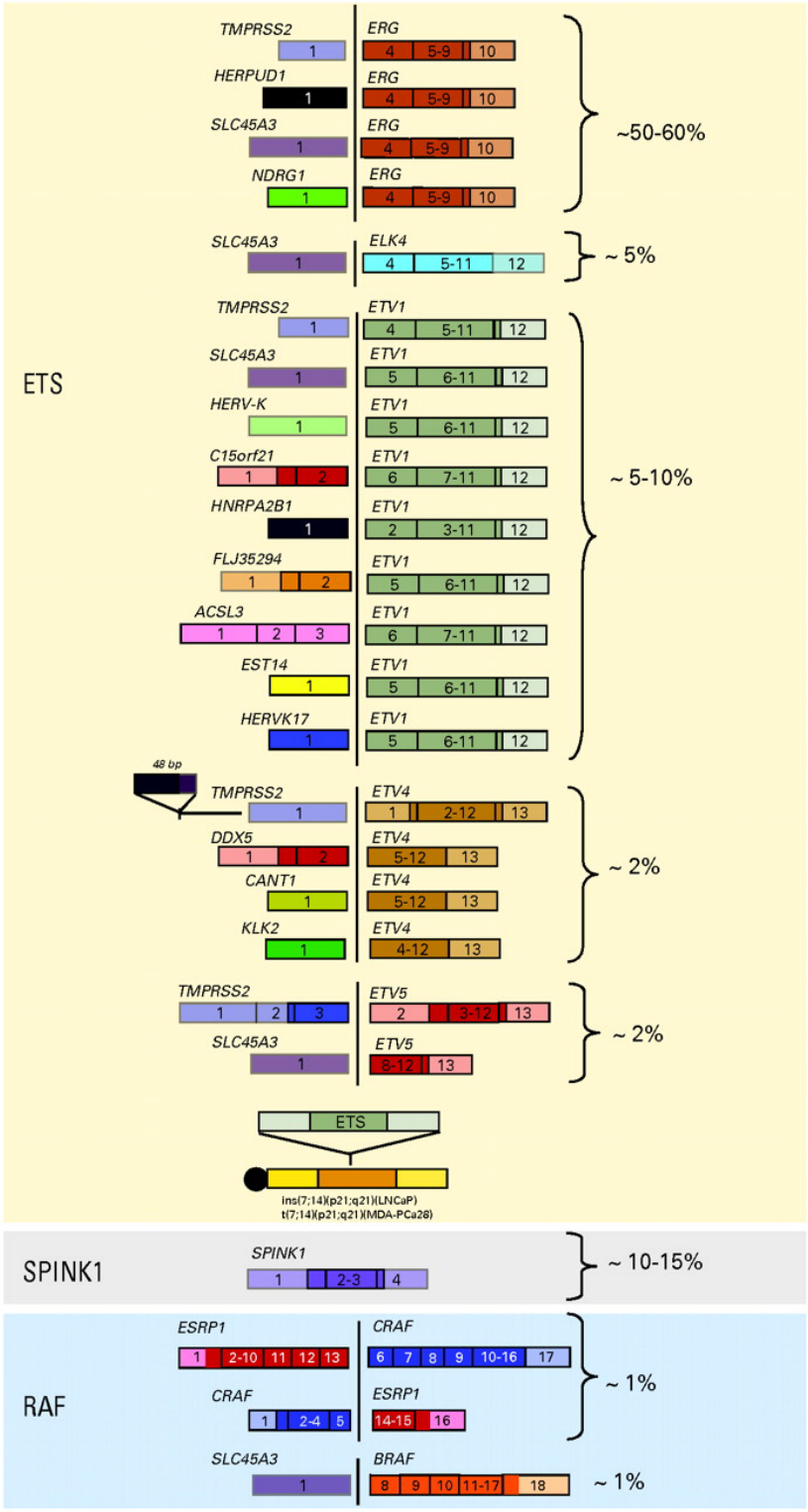

Figure 8: Prostate cancer gene fusion classification. Gene fusions are divided into three groups ETS fusion gene positive, negative and RAF kinase fusions. Percentages reflect the estimated frequency of each subtype according to the literature [27].

Aim of the thesis

A recent study from our lab demonstrated that approximately the same number of genes is simultaneously up and down-regulated by EWS-FLI1. The most striking difference was that the results showed an enrichment of consensus ETS binding sites in the promoters of activated genes, while the ETS binding motif was clearly under-represented in EWS-FLI1 repressed promoters. Furthermore, we could show that the second most common transcription factor binding motif after ETS in EWS-FLI1 activated genes was the E2F consensus site.

Since E2Fs are major regulators of cell cycle progression, which in turn is a hallmark of cancer, we aimed to functionally define the transcriptional network of factors binding to EWS-FLI1 regulated E2F target genes, and to molecularly define the mode of action used by EWS-FLI1 and E2F and their consequences on cell cycle progression.

The specific aims were:

- To physically confirm the simultaneous binding of EWS-FLI1 and members of the E2F family to EWS-FLI1 activated target genes by ChIP-seq for FLI1, E2F3 and E2F4 in collaboration.
- To define by mutation analysis EWS-FLI1, E2F3 and E2F4 target sites within ChIP-seq hits in the promoters of EWS-FLI1 target genes and to analyse the functional consequences in reporter gene assays performed in Ewing Sarcoma cells in the presence and absence of EWS-FLI1.
- To characterize the composition of transcription factor complexes occupying the promoters of these genes with respect to E2F3, E2F4, hyper- and hypophosphorylated pocket proteins RB1, RBL1/p107, and RBL2/p130 in a time resolved manner after modulation of EWS-FLI1.
- To modulate the individual and combined expression of EWS-FLI1, E2F3, and E2F4 by RNA interference and/or ectopic expression, and to test for the functional consequences on promoter activity of these genes and cellular proliferation/cell cycle.

- To study if co-localization and co-occupancy of promoters by EWS-ETS and E2F factors would also be found in Prostate Cancer cell lines harboring a similar gene fusion resulting in a transcription factor which binds as well to ETS binding motifs.

2 Material and Methods

2.1 Material

2.1.1 Media

Luria Broth (LB):

1% Trypton

1% NaCl

0,5% Yeast-extract

sterilization by autoclaving

For Agar Dishes:

1% Trypton

1% NaCl

0,5% Yeast extract

1,5% Agar

Ampicillin was supplied after cooling down the autoclaved agar to 50°C.

Pour out the solution into petri-dishes.

Terrific Broth (TB):

900ml H_20

12g Tryptone

24g Yeast-extract

4ml Glycerol

sterilization by autoclaving

the following filer-sterilized supplement was added prior to use:

100ml of 0,17M KH_2PO_4, 0,72M K_2HPO_4

NZY+ Broth:

10 g of NZ amine (casein hydrolysate)

5 g of yeast extract

5 g of NaCl

pH 7.5

per liter

sterilization by autoclaving

the following filer-sterilized supplements were added prior to use:

12.5 ml of 1 M MgCl2

12.5 ml of 1 M MgSO4

20 ml of 20 % (w/v) glucose

RPMI 1640 with GlutaMAX^{Tm}-I: Invitrogen, Groningen, Netherlands

10% fetal calf serum (FCS Gold, PAA Laboratories, Linz, Austria) and 100000 Units/l penicillin / streptomycin (PAA Laboratories, Linz, Austria)

Opti-MEM: Invitrogen, Groningen, Netherlands

DMEM, High Glucose, GlutaMAX™: Invitrogen, Groningen, Netherlands

4500 mg/L glucose, 4mM L-glutamine and 110 mg/L sodium pyruvate

10% fetal calf serum (FCS Gold, PAA Laboratories, Linz, Austria) and 100000 Units/l penicillin / streptomycin (PAA Laboratories, Linz, Austria)

2.1.2 Reagents:

Trypsin / EDTA: PAA Laboratories, Linz, Austria

Accutase: PAA Laboratories, Linz, Austria

Puromycin: Sigma, St. Louis, USA

Ampicillin: Biomol, Hamburg, Germany

Doxycycline: Sigma, St. Louis, USA

Blasticidin: Invitrogen, Groningen, Netherlands

Zeocin: Cayla, Toulouse, France

Dimethyl pimelimidate dihydrochloride (DMP): Sigma, St. Louis, USA

2.1.3 Buffers

PBS: 137mM NaCl; 3mM KCl; 6,5mM Na_2HPO_4-$2H_2O$; 1,5mM KH_2PO_4

TBS: 50mM Tris, 150mM NaCl, pH 7,5

TBS-T: 50mM Tris, 150mM NaCl, 0,1% Tween 20; pH 7,5

2x sample buffer:

20% (v/v) glycerol

6% ß-mercaptoethanol

3% SDS

125mM Tris-Cl pH 6,8

a few bromphenol blue crystals

Laemmli buffer:

15,1g Tris

72g glycine

25ml 20% SDS

per 1 liter

Transfer buffer:

14g glycine

3g Tris

20% methanol

per 1 liter

Ponceau S staining solution (10x stock):

2g Ponceau S

30g trichloroacetic acid

30g 5-sulfosalicylic acid

ad 100ml

Loading Dye:

4M Urea

80mM EDTA

10% Saccharose

0,25% BPB

TBE:

5,4g Tris Base

2,75g Boric Acid

2ml 0,5M EDTA/pH8

per 1 liter

DMP solution:

0.01032g DMP/2 ml Na-Borate

IP-Lysis Buffer:

50 mM Tris (pH 8,0)

400 mM NaCl

0,5% NP40

1 tablet Protease Inhibitor cocktail “complete” (Roche, Basel, Switzerland) disvolved in 50ml PBS

0,2M NaBorate pH(9): 20g in 500ml H20

125mM Tris: 25ml 1M +75ml H20

0,1M Glycin: 3,7g in 500ml H20

Buffer 1A for Chromatin Immunoprecipitation:

0.1% SDS

1% Triton X-100

2mM EDTA

20mMTris HCl

500mM NaCl

pH8.1

Blocking solution:

Licor blocking reagent (Roche, Basel, Switzerland) in maleic acid buffer (100mM Maleic Acid, 150 mM NaCl, pH= 7.5, sterile).

2.1.4 Bacterial strains

JM109: *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, ∆(*lac-pro*AB), [F’, *tra*D36, *pro*AB, *lac*I^qZ∆M15], (Promega, Madison, USA)

Sure2 Supercompetent Cells: e14-(McrA-) Δ*(mcrCB-hsdSMR-mrr)171 endA1 gyrA96 thi-1 supE44 relA1 lac recB recJ sbcC umuC*::Tn*5* (Kanr) *uvrC* [F’ *proAB lacI*q*ZΔM15* Tn*10* (Tetr) Amy Camr], (Stratagene, La Jolla, USA)

2.1.5 Cell lines

ASP14: Ewing tumor cell line established from A673 parental cell line by Javier Alonso (Laboratorio de Patología Molecular de Tumores Sólidos Infantiles, Departamento de Biología Molecular y Celular del Cáncer, Instituto de Investigaciones Biomédicas, Madrid, Spain) (expresses EWS-FLI1 type 1, p53 mutant). Inducible cell line, Doxycycline induces shRNA against EWS-FLI1 [95]

VCaP: TMPRSS2-ERG expressing prostate cancer cell line established from a vertebral bone metastasis from a patient with hormone refractory prostate cancer by the lab of Pienta KJ [96].

HeLa: Cell line established from epidermoid carcinoma of the cervix by the group of Gey GO [97].

2.1.6 Plasmids

2.1.6.1 Existing plasmids

pMaxGFP Used for monitoring transfection efficiency. Amaxa GmbH, Cologne, Germany

pGL4.10: Firefly luciferase reporter vector. Promega, Madison, USA

pCMV: CMV promoter based mammalian expression (constructed by Suzanne Baker, John's Hopkins, Baltimore)

pcDNA3-E2F1: Mammalian expression vector encoding human E2F1 (Gift from Nevins JR, Department of Molecular Genetics and Microbiology, Institute for Genome Sciences and Policy, Duke University Medical Center, NC, USA)

pBSK-E2F3: Mammalian expression vector encoding human E2F3 (Gift from Nevins JR, Department of Molecular Genetics and Microbiology, Institute for Genome Sciences and Policy, Duke University Medical Center, NC, USA) [98]

pcDNA3-E2F4: Mammalian expression vector encoding human E2F4 (Constructed by Claude Sardet, Whitehead Institute, Cambridge, MA, USA)

pRETROSuperScrambled: pSUPER-based retroviral mammalian expression vector. (Gift from Eiji Hara, The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan)

shE2F3: Adtrack based shRNA expression plasmid under the control of U6 promoter encoding shRNA against E2F3 (Gift from Nevins JR, Department of Molecular Genetics and Microbiology, Institute for Genome Sciences and Policy, Duke University Medical Center, NC, USA)

shE2F1: Adtrack based shRNA expression plasmid under the control of U6 promoter encoding shRNA against E2F1 (Gift from Nevins JR, Department of Molecular Genetics and Microbiology, Institute for Genome Sciences and Policy, Duke University Medical Center, NC, USA)

2.1.6.2 Plasmids established in the course of this thesis

pGL4.10-ATAD2: ATAD2 reporter gene construct that encodes ATAD2 Promoter Region -368 / +202

pGL4.10-E2F3: E2F3 reporter gene construct that encodes E2F3 Promoter Region -272 / 327

pGL4.10-RRM2: RRM2 reporter gene construct that encodes RRM2 Promoter Region -463 / 191

pGL4.10-RFC2: RFC2 reporter gene construct that encodes RFC2 Promoter Region -400 / 87

pGL4.10-VRK1: VRK1 reporter gene construct that encodes VRK1 Promoter Region -269 / 100

pGL4.10-MFL1IP: MFL1IP reporter gene construct that encodes MFL1IP Promoter Region -251 / 70

pGL4.10-GEMIN4: GEMIN4 reporter gene construct that encodes GEMIN4 Promoter Region -275 / 87

pGL4.10-CDK2: CDK2 reporter gene construct that encodes CDK2 Promoter Region -118/+458

pGL4.10-SKP2: SKP2 reporter gene construct that encodes SKP2 Promoter Region -240 /348

pGL4.10-RAD51: RAD51 reporter gene construct that encodes RAD51 Promoter Region -186 / 164

pGL4.10-PRKC1: PRKC1 reporter gene construct that encodes PRKC1 Promoter Region -169 / 235

2.1.7 Oligonucleotides

ChIP-PCR Primers:

ATAD2 -1678/-1470 fwd: CCCAGACATTGCATTCTTCA

ATAD2 -1678/-1470 rev: GAGGCCAATGAGAACAGAGC

ATAD2 -350/-240 fwd: CAGGGGTGGGGAGGAGACGC

ATAD2 -350/-240 rev: GAGCGGTGCGTAGCCCGTTT

ATAD2 -117/18 fwd: GCCCGGCCTCCTTCGCTCTA

ATAD2 -117/18 rev: GGCGCCACAAGCTCCGCGCCA

ATAD2 26/129 fwd: GAGCGCGGAAGAGCCAGAG

ATAD2 26/129 rev: GCTGCTGCGGAGAACCACCA

E2F3 -1457/-1334 fwd: AAGGAGTCCTAGCCTGATCTGA

E2F3 -1457/-1334 rev: TGAGGATTGCAACACCTTGA

E2F3 -272/-149 fwd: TCAAGGAGGCCTATGCAAAT

E2F3 -272/-149 rev: GGCCGCTACCTCCTTACTTC

E2F3 -132/-62 fwd: CGGGTTGAGGGGCGGGGATA

E2F3 -132/-62 rev: TGCAACGGATTGCGAGGCGG

E2F3 131/262 fwd: CCAGAGCCCCGATTATTTTT

E2F3 131/262 rev: GCAGTCGGAGTTTCCAAGTC

RAD51 -4270/-4017 fwd: AGGCAGGAGTATCGCTTGAA

RAD51 -4270/-4017 rev: AGTGTGGTGGCTCACACTTG

RAD51 -93/104 fwd: CGTCTTGGGTTAGCGCGCAG

RAD51 -93/104 rev: GTTCCCAGCTGCACGCCTCG

RAD51 -51/65 fwd: ATCCGGGAGGCGGGGATACG

RAD51 -51/65 rev: TAGGGCTCGGTCTCTGGCCG

RAD51 -51/106 fwd: ATCCGGGAGGCGGGGATACG

RAD51 -51/106 rev: CAGTTCCCAGCTGCACGCCT

RAD51 45/146 fwd: CGGCCAGAGACCGAGCCCTA

RAD51 45/146 rev: TCGCTTGCCCCAGCCTTCTG

GEMIN4 -3609/-3486 fwd: GGAGGCTACTGTGGAGACCA

GEMIN4 -3609/-3486 rev: ATGACCCTGGACACTCAAGC

GEMIN4 -236/-47 fwd: GTTACCGGGTGAGGGTGAAT

GEMIN4 -236/-47 rev: GCAGTCCTCACGAACGAG

GEMIN4 -153/8 fwd: GGTGCGGAGGGGTCTAGT

GEMIN4 -153/8 rev: TTAGGCCTGCTCACAACCTC

GEMIN4 -130/76 fwd: ACGTCCGGGTACCTGAGGGC

GEMIN4 -130/76 rev: TCCGAGAACTCGAACGCGGC

ChIP-PCR on promoter constructs Primers:

pGL4.10 rev: AACAGTACCGGATTGCCAAG

E2F3 fwd:	GCGTAAACCGTATCCCTTCA
ATAD2 fwd:	CAGCAAGCAGGTGTCAACAG
pGL4.10-GEMIN4 rev:	CTCGAAGTACTCGGCGTAGG used with GEMIN4 -236/-47 fwd

Site directed mutagenesis primers:

ATAD2 Ets mt fwd:	CGCAGCTCTGGCTCTTTATAGCTCCGAATTCTGGCGCC
ATAD2 Ets mt rev:	GGCGCCAGAATTCGGAGCTATAAAGAGCCAGAGCTGCG
ATAD2 E2F mt fwd:	CGCGCTCCGAATTCTGTTACCACAAGCTCCGCGC
ATAD2 E2F mt rev:	GCGCGGAGCTTGTGGTAACAGAATTCGGAGCGCG
ATAD2 E2F mt fwd:	CCCGCCGCCGTCCCTTACCAAAATTCCAAACGG
ATAD2 E2F mt rev:	CCGTTTGGAATTTTGGTAAGGGACGGCGGCGGG
E2F3 Ets mt fwd:	CATTGTCAGCAGCAGCTATATGGAGCCATTTTTCAGCTGCC
E2F3 Ets mt rev:	GGCAGCTGAAAAATGGCTCCATATAGCTGCTGCTGACAATG
E2F3 Ets mt fwd:	GAGAGGGGGCTCTATAGCGCCGGGCGG
E2F3 Ets mt rev:	CCGCCCGGCGCTATAGAGCCCCCTCTC
RAD51 Ets mt fwd:	GCGCGCAGGGCTATAGCGGGGAGAAGGCGG
RAD51 Ets mt rev:	CGCGCGTCCCGATATCGCCCCTCTTCCGCC
RAD51 Ets mt fwd:	GGAGAGTGCGGCGCTATACGAGGCGTGCAGCTG
RAD51 Ets mt rev:	CCTCTCACGCCGCGATATGCTCCGCACGTCGAC
RAD51 E2F mt fwd:	CTGGGCGAGAGGGTTTGTTAGGAATTCTGAAAGCCGCC
RAD51 E2F mt fwd:	GACCCGCTCTCCCAAACAATCCTTAAGACTTTCGGCGG
GEMIN4 Ets mt fwd:	CCGCTGGGACCCCTATAGAGGGGCCGGGC

GEMIN4 Ets mt rev: GGCGACCCTGGGGATATCTCCCCGGCCCG

GEMIN4 Ets mt fwd: GGGAGGGCTCTGCCTATAGGCGGCGCTGTGC

GEMIN4 Ets mt rev: CCCTCCCGAGACGGATATCCGCCGCGACACG

GEMIN4 E2F mt fwd: CGGCGCTGTGCGCTTGTTACGCTCGTTCGTGAGG

GEMIN4 E2F mt rev: GCCGCGACACGCGAACAATGCGAGCAAGCACTCC

GEMIN4 E2F mt fwd: CGTGCCGTGCGTCCCTTACCGCGTTCGAGTTCTC

GEMIN4 E2F mt rev: GCACGGCACGCAGGGAATGGCGCAAGCTCAAGAG

MWG pGL-series Standard primers:

pGL rev: CTTTATGTTTTTGGCGTCTTCC

pGL3 for: CTAGCAAAATAGGCTGTCCC

2.1.8 Antibodies

Anti-E2F3 (C-18): rabbit polyclonal antibody against the C-terminus of human E2F3. (Santa Cruz Biotechnology Inc., Santa Cruz, USA, sc-878).

Western Blot Dilution: 1:400

ChIP: 1,5µg per reaction

Anti-E2F4 (C-20): rabbit polyclonal antibody against the C-terminus of human E2F4. (Santa Cruz Biotechnology Inc., Santa Cruz, USA, sc-866).

Western Blot Dilution: 1:500

ChIP: 1,5µg per reaction

Anti-E2F1 (C-20): rabbit polyclonal antibody against the C-terminus of human E2F1. (Santa Cruz Biotechnology Inc., Santa Cruz, USA, sc-193).

Western Blot Dilution: 1:200

ChIP: 1,5µg per reaction

Anti-FLI1: rabbit polyclonal antibody against the C-terminus of human FLI1. (MyBiosource, San Diego, California, USA, MBS300723).

Western Blot Dilution: 1:400

ChIP: 3µg per reaction

Anti-RB (C-15): rabbit polyclonal antibody against the C-terminus of human RB. (Santa Cruz Biotechnology Inc., Santa Cruz, USA, sc-50).

Western Blot Dilution: 1:200

ChIP: 3µg per reaction

Anti-p130 (C-20): rabbit polyclonal antibody against the C-terminus of human p130. (Santa Cruz Biotechnology Inc., Santa Cruz, USA, sc-317).

Western Blot Dilution: 1:200

ChIP: 3µg per reaction

Anti-p107 (C-18): rabbit polyclonal antibody against the C-terminus of human p107. (Santa Cruz Biotechnology Inc., Santa Cruz, USA, sc-318).

Western Blot Dilution: 1:200

ChIP: 3µg per reaction

Anti-DP1 (K-20): rabbit polyclonal antibody against the N-terminus of human DP1. (Santa Cruz Biotechnology Inc., Santa Cruz, USA, sc-610).

Western Blot Dilution: 1:200

ChIP: 3µg per reaction

Anti-Rabbit IgD, DyLight^{TM}800: Goat Anti-Rabbit antibody reacts with heavy chains of rabbit IgG and with light chains of most rabbit immunoglobulins (Pierce Biotechnology Inc., Rockford, USA).

Dilution: 1:10000

Anti-Mouse IgD, DyLight^{TM}800: Goat Anti-Rabbit antibody reacts with heavy chains of mouse IgG and with light chains of most mouse immunoglobulins (Pierce Biotechnology Inc., Rockford, USA).

Dilution: 1:10000

2.2 Methods

2.2.1 DNA Methods

2.2.1.1 Cloning of gene reporter constructs

PCR was performed for 25 cycles in a total volume of 50µl with 10pmol of the corresponding primers, 10mM dNTPs (Promega, Madison, USA), 5µl 5xPhusion HotstartII Buffer (Finnzymes, Espoo, Finland), 1µl Phusion Hotstart II (Finnzymes, Espoo, Finland) and 100ng of genomic DNA were used as a template. PCR products were digested with KpnI and NheI restriction enzymes (NEB, Ipswich, USA) using Buffer1 in the presence of BSA over night at 37°C. Gel purification was performed with ZymocleanTM Gel Recovery Kit (Zymo Research, Orange, USA). pGL4.10 was digested with KpnI and NheI and dephosphorylated with CIAP (NEB, Ipswich, USA) at 37°C for 30min.

Ligation was performed overnight at RT with T4 Ligase and the provided T4 Ligase Buffer (Promega, Madison, USA).

VRK1 was cloned using KpnI and HindIII and GEMIN4 with NheI and XhoI.

2.2.1.2 Site directed Mutagenesis

Site directed Mutagenesis was performed using QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA, 200523) according to manufacturer's instructions.

2.2.1.3 Transformation of competent E.coli (JM109)

DNA transformation of competent E.coli JM109 (Promega, Madison, USA) was permormed according to manufacturer's instructions.

2.2.1.4 Transformation of competent E.coli (Sure2 Supercompetent Cells)

DNA transformation of competent E.coli Sure2 Supercompetent Cells (Stratagene, La Jolla, USA, 200523) was permormed according to manufacturer's instructions.

2.2.1.5 Mini Prep

2ml of 10mg/ml ampicillin containing LB were inoculated with a single colony and incubated overnight at 37°C with shaking. For Mini Preps the Quiagen MiniPrep kit (Quiagen, Austin, USA) was used according to the manufacturer's instructions.

2.2.1.6 Restriction digest of gene reporter plasmids

500ng DNA of plasmids was digested with KpnI and NheI in Buffer1 (NEB, Ipswich, USA) in the presence of BSA at 37°C for 4h. Restriction reactions were analyzed on an 1% Agarose Gel. Plasmids containing the corresponding fragment were sent for sequencing.

2.2.1.7 Sequencing

Sequencing was done at MWG Biotech (Ebersberg, Germany). The sequences were blasted against the NCBI human genomic plus transcript database.

2.2.1.8 Maxi Prep

250ml of 10mg/ml ampicillin containing LB were inoculated with a preculture of the corresponding plasmid and incubated overnight at 37°C with shaking. For Maxi Preps, the Quiagen Endotoxin free MaxiPrep kit (Quiagen, Austin, USA) was used according to the manufacturer's instructions.

2.2.1.9 Chromatin Immunoprecipitation followed by PCR:

Chromatin Immunoprecipitation was performed using the MAGnify™ Chromatin Immunoprecipitation System (Invitrogen, Groningen, The Netherlands) according to the manufacturers instructions with minor modifications. Cells were crosslinked with 1% formaldehyde at room temperature for 10 minutes, the reaction was not stopped with Glycin. The cells were then sheared with a Bioruptor UCD200 4 times 7 minutes of alternating 30sec sonication and 30 sec break to achieve an average shearing size of 600bp. The antibodies used were: anti-FLI1 antibody (MyBiosource, San Diego, California, USA, MBS300723), anti-E2F3 (Santa Cruz Biotechnology, Santa Cruz, USA sc-878), anti-E2F4 (Santa Cruz Biotechnology Inc., Santa Cruz, USA, sc-866), anti-RB (Santa Cruz Biotechnology Inc., Santa Cruz, USA, sc-50) and anti-p130 (Santa Cruz Biotechnology Inc., Santa Cruz, USA, sc-317). Please refer to 2.1.8. for the amounts of antibody used per ChIP. Washing steps after chromatin incubation with the Ab coupled beads were extended to 20min each, and an additional washing step between IP Buffer1 and 2, was applied using Buffer 1A (as described in 2.1.3.).

PCR was performed for 35 cycles in a total volume of 30µl with 10pmol of the corresponding primers, 10mM dNTPs (Promega, Madison, USA), 5µl 5xPhusion HotstartII Buffer (Finnzymes, Espoo, Finland), 0.3µl Phusion Hotstart II (Finnzymes, Espoo, Finland) and 3µl of ChIP Eluate. Sybr Green PCR for ChIP on promoter constructs and pocket proteins was performed using Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas) according to manufacturer's instructions.

2.2.1.10 Chromatin Immunoprecipitation and Sequencing.

Chromatin immunoprecipitation (ChIP) was performed using a ChIP-IT kit from Active Motif (Carlsbad, CA), following the manufacturer's instructions with minor modifications. Briefly, A673 cells were crosslinked with 1% formaldehyde at room temperature for 15 minutes. The cells were then sheared with a VirSonic 100 sonicator for 20 cycles of 10 x 1-second pulses. The chromatin was immunoprecipitated overnight at 4°C. The antibodies used were: anti-FLI1 antibody (sc-356), and anti-E2F3 (sc-878) (Santa Cruz Biotechnology, Santa Cruz, CA). A mixture of Protein-G and Protein-A agarose beads was used. After reversal of crosslinking at 65^{o}C overnight, the ChIP DNA was purified using spin columns provided by the kit. For ChIP-seq, the ChIP DNA was prepared, amplified, and

analyzed on an Illumina G1 Genome Analyzer (San Diego, CA), following manufacturer's protocols.

2.2.2 Protein Methods

2.2.2.1 SDS- Polyacrylamid Gel Electrophoresis

The SDS- polyacrylamid gel consists of two different layers of gels, the stacking gel which is always a 6% gel and the separating gel which varies between 6 and 12,5%.

Separating gel:

	6%	**8,5%**	**12,5%**
30%Acrylamid / 0,8% Bis	1,05ml	1,4ml	2,1ml
H_2O	2,625ml	2,275ml	1,575ml
1,5M Tris pH8,8	1,25ml	1,25ml	1,25ml
20% SDS	25µl	25µl	25µl
10% APS	50µl	50µl	50µl
TEMED	6µl	6µl	6µl

Stacking gel:

30%Acrylamid / 0,8% Bis	415µl
H_2O	1,7ml
1M Tris pH6,8	315µl
20% SDS	12,5µl
10% APS	25µl
TEMED	2,5µl

The cell pellet was resuspended in PBS and the same amount of 2x Sample Buffer was added. After boiling the samples 10min at 95°C and centrifugation they were

loaded on the gel, which was run at 40mA till the bromophenol blue front was not visible any more.

2.2.2.2 Western Blot

The transfer unit, consisting of a sponge, three 3 pieces of 3MM paper, the gel, a nitrocellulose membrane, three 3 pieces of 3MM paper and a sponge (everything presoaked with transfer buffer), was put in the blotting stock. The transfer was run at 400mA for 90min on ice.

For a first staining, the membrane was incubated in PonceauS solution for 5 to 10min and scanned afterwards. To block unspecific binding, the membrane was incubated in 1% blocking solution for 1 hour at RT. The primary antibody was diluted in 0,5% blocking solution, added to the membrane and incubated overnight at 4%. The membrane was then washed twice with TBST for 10min and once with 0,5% blocking solution. The secondary antibody was diluted in 0,5% blocking solution, added to the membrane and incubated for 1 hour at RT: After washing the membrane three times with TBST and once with PBS for 15min each, it was scanned using the Li-cor Odysee Infrared Imaging System (Li-cor Biosciences, Lincoln, USA).

2.2.2.3 Co-Immunoprecipitation with Ab-Crosslinking:

For Co-Immunoprecipitation with Ab-Crosslinking Dynabeads (Invitrogen, Groningen, The Netherlands)) were incubated with the Ab over night at 4°C. After washing with 0,2M Na-Borate (pH 9,0), the beads were crosslinked with the antibody using Dimethyl pimelimidate dihydrochloride (for solution preparation please refer to 2.1.3). Washing with 250 mM Tris and preelution using 0,1 M Glycine (pH 2.0); was followed by incubation of the cell lysate over night (IP lysis buffer as described in 2.1.3) at 4°C. After washing with IP lysis buffer, elution was performed using 0,1 M Glycine (pH 2.0).

2.2.3 Cell culture techniques

A673 human cell line was routinely grown in DMEM, high glucose (4,5g/l), GlutaMAX^{Tm}-I medium (Invitrogen, Groningen, The Netherlands) supplemented with 10% fetal calf serum (FCS Gold, PAA Laboratories, Linz, Austria), 100.000 U/l penicillin/streptomycin (PAA Laboratories, Linz, Austria) in 5% CO_2 at 37°C.

2.2.3.1 Transfection

Cells were seeded in a 75cm² flask to 50-80% confluency one day before transfection. DMEM medium was removed and cells were incubated in Opti-MEM I at least one hour prior to transfection. Transfection was performed with Lipofectamine and Plus reagent (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions in serum-free OptiMEM I medium (Invitrogen, Groningen, The Netherlands) using at most 4µg DNA. After incubation for 4 hours the serum-free medium was replaced by supplemented DMEM medium.

2.2.3.2 Transfection for gene reporter assays

Cells were seeded in a 24well plate to 20-30% confluency one day before transfection. DMEM medium was removed and cells were incubated in Opti-MEM I at least one hour prior to transfection. Transfection was performed with Lipofectamine and Plus reagent (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions in serum-free OptiMEM I medium (Invitrogen, Groningen, The Netherlands) using 50ng of the reporter gene plasmid, 50ng of pmaxGFP and 150ng shRNA if used. After incubation for 4 hours, the serum-free medium was replaced by supplemented DMEM medium. If applied, 1µg/ml doxycycline was added 48h after transfection.

2.2.3.3 Reporter Gene Assays

Gene repoter assays were performed 96h after transfection if doxycycline was applied, otherwise 48h after transfection. Reporter gene assays were performed using the Bright-Glo™ Luciferase Assay System (Promega, Madison, USA) according to the manufacturer's instructions.

2.2.3.4 Cell Cycle Assay

Cell Cycle analysis was performed using the Cycletest™ Plus DNA Reagent Kit (Becton Dickinson, New Jersey, USA) according to manufacturers instructions.

2.2.3.5 Double thymidine block

Complete DMEM medium with 2mM thymidine was added to the cells for 16h, then the cells were washed twice with DMEM and incubated in normal DMEM for 8h,

followed by a second thymidine block for 16h. Cells were harvested and subjected to either cell cycle assay or ChIP.

2.2.4 Bioinformatic Methods

2.2.4.1 Gene Expression Analysis

Microarray analysis. RNA was extracted at five timepoints, 0h, 18h, 36h, 53h, 72h, and subjected to microarray analysis where 0h marks the timepoint when doxycycline was added, and 18h the time point when modulation of EWS/FLI1 protein was first observed as determined by immunoblot analysis. RNA was hybridized to Affymetrix HGU-133-A2 arrays (Affymetrix, Inc., Santa Clara, CA). cRNA target synthesis and GeneChip® processing were performed according to standard protocols (Affymetrix, Inc., Santa Clara, CA). Processing of CEL files, normalizing and filtering was done in R statistical environment using Bioconductor packages [99]. Microarray data from the knockdown analysis was submitted to GEO – accession number: GSE27524. Each time-point was replicated at least twice.

Affymetrix CEL files were read into R statistical environment and normalized using the “gcrma” algorithm [100]. Probesets with very low expression values across all samples (R package “panp”) were filtered out. Subsequently, probesets associated with the same gene identifier were averaged and merged to one symbol, yielding 12928 unique genes. Principal component analysis was performed using the GNU scientific library Singular Value Decomposition routines. Pearson correlation coefficients with $|r| > 0.8$ of comparing individual genes with the first three principal components were used to identify significantly correlated genes.

2.2.4.2 Sequence Data Analysis

Sequence reads were mapped to the human reference genome (NCBIv36, Hg18) using Illumina’s extended Eland alignment program. Reads starting at identical positions, as well as low quality reads with more than two deviations from the reference or an alignment score less than 25 were removed from the resulting datasets. Local read densities were then estimated by counting coverage of read-events for each nucleotide in the genome, where the oriented reads were extended to the insert length (100bp), which was size-selected during library preparation.

P-values were used to identify significantly increased read densities. They were estimated based on the cumulative Poisson distribution, where the local emission coefficient $\lambda(x)$ was estimated from input (non-IP) data using the average read densities of windows centered around *x* of sizes 1bp, 100bp, 1000bp, respectively. Of those, the most conservative (largest) estimate max $\lambda_i(x)$ was used in order to minimize the false discovery rate. Discrete enriched regions were identified using the following heuristic: a *continuous* stretch of DNA was called significantly enriched if the following conditions were met simultaneously: $p < 10^{-9}$ anywhere within that region, and $p <= 10^{-6}$ everywhere else. Subsequently, distinct significant regions were merged into a single region if they were less than ½ fragment size (50bp) apart. Finally, regions determined in this way smaller than the median fragment length (100bp) were rejected.

The discrete regions of enrichment were analyzed for conservation by reporting the maximum phast-score (vertebrate, 44-way conservation scores downloaded from UCSC, www.genome.ucsc.edu) within the discrete regions of enriched read density.

2.2.4.3 Identification of putative EWS/FLI1, E2F3 regulatory target genes

Putative regulatory targets of EWS-FLI1 and E2F3 binding were identified based on the closest gene heuristic, this is, for each binding site, the closest RefSeq transcript with a unique Entrez gene identifier was identified. In cases where multiple RefSeq transcripts mapped to the same Entrez gene identifier, the longest transcript was selected. Gene ontology analysis of the genes was made with custom in-house software. Pathway analysis was done using DAVID [101].

2.2.4.4 DNA Motif Analysis

Coordinates of sequences similar to known transcription factor binding site motifs were identified using a matrix based approach [102]. The frequency of known motifs (Transfac 11.4 database) present in regions of enriched read density was counted and compared to their respective frequency in (a) the entire genome or (b) regions selected randomly from the genome. For (b), sequencing data for non-selected (input) DNA was used to generate the random location distribution. P-values for over-representation were derived using (for a) Fisher's exact test or (for b) by counting the

number of random iterations, where the frequency of a given motif in the random set was larger or equal to the frequency observed in the ChIP-seq dataset.

Co-enrichment of other transcription factors around a given motif was estimated by enlarging a hit for that motif up- and downstream +/-100 bases, counting the number of co-occurrences and comparing this number to their respective frequency in the entire genome, again using Fisher's Exact test to calculate P-values.

3 Results:

3.1 Physical confirmation of simultaneous binding of EWS-FLI1 and members of the E2F family

In one of our earlier studies, Kauer et al. reported that ETS and E2F binding sites significantly co-occur on promoters of EWS-FLI1 activated genes [11]. To validate this co-binding, chromatin immunoprecipitation followed by DNA sequence analysis (ChIP-seq) was performed in collaboration with the lab of Paul Meltzer at the Genetics Branch, National Cancer Institute, NIH, USA with antibodies specific for EWS-FLI1, E2F3 and E2F4 in the A673 Ewing Sarcoma cell-line. Our decision to study E2F3 rather than E2F1 is primarily based on their induction in mesenchymal stem cells after EWS-FLI1 transduction in our own experiments and from Riggi et al. [17]. While E2F1 was only slightly induced in our own data and not at all in Riggi's data, E2F3 was up regulated in both datasets. The basis for additionally analyzing E2F4 binding was the fact that E2F4 is the only E2F family member expressed in the presence and absence of EWS-FLI1. Since FLI1 is not expressed in Ewing Sarcoma cells a FLI1 antibody was used to precipitate EWS-FLI1.

Short read sequencing generated 27 million EWS/FLI1 high quality sequence tags which uniquely aligned to the human genome, 12.5 million E2F3 tags and 9.5 million E2F4 tags. In addition, non-selected input DNA (13.5 million aligned tags) was sequenced in order to compensate for potential local bias in tag density.

An analysis of tag densities identified 16386 EWS-FLI1, 4303 E2F3 and 1431 E2F4 discrete binding regions with significantly (up to 100-fold) increased read densities. Both EWS/FLI1 and E2F3 binding regions demonstrated a high level of conservation in a 44-way vertebrate comparison (Figure 9). More than 90% of the E2F3 and E2F4 binding regions had a maximum conservation score larger than 0.5, in 75% of regions the peak score within the region was at its maximum value 1. For EWS-FLI-1 the corresponding numbers were 78% with conservation score larger than 0.5 and almost 60% with score 1. EWS-FLI1 binding sites in close proximity to transcription start sites (<4kb) showed an even higher conservation (83% >0.5, 65% score=1) as compared to distant binding sites (>4kb) (66% >0.5, 45% score=1). These values are significantly higher in comparison to randomized regions, where only approximately 40% of regions reach a 0.5 peak conservation score, and 15% a score of 1.

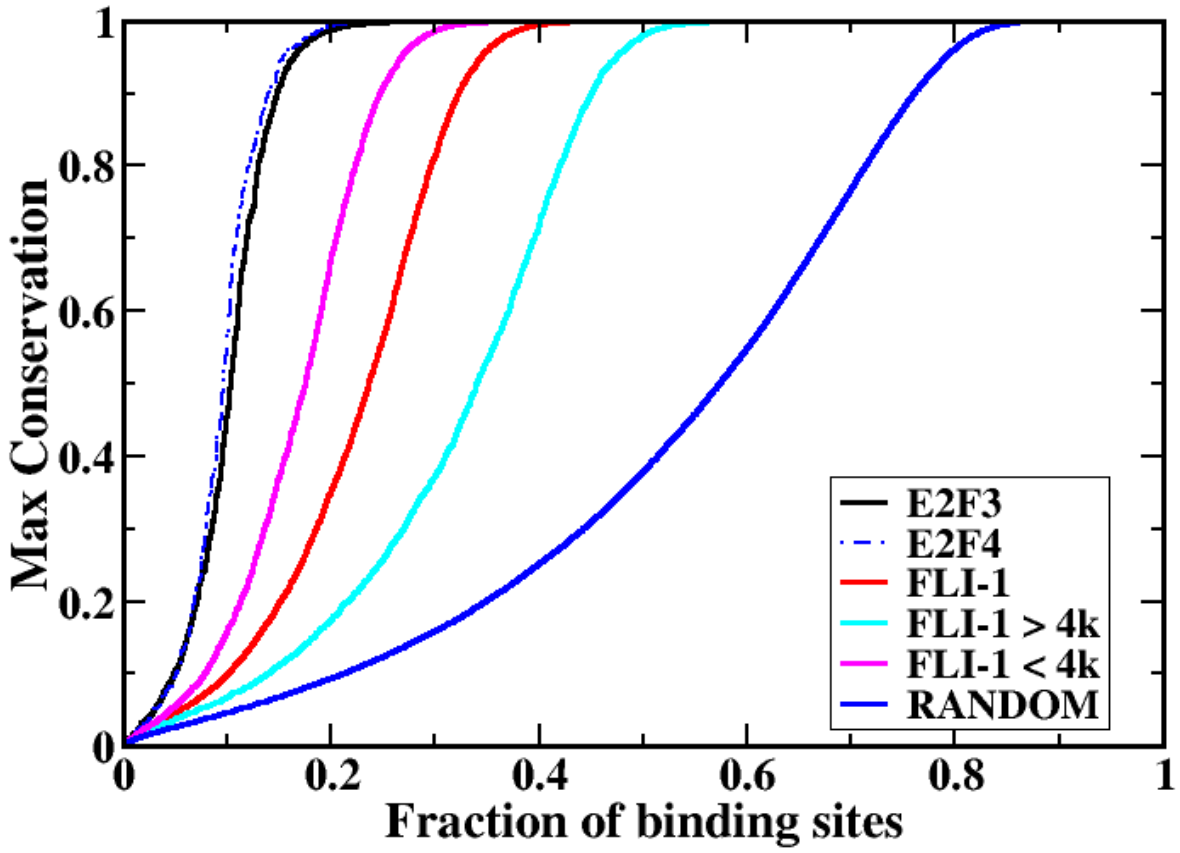

Figure 9: Genome wide characterization of the conservation score of binding sites. Fraction of binding regions with a maximal conservation smaller or equal to the value on the ordinate. E2F4 binding regions are most highly conserved, over 80% of all regions show the highest level of conservation. EWS-FLI-1 binding regions, too, show a high degree of conservation, regardless of whether they are located within proximal promoter regions or in distal enhancer regions. As a reference, the random curve estimates the behavior of unselected regions in the genome, it was obtained by randomizing the location, but not the shape, of EWS-FLI-1 binding regions using the density of read tags from the input lanes to model the location probability distribution.

Based on a “closest gene” heuristic, which associated each binding region to the closest gene regardless of distance, 3776, 1373 and 8204 unique gene identifiers were associated with E2F3, E2F4 and EWS-FLI1 binding sites, respectively.

Strikingly more than 50% of the E2F3 binding regions overlapped with those of EWS-FLI-1 (Figure 10), indicating that both factors frequently co-localize in the genome and, therefore, potentially interact in regulating their targets. The observed overlap was approximately 75-times more frequent than would have been attributable to mere chance when compared to a theoretical flat genomic background. 87% of E2F4 binding regions were also occupied by E2F3 and EWS-FLI1.

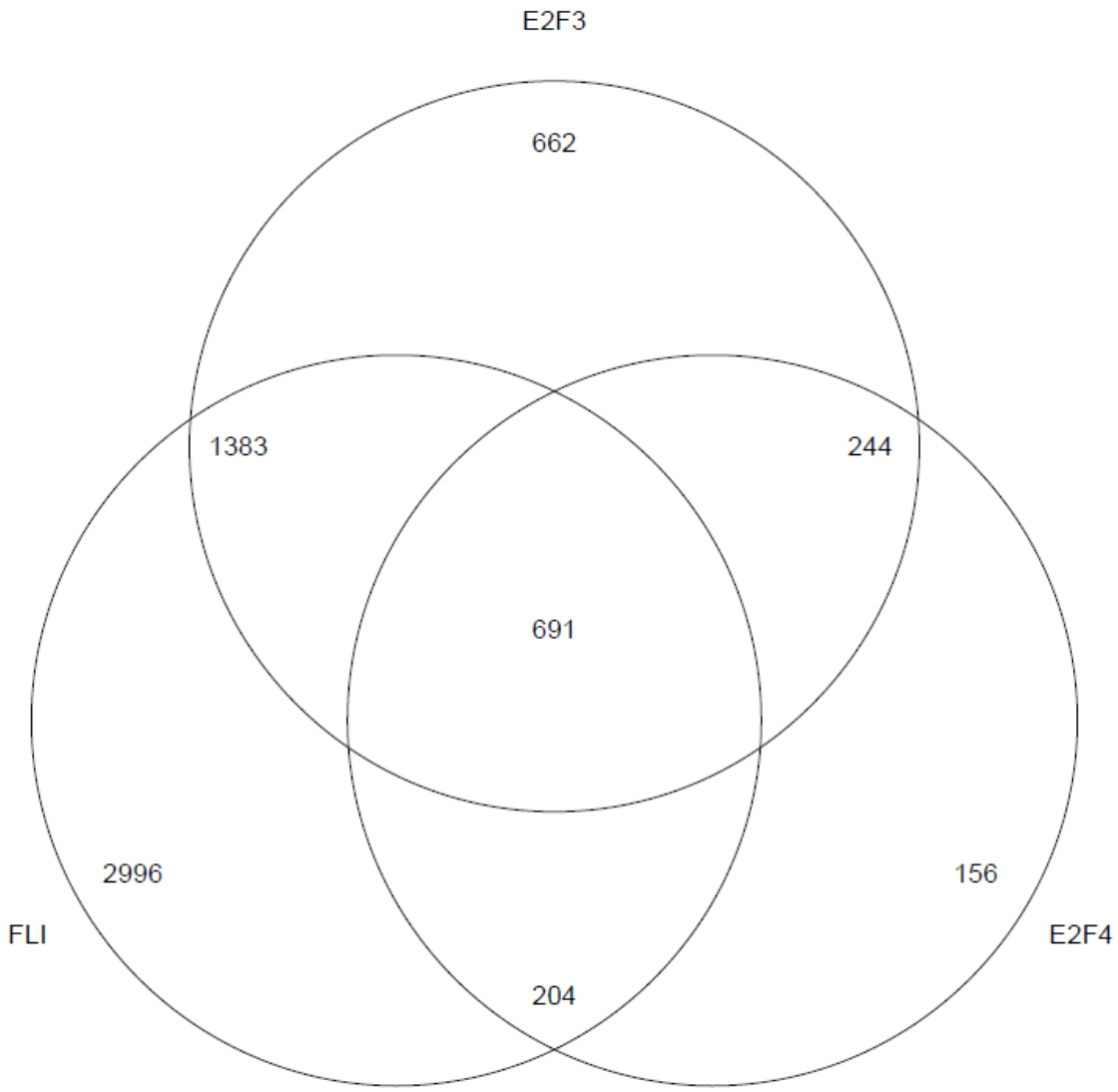

Figure 10: Venn diagram of E2F3, E2F4 and FLI1 ChIP-Seq hits in the promoter region (<1kb +- transcription start site) of target genes

Individually, E2F3 as well as EWS-FLI-1 had a substantial bias towards binding within promoter regions (Figure 11): E2F3 and EWS-FLI-1 binding regions were 50 fold (1419 of 4304) and 18 fold (2775 of the 16383 regions) more likely to be located within 1kb upstream of transcription start sites compared to an “average” 1kb region in the genome.

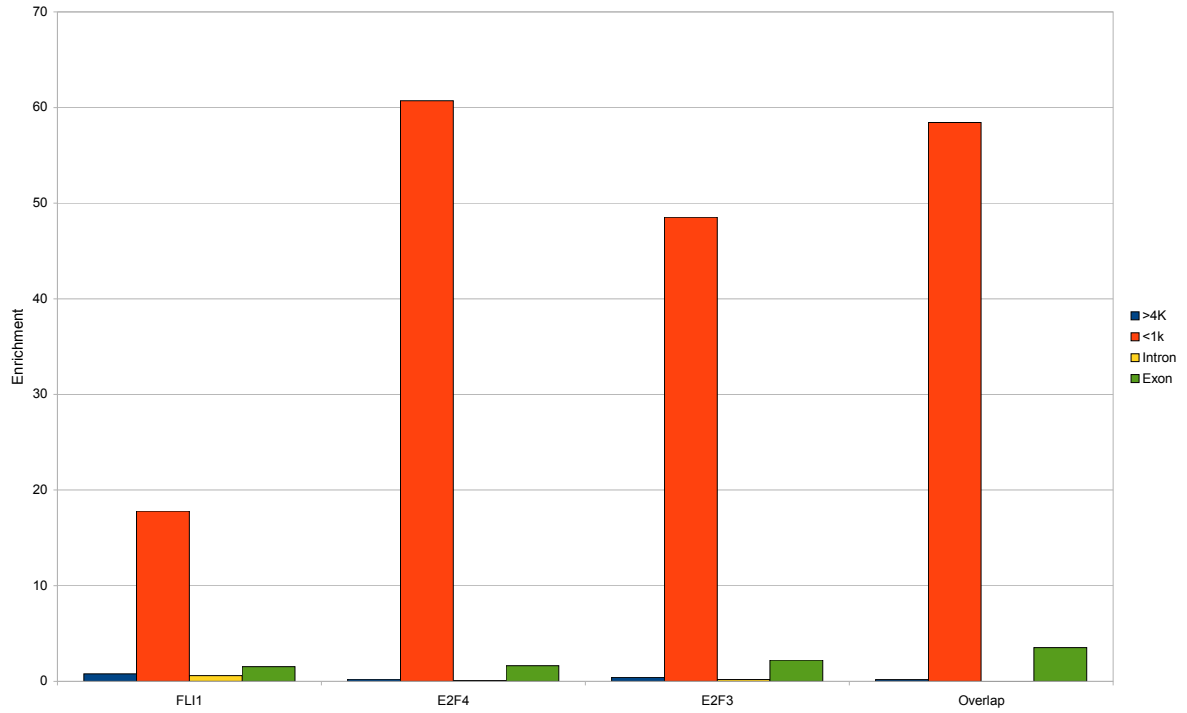

Figure 11: E2F3, E2F4 and EWS-FLI1 bind significantly to promoter regions. Enrichment of binding sites in the intergenic region (>4k), proximal promoter (<1k), intron and and exons.

In accordance with the observed co-localization in the ChIP-seq data, an *in silico* analysis of transcription factor site recognition matrices revealed a very strong cross-enrichment of E2F and ETS motifs [102]. Figure 12 shows the frequency of EWS-FLI1 and E2F binding relative to ETS binding motifs. E2F3, E2F4 (not depicted in Figure 12) and EWS-FLI1 preferentially bind to regions with ETS binding sites.

In addition, E2F motifs were 36-fold over-represented within EWS/FLI1 binding regions and ETS motifs were 60-fold over-represented within E2F3 binding regions. Simultaneous presence of the ETS motif with the E2F motif almost doubled the probability of observing E2F3 binding compared to promoters containing an E2F recognition motif only.

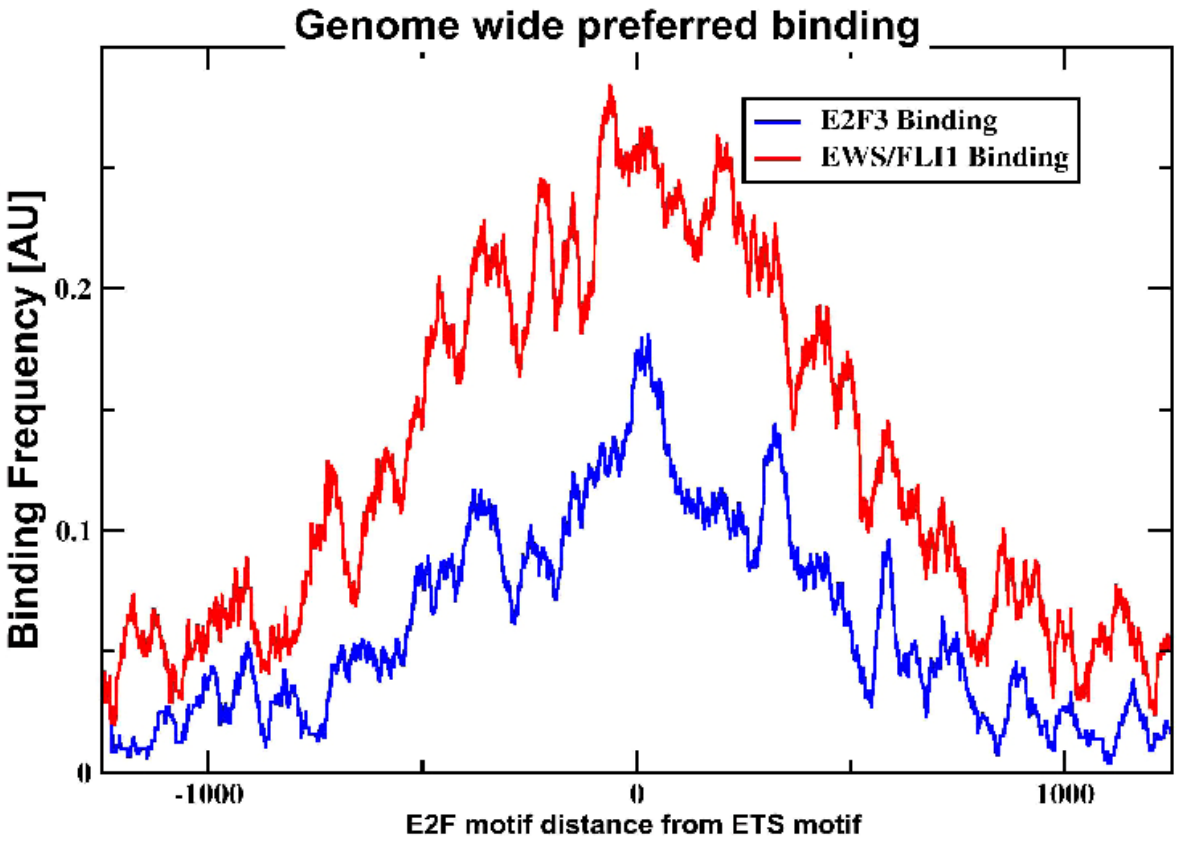

Figure 12: Genome wide preferred binding of E2F and EWS-FLI1 in regard to ETS binding motifs. The X-axis represents the E2F motif distance from the ETS motif in bps and the Y-axis represents the binding frequency of E2F3 and EWS/FLI1.

3.2 EWS-FLI1 gene expression profile shows a better overlap with the E2F3 than with the E2F1 signature

Gene expression profiles upon conditional EWS-FLI1 knockdown in A673 cells revealed that several E2F transcription factors belong to a class of genes early down regulated upon knock down of EWS-FLI1. Among the subgroup of activating E2F factors E2F1 and E2F3 were down regulated upon depletion of EWS-FLI1. Since a significant number of genes are co-operatively bound by EWS-FLI1 and E2F factors, we decided to analyse the overlap of the EWS-FLI1 signature with the signature of E2F1 and E2F3. We therefore performed knock downs of E2F1 and E2F3 in A673 cell lines and subjected the cells to gene expression profiling. Comparison of the gene-expression profile upon conditional EWS-FLI1 knockdown with data from E2F1 and E2F3 knockdowns in the same cell line revealed that 966 genes were down regulated upon EWS-FLI1 depletion (threshold -1), 1266 genes (threshold -0.7) were decreased upon knockdown of E2F3 and expression levels of 482 genes (threshold -0.7) declined after E2F1 knockdown. While EWS-FLI1 activated genes mainly

annotate to cell cycle genes, E2F3 annotate to angiogenesis related clusters and E2F1 to extracellular space. 50 genes are activated by both EWS-FLI1 and E2F3 and annotate to cell cycle genes. The overlap of EWS-FLI1 and E2F1 genes is only about half (24 genes) which are related to disulfide bonds and extracellular space. Although 224 genes are co-activated by the cell cycle factors E2F1 and E2F3, they are not annotated as cell cycle genes, but rather extracellular space and disulfide bonds. The data suggests, that E2F3 and EWS-FLI1 share a larger number of target genes compared to EWS-FLI1 and E2F1, and, even more important, EWS-FLI1 target genes and shared EWS-FLI1/E2F3 target genes both annotate to cell cycle genes. The fact that depletion of E2F3 or E2F1 did not result in significant changes in cell cycle associated genes, while EWS-FLI1 knock down, or combinatorial knock down of EWS-FL11 and E2F3 does, implies that EWS-FLI1 might be the major cell cycle regulator in Ewing Sarcoma.

3.3 Validation of target genes using gene reporter assays

The analysis so far indicated preferential co-localization of EWS-FLI1 and E2F3 on proximal promoters of activated genes, but regulatory activity of both factors *in vitro* remained to be shown. Therefore, promoter fragments of ten randomly selected genes with proximal EWS-FLI1, E2F3 and E2F4 binding regions were cloned into a luciferase reporter vector and tested for EWS/FLI1 dependent activity by firefly luciferase reporter assays in A673 cells. All ten constructs demonstrated a significant two- to three-fold reduction of the reporter activity 48 hours after conditional EWS-FLI1 knockdown, while the promoter of an expressed gene without any EWS-FLI1 or E2F ChIP-seq signal and the empty vector control did not respond to EWS-FLI1 modulation (Fig. 13).

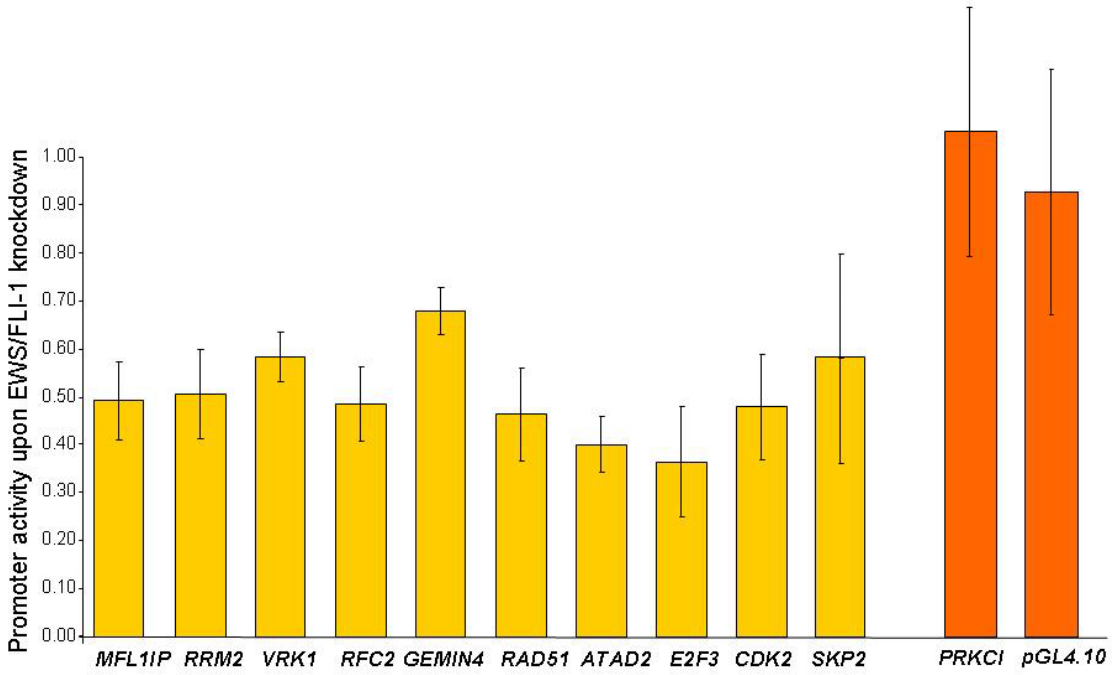

Figure 13: Firefly luciferase reporter assays for 10 arbitrarily chosen genes identified by ChIP-seq as EWS-FLI1 and E2F target genes. Promoter fragments (*CDK2*:-122/+458; *E2F3*: -272/+327; *RAD51*: -186/+164; *VRK1*: -269/+100; *RFC2*: -400/+25; *ATAD2*:-368/+202; *RRM2*: -463/+191; *GEMIN4*: -275/+87; *MFLI1P*: -251/+70, *SKIP2*: -240/348) were cloned into the pGL4.10 vector (Promega) and tested for responsiveness to conditional EWS-FLI1 knockdown in A673 Ewing Sarcoma cells 48h after doxycycline induced EWS-FLI1 shRNA induction. As negative controls, promoter activities of an expressed gene that does not show a change in mRNA expression after the EWS-FLI1 knockdown (*PRKCI:* -139/265), and of the empty vector (pGL4.10) are shown. The Y-axis represents the promoter activity relative to control conditions. Means and standard deviations of at least three independent experiments, each performed in triplicate, are shown.

3.4 Target gene selection

Of the ten target genes (mentioned in chapter 3.3) which show ChIP-seq hits for EWS-FLI1, E2F3 and E2F4 and are down regulated upon knockdown of EWS-FLI1, we arbitrarily chose *E2F3*, *RAD51, ATAD2* and *GEMIN4* to study the interplay of EWS-FLI1 with E2F3 and E2F4. The promoter regions of these genes harboring an overlapping ChIP-seq hit for E2F3, E2F4 and EWS-FLI1 were cloned in a firefly luciferase gene reporter construct. Transcription factor binding sites (TFBS) within these regions were identified using the ConSite algorithm [103]. *In silico* analysis confirmed the Chip-seq data. Since several TFBS were found, a conservation score higher than 85% was used as a cut off criteria for E2F binding sites. For ETS binding sites the CGGAAG motif was also required. TFBS fulfilling these criteria were

mutated individually and simultaneously. The resulting constructs were tested in gene reporter assays and analyzed for their promoter occupancy by EWS-FLI1, E2F3 and E2F4. For E2F3 see chapter 3.5, for RAD51 3.6, for GEMIN4 3.7 and for ATAD2 3.8.

E2F3 and RAD51 were studied in more detail to gain better insight into the regulatory mechanisms used by EWS-FLI1 and E2F factors.

3.5 E2F3 is a direct target of EWS-FLI1 and E2Fs

Previously published gene expression profiling data using the doxycycline inducible EWS-FLI1 shRNA cell line A673 before and after EWS-FLI1 knockdown, indicate that E2F3 is a target of EWS-FLI1 [11]. To further study the mechanisms of E2F3 promoter regulation by EWS-FLI1, the promoter region of E2F3 was cloned in a gene reporter construct. Gene reporter assays were performed in A673 cells before and after knockdown of EWS-FLI1. Knockdown of EWS-FLI1 or mutation of the upstream located 96% scoring ETS-binding site led to a reduction of luciferase activity by 62%. While the mutation of the downstream 89% scoring ETS-binding site led only to a 32% reduction in reporter activity, combinatorial mutation of both ETS-binding sites lead to a decrease of luciferase activity by 77% (Fig.14). Consequently we wanted to validate the direct binding of EWS-FLI1 to the E2F3 promoter using ChIP for EWS-FLI1 followed by PCR. Binding of EWS-FLI1 to both ETS binding sites is demonstrated in Fig. 15, which shows it to be weakened (for the upstream TFBS) or decreased (for the downstream TFBS) upon knockdown of EWS-FLI1. A third PCR which covers an upstream promoter region of E2F3 without E2F or ETS binding sites shows no binding. These results indicate a direct regulation of E2F3 via the binding of EWS-FLI1.

E2F3

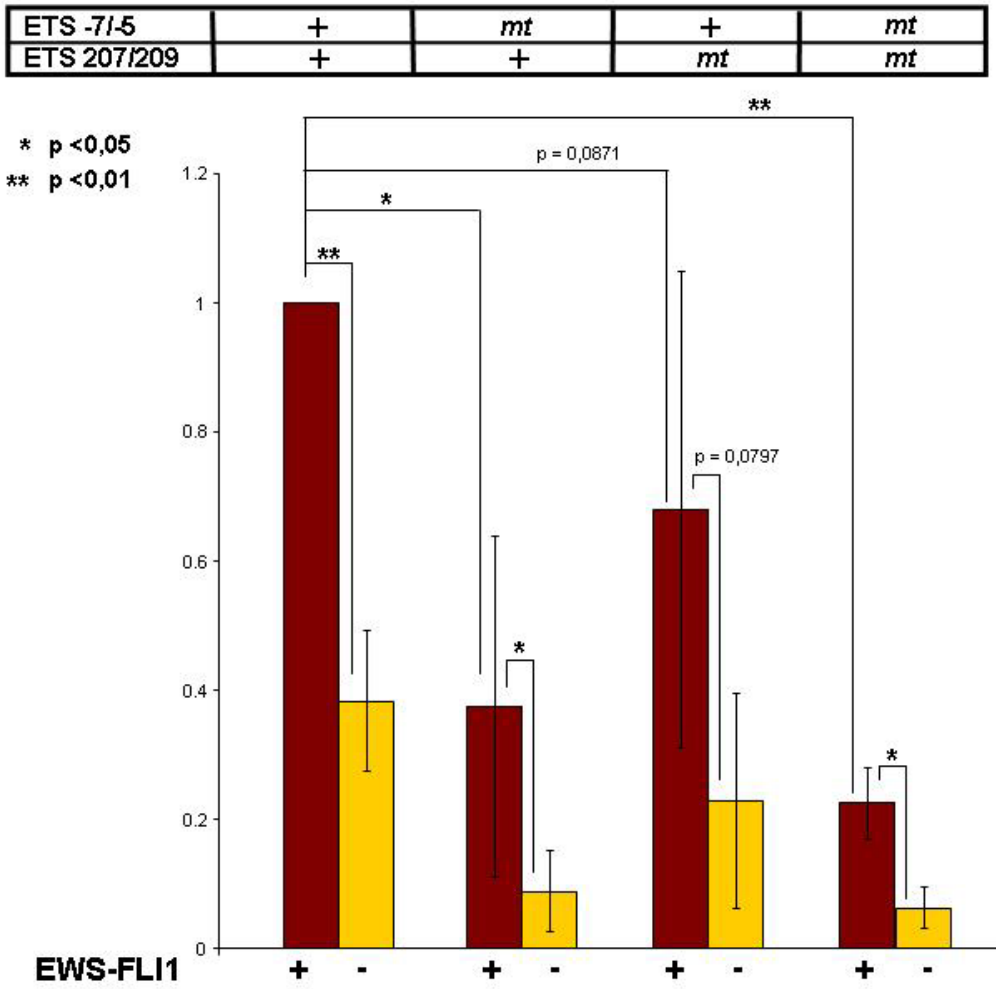

Figure 14: *E2F3* promoter study. Firefly luciferase reporter assays for E2F3 wildtype and mutant promoter construct. Promoter fragment (-272/+327) was cloned into the pGL4.10 vector (Promega) and tested for responsiveness to conditional EWS-FLI1 knockdown in A673 Ewing Sarcoma cells. ETS binding sites at -7/-5 and/or 207/209 were mutated. Fold changes in reporter activity of wild type and mutant E2F3 reporter constructs in the presence (+) and doxycycline-induced absence (-) of EWS/FLI1 48h after EWS/FLI1 shRNA induction. Y-axis presents promoter activity relative to untreated control conditions. Data represent means and standard deviations of at least three independent experiments, each performed in triplicate.

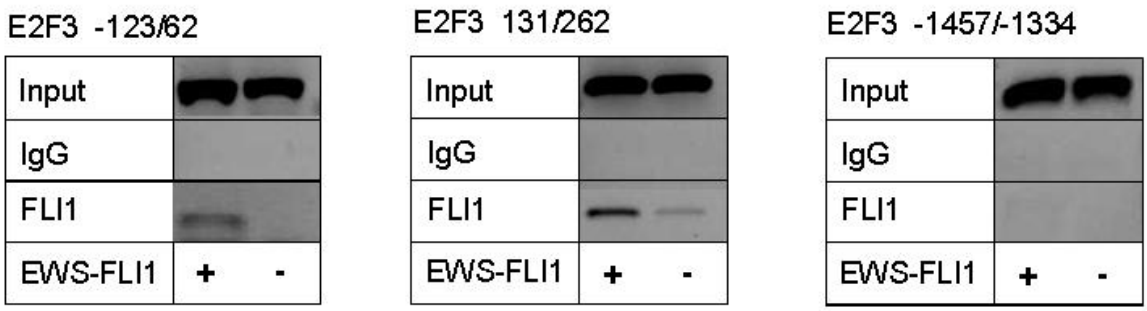

Figure 15: Occupancy of the *E2F3* promoter by EWS-FLI1. To monitor binding of EWS-FLI1 to the promoter region of *E2F3*, chromatin immunoprecipitation was performed in A673 Ewing Sarcoma cells. Cells were either left untreated (+) for control, or were treated for 48h with doxycycline to induce knockdown of EWS-FLI1 (-). Two regions containing putative ETS binding sites (-123/62 and 131/262) and a region 1.4kb upstream of the TSS (-1457/-1334) not containing E2F or ETS binding sites were amplified.

3.6 Combinatorial binding of EWS-FLI1 and E2F is necessary for RAD51 promoter regulation.

As previously mentioned RAD51 was chosen for in depth promoter studies (see chapter 3.4). Therefore TFBS were analyzed and revealed a E2F binding site with a conservation score of 92% near the transcription start site (+5) and two ETS binding sites. The upstream ETS binding site had a conservation score of 92% and the downstream ETS TFBS of 86%. Gene reporter studies with wildtype and mutation of the ETS and E2F binding sites within the *RAD51* promoter were performed in A673 cells before and after knockdown of EWS-FLI1 (Fig. 16A). Depletion of EWS-FLI1 led to a reduction of promoter activity by 55%. While the single mutation of the ETS binding sites led to no or only slight reduction, the combinatorial mutation of both ETS binding sites resulted in a 45% reduction of transcriptional activity, indicating that both ETS binding sites are equally able to bind EWS-FLI1 and keep *RAD51* active. However, mutation of the single E2F binding site resulted in similar luciferase activity as the wildtype construct, even in the absence of EWS-FLI1. Consequently, binding of a member of the E2F family is necessary for the regulation of *RAD51* by EWS-FLI1 and is therefore dependent on both EWS-FLI1 and E2F. Furthermore, the triple mutation of both ETS binding sites and the E2F binding sites showed no regulation in the presence and absence of EWS-FLI1 compared to the wildtype construct.

Also we wanted to confirm the direct binding of EWS-FLI1 to the *RAD51* promoter by ChIP. Figure 16B shows ChIP-PCRs for both ETS binding sites, the upstream TFBS

is bound by EWS-FLI1 in the presence of EWS-FLI1, but not in its absence. The downstream ETS binding site is bound in both conditions but significantly reduced in the EWS-FLI1 knockdown condition. The control PCR covering a *RAD51* promoter fragment 4kb upstream of the transcription start site shows no binding of EWS-FLI1. As a consequence of these findings we suggest that EWS-FLI1 is directly regulating *RAD51*, but needs assistance from E2F family members.

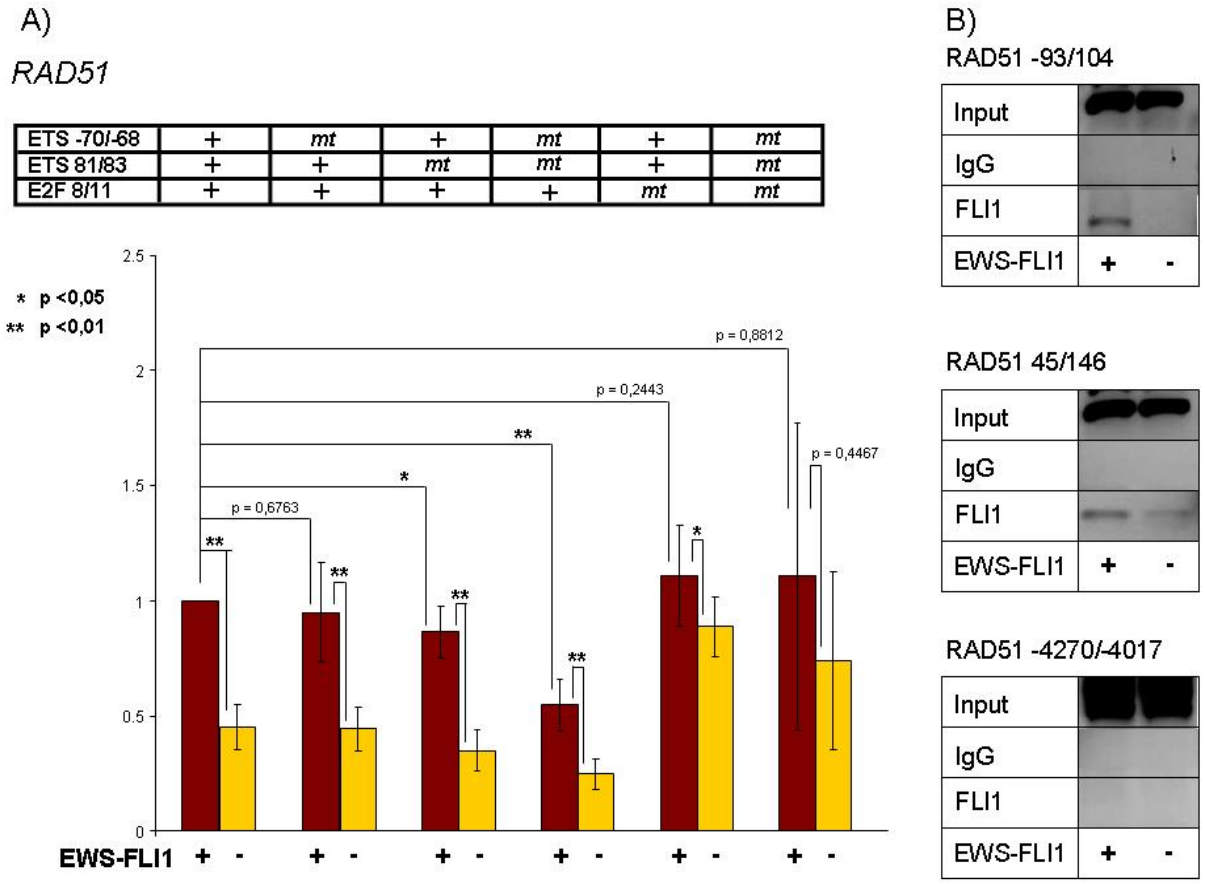

Figure 16: *RAD51* promoter study. A) Fold changes in reporter activity of wildtype and mutant RAD51 reporter constructs in the presence (+) and doxycycline-induced absence (-) of EWS/FLI1 48h after EWS/FLI1 shRNA induction. ETS binding sites in the *RAD51* promoter at -70/-68 and 81/83 and one E2F binding site in the *RAD51* promoter at 8/11 were mutated. Y-axis presents promoter activity relative to untreated control conditions. Data represent means and standard deviations of at least three independent experiments, each performed in triplicate. B) To monitor binding of EWS-FLI1 to the promoter region of *RAD51*, chromatin immunoprecipitation was performed in A673 Ewing Sarcoma cells. Cells were either left untreated (+) for control, or were treated for 48h with doxycycline to induce knockdown of EWS-FLI1 (-). Two regions containing putative ETS binding sites (-93/104 and 45/146) and a region 4kb upstream of the TSS (-4270/-4017) not containing E2F or ETS binding sites were amplified

3.7 GEMIN4 promoter regulation by E2F and EWS-FLI1

For *GEMIN4,* individual mutations of each of two ETS binding sites (at -176 and at -92), and of one of two putative E2F binding motifs (at -70) only insignificantly (p= 0,1068, p=0,4017 and p=0,065) reduced basal promoter activity in A673 cells. In contrast, ablation of the second E2F motif at +55, and combinatorial mutation of both ETS sites or both E2F sites significantly reduced *GEMIN4* promoter activity to between 50-60% (p<0,01 and p<0,05). In all instances, the modulatory response to EWS/FLI1 knockdown lost significance, except for mutation of the ETS site at -92 (Fig. 17). These results are compatible with both ETS and E2F sites being involved in *GEMIN4* transcriptional regulation, and confirm the dependence of promoter response to EWS/FLI1 modulation on the loss of availability and binding of E2F3.

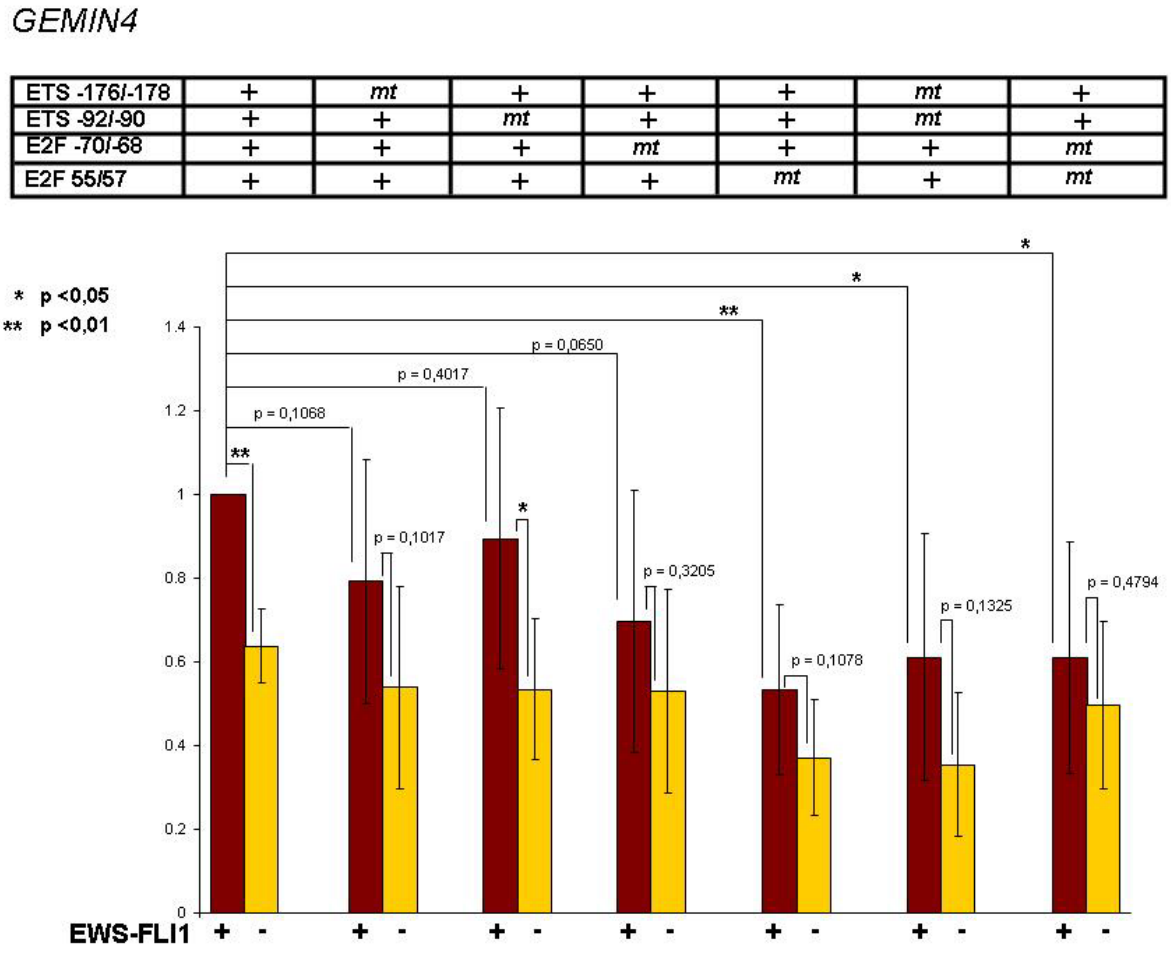

Figure 17: *GEMIN4* promoter study. Fold changes in reporter activity of wildtype and mutant GEMIN4 reporter constructs in the presence (+) and doxycycline-induced absence (-) of EWS/FLI1 48h after EWS/FLI1 shRNA induction. ETS binding sites in the *GEMIN4* promoter at -176/-178 and -92/-90 and E2F binding sites at -70/-68 and 55/57 were mutated. Y-axis represents promoter activity

relative to untreated control conditions. Data represent means and standard deviations of at least three independent experiments, each performed in triplicate.

To verify the ChIP-seq results for EWS-FLI1 and E2F3, standard ChIP followed by PCR was performed for the *GEMIN4* promoter. Reduction of promoter occupancy by EWS-FLI1 and E2F3 upon knockdown of EWS-FLI1 was demonstrated. A control region where both EWS-FLI1 and E2F3 should not bind shows no PCR product (Fig. 18).

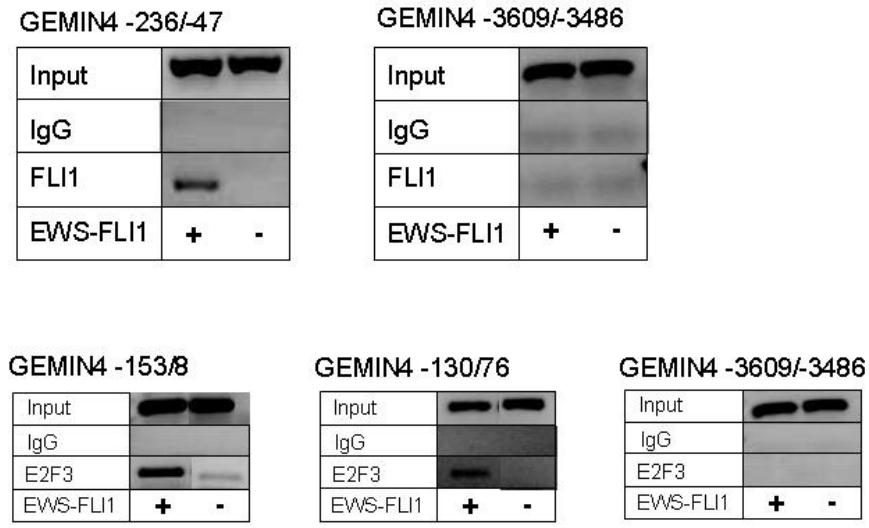

Figure 18: Occupancy of the*GEMIN4* promoter by EWS-FLI1 and E2F3. To monitor binding of EWS-FLI1 and E2F3 to the promoter region of *GEMIN4*, chromatin immunoprecipitation was performed in A673 Ewing Sarcoma cells. Cells were either left untreated (+) for control, or were treated for 48h with doxycycline to induce knockdown of EWS-FLI1 (-). Two regions containing putative E2F (-153/8 and -130/76) binding sites, one region containing putative ETS binding sites (-136/-47) and a region 4kb upstream of the TSS neither containing E2F or ETS binding sites (-3609/-3486) were amplified.

3.8 Regulation of ATAD2 is dependent on EWS-FLI1 and E2F

For *ATAD2*, individual mutation of either the single ETS core motif at position +33 or a highly conserved E2F site at -267 reduced the response by approximately 50%, while perturbation of a second E2F core motif at +14 lowered the intensity only by about 25% (Fig. 19). In all single mutation instances, the promoter fragments retained their responsiveness to EWS-FLI1 knockdown. In contrast, the triple mutation disrupting ETS and E2F sites together lowered promoter activity in the presence of EWS-FLI1 to a level similar to that observed after EWS-FLI1 knockdown in the wildtype construct, thus rendering the *ATAD2* promoter completely unresponsive to

EWS/FLI1 modulation. This result suggests that both ETS and E2F motifs contribute to *ATAD2* promoter regulation by EWS/FLI1.

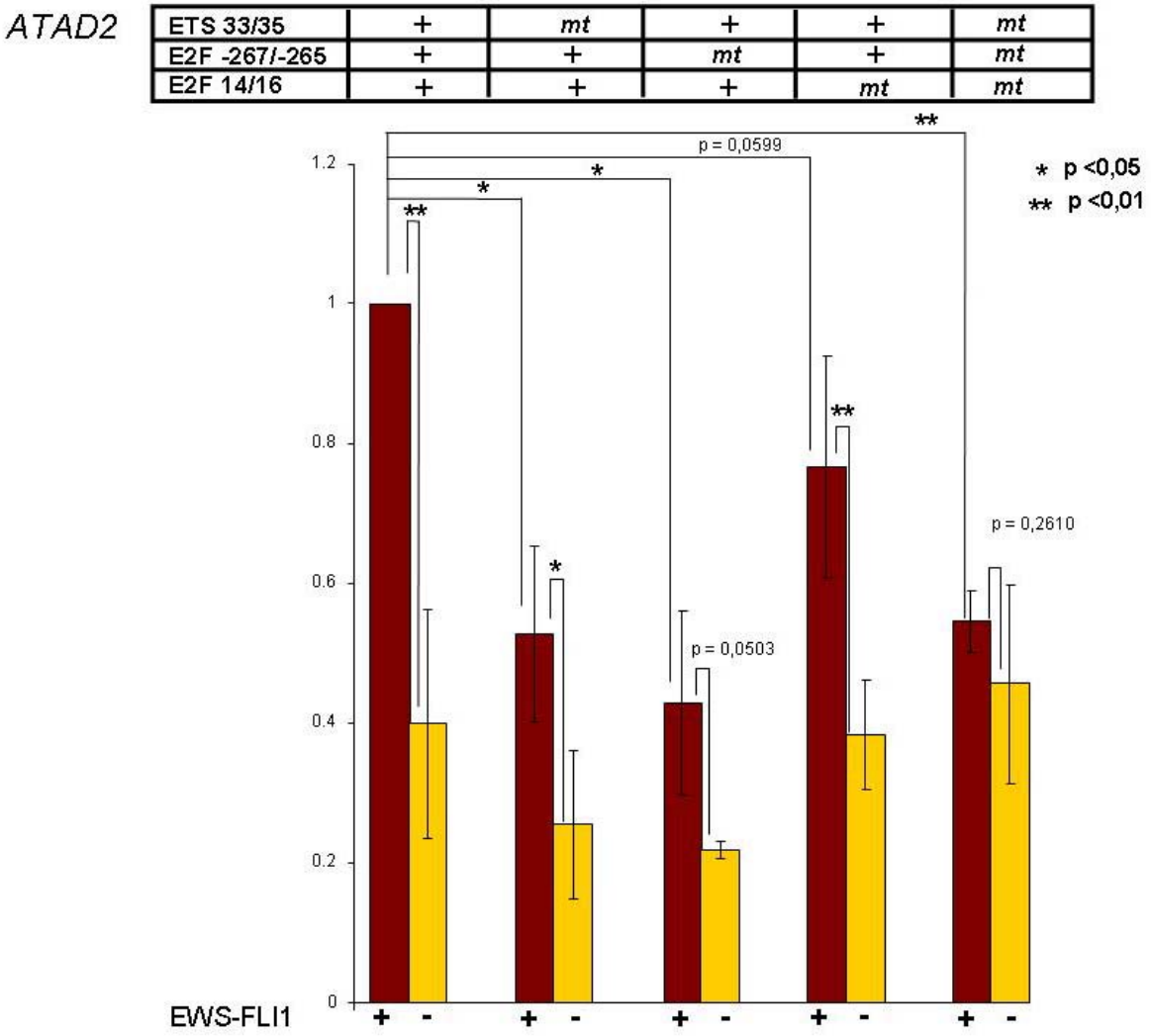

Figure 19: *ATAD2* promoter study. Fold changes in reporter activity of wildtype and mutant ATAD2 reporter constructs in the presence (+) and doxycycline-induced absence (-) of EWS/FLI1 48h after EWS/FLI1 shRNA induction. Y-axis presents promoter activity relative to untreated control conditions. ETS binding site in the *ATAD2* promoter at 33/35 and 81 E2F binding sites at -267/-265 were mutated. Data represent means and standard deviations of at least three independent experiments, each performed in triplicate.

To verify the ChIP-seq results for EWS-FLI1 and E2F3, standard ChIP followed by PCR was performed for the *ATAD2* promoter. Reduction of EWS-FLI1 and E2F3 binding upon knockdown of EWS-FLI1 was demonstrated. A control region where both EWS-FLI1 and E2F3 should not bind shows no PCR product (Fig. 20).

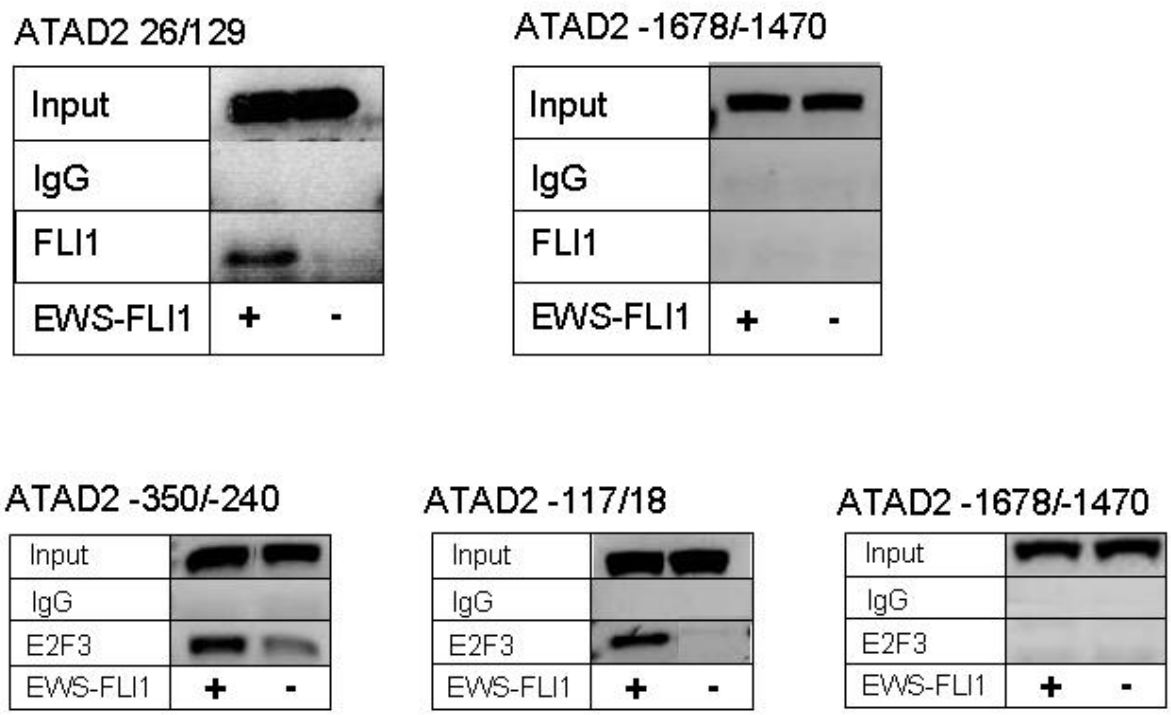

Figure 20: Occupancy of the *ATAD2* promoter by EWS-FLI1 and E2F3. To monitor binding of EWS-FLI1 and E2F3 to the promoter region of ATAD2, chromatin immunoprecipitation was performed in A673 Ewing Sarcoma cells. Cells were either left untreated (+) for control, or were treated for 48h with doxycycline to induce knockdown of EWS-FLI1 (-). Two regions containing putative E2F binding sites (-350/-240 and -117/18), one region containing putative ETS binding sites (26/129) and a region 4kb upstream of the TSS (-1678/-1470) not containing E2F or ETS binding sites were amplified.

3.9 E2F3 is exchanged for E2F4 after knockdown of EWS-FLI1

According to the before mentioned ChIP-seq data, the activating E2F factor E2F3 and the repressive E2F factor E2F4 bind to the same promoters of approximately 140 genes. Because of their supposed counteracting effects we wanted to investigate why they are jointly binding the same promoters. Therefore, we performed ChIPs for E2F3 and E2F4 before and at different time points after the doxycycline induced knockdown of EWS-FLI1 in A673 cells and exemplarily investigated the occupancy of the *E2F3* and *RAD51* promoters.

ChIP assays revealed strong binding of E2F3 and a weak occupancy of the *E2F3* promoter by E2F4 in the presence of EWS-FLI1. With increasing time and reduction of EWS-FLI1, the occupancy of the *E2F3* promoter by E2F3 itself decreased while E2F4 increased. While this exchange of E2F3 for E2F4 on the *E2F3* promoter happened gradually over time with a complete exchange 48h after knockdown of EWS-FLI1, the occupancy of the 5'UTR of *E2F3* by E2F4 vanished at 48h after

knockdown of EWS-FLI1 (Fig. 21A). The binding pattern of E2F3 and E2F4 on the *RAD51* promoter resembled the *E2F3* promoter occupancy (Fig. 21B) except that E2F3 binding decreased to a higher degree earlier in the time course of EWS-FLI1 knockdown, while E2F4 increased at a later time point.

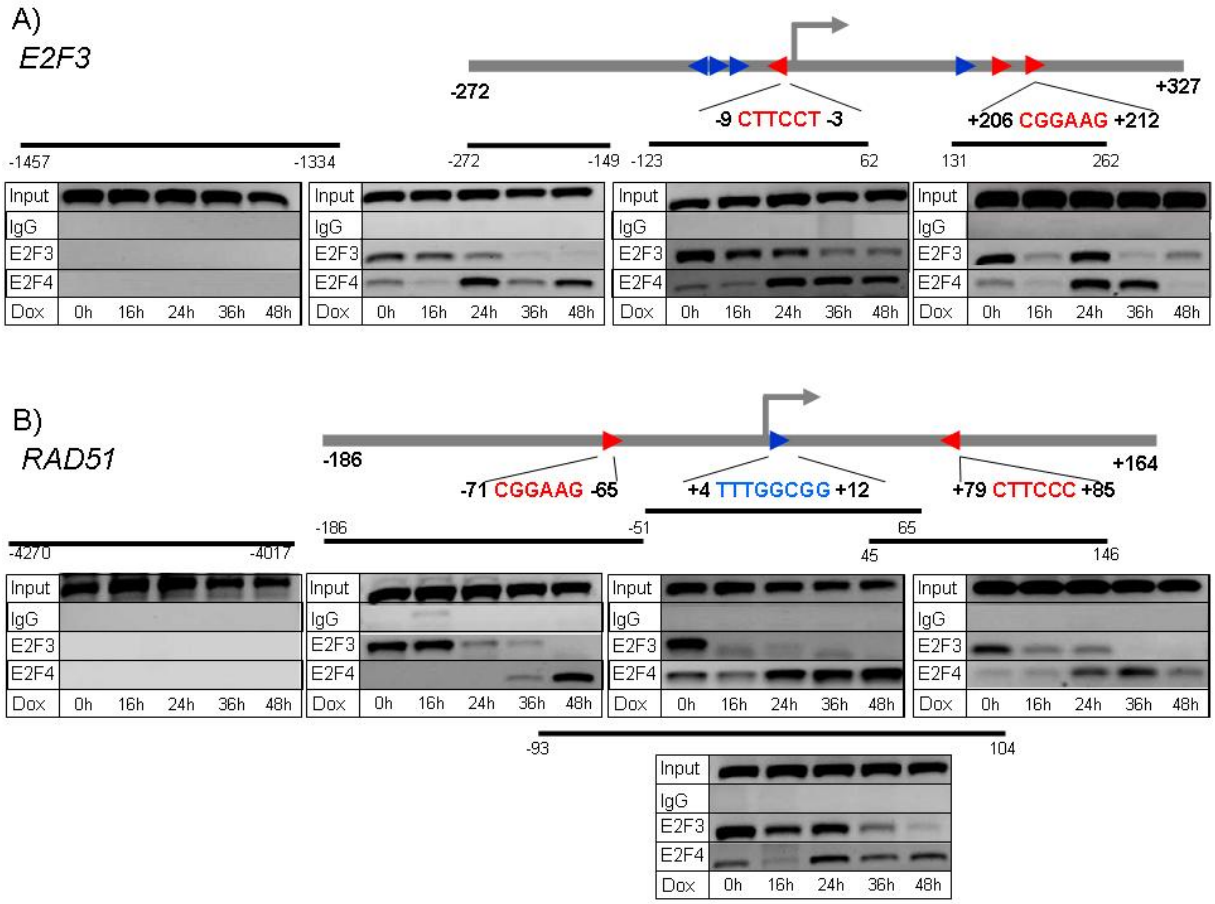

Figure 21: Occupancy of the *E2F3* and *RAD51* promoters by E2F3 and E2F4. Chromatin Immunoprecipitations were performed in A673 cells before and at several time points after induction of EWS-FLI1 knockdown. A) Three overlapping regions of the *E2F3* promoter containing E2F and ETS binding sites (-272/-149, -123/62 and 131/262) and a region 1.4kb upstream of the TSS (-1457/-1334) not containing E2F or ETS binding sites were amplified. B) Four overlapping regions of the *RAD51* promoter containing E2F and ETS binding sites (-186/-51, -93/104, -51/65 and 45/146) and a region 4kb upstream of the TSS (-4270/-4017) not containing E2F or ETS binding sites were amplified. Red arrows indicate an ETS binding site, blue arrows an E2F binding site.

3.9.1 The E2F3/E2F4 exchange is independent of cell cycle

Considering the observations of Hu et al. [77] that knockdown of EWS-FLI1 modulates cell cycle, thereby inducing cell cycle arrest, and those of Takashi et al. who demonstrated a cell cycle dependent occupancy of several promoters by E2F4 [59], we wanted to examine whether the replacement of E2F3 for E2F4 might be due

to the G1 arrest induced by EWS-FLI1 knock down. Cell cycle analysis before and at several time points after knockdown of EWS-FLI1 in A673 cells revealed increasing G1 arrest over time, starting with 47% and resulting in a maximum of 85% cells in G1 arrest after 48h (Fig. 22). To analyze E2F3 binding in the presence of EWS-FLI1 but in a comparable G1 arrest situation like the absence of EWS-FLI1 we induced G1 arrest with two different methods resulting in a 65% G1 arrest using a double thymidine block approach and a 77% G1 arrest by transfection of p57, which inhibits all G1/S-phase cyclin-CDK complexes [104]. ChIP experiments were performed using an antibody against E2F3 in untreated control cells, in cells with double thymidine block, and in cells transfected with p57. Both cell cycle inhibiting treatments had no influence on the occupancy of the *E2F3* and *RAD51* promoter by E2F3, indicating that the observed exchange is not due to cell cycle arrest but dependent on EWS-FLI1 (Fig 23).

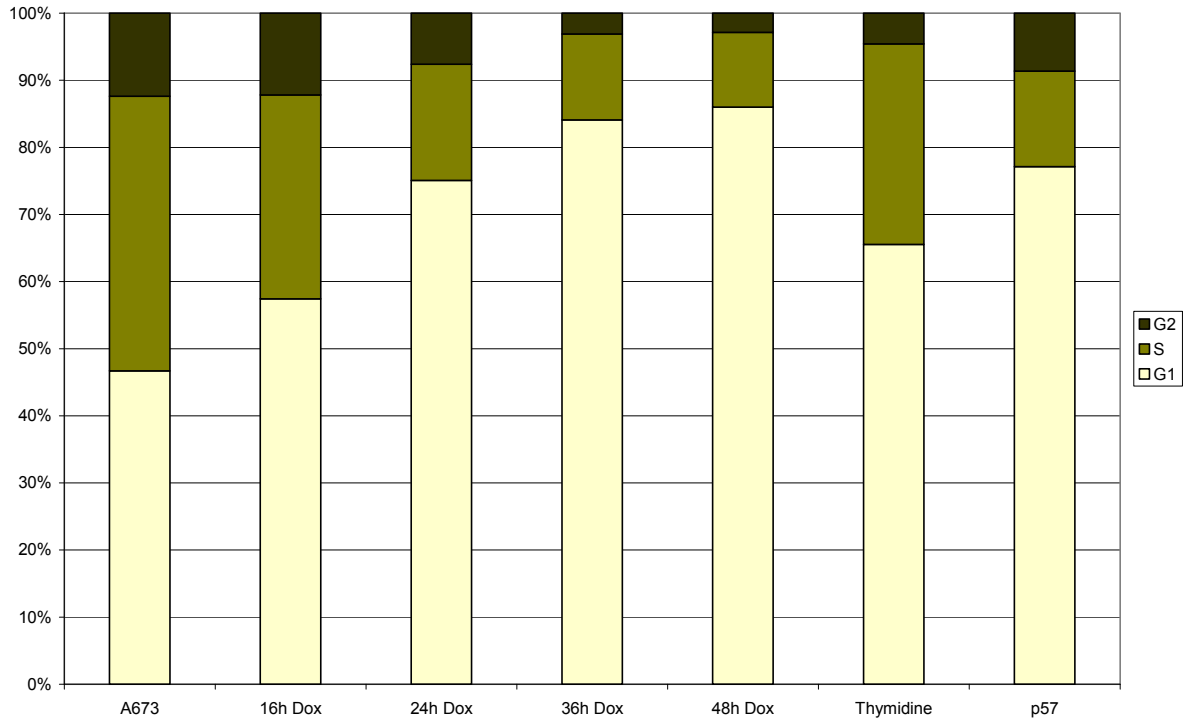

Figure 22: Cell cycle analysis. A673 cells were either left untreated, treated with doxycycline for 16h, 24h, 36h or 48h, treated with thymidine or transfected with p57. The diagram shows a representative experiment.

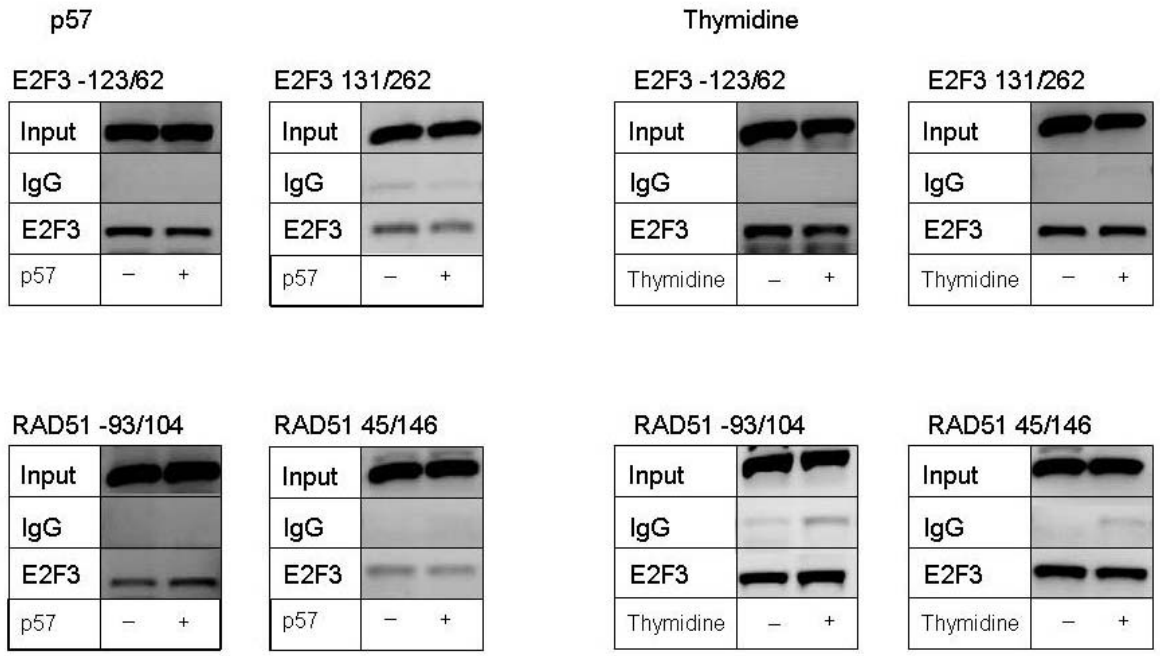

Figure 23: Occupancy of the *E2F3* and *RAD51* promoter by E2F3 before and after cell cycle arrest induction. To monitor binding of E2F3 to the promoter region of E2F3 and RAD51 with and without induction of a G1 arrest, chromatin immunoprecipitation was performed in A673 Ewing Sarcoma cells. Cells were either left untreated (-) for control, or were treated with thymidine or transfected with a p57 expression plasmid (+). Two regions for both E2F3 and RAD51 containing putative E2F binding sites were amplified.

To further test functional consequences of a thymidine induced cell cycle arrest, we performed gene reporter assays with *RAD51* and *E2F3* promoter constructs. Figure 24 shows that treatment of cells with thymidine and subsequent cell cycle arrest had no effect on the luciferase activity of all tested promoter constructs compared to untreated controls.

Taken together these results suggest that not only the exchange of E2F3 for E2F4 is cell cycle independent, but also the transcriptional activity of the E2F3 and RAD51 promoter is not changing upon induction of a G1 arrest.

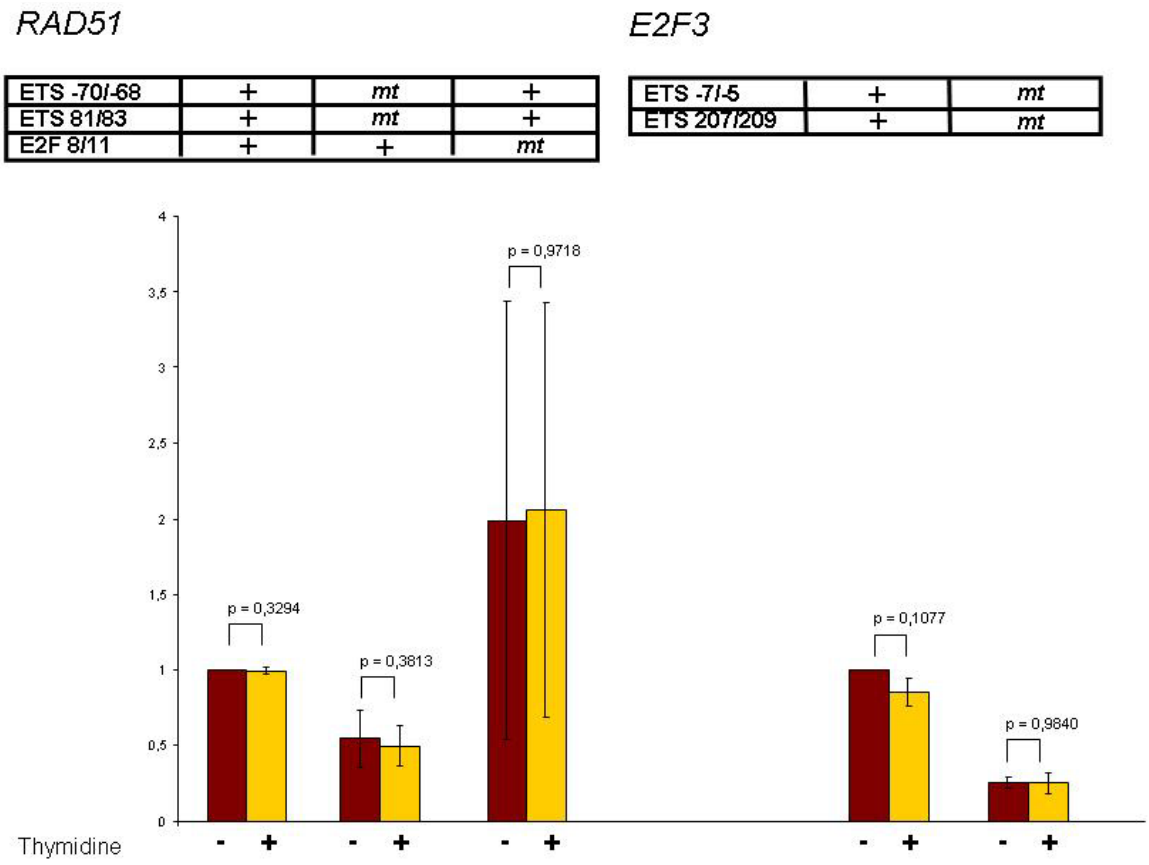

Figure 24: *E2F3* and *RAD51* promoter construct activity is not changing upon G1 arrest induction. Firefly luciferase reporter assays for *RAD51* and *E2F3* wildtype and mutant promoter constructs. Fold changes in reporter activity of wild type and mutant *RAD51* and *E2F3* reporter constructs in the untreated control (-) and Thymidine treated (+) A673 cells. ETS binding sites in the *RAD51* promoter at -70/-68 and 81/83 and in the *E2F3* promoter at -7/-5 and 207/209 and one E2F binding site in the *RAD51* promoter at 8/11 were mutated. Y-axis presents promoter activity relative to untreated control conditions. Data represent means and standard deviations of at least three independent experiments, each performed in triplicate.

3.10 EWS-FLI1 is the main cell cycle regulator

It was previously described that knockdown of EWS-FLI1 induces a strong cell cycle arrest. Based on these data and the fact that E2F1 and E2F3 are known to promote cell cycle progression from G1 to S phase, we were interested in whether knockdown of E2F1 and or E2F3 might enhance a G1 arrest [76, 77].

As depicted in Figure 25A, already 50% of A763 cells are in G1 under control treatment conditions. Knock down of either E2F1, or E2F3 or both simultaneously in A673 cells did not change the percentage of cells in G1 phase.

These data suggest that up-regulation of the cell cycle regulators E2F1 and E2F3 has no effect on cell cycle phase distribution. Therefore we conclude that EWS-FLI1 is the major regulator of cell cycle in Ewing Sarcoma.

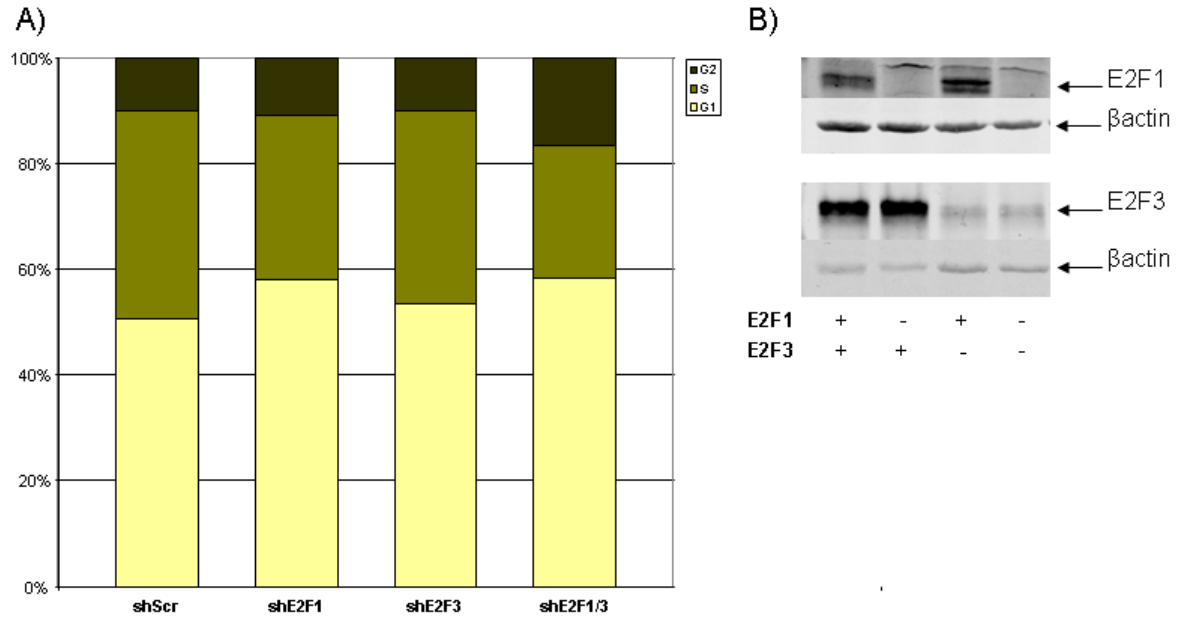

Figure 25: Cell cycle analysis after E2F modulation. A) A673 cells were transfected either with shScrambled as a control, shE2F1, shE2F3 or shE2F1+shE2F3. Diagram shows a representative experiment. B) Western Blot for A673 cells transfected either with shScrambled as a control, shE2F1, shE2F3 or shE2F1+shE2F3.

3.11 E2F3 binding is dependent on EWS-FLI1 binding

To test if EWS-FLI1 binding directly affects E2F3 recruitment to their shared target gene promoters, we assessed E2F3 occupancy of wildtype and ETS binding site mutated promoters of *E2F3* and *GEMIN4* on transfected constructs by chromatin immunoprecipitation. Prohibition of ETS factor binding to the *E2F3* promoter by mutation of the conserved ETS binding sites at -7 and +207 led to a significant decrease of E2F3 binding in A673 but not in HeLa cells used as an EWS/FLI1 negative control. A similar result was obtained for the binding of E2F3 to the *GEMIN4* promoter when the two ETS binding motifs at -176 and -92 were disrupted (Fig. 26). This suggests that EWS-FLI1 binding is necessary for the recruitment of E2F3 at these sites on both tested promoters.

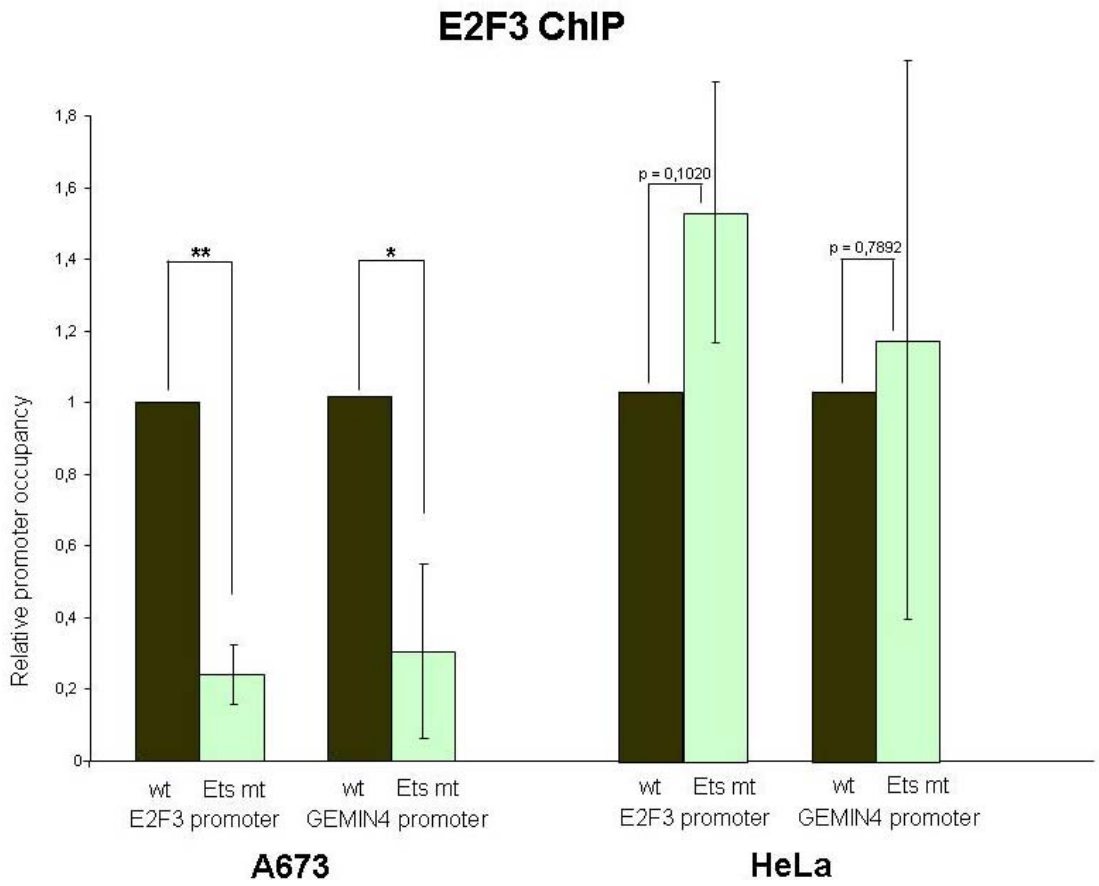

Figure 26: Occupancy of the *E2F3* and *GEMIN4* promoter by E2F3 in the presence and absence of an intact ETS binding site. Binding of E2F3 to the promoters of *E2F3* and *GEMIN4* is significantly reduced in the Ewing Sarcoma cell line A673 when the ETS recognition site is mutated in comparison to the wildtype sequence. In HeLa cells, mutation of the ETS recognition site does not change E2F3 binding.

3.12 Pocket proteins reflects binding pattern of their interaction partners

Pocket proteins are, as described in chapter 1.2.4, regulators of E2F factors and thereby of the cell cycle. According to the literature E2F3 forms a complex with RB, and E2F4 with p130. Therefore, we were interested in whether RB and p130 show the same promoter occupancy pattern as their interaction partners [56, 57]. To test this, we assessed E2F3, E2F4, RB and p130 promoter occupancy by ChIP followed by quantitative RT-PCR (Fig. 27). Although the results showed a high variability between the different promoters, overall RB binding to the various tested promoters was reduced upon knockdown of EWS-FLI1 as was binding of its interaction partner E2F3. In contrast binding of p130 in combination with E2F4 was induced after EWS-FLI1 depletion. These findings indicate that RB and p130, the regulatory pocket

proteins of E2F3 and E2F4, show the same promoter occupancy as their interaction partners and that the pocket proteins are involved in the regulation of the tested genes.

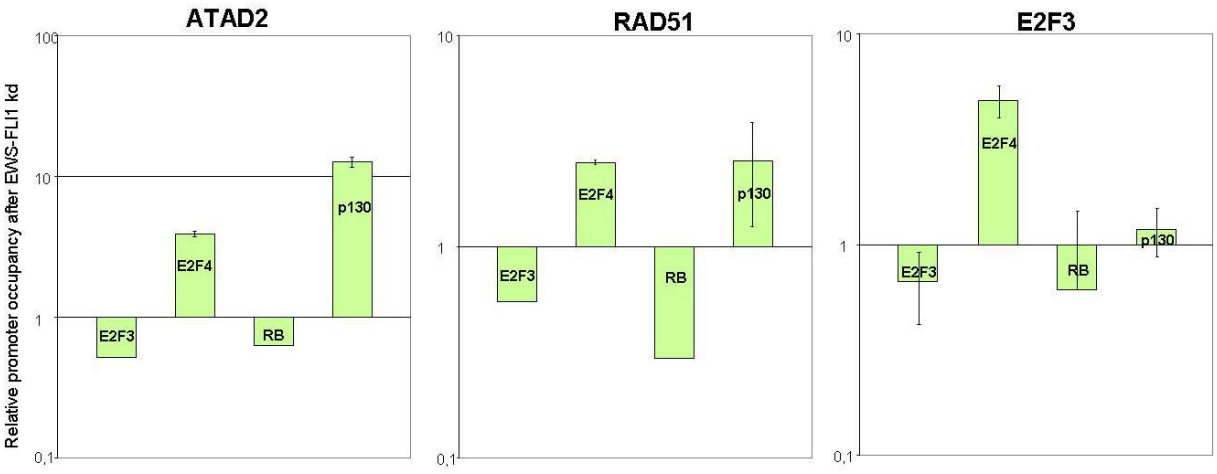

Figure 27: Occupancy of target gene promoters by pocket proteins and E2Fs. The occupancy by RB along with E2F3 of the promoter of ATAD, RAD51 and E2F3 was reduced upon knockdown of EWS-FLI1. Binding of p130 along with E2F4 was enhanced upon EWS-FLI1 depletion.

3.13 Complex formation in Ewing Sarcoma

According to our results it is clear that EWS-FLI1 leads to the exchange of E2F3 for E2F4 and that binding of EWS-FLI1 is necessary for this exchange. Furthermore ChIPs for pocket proteins revealed that they bind together with their interaction partner to the tested promoters, suggesting that pocket proteins are also involved in this regulatory mechanism. However, so far, it is not known how this exchange is achieved. One possible mechanism for this interplay could be a physical interaction of the EWS-FLI1 protein with any of the E2F/pocket protein complex members. In order to test for complex formation between the different components, we performed Co-Immunoprecipitations (Co-IP) for the different components followed by Western Blots.

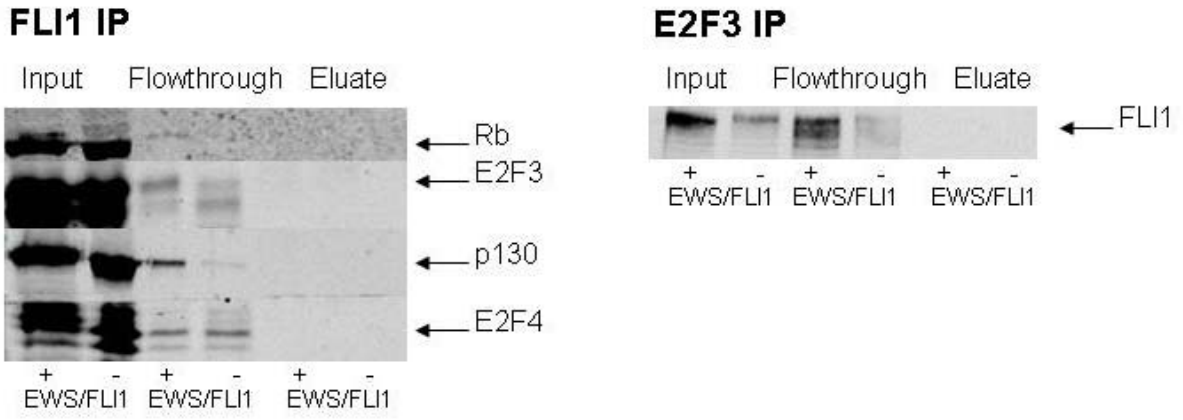

Figure 28: EWS-FLI1 does not form a complex with the tested E2F factors or pocket proteins. Co-IPs in the presence and absence of EWS-FLI1 for FLI1 and E2F3 were performed as well as Western Blots using E2F3, E2F4, RB, p130 and FLI1 Abs.

Co-IPs for FLI1 showed no interaction with any of the tested possible complex partners. E2F3 IP followed by a Western Blot probing with a FLI1 antibody did not reveal a signal either (Fig. 28).

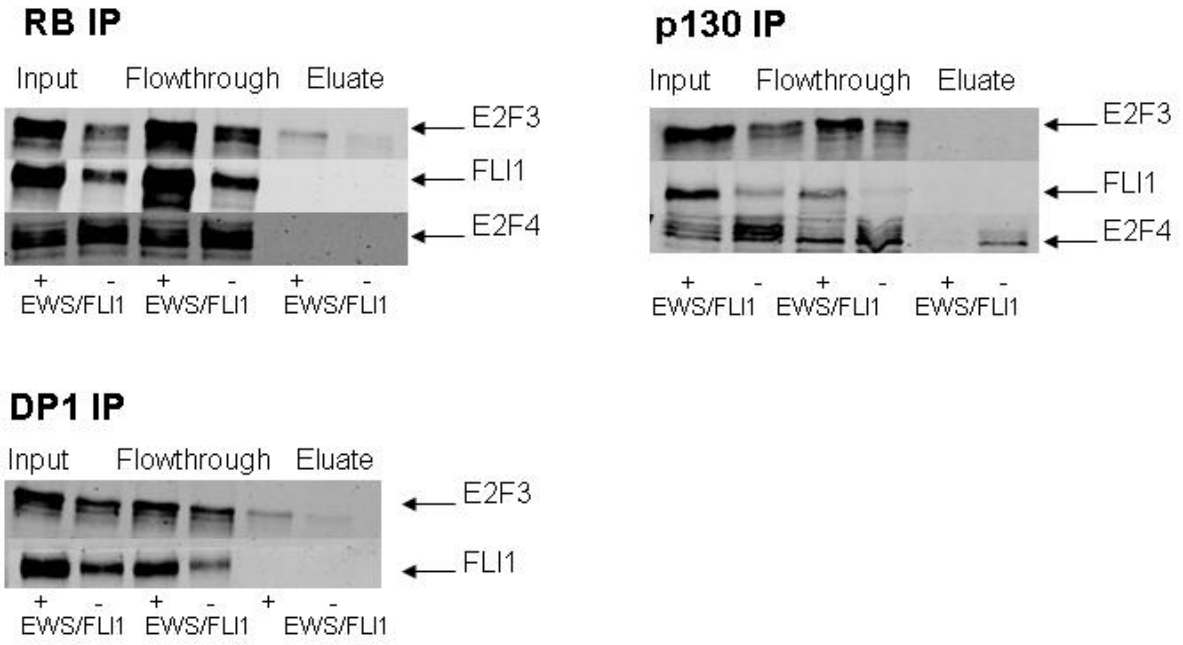

Figure 29: Pocket proteins form complexes with E2F factors. Co-IPs in the presence and absence of EWS-FLI1 for RB, p130 and DP1 were performed as well as Western Blots using E2F3, E2F4, p130 and FLI1 Abs.

RB IP validated the interaction with E2F3 in the presence of EWS-FLI1, but not in its absence, which is a consequence of the down-regulation of E2F3 upon knock down of EWS-FLI1 (Fig. 29). No obvious interaction of RB with FLI1 or E2F4 was detected. p130 IP confirmed the complex with E2F4 in the absence of EWS-FLI1 but not in the presence. This is in line with the upregulation of p130 upon knock down of EWS-FLI1 and the increased E2F4 and p130 binding after depletion of EWS-FLI1 on target gene promoters. A complex formation of p130 with FLI1 or E2F3 was not identified. DP1 interacted with E2F3 in the presence of EWS-FLI1, but in neither case with p130 or FLI1.

These results suggest that the EWS-FLI1 protein does not interact directly with members of the E2F and pocket protein family.

3.14 E2F1 does not regulate EWS-FLI1 target genes bound by E2F factors

E2F1 is known to be a strong transcriptional activator and also an EWS-FLI1 activated gene. It has previously been reported that E2F3 and E2F1 differ in their target genes and subsequent functions [105]. To test whether this is also the case in Ewing Sarcoma, we performed gene reporter assays. More specifically, E2F1 or E2F3 were knocked down and luciferase activity of the *E2F3* and *RAD51* promoters were tested. Both promoter constructs showed that the depletion of E2F1 had no significant effect on luciferase activity. In contrast, depletion of E2F3 led to a decrease of *RAD51* promoter activity by 50% and a reduction by 37% of the *E2F3* promoter activity (Fig. 30).

These data suggest that E2F1 does not regulate the EWS-FLI1 target genes investigated in this study.

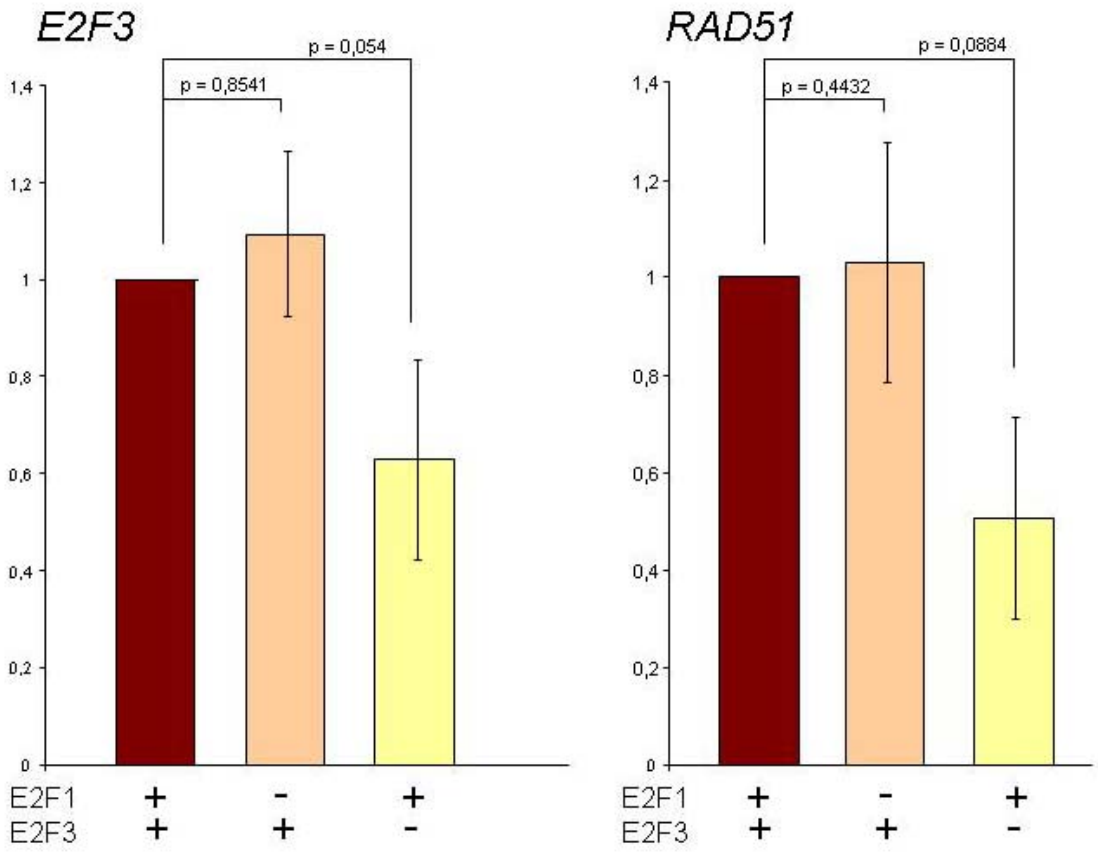

Figure 30: *E2F3* and *RAD51* promoter construct activity is reduced upon knock down of E2F3, not by E2F1. Fold changes in reporter activity of wild type E2F3 and RAD51 reporter constructs in the presence (+) and absence (-) of E2F1 or E2F3. Y-axis presents promoter activity relative to untreated control conditions. Data represent means and standard deviations of at least three independent experiments, each performed in triplicate.

3.15 EWS-ETS driven cancers share the same regulatory mechanism

Oncogenic fusion genes involving an ETS transcription factor have been described in a number of different cancer types, like in acute lymphoblastic leukemia and prostate cancer [27, 28]. In our model we propose that EWS-FLI1 binding is essential for E2F binding. Since EWS-FLI1 and for example the prostate cancer fusion protein TMPRSS2-ERG both bind to ETS binding sites, we wanted to elucidate whether a similar mechanism regulates target genes in prostate cancer.

For this reason we integrated data of published gene expression profiles from a TMPRSS2-ERG knockdown experiment in the prostate cancer cell line VCaP into our own data set [106, 107]. We identified a shared set of ETS fusion gene binding sites

in Ewing Sarcoma and prostate cancer which exhibit colocalization with E2F3 in both tumor types. In addition we found a significant overlap in these two systems in gene expression responses to the depletion of their respective ETS fusion oncoproteins.

To functionally validate the *in silico* data we assessed E2F3 occupancy of wildtype and ETS binding site mutated promoters of *E2F3* and *GEMIN4* on transfected constructs by ChIP as described in 3.11. Mutation of the ETS binding motifs and consequently prevention of TMPRSS2-ERG binding to the promoters of E2F3 and GEMIN4 resulted in decreased E2F3 binding in the prostate cancer cell line VCaP (Fig. 31).

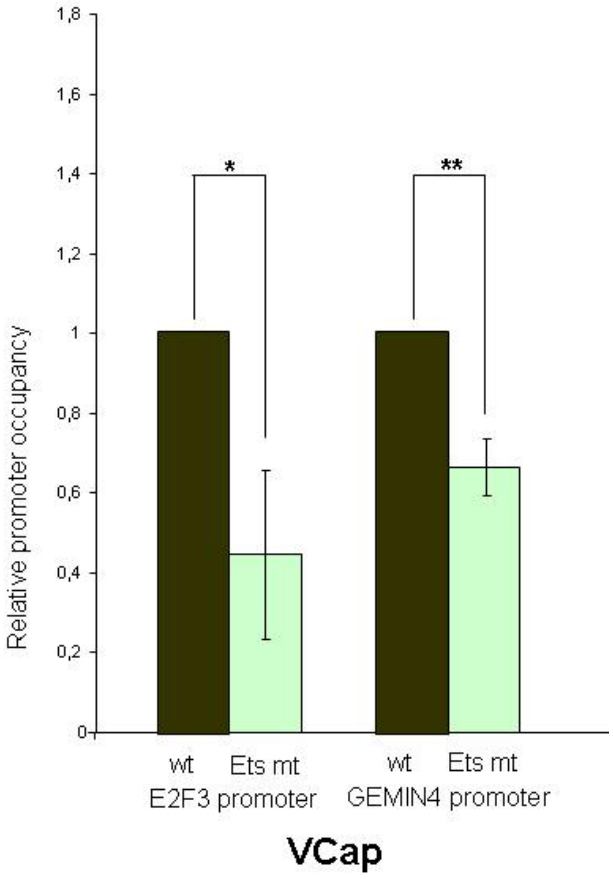

Figure 31: Occupancy of the *E2F3* and *GEMIN4* promoter by E2F3 in the presence and absence of an ETS binding site in a prostate cancer cell line. Binding of E2F3 to the promoters of *E2F3* and *GEMIN4* is significantly reduced in the prostate cancer cell line VCaP when the ETS recognition site is mutated in comparison to the wild type sequence.

These data suggest that, just like in Ewing Sarcoma the binding of the ETS fusion gene in prostate cancer cells is necessary for the recruitment of the cell cycle regulator E2F3.

4 Discussion

EWS-FLI1 dependent regulation of E2F

A major hallmark of oncogenesis is the deregulation of cell cycle genes in order to promote proliferation of cancer cells [108]. According to our ChIP-seq data, EWS-FLI1 binds to several members of the E2F transcription factor family which are major regulators of the cell cycle. Here, we demonstrated direct binding of EWS-FLI1 to the *E2F3* promoter via ChIP-PCR, the ability of EWS-FLI1 to transcriptionally activate *E2F3* was proven by gene reporter assays. Strikingly, *E2F3* belongs to the group of immediate early EWS-FLI1 responsive genes. Additionally, ChIP assays showed binding of E2F3 to its own promoter indicating that EWS-FLI1 further drives the up-regulation of E2F3 by increasing E2F3 levels, followed by binding of E2F3 to its promoter and subsequent transcriptional activation. Together with the highly significant ChIP-seq overlap, this suggests that EWS-FLI1 and E2F3 are components of a feed-forward loop. More specifically, the transcription factor EWS-FLI1 up-regulates the transcription factor E2F3, followed by the combinatorial binding of EWS-FLI1 and E2F3 on their shared target genes including *E2F3* thereby jointly activating their transcription [109]. This assumption is strengthened by our observation that mutation of the ETS binding site in promoters regulated by EWS-FLI1 in A673 results in decreased E2F3 occupancy. We therefore conclude that the binding of EWS-FLI1 is indeed necessary for the binding of E2F3.

Furthermore, by active recruitment of E2F3 by EWS-FLI1 to promoters of their shared target genes, EWS-FLI1 binding leads to E2F4 replacement by E2F3 and activates gene expression of the corresponding genes.

The results of an earlier study by Freedman et al. in fact support our model. In their study they demonstrated that E2F factors can either bind to distinct activating or repressive E2F sites such as in the Cdc2 promoter [110, 111] or, alternatively, activating and repressing E2Fs can bind to the same binding site as in the Cdc6 promoter. In the latter case interaction with further binding partners determines the fate of target gene expression. For both types of promoters, Freedman et al. performed ChIP assays on promoter constructs at the G1/S transition of the cell cycle. By mutation of the activating E2F binding site, they showed binding of E2F4 to the repressive E2F site, whereas the introduction of a YY1 binding element diminished E2F4 binding and enabled E2F3 to bind to Cdc2 and Cdc6 promoters.

Mutation of the YY1 binding site again shifted binding from the activating E2F3 to the repressing E2F4, indicating that the regulation is not determined by the sequence of the binding site or the cell cycle status, but by the context and the binding partners of E2F [111]. Since ETS binding sites significantly co-occur with E2F binding sites on EWS-FLI1 activated genes and ChIP-seq hits for EWS-FLI1, E2F3 and E2F4 overlap, we propose that EWS-FLI1 is such an activating co-factor and thereby regulating E2F target genes.

A recent study by Li et al. showed an androgen-dependent switch of E2F1 and E2F4 on the cyclin B promoter in prostate stromal cells. Stimulation with androgens leads to a displacement of E2F1 for E2F4 by the androgen receptor, resulting in recruitment of repressive complexes. The exchange coincides with direct interaction of the androgen receptor and E2F1. In contrast to our results, interaction with members of the pocket protein family and binding of E2F3 to the promoter was not observed. The involvement of E2F1 in contrast to E2F3 plus pocket proteins in our study might be due to the different cellular systems. While Li et al. used non-cancerous prostate stromal cells, we investigated a Ewing Sarcoma and a prostate cancer cell line, both harboring an ETS driven oncogenic transcription factor. Li and colleagues also analyzed the involvement of cyclin B in LNCaP cells, an ETS fusion positive prostate cancer cell line, and did not observe a response due to androgen treatment [112].

Our data are consistent with a model in which EWS-FLI1 actively recruits an activating E2F factor thereby replacing a repressing E2F factor and in which EWS-FLI1 binding is essential for E2F binding.

E2F recruitment to shared target gene promoters

The actual mechanism by which EWS-FLI1 recruits E2F3 still remains unclear. Data from our ChIP-seq study showed that E2F and EWS-FLI1 binding sites are distributed in a specific pattern. The length of DNA between E2F and EWS-FLI1 binding motifs is in line with the length of DNA associated with single nucleosome plus one (or two) linker DNA sequences. This suggests that geometric constraints or a form of nucleosome-mediated cooperativity contribute to the selection of referred configurations [113].

It is conceivable that the binding of EWS-FLI1 leads to a change in nucleosome formation, opening up the chromatin structure and resulting in the binding of E2F3. The ability of ETS transcription factors to bend DNA and enhance promoter activation has already been described for ETS1. For instance, it has been reported that binding of either p51 or p42 isoforms of ETS1 to the Stromelysin 1 promoter results in different DNA bending and consequently promoter activation. While p51 induces a bend directed away from the protein–DNA interface and fully trans-activates the Stromelysin-1 promoter, p42 generates an opposite bend and activates the promoter only weakly [114].

The regulation of pocket proteins by EWS-FLI1 would be another possible mechanism. According to our data, Rb is regulated along with its binding partner E2F3 and p130 along with E2F4, emphasizing their cooperativity. Although we could not show that EWS-FLI1 is in a complex with the pocket proteins or E2F3 or E2F4, it is still possible that it interacts with Rb leading to the release of E2F3 from the repressive Rb-E2F-HDAC complex, thereby inducing expression of target genes [68]. This notion is further supported by a study reporting that the N-terminal region of EWS-FLI1 associates with HDAC1 to form a complex with p53 and inhibit its function [115]. Supposedly EWS-FLI1 interacts with HDAC in the Rb-E2F complex and either disrupts the complex, leading to target gene expression, or uses its interaction with HDAC to attract the complex to its own target genes.

Another possible interaction partner mediating the complex formation of EWS-FLI1 and E2F family members might be host cell factor 1 (HCF-1). Unpublished data from Jeff Toretsky's lab revealed an interaction of EWS-FLI1 with HCF-1 (personal communication). HCF-1 is not binding directly to DNA but shows chromatin association activity via its Kelch domain, which promotes binding of HCF to the HCF-1 binding motif (HBM) in DNA binding proteins. A single mutation within this domain leads to loss of binding and G1 arrest. Strikingly, pRB inactivation leads to a release from the G1 arrest, suggesting that HCF-1 regulates G1 phase progression via counteraction of one or several pocket proteins. Furthermore, it was demonstrated that HCF-1 is associated with several members of the E2F family, both with activators and repressors [116-119]. Together, this suggests that HCF-1 is involved in E2F mediated transcriptional activation and repression, and therefore may represent an intriguing candidate for the missing link between EWS-FLI1 and E2F family members.

Although the actual mechanism by which EWS-FLI1 recruits E2F3 and replaces E2F4 in the progenitor cell of Ewing Sarcoma still remains unknown, we propose several possible alternative mechanisms: DNA bending due to binding of EWS-FLI1, regulation of pocket proteins, or interaction with a complex partner of E2F factors such as HCF-1.

EWS-FLI1 deregulates the cell cycle

A previous study reported that knock down of EWS-FLI1 arrests cells in G1 [76, 77]. Due to the fact that E2Fs are major regulators of cell cycle progression we wanted to explore the consequences of their depletion or of overexpression on cell cycle arrest. We found that in Ewing Sarcoma cell lines, EWS-FLI1 is the master regulator of the cell cycle and neither overexpression nor depletion of E2F factors affects the cell cycle phase distribution. This is in line with our gene expression profiling of A673 cells with E2F1, E2F3 or EWS-FLI1 knockdown. Based on these data EWS-FLI1 activated targets annotate to cell cycle genes. Genes activated by both EWS-FLI1 and E2F3 also annotate to cell cycle genes, whereas genes down-regulated after knock down of E2F1 and E2F3 individually and simultaneously do not annotate to cell cycle, indicating that EWS-FLI1 is required for cell cycle regulation.

Additionally no cell cycle induced occupation of promoters by E2F4 as reported by Takahashi et al. could be shown in Ewing Sarcoma [59]. Enforced cell cycle arrest of Ewing Sarcoma cells in the presence of EWS-FLI1 did not show reduced binding of E2F3, suggesting that the exchange of E2F3/4 is not a cell cycle artifact, but rather EWS-FLI1 dependent.

Therefore, we propose a model in which EWS-FLI1 actively recruits E2F3 to target genes in the progenitor cell of Ewing Sarcoma and induces an irreversible switch in the gene expression profile which can only be altered through the depletion of EWS-FLI1 but not completely through modulation of E2F family members.

Results from our previous study already revealed that EWS-FLI1 activated genes are significantly enriched in cell cycle functions [11]. Here we could validate a number of cell cycle regulating genes to be direct targets of EWS-FLI1 and additionally, also to be regulated by E2F3. One of these target genes is *CDK2*, a master regulator of G1/S transition (see chapter 1.2.2). CDK2 and its corresponding cyclin phosphorylate and inactivate Rb, thereby releasing it from the repression complex with E2F family members and allowing the transcription of genes necessary for driving the G1/S

transition. Recently it has also been shown that loss of CDK2 results in enhanced neuronal differentiation, whereas in Ewing Sarcoma CDK2 is overexpressed and differentiation is decreased [1, 76, 77, 120].
SKP2 overexpression is associated with a variety of cancers and is involved in G1/S transition [121]. Recently, our lab reported on a tumor suppressive mechanism in Ewing Sarcoma mediated through FOXO1, which is in turn negatively regulated by SKP2 and also CDK2. In this study we show that *SKP2* is a direct target of EWS-FLI1 (see chapter 3.3), resulting in enhanced cell proliferation as well as suppression of a tumor suppressor in Ewing Sarcoma. As already discussed, E2F3, along with several other E2F family members is as well regulated by EWS-FLI1 and E2F3.

In line with the data from Kauer et al., we report here that EWS-FLI1 actively deregulates cell cycle progression in Ewing Sarcoma. Additionally, it deregulates E2F family members which are themselves major regulators of the cell cycle [11, 53]. This mechanism leads to enhanced proliferation, consequently tumor growth and ultimately tumor progression. Additionally we suggest that EWS-FLI1 actively recruits E2F3 to target genes in the cell of origin of Ewing Sarcoma and induces an irreversible switch in the gene expression profile. In fact, only the depletion of EWS-FLI1 seems to be able to reverse that profile.

ETS fusion driven cancers – prostate cancer

Since binding of EWS-FLI1 to the ETS binding site is the initial step and the prerequisite of our proposed model, we decided to elucidate whether the mechanism found in Ewing Sarcoma is also applicable to another ETS fusion driven cancer. ETS oncogenic fusion genes are known to be involved in several types of cancer. One of those, with the most frequent ETS fusions, is prostate cancer, in which more than 50% of cases show a rearrangement involving *TMPRSS2* and an ETS transcription factor [27].
Strikingly, integrating data of published gene expression profiles from a TMPRSS2-ERG knockdown experiment in VCaP into our own data set, identified a shared set of ETS fusion gene binding sites in Ewing Sarcoma and prostate cancer which exhibit colocalization with E2F3 in both tumor types [106, 107]. The importance of this result was strengthened by our demonstration of a significant overlap in the gene expression responses of these two systems to depletion of their respective ETS

fusion oncoproteins. Additionally, we demonstrated that the binding of the ETS fusion gene TMPRSS2-ERG is necessary for the recruitment of the cell cycle regulator E2F3 in the prostate cancer cell line VCaP.
These observations suggest that the functional EWS-FLI1/E2F3 module in Ewing Sarcoma is shared to a significant extent between these diseases and that it may be in part responsible for the oncogenic activity of their respective ETS factors.

Conclusion

Understanding the mechanisms of EWS-FLI1 driven gene regulation is essential to understand Ewing Sarcomagenesis and is the key to develop new treatment strategies.
In this thesis I confirm that EWS-FLI1 is a transcriptional regulator via direct and indirect mechanisms. More specifically I show that EWS-FLI1 up-regulates the transcription factor E2F3, followed by the combinatorial binding of EWS-FLI1 and E2F3 on their shared target genes. Interestingly both factors bind within the E2F3 promoter region itself confirming the establishment of a feed-forward loop.
Furthermore, by active recruitment of E2F3 by EWS-FLI1 to promoters of their shared target genes, EWS-FLI1 binding leads to E2F4 replacement for E2F3, in the progenitor cell of Ewing Sarcoma, and activates gene expression of the corresponding genes (Fig. 32).
Strikingly, the functional E2F/ETS transcriptional module detected in Ewing Sarcoma for the chimeric ETS factor EWS-FLI1 was found to be also relevant to TMPRSS2-ERG expressing prostate cancer cells. Our findings in a paediatric tumor may therefore also be of relevance to one of the most frequent human cancers and suggest that an ETS-E2F transcriptional module may be a general feature of ETS driven cancers.

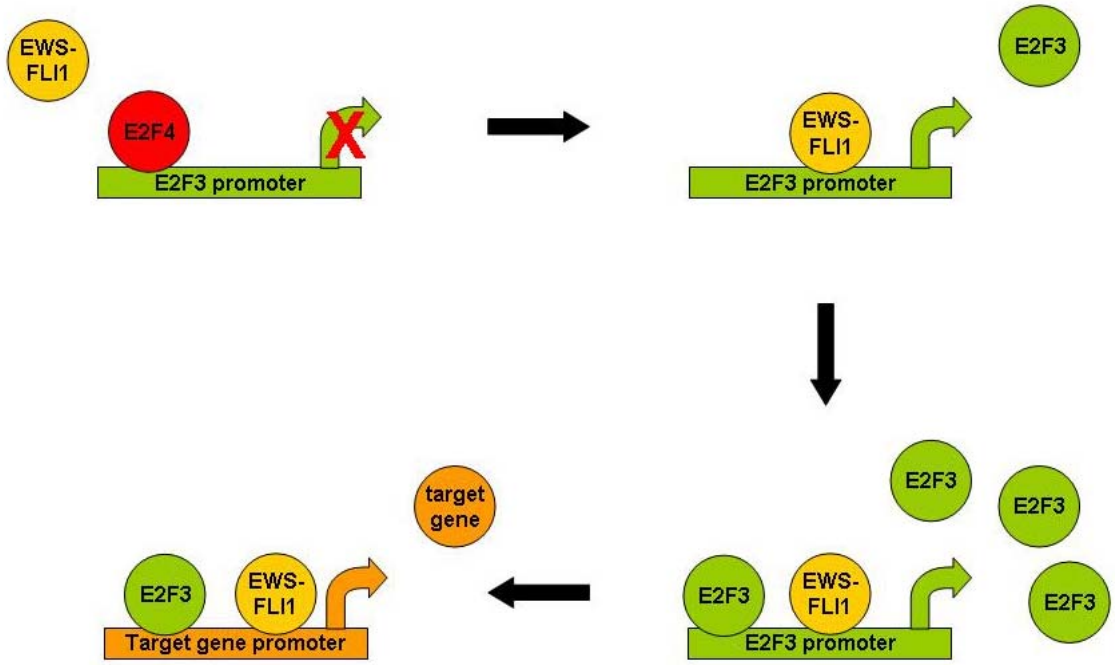

Figure 32: Model of target gene regulation by EWS-FLI1 and E2F factors. EWS-FLI1 replaces E2F4 by E2F3 on their shared target genes including *E2F3,* up-regulates the transcription factor E2F3, followed by the combinatorial binding of EWS-FLI1 and E2F3 on further target genes.

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