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DISSERTATION

Titel der Dissertation

„The Effect of EWS and its Oncogenic Derivative EWS-FLI1 on Transcriptional and Post-transcriptional Gene Regulation in Ewing Sarcoma“

Verfasserin

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Summary

Ewing sarcoma (ES) are characterized in 85% of cases by the expression of the oncogenic fusion protein EWS-FLI1 which results from the fusion of two genes, EWS and FLI1, upon chromosomal translocation. While FLI1 is an ETS transcription factor, EWS belongs to the evolutionarily conserved FET family of RNA-binding proteins. So far, functional studies of oncogenic EWS fusion proteins have concentrated on their presumed role as aberrant ETS transcription factors. However, chimeric FET proteins are usually co-expressed with their intact counterparts and previous protein interaction studies have shown that EWS and EWS-FLI1 interact with each other. Therefore, it is possible that functional interference with normal EWS function accounts for part of the oncogenic activities of EWS-FLI1. However, the role of EWS in post-transcriptional and transcriptional gene regulation is poorly defined. Since FET proteins associate with a number of RNA processing factors, we investigated the influence of modulated EWS expression on genome wide transcript expression and exon composition in ES cell lines using Affymetrix HuEx-1.0stv2 arrays. For that purpose, we established two cellular model systems: a unique ES cell line lacking endogenous EWS was studied upon stable restoration and re-knockdown of EWS expression (restoration model), and an ES cell line with stable EWS knockdown was analyzed upon inducible RNAi-mediated EWS-FLI1 suppression (knockdown model).

Regarding differential expression of whole transcripts, a number of genes with expression level variations upon modulated EWS expression were identified in both cellular systems. Differential gene expression in the presence and absence of EWS might be explained by two models of either functional competition or functional interaction between EWS and the fusion gene, and genes from the overlap between the two data sets were classified accordingly depending on their EWS and EWS-FLI1 dependent expression patterns. 5´-RACE PCR experiments excluded the possibility of EWS dependent alternative transcription start site selection as a potential cause of altered transcript stability to account for the observed differential RNA expression effect.

In addition, we found significant differences in exon composition of genes in each of the two cellular systems, but almost no overlap between the two models. The observed alternative exon composition patterns were verified by RT-qPCR of candidate genes.

The phenotypic relevance of EWS expression in ES cells was examined involving two different functional assays. EWS expression was found to improve anchorage-independent growth in soft agar colony formation assays while no significant effect could be detected concerning anchorage-dependent growth in proliferation assays.

Taken together, the results described in this thesis are in line with published evidence for an involvement of EWS in transcriptional and post-transcriptional regulation. However, the differences detected between the two cellular model systems emphasize dependence of EWS function and interaction with EWS-FLI1 on the cellular background.

Zusammenfassung

Ewing Sarkome (ES) sind in 85% der Fälle durch das onkogene Fusionsprotein EWS-FLI1 charakterisiert. Dieses entsteht durch die Fusion zweier Gene, EWS und FLI1, auf Grund einer chromosomalen Translokation. Während FLI1 ein ETS Transkriptionsfaktor ist, gehört EWS zur evolutionär konservierten FET Familie der RNA-bindenden Proteine. Bislang haben sich funktionelle Studien der onkogenen EWS-Fusionsproteine immer auf deren mutmaßliche Rolle als veränderte ETS Transkriptionsfaktoren bezogen. Allerdings werden chimäre FET-Proteine normalerweise gemeinsam mit ihren intakten Gegenstücken exprimiert und Proteininteraktions-Studien haben gezeigt, dass EWS und EWS-FLI1 miteinander interagieren. Folglich ist es denkbar, dass ein Teil der onkogenen Aktivität von EWS-FLI1 in der funktionellen Interferenz mit der normalen Funktion von EWS besteht. Die exakte Rolle von EWS in der transkriptionellen und post-transkriptionellen Genregulation ist jedoch bislang nicht gut definiert. Da FET Proteine mit einer großen Anzahl RNA-Prozessierungsfaktoren assoziiert sind, wurde im Rahmen dieser Arbeit der Einfluss modulierter EWS-Expression sowohl auf die Genexpression als auch die Exon-Zusammensetzung von Transkripten in ES Zelllinien mittels Affymetrix HuEx-1.0stv2 Arrays untersucht. Zu diesem Zweck wurden zwei verschiedene zelluläre Modelle etabliert: In einer einzigartigen ES Zelllinie ohne endogenes EWS wurde stabile EWS-Expression wiederhergestellt (Wiederherstellungs-Modell). Eine andere ES Zelllinie wurde nach stabiler Stilllegung von EWS und induzierbarer, RNAi-vermittelter Suppression von EWS-FLI1 untersucht (Suppressions-Modell).

Betreffend differenzieller Expression ganzer Transkripte, konnten mehrere Gene mit EWS-abhängiger Variation des Expressions-Niveaus in beiden zellulären Systemen identifiziert werden. Die differenziellen Genexpressionsmuster lassen sich durch zwei Modelle funktioneller Konkurrenz bzw. Interaktion zwischen EWS und dem Fusionsprotein erklären. Die Gene aus der Überschneidungsmenge der beiden zellulären Modelle konnten dementsprechend anhand ihrer EWS- und EWS-FLI1-abhängigen Expressionsmuster klassifiziert werden. Mittels 5‘-RACE PCR Experimenten wurde die Möglichkeit alternativer Transkriptionsstartpunkt-Selektion in Abhängigkeit von EWS als möglicher Grund veränderter Transkriptstabilität, die die beobachteten differenziellen RNA-Niveaus verursacht, ausgeschlossen.

Außerdem konnten signifikante Unterschiede in der Exon-Zusammensetzung mehrerer Gene in beiden zellulären Modellen festgestellt werden. Jedoch war keine Überlappung der beiden Datensätze feststellbar. Diese beobachteten alternativen Exon-Zusammensetzungen konnten mit Hilfe von RT-qPCR verifiziert werden.

Die phänotypische Relevanz von EWS in ES Zellen wurde anhand zweier verschiedener funktioneller Tests untersucht. In Soft-Agar Colony Assays konnte gezeigt werden, dass EWS die Oberflächen-unabhängige Koloniebildung fördert. Proliferations-Versuche hingegen zeigten, dass EWS keinen Effekt auf das Oberflächen-abhängige Zellwachstum hat.

Zusammengefasst unterstützen die hier beschriebenen Resultate bereits veröffentlichte Hinweise für eine Beteiligung von EWS an transkriptioneller und post-transkriptioneller Genregulation. Die beobachteten Unterschiede zwischen den beiden untersuchten Zellmodellen unterstreichen aber den Einfluss des zellulären Hintergrundes auf die EWS Funktion und dessen Interaktion mit EWS-FLI1.

1. Introduction

1.1 The FET Protein Family

Since the identification of EWS as the gene product of the Ewing sarcoma breakpoint region 1 (*EWSR1*) by Delattre et al. in 1992 (Delattre, Zucman et al. 1992) three more proteins have been identified, which form an evolutionarily conserved family of putative RNA-binding proteins: FUS/TLS/Pigpen/hnRNP P2 (Crozat, Aman et al. 1993; Rabbitts, Forster et al. 1993; Zinszner, Albalat et al. 1994; Alliegro and Alliegro 1996) and TAF15/hTAFII68/TAF2N/RPB56 (Bertolotti, Lutz et al. 1996; Hackl and Luhrmann 1996) as well as the *Drosophila* ortholog Cabeza/SARFH (sarcoma-associated RNA-binding fly homolog) (Immanuel, Zinszner et al. 1995; Stolow and Haynes 1995). All members of this protein family, which is generally referred to as FET (FUS/TLS, EWS, TAF15) family of proteins, are structurally and functionally related and share extensive sequence similarity and identity (Guipaud, Guillonneau et al. 2006) (figure1). Besides, recent

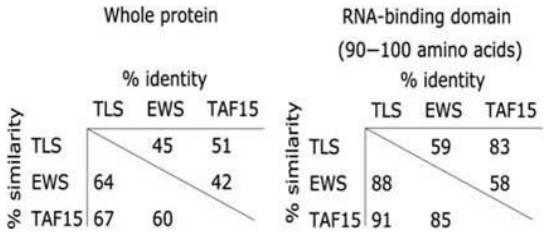


Figure 1. Comparison of amino acid similarity and identity over the whole protein and within the RBD for FUS/TLS, EWS and TAF15. (from Tan and Manley, 2009)

descriptions of a new subtype of bone sarcoma characterized by the BCOR-CCNB3 gene fusion suggest the FET protein family to be still expanding (Pierron, Tirode et al. 2012). The proteins are almost ubiquitously expressed and mostly localized to the nucleus (Andersson, Stahlberg et al. 2008). Due to genomic translocations, all known FET proteins have been found to be fused to transcription factors thereby contributing to the genesis of a variety of human sarcomas and acute leukemias. The resulting fusion proteins function as aberrant transcription factors which is the reason for most mechanistic studies so far to concentrate on the identification of downstream target genes. However, there is evidence for an interaction between EWS and its oncogenic derivative EWS-FLI1 (Spahn, Siligan et al. 2003) which supports the hypothesis that the oncogenic fusion proteins exert a dominant-negative effect on normal FET protein functions. Furthermore, FET proteins were found to interact with each other (Pahlich, Quero et al. 2009) implicating them to be engaged in the same protein complexes and cellular processes [for review see (Law, Cann et al. 2006; Tan and Manley 2009; Kovar 2011)] although their precise functions still remain poorly characterised. Thus, the following chapter will give a detailed summary of the current scientific knowledge concerning the biologic role of FET family proteins.

1.1.1 Functional Motifs

The FET protein family is defined by akin composition of functional domains (figure2): The amino-terminus or activation domain (AD) is enriched for serine, tyrosine, glycine, and glutamine (SYGQ) residues and functions as potent transcriptional activation domain (May, Lessnick et al. 1993; Prasad, Ouchida et al. 1994; Zinszner, Albalat et al. 1994; Bertolotti, Bell et al. 1999) when fused to the DNA-binding domain of a transcription factor as a result of genomic translocation. Despite similar glutamine-rich elements observed in various transcription factors, their function in RNA-binding proteins is still unclear (Stolow and Haynes 1995).

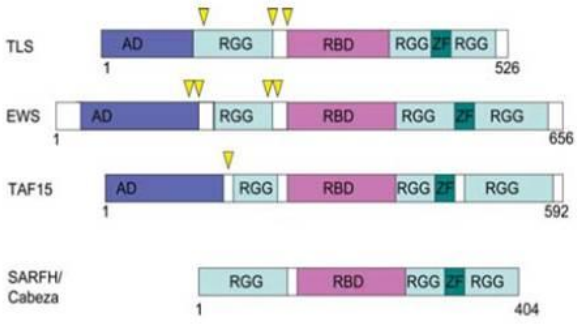


Figure 2. Domain structure of FET and Cabeza/SARF proteins. AD - Activation domain, RGG boxes, RBD - RNA binding domain, ZF - C2/C2 zinc finger domain, arrowheads depict sarcoma breakpoint regions. (from Tan and Manley, 2009)

The carboxy-terminus of FET proteins contains one RNA-binding domain (RBD) also referred to as RNA-recognition motif (RRM), comprising approximately 80-90 amino acids, with three adjoining arginine-glycine-glycine (RGG)-boxes (Burd and Dreyfuss 1994), one C_2/C_2 zinc finger motif (ZF) (Morohoshi, Ootsuka et al. 1998) and a conserved nuclear import and retention signal (C-NLS). The basic C-NLS comprises 18 amino acid residues (PGKMDKGEHRQERRDRPY) and shares no homology to other known NLS sequences but seems to be highly conserved within the FET family of proteins (Zakaryan and Gehring 2006).

RGG-boxes are defined as closely spaced arginine-glycine-glycine repeats separated by other, often aromatic, amino acids. RGG-boxes usually occur in combination with other types of RNA-binding domains (Kiledjian and Dreyfuss 1992) and are thought to facilitate binding since their RNA binding is relatively sequence unspecific (Burd and Dreyfuss 1994).

The zinc-finger domain is the eponymous structural characteristic of a family of DNA-binding proteins but it has also been found in a number of RNA-binding proteins, e.g. TFIIIA and RNA-polymerases, without there being an obvious difference in the amino acid composition between predominantly DNA and RNA binding zinc fingers (Theunissen, Rudt et al. 1992). C_2/C_2 zinc fingers similar to the one present in FET family proteins have been observed in ribosomal as well as pre-rRNA-processing proteins (Chan, Suzuki et al. 1993; Hess, Stanford et al. 1994).

The high level of conservation between the members of the FET family of proteins is most apparent in the RBD (Tan and Manley 2009). RBDs consist of four antiparallel β-strands and two perpendicularly oriented α-helices (Burd and Dreyfuss 1994). Like in other RNA-binding proteins the FET-RBD contains two sub-domains, the octameric RNP-1 and the hexameric RNP-2, which are highly conserved and locate to the two central β-strands, β1 and β3. The charged and aromatic side chains of RNP-1 and RNP-2 are exposed and are thought to make contact with bound RNA molecules via hydrogen bonds and ring stacking. However, the FET-RBD differs from the typical RBD in two considerable ways: First, the highly conserved aromatic residue at position +3 of RNP-1 is substituted for a negatively charged amino acid, namely glutamic or aspartic acid. Second, loop 2, which is usually 3-4 amino acids long, is extended and contains 12 amino acids in FET proteins (Stolow and Haynes 1995; Hackl and Luhrmann 1996).

There are some structural variations between the FET protein family members, albeit their highly analogous domain composition. The amino-terminus of EWS is rich in proline and threonine and encompasses multiple degenerate hexapeptide repeats with the consensus SYGQQS, where the tyrosine at the second position is absolutely and the glutamine at the fourth position is highly conserved (Feng and Lee 2001; Ng, Potikyan et al. 2007). This region shares 40% homology to the carboxy-terminus of the RNA polymerase II largest subunit and its high content of glutamine and proline is reminiscent of activation domains of various transcription factors (Delattre, Zucman et al. 1992). Systematic computational as well as mutational analysis revealed this region to be intrinsically disordered and the aromatic side chains of the conserved tyrosine residues to be required for the transactivating properties of the AD (Ng, Potikyan et al. 2007). In addition, one IQ domain is localized at the carboxy-terminal end of the EWS amino-terminal domain (Deloulme, Prichard et al. 1997). This domain was found to bind to the splicing factor/transcriptional repressor SF1/ZFM1 [(Zhang, Paley et al. 1998) see figure3].

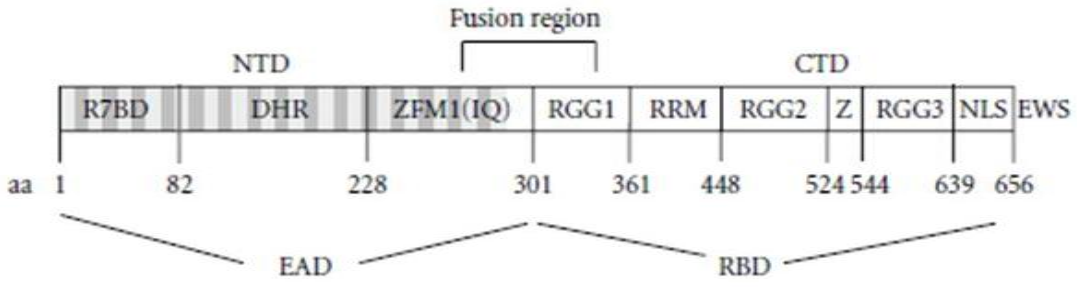


Figure 3. Protein structure of EWS. NTD - amino-terminal domain; CTD - carboxy-terminal domain; R7BS - RPB7-binding domain; DHR – Degenerate hexapeptide repeats; ZFM1(IQ) - IQ domain binding to ZMF1; RGG 1, 2, 3 – Arginine/Glycin/Glycin-boxes 1, 2, 3; RRM – RNA recognition motif; Z – Zinc finger domain; NLS – Nuclear localisation signal; aa – amino acid; EAD – EWS activation domain; RBD – RNA-binding domain; Fusion region – Region disrupted by the fusion to transcription factor moieties upon EWS-rearrangement. (from Kovar, 2011)

In TAF15, the RGG-boxes are not as closely spaced as in the other two FET proteins and overlap with 17 perfect and 3 imperfect GGYGGDR-repeats (Burd and Dreyfuss 1994; Bertolotti, Lutz et al. 1996; Morohoshi, Arai et al. 1996). Whether this protein motif plays a role in RNA-binding or not remains to be investigated.

The *Drosophila melanogaster* ortholog SARFH/Cabeza shares homology to the carboxy-terminus of the remaining FET proteins which is most apparent in the RRM. However, there is very limited sequence identity in the amino-terminal region (Immanuel, Zinszner et al. 1995; Stolow and Haynes 1995). Nonetheless, the amino-terminus of SARFH/Cabeza contributes to the transformation of NIH3T3 cells when fused to CHOP instead of TLS (or EWS), although it does not activate transcription in neither mammalian nor *Drosophila* cells (Immanuel, Zinszner et al. 1995).

1.1.2 Regulation of FET proteins

FET proteins contain various sites for post-translational modifications such as phosphorylation and arginine methylation (Tan and Manley 2009). Post-translational modifications at or near the RBD or RGG motifs are thought to affect protein stability, RNA- or DNA-binding, protein-protein interactions or subcellular localization (Burd and Dreyfuss 1994).

1.1.2.1 Phosphorylation

EWS and FUS/TLS are targeted by a number of Ser/Thr kinases. ATM, a member of the PIKK family of protein kinases, phosphorylates FUS/TLS - but not FUS-CHOP – at Ser 42 *in vivo* upon induction of DNA double-strand breaks (Gardiner, Toth et al. 2008). Likewise, EWS and EWS-FLI1 are phosphorylated at Thr 79 by JNK and p38α/p38β MAPK, respectively, in response to DNA damage. In comparison to the strong phosphorylation after DNA damage, the same residue is weakly phosphorylated by ERK1 and ERK2 after mitogen induction (Klevernic, Morton et al. 2009).

Additionally, the EWS-IQ domain is phosphorylated at Ser 266 by PKC which prevents binding of EWS to RNA. This suggests that the amino-terminal domain of EWS is involved in the regulation of the binding activity of its carboxy-terminal domain (Deloulme, Prichard et al. 1997). FUS/TLS was found to be another substrate of PKC phosphorylated at Ser 256 (Perrotti, Bonatti et al. 1998) which prevents it from proteasome-mediated degradation (Perrotti, Iervolino et al. 2000).

Moreover, FUS/TLS and TAF15 have been found substrates for Tyr phosphorylation. FUS/TLS is tyrosine phosphorylated in FGF-, EGF- and PDGF-stimulated cells and localises to the cytoplasm

(Klint, Hellman et al. 2004). In contrast, TAF15 phosphorylation on Tyr residues by v-Src causes its localization to the nucleus and thus increases transactivation activity of TAF15 (Lee, Kim et al. 2004).

1.1.2.2 O-linked-β-N-acetylglucosaminylation

The amino-terminus of EWS present in the fusion protein EWS-FLI1 is post-translationally modified by O-linked-β-N-acetylglucosaminylation affecting the same sites as Ser/Thr phosphorylation. This is supposed to render the two modifications mutually exclusive thereby modulating transactivation activity of EWS-FLI1 (Bachmaier, Aryee et al. 2009).

1.1.2.3 Methylation

Post-translational methylation of arginine residues is frequently found in RNA-binding proteins and is thought to affect protein-RNA and protein-protein interactions as well as protein stability and subcellular localization (Liu and Dreyfuss 1995). The structure of arginine allows for mono- or di-methylation, which can be symmetric or asymmetric. Type I protein arginine methyltransferases (PRMTs) mono- and asymmetrically methylate arginine residues, whereas mono- and symmetric methylation is administered by Type II PRMTs. FET proteins have been found to be substrates of PRMT1, a type I PRMT. Accordingly, FUS/TLS contains over 20 asymmetrically methylated arginine residues within its RGG motifs (Rappsilber, Friesen et al. 2003). Likewise, the RGG sites of EWS are extensively methylated, with infrequent mono-methylation events among the predominantly asymmetrically dimethylated arginines (Belyanskaya, Gehrig et al. 2001; Pahlich, Bschir et al. 2005). This methylation was found to impact cellular localization and transcriptional activity of EWS through nuclear exclusion and exposure on the cell surface (Belyanskaya, Delattre et al. 2003; Araya, Hiraga et al. 2005). Similarly, the modifications of arginine residues in TAF15 were shown to affect its gene regulatory functions and cellular localization in vivo. Arginine methylation resulted in nuclear localization of TAF15, whereas the inhibition of this modification resulted in cytoplasmic concentration of TAF15 into dense foci identified as stress granules (Jobert, Argentini et al. 2009).

A brain-specific variant of EWS resulting from alternative splicing (Melot, Dauphinot et al. 2001) was also found to be methylated by a Type I PRMT, PRMT8, which is located to the cell surface (Pahlich, Zakaryan et al. 2008). However, the functional significance of this modification remains unclear.

1.1.3 Putative and Validated Functions

The FET family proteins are considered to be multifunctional RNA-binding proteins which integrate gene expression into a tightly regulated cellular machinery rather than a plain succession of transcription, RNA processing, transport and translation (Keene and Lager 2005; Law, Cann et al. 2006). According to their stability, almost ubiquituous expression and type of promoter FET proteins were at first regarded as housekeeping genes (Plougastel, Zucman et al. 1993; Aman, Panagopoulos et al. 1996; Morohoshi, Ootsuka et al. 1998). Meticulous analysis of expression patterns and subcellular localization, however, points to partially overlapping but unique, cell type specific functions of EWS, FUS/TLS and TAF15 (Andersson, Stahlberg et al. 2008).

The FET proteins are implicated in various cellular processes such as transcription (Immanuel, Zinszner et al. 1995; Bertolotti, Lutz et al. 1996; Hoffmann and Roeder 1996; Bertolotti, Melot et al. 1998; Zhang, Paley et al. 1998; Tan and Manley 2010), splicing (Deloulme, Prichard et al. 1997; Knoop and Baker 2000; Chansky, Hu et al. 2001; Lerga, Hallier et al. 2001), micro-RNA (miRNA) processing (Gregory, Yan et al. 2004), RNA transport (Calvio, Neubauer et al. 1995; Zinszner, Sok et al. 1997), signalling (Andersson, Stahlberg et al. 2008) and maintenance of genomic integrity (Hicks, Singh et al. 2000; Spahn, Petermann et al. 2002; Li, Watford et al. 2007; Wang, Arai et al. 2008). Nevertheless, their particular functions remain poorly characterized.

1.1.3.1 Nucleic acid binding specificities of FET proteins

FET proteins have been shown to bind RNA as well as single-stranded and even double-stranded DNA (Crozat, Aman et al. 1993; Ohno, Ouchida et al. 1994; Prasad, Ouchida et al. 1994; Bertolotti, Lutz et al. 1996; Hackl and Luhrmann 1996; Zinszner, Sok et al. 1997; Perrotti, Bonatti et al. 1998), a capability found in a comparably small number of proteins (Cassiday and Maher 2002). Additionally, EWS has been found to specifically target G-quadruplex DNA and RNA *in vitro* via the arginine residue of its carboxy-terminal RGG-motif (Takahama, Kino et al. 2011). G-quadruplexes are DNA secondary structures formed by single-stranded, G-rich human telomeric DNA (Sen and Gilbert 1988; Dai, Carver et al. 2008). Intriguingly, FUS/TLS was also found to bind to telomeres (Dejardin and Kingston 2009). The sequence-specificity of FET protein binding to RNA and DNA has been studied by several research groups so far. Shortly after its identification, EWS was found to bind to poly G and poly U sequences via its carboxy-terminal RGG domain (Ohno, Ouchida et al. 1994). Successive studies established similar binding affinities for FUS/TLS. An *in vitro* systematic evolution of ligands by exponential enrichment (SELEX) experiment (Tuerk and Gold 1990) implied FUS/TLS to bind GGUG-containing RNAs through its RBD and RGG motifs

(Lerga, Hallier et al. 2001). Furthermore, FUS/TLS was recently found to bind small, single-stranded low-copy-number RNAs tethered to the promoter of cyclin D1 (Wang, Arai et al. 2008).

As mentioned above, FET proteins also bind single-stranded and double-stranded DNA. FUS/TLS was shown to be involved in D-loop formation, a process essential for DNA repair and homologous recombination (Baechtold, Kuroda et al. 1999; Bertrand, Akhmedov et al. 1999). DNA-binding of FET proteins is most likely mediated by the C_2/C_2 zinc finger, a domain frequently found in transcription factors. There are, however, reports of RBDs mediating DNA-binding in a sequence-specific manner (Ding, Hayashi et al. 1999). On the other hand, the FET zinc finger domain resembles those present in ZIS, which is a protein involved in splicing (Ladomery and Dellaire 2002). In addition, the results of Iko et al. strongly suggest the zinc finger domain of FUS/TLS to bind to RNA, whereas the RBD exhibited no apparent interaction (Iko, Kodama et al. 2004). The RGG-boxes present in FET proteins are thought to increase binding affinity rather than specificity and may serve as sites of post-translational modifications that regulate RNA binding (Burd and Dreyfuss 1994).

Correspondingly, Cabeza/SARFH was found to localize to sites of active RNA polymerase II transcription and could be shown to bind RNA *in vitro* involving the RBD (Immanuel, Zinszner et al. 1995; Stolow and Haynes 1995).

So far, little is known about the DNA/RNA binding specificities of TAF15 and still further investigations are required to learn more about the specificity, affinity and function of the nucleic acid-binding domains of the remaining FET proteins. Promising results concerning global RNA-targets for human FET proteins were recently obtained from large-scale PAR-CLIP experiments, which detected preferential binding of FET proteins near splice acceptor sites (Hoell, Larsson et al. 2011).

1.1.3.2 FET proteins and RNA Transcription

The initial connection between FET proteins and gene expression originated from the findings that the amino-terminal SYGQ-rich domains of EWS and FUS/TLS function as potent transcriptional activation domains in their respective oncogenic fusions (May, Lessnick et al. 1993; Prasad, Ouchida et al. 1994). Subsequently, the same region of TAF15 was shown to possess transactivation potential (Bertolotti, Bell et al. 1999). A plethora of scientific findings have since then supported the suggested role for FET family proteins in transcription.

1.1.3.2.1 Direct regulation of transcription by FET proteins

Transcription factor (TF) IID contains the TATA binding protein (TBP), various TBP associated factors (TAFs) in stoichiometric or sub-stoichiometric ratios, and non-TBP associated co-activators. The resulting variations in TFIID composition are thought to affect promoter choice, recruitment of processing factors and transcription (Brou, Chaudhary et al. 1993). Each of the FET proteins was found to co-purify with functionally distinct fractions of the TFIID complex in a sub-stoichiometric ratio strongly suggesting a common role in transcription initiation and elongation (Bertolotti, Lutz et al. 1996; Bertolotti, Melot et al. 1998). In fact, EWS and TAF15 have been found to contact the same TAFs in a direct protein-protein interaction assay but could not be co-purified, suggesting the presence of EWS and TAF15 in the same TFIID complex to be mutually exclusive (Bertolotti, Melot et al. 1998).

Furthermore, FUS/TLS, EWS and TAF15 have been shown to associate with subunits of RNA polymerase (RNAP) II itself thereby possibly affecting RNAPII mediated transcription (Bertolotti, Lutz et al. 1996; Bertolotti, Melot et al. 1998). Consistently, Cabeza/SARFH was also found to associate with transcribed chromatin in *Drosophila* (Immanuel, Zinszner et al. 1995) which further supports an evolutionarily conserved involvement of FET proteins in RNA transcription.

hsRPB7 is the seventh largest subunit of RNAPII and together with hsRPB4 forms a sub-complex that may function in promoter recognition, similar to the stress-specific prokaryotic σ factor (Petermann, Mossier et al. 1998). TAF15 was shown to bind directly to hsRPB3, hsRPB5 and hsRPB7, whereas germline EWS was only able to interact with hsRPB3 (Bertolotti, Melot et al. 1998). Carboxy-terminally truncated EWS, however, was shown to interact with hsRPB4 and hsRPB7 (Petermann, Mossier et al. 1998; Zhou and Lee 2001) (see model in figure 4).

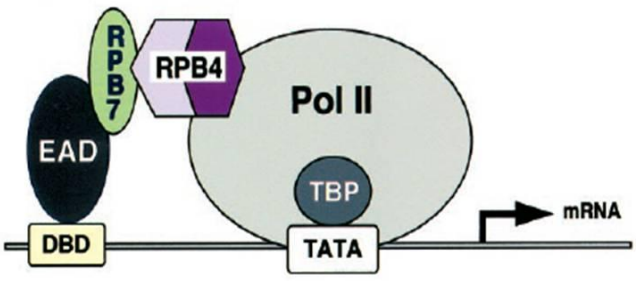


Figure 4. Model for EWS activation domain (EAD)-mediated transactivation. Only select components of the transcription complex are depicted for simplicity reasons. The EAD binds the promoter via the DNA binding domain of the fusion protein and directly contacts RPB7. Recruitment of RPB4/7 into RNAPII is required for efficient transactivation. EAD – EWS-activation domain; DBD – DNA-binding domain; RPB4/7 – RNA polymerase subunit B4/7; Pol II – RNA polymerase II; TBP – TATA-binding protein; TATA – TATA-box. (modified from Zhou and Lee, 2001)

Nevertheless, it is not clear whether the respective FET fusion proteins of EWS and TAF15 are also able to interact with RNAPII. Although the involved amino-terminal region of EWS is also present in EWS-FLI1, Bertolotti et al. could find no proof for an interaction between the fusion protein and RNAPII (Bertolotti, Melot et al. 1998). In contrast, the results of Petermann et al. suggest not only an interaction between EWS-FLI1 and RNAPII but they claim that overexpression of recombinant RBP7 causes increased EWS-FLI1 mediated transactivation (Petermann, Mossier et al. 1998). Indeed, the function of the amino-terminal domain of germline FET-proteins is still poorly characterized. Experiments involving artificial fusions of FET proteins and DNA-binding domains revealed that the FET RBD strongly and specifically inhibited transactivation of the AD *in cis* (Li and Lee 2000; Rossow and Janknecht 2001). Subsequently, it was demonstrated that multiple RGG-motifs within the RBD are not only sufficient to repress the AD *in cis* but can also achieve this effect *in trans* upon dimer formation (Alex and Lee 2005).

1.1.3.2.2 Indirect regulation of transcription by FET proteins

Besides directly associating to general transcription factors and RNAPII, FET proteins might possibly affect transcription regulation by addressing interacting activators or repressors. FUS/TLS has been identified to be one of a few proteins that bind to nuclear hormone receptors without diminishing their ability to bind DNA thus influencing transcription activation of various steroid and thyroid hormone and retinoid receptors (Powers, Mathur et al. 1998). The interaction between the transcription factor Spi-1/PU.1 and FUS/TLS *in vivo* reduces the DNA-binding and transactivating ability of Spi-1/PU.1 (Hallier, Lerga et al. 1998). Additionally, FUS/TLS has been reported to act as coactivator in NFκB-mediated transcription (Uranishi, Tetsuka et al. 2001). EWS, on the other hand, was found to have a positive effect on CBP/p300-dependent transcription activation via the transcription factors HNF-4 (Araya, Hirota et al. 2003) and OCT-4 (Lee, Rhee et al. 2005). HNF-4 is enriched in the liver and belongs to the nuclear receptor superfamily while OCT-4 is known to be involved in maintaining an undifferentiated totipotent state in germ cells and embryonic stem cells. The interaction between EWS and p300 is blocked by serine-threonine kinase receptor-associated protein (STRAP) which inhibits the activating effect on HNF-4 and OCT-4 (Anumanthan, Halder et al. 2006). In addition, EWS inhibits BRN3A-dependent transcription, which is thought to promote development of the nervous system (Thomas and Latchman 2002).

Hume et al. demonstrated that EWS binds directly to the promoter of the CSF-1 receptor (CSF1R) gene and the consensus binding sites for the myeloid zinc finger protein 1 (Mzf1) *in vivo* and *in vitro* whereas for FUS/TLS the same behaviour could only be observed *in vitro*. These findings

suggest proteins of the FET family to be involved in transcriptional start site recognition of TATA-less mammalian promoters (Hume, Sasmono et al. 2008).

As mentioned earlier, Takahama et al. established EWS as capable of binding G-quadruplexes which are known to be telomeric structures (Takahama, Kino et al. 2011) but have also been suggested to be formed by G-rich sequences near transcription start sites (Sen and Gilbert 1988; Verma, Halder et al. 2008). This implicates once more a role for FET proteins in transcription control. Indeed, the Akt/PKB substrate PRAS40 was recently identified as EWS target gene (Huang, Nakai et al. 2012). The PRAS40 3'UTR is G-rich and could potentially form a G-quadruplex structure. EWS was found to associate directly with PRAS40 3'UTR thus negatively regulating PRAS40 expression which resulted in reduced proliferation and metastatic potential of Ewing Sarcoma cell lines (Huang, Nakai et al. 2012).

Dominant point mutations in two RNA-binding proteins, TDP-43 and FUS/TLS, were found to cause a subset of familial amyotrophic lateral sklerosis (ALS), an incurable neurodegenerative disease affecting motoneurons (Kim, Shanware et al. 2010). Single nucleotide changes within the highly conserved last 13 amino acids of FUS/TLS resulted in increased cytoplasmic localization of aggregated FUS/TLS (Kwiatkowski, Bosco et al. 2009; Vance, Rogelj et al. 2009). The exact role of FUS/TLS in familial ALS remains to be elucidated, however, the convergent phenotypes resulting from mutations in FUS/TLS and TDP-43 point to their being elements of the same pathway. Indeed, FUS/TLS and TDP-43 were found to form a functional complex which co-regulates expression of histone deacetylase (HDAC) 6 mRNA (Kim, Shanware et al. 2010).

Interestingly, FUS/TLS represses transcription by RNAPIII via controlling the access of TBP and the RNAPIII machinery to target genes (Tan and Manley 2010). RNAPIII transcribes small untranslated RNAs, such as 5S rRNA and U6 snRNA. Thus, FUS/TLS now lists with a number of cancer-related factors like Rb and p53 that are capable of controlling multiple polymerases which suggests a more complex role for FET proteins in context of the transcriptome (Tan and Manley 2010).

1.1.3.3 FET Proteins and Splicing

FET proteins have been suggested to function in splicing almost as long as they have been known. FUS/TLS was identified by nano electrospray mass spectrometry as hnRNP P2 of the H complex that assembles specifically on pre-mRNAs *in vitro* (Calvio, Neubauer et al. 1995). Accordingly, FUS/TLS was subsequently found to specifically bind to the 3' splice site during the second step of pre-mRNA splicing (Wu and Green 1997). FUS/TLS and EWS were shown to

participate in the same complex with the splicing factor polypyrimidine-tract-binding-protein associated factor (PSF) (Deloulme, Prichard et al. 1997). Correspondingly, FUS/TLS was subsequently identified as component of a large protein complex containing hyperphosphorylated RNAPII, U1 snRNP, $p54^{nrb}$, PSF and various transcription elongation factors located at the 5' splice site (Kameoka, Duque et al. 2004) and – as already mentioned above - specifically binds to the GGUG motif commonly found in 5' splice sites (Lerga, Hallier et al. 2001). The presence of FET proteins in the spliceosome was confirmed involving large-scale purification of intact functional spliceosomes as well as mass spectrometric tools (Rappsilber, Ryder et al. 2002; Zhou, Licklider et al. 2002).

FET proteins were found to associate with a variety of SR proteins and other splicing factors. As mentioned earlier, all FET proteins associate with the splicing factor/transcriptional repressor SF1/ZMF1 (Zhang, Paley et al. 1998). FUS/TLS was shown to interact with the SR proteins TLS-associated serine-arginine protein (TASR) and SC35 via its carboxy-terminal RGG motifs (Yang, Embree et al. 1998; Lerga, Hallier et al. 2001). It also associates with SRp75, SRm160 and PTB (Meissner, Lopato et al. 2003) as well as the hnRNPs A1 and C1/C2 (Zinszner, Albalat et al. 1994). Moreover, EWS and EWS-FLI1 were proven to associate with U1C which – being one of three human U1 small nuclear ribonucleoprotein-specific proteins – interacts with various other splicing factors and plays an important role in spliceosome formation (Knoop and Baker 2000). The carboxy-terminal domains of EWS and FUS/TLS were found to interact with the splicing activator YB-1 thereby recruiting YB-1 to hyperphosphorylated RNAPII (Chansky, Hu et al. 2001). Dutertre and co-workers reported the interaction between EWS and YB-1 to be inhibited after exposure to various inducers of genotoxic stress, which resulted in the cotranscriptional skipping of several exons of the MDM2 gene coding for the main p53 ubiquitin ligase (Dutertre, Sanchez et al. 2010). TAF15 was currently found to participate in interactions with a subset of the spliceosomal U1 snRNP complex (Leichter, Marko et al. 2011) suggesting that the TAF15-U1 snRNP complex serves a similar function to the one proposed for TIA-1-U1 snRNP, which is thought to participate in the selection of a specific subset of pre-mRNAs for splicing (Forch, Puig et al. 2002).

The most recent results on this topic strongly support all of the above listed evidence for a role of FET proteins in pre-mRNA splicing. EWS depletion was reported to result in alternative splicing changes of genes involved in DNA damage repair and genotoxic stress signalling, including ABL1, CHEK2 and MAP4K2. Accordingly, depletion of EWS caused a reduction of cell viability and proliferation upon UV irradiation, an effect that was reversed upon restoration of c-ABL expression (Paronetto, Minana et al. 2011).

The idea of pre-mRNA processing and transcription as an integrated pathway rather than two separate processes has been established over the previous years (Bentley 1999; Hirose and Manley 2000; de Almeida and Carmo-Fonseca 2008; Moore and Proudfoot 2009) and it is very likely that they are linked by multifunctional proteins such as the FET family of proteins (Tan and Manley 2009). In the recent years considerable evidence was collected supporting the role of FET proteins as mediators between the individual processes of gene expression, which has already been discussed above to some extent. The amino-terminal domain of EWS was found to interact with BARD1 (Spahn, Petermann et al. 2002). BARD1 is known to be associated with BRCA1 and has been shown to interact with the polyadenylation factor CstF-50 (Kleiman and Manley 1999). The BARD1/BRCA1/CstF-50 complex mediates a transient inhibition of polyadenylation after DNA damage (Kleiman and Manley 2001; Kim, Li et al. 2006). Furthermore, FUS/TLS has been found to interact with CIP29, a protein associated with the DEAD box RNA helicase DDX39 (Sugiura, Sakurai et al. 2007). DEAD-box proteins, like DDX39, have been functionally implicated in diverse processes including RNA transcription, RNA splicing, mRNA transport, translation initiation and cell cycle regulation (Rocak and Linder 2004; Rosner and Rinkevich 2007). Interestingly, EWS-FLI1 but not EWS was found in association with RNA helicase A (Toretsky, Erkizan et al. 2006). FUS/TLS and Cabeza/SARFH have been found to localize to sites of active transcription and were suggested to function in transcriptional regulation together with other RNPs (Calvio, Neubauer et al. 1995; Immanuel, Zinszner et al. 1995). In addition, all human FET proteins have been found to be associated with distinct subpopulations of TFIID complexes (Bertolotti, Lutz et al. 1996; Bertolotti, Melot et al. 1998). Consequently, Kameoka et al. found FUS/TLS to be part of large RNAPII-snRNP complexes involving various transcription and splicing factors such as PSF, U1 snRNP and p54nrb (Kameoka, Duque et al. 2004).

1.1.3.4 FET proteins and RNA transport

FET proteins are localised mostly to the nucleus corresponding to their proposed functions in gene regulation and transcription (Hackl and Luhrmann 1996; Alliegro and Alliegro 1997; Zinszner, Immanuel et al. 1997; Andersson, Stahlberg et al. 2008). Hackl and Lührmann reported a subnuclear localization of TAF15 to speckles (Hackl and Luhrmann 1996) while Andersson et al. observed a smooth nuclear localization of FET family proteins in cultured human cells with FUS/TLS and TAF15 but not EWS showing also a diffuse distribution in the cytoplasm (Andersson, Stahlberg et al. 2008). Zinszner et al., on the other hand, reported a change in nucleic distribution of FUS/TLS from a diffuse pattern to an accumulation into dense nuclease-resistant aggregates upon inhibition of RNAPII mediated transcription (Zinszner, Immanuel et al. 1997). In

fact, there is evidence that FET proteins shuttle between the nucleus and the cytoplasm and are involved in RNA transport. FUS/TLS was identified as being identical to hnRNP P2 (Calvio, Neubauer et al. 1995) which corresponds to the observation that FUS/TLS shuttles between the nucleus and the cytoplasm in complexes containing mRNA and various hnRNPs (Zinszner, Sok et al. 1997). Besides, FUS/TLS was found to participate in mRNA sorting to dendritic spines upon mGluR5 activation in mouse hippocampal pyramidal neurons (Fujii, Okabe et al. 2005; Fujii and Takumi 2005). Thus, FET proteins are implicated in the distribution of cytoplasmic factors for local translation.

Belyanskaya and co-workers found EWS shuttling from the nucleus to the cell surface depending on its methylation status (Belyanskaya, Gehrig et al. 2001; Belyanskaya, Delattre et al. 2003). However, the relevance of this finding remains to be ascertained.

1.1.3.5 FET proteins and maintenance of genomic integrity

A close interdependence of transcription and genome stability has been observed in *S. cerevisiae*, where proteins that function at the interface of RNA metabolism and transcription affect genomic stability by regulating transcription-associated recombination (Luna, Jimeno et al. 2005).

Similarly, FUS/TLS was found to promote homologous DNA pairing and D-loop formation which are key processes in homologous recombination (Baechtold, Kuroda et al. 1999; Bertrand, Akhmedov et al. 1999). Subsequently, EWS was observed to interact with BRCA1-associated BARD1 (Spahn, Petermann et al. 2002). In addition, FET proteins together with the splicing factor PSF were shown to bind specifically to homologous DNA *in vitro* (Guipaud, Guillonneau et al. 2006) which hints to a role for FET proteins in the DNA repair pathway. This agrees with the above mentioned finding of Gardiner et al. that FUS/TLS but not FUS-CHOP is phosphorylated after induction of DNA damage (Gardiner, Toth et al. 2008). Correspondingly, the knockout of FUS/TLS in mice resulted in a pleiotropic effect on B-lymphocyte development, B-cell activation and genomic stability - resulting from defects in chromosomal pairing and enhanced radiation sensitivity – which is reminiscent of disruptions of *c-Abl* and *Atm* (Tybulewicz, Crawford et al. 1991; Hardin, Boast et al. 1995; Barlow, Hirotsune et al. 1996; Xu and Baltimore 1996; Hicks, Singh et al. 2000; Kuroda, Sok et al. 2000). Both, c-ABL and ATM kinase are activated upon DNA damage and target FUS/TLS which likely explains why FUS/TLS-deficient cells display high levels of genomic instability (Perrotti, Bonatti et al. 1998; Gardiner, Toth et al. 2008). Additionally, FUS/TLS is thought to link DNA repair and cell cycle by transcriptionally repressing cyclin D1 via

recruiting a non-coding RNA that inhibits CBP/p300 at the cyclin D1 promoter (Wang, Arai et al. 2008).

Accordingly, EWS knockout in mice resulted in a similar phenotype including defects in pre-B lymphocyte development, reduced meiotic recombination, defective gamete maturation and sensitivity to ionizing radiation (Li, Watford et al. 2007). However, EWS and FUS/TLS are not functionally redundant since molecular details of EWS and FUS/TLS deficiencies are different. EWS-null spermatocytes displayed reduced number of meiotic crossovers and high levels of unsynapsed XY sex chromosomes while FUS/TLS-knockout resulted in defective autosomal bivalent formation (Kuroda, Sok et al. 2000; Li, Watford et al. 2007). Decreased meiotic crossovers could likely be an indirect consequence of XY asynapsis which strongly suggests a direct role for EWS in meiotic recombination (Li, Watford et al. 2007). Furthermore, EWS deficiency resulted in infertility of both sexes while loss of FUS/TLS only caused male sterility (Kuroda, Sok et al. 2000; Li, Watford et al. 2007). In zebrafish, morpholino-induced knockdown of EWS orthologs caused developmental defects, apoptosis of pre-neural cells and embryonic lethality which was confirmed by results from HeLa cells, where siEWS also induced mitotic defects and apoptosis (Azuma, Embree et al. 2007). Together, these results implicate specific, non-redundant and evolutionarily conserved roles for FET family proteins in DNA repair and surveillance of genomic integrity.

1.1.3.6 FET proteins and miRNA processing

EWS has recently been suggested to be involved in the micro RNA (miRNA) mediated gene regulation since it was found in a complex together with the nuclear RNase III DROSHA, RNA helicases, doublestrand RNA-binding proteins and hnRNPs (Gregory, Yan et al. 2004). DROSHA cleaves primary (pri-)miRNA transcripts in the nucleus generating pre-miRNAs of about 60-70nt length thereby initiating miRNA processing and transport to the cytoplasm (Lee, Ahn et al. 2003). Subsequently, pre-miRNAs are processed by another RNase III enzyme, Dicer, to generate the final ~22nt miRNAs which guide RISC-mediated mRNA degradation (Bernstein, Caudy et al. 2001; Grishok, Pasquinelli et al. 2001; Hutvagner, McLachlan et al. 2001; Bartel 2004). Furthermore, FET proteins have been found to colocalize to stress granules with components of the miRNA processing machinery (Leung, Calabrese et al. 2006; Andersson, Stahlberg et al. 2008) which could hint to functions in the regulation of post-transcriptional gene expression during normal and stress-induced situations. Moreover, deregulation of miRNA-mediated gene-regulation has been implicated to play a major role in cancer development as miRNAs are key-regulators of many biological processes such as development, cell proliferation, differentiation and apoptosis

(Lagos-Quintana, Rauhut et al. 2003; Thomson, Newman et al. 2006; Sassen, Miska et al. 2008; Riedmann and Schwentner 2010). However, the functional significance of the association between EWS and DROSHA for the regulation of miRNAs remains to be determined.

Furthermore, FET proteins could also be involved in the generation of miRNAs via their involvement in the splicing process. Most miRNA genes are located in noncoding regions of the genome but about 25% of human miRNAs have been identified in introns of protein-coding genes (Bartel 2004; Ying and Lin 2004). Thus, one might speculate that FET proteins not only link transcription to splicing but also splicing to miRNA generation from gene introns (Kovar 2011).

1.2 Ewing Tumors

Sarcomas are a class of mostly sporadic, relatively rare but aggressive malignancies with a high tendency towards dissemination and relapse that constitute less than 10% of human malignancies but about 15% of paediatric cancers (Riggi, Cironi et al. 2007). The cellular origin of these tumors remains largely unknown but there is evidence that they develop from mesenchymal progenitor cells as a result of genetic alterations (Riggi, Cironi et al. 2007). Soft tissue sarcomas were found to affect more adults whereas bone sarcomas are more common in children and adolescents (Riggi, Cironi et al. 2007; Osuna and de Alava 2009). The identification of characteristic genetic aberrations for the specific sarcoma subtypes has advanced the scientific and clinical understanding of these tumors (Bennicelli and Barr 2002). The finding that sarcomas can rather be defined by their molecular pathology than by their organ or tissue of origin has enabled the development of more efficient targeted therapies (Helman and Meltzer 2003). Ewing sarcoma (ES) result from genomic translocations fusing the 5' part of a FET family protein to the 3' portion of an ETS-family transcription factor, thereby giving rise to an aberrant transcription factor (Law, Cann et al. 2006).

1.2.1 Pathogenesis, Characteristics and Tissue of Origin

Ewing sarcoma was first described by the American pathologist James Ewing (figure 5) in 1921 as a diffuse endothelioma of the bone (Ewing 1921). ES are the second most common bone malignancy in children and young adults and comprise a class of poorly differentiated tumors including Ewing sarcomas and primitive peripheral neuroectodermal tumors (PPNETs) (Riggi, Cironi et al. 2007). ES affects slightly more males than females (male: female ratio = 1.2:1) and is more frequent in Caucasians than Asians, African-Americans or Africans with a peak incidence around 15 years of age (Bernstein, Kovar et al. 2006; Jawad, Cheung et al. 2009). Typical sites of primary ES are the pelvic bones, the long bones of the lower extremities and the bones of the chest wall with primary metastases in lung, bone and/or bone marrow detectable in about 25% of patients (Bernstein, Kovar et al. 2006) (figure 6). The initial symptoms include intermittent strain-like pain and a palpable mass which often lead to misdiagnosis since the onset of symptoms was often reported to coincide with minor trauma

Figure 5. James Ewing, about 1890

occurring at approximately the same time (Widhe and Widhe 2000). Thus, plain radiograph is usually performed as initial measure, however, the most precise definition of the local extent of disease can be achieved by magnetic resonance imaging (figure 7) while the definitive diagnostic test still is biopsy (Widhe and Widhe 2000).

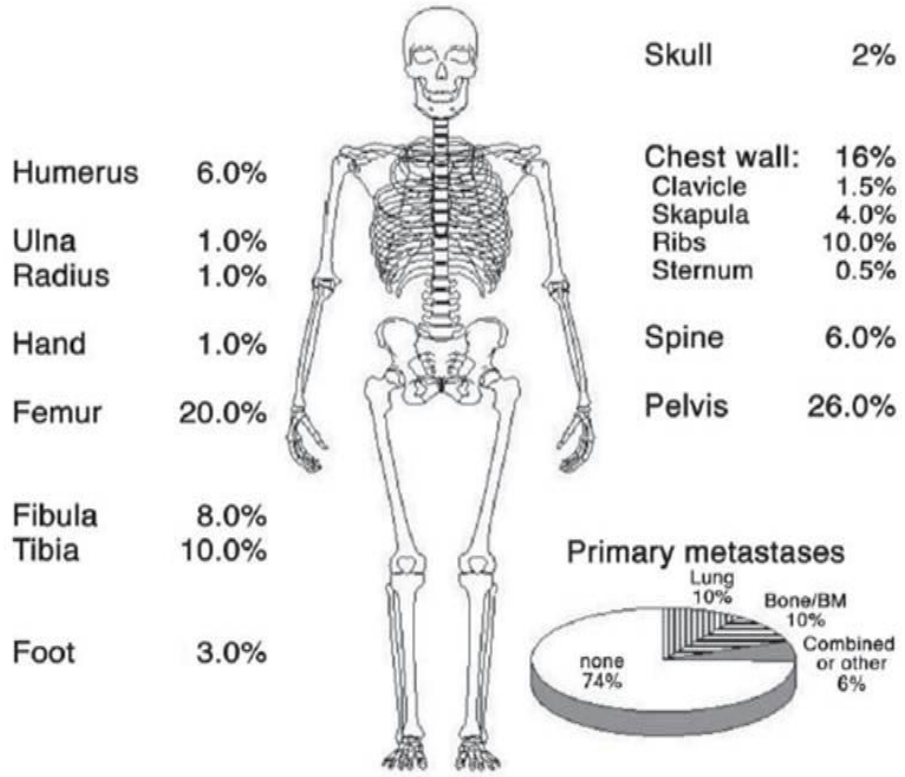


Figure 6. Primary tumor sites in Ewing tumors. (from Bernstein and Kovar, 2006)

Characteristically, ES consists of small round cells with a high nuclear to cytoplasm ratio that are arrayed in sheets (Bernstein, Kovar et al. 2006). Due to their similar cell morphology ES, neuroblastoma, alveolar rhabdomyosarcoma and lymphoblastic lymphoma are also referred to as small round blue cell tumors (Riggi, Cironi et al. 2007). Additionally, ES cells strongly express the cell surface marker p30/32^{MIC2} (CD99) and exhibit immunoreactivity for vimentin (Dierick, Roels et al. 1993; Weidner and Tjoe 1994). However, the histologic and immunophenotypic features of ES resemble to varying extent other paediatric small round blue cell tumors. Thus, detection of characteristic translocations and other genetic aberrations involving fluorescence in situ hybridisation (FISH) and/or RT-PCR are often necessary for definitive diagnosis.

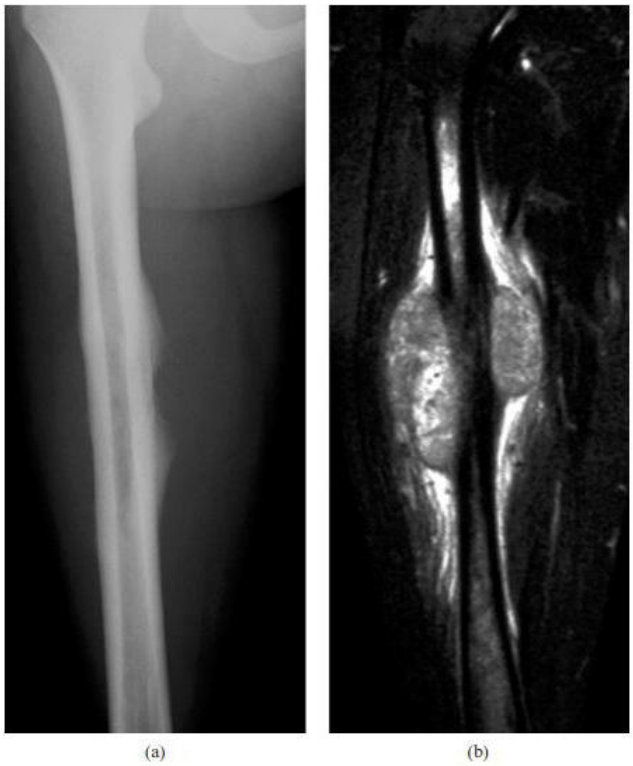


Figure 7. Ewing tumor in the femoral diaphysis. (a) anteroposterior radiograph. (b) Coronal short tau inversion recovery MRI. (O'Donnell 2003)

The karyotype of ES is relatively simple harbouring only few numerical and structural aberrations (Bernstein, Kovar et al. 2006). All ESs are characterized by reciprocal translocations resulting in the expression of one of several fusion proteins, the most prominent being EWS-FLI1 (Turc-Carel, Aurias et al. 1988; Delattre, Zucman et al. 1992) followed by EWS-ERG and the much less frequent EWS-ETV-1,EWS-E1AF, EWS-FEV and FUS-ERG (Sorensen, Lessnick et al. 1994; Kovar 1998; Law, Cann et al. 2006; Riggi, Cironi et al. 2007; Mackintosh, Madoz-Gurpide et al. 2010)(for more detail see also section 1.2.2). Additional numerical and structural aberrations with prognostic relevance have been observed in ES, the most frequent being gain of chromosomes 8 and 12, loss of 9p and 16q and the non-reciprocal translocation t(1;16)(q12;q11.2) causing gain of 1q (Mugneret, Lizard et al. 1988; Armengol, Tarkkanen et al. 1997; Hattinger, Rumpler et al. 1999; Lopez-Guerrero, Pellin et al. 2001; Hattinger, Potschger et al. 2002).

The introduction of EWS-ETS fusion proteins into diverse cellular systems evoked variant responses, reaching from growth arrest over induction of apoptosis to dedifferentiation (Thompson, Teitell et al. 1999; Deneen and Denny 2001; Lessnick, Dacwag et al. 2002; Zwerner, Guimbellot et al. 2003). Accordingly, the cellular context is a major determinant since development of ES requires cells with a certain degree of plasticity and permissiveness for EWS-FLI1-induced transformation. Nevertheless, the actual tissue of origin for ES is still unclear. Bone marrow human mesenchymal stem cells (MSCs) are considered a probable cellular source since most ESs occur in bone and soft tissue. In fact, the gene expression profile of ES cells after knockdown of EWS-FLI1 was most similar to that of mesenchymal progenitor cells (Tirode, Laud-Duval et al. 2007; Kauer, Ban et al. 2009). This corresponds well with the finding that expression of EWS-FLI1 in human MSCs induced a gene expression profile resembling that of ES (Riggi, Suva

et al. 2008). Kauer et al. obtained an EWS-FLI1-specific gene expression pattern from the comparison of EWS-FLI1 knockdown in five ES cell lines and 59 primary ESs using mesenchymal progenitor cells as a reference tissue (Kauer, Ban et al. 2009). Previously, Hancock et al. acquired an expression signature based on a meta-analysis of EWS-FLI1 dependent gene expression data from different models obtained from various expression profiling platforms (Hancock and Lessnick 2008). However, the resulting profiles of the two studies share only little significant overlap although the overall overlap between the gene lists affected by EWS-FLI1 is strong. This is most likely due to the use of different reference tissues (Kovar 2010).

Moreover, EWS-ETS fusion proteins were shown to block osteogenic and adipogenic differentiation which is consistent with the observed undifferentiated phenotype of Ewing tumor cells (Torchia, Jaishankar et al. 2003). The generation of ES-like tumors from murine MSCs expressing EWS-FLI1 has already been achieved in mice (Castillero-Trejo, Eliazer et al. 2005; Riggi, Cironi et al. 2005). Mouse bone marrow-derived mesenchymal progenitor cells (MPCs) were shown to maintain expression of retrovirally introduced EWS-FLI1 and formed tumors upon injection into severe combined immunodeficient (SCID) mice (Riggi, Cironi et al. 2005). These tumors were found to display hallmarks of ES, such as round cell phenotype, expression of CD99 and NSE, growth dependence to IGF-R1 and altered expression of several known EWS-FLI1 target genes (Riggi, Cironi et al. 2005). Suva and colleagues identified a subpopulation of $CD133^{+}$ tumor cells with the capability to initiate and sustain tumor growth through the course of serial transplantations in immunodeficient mice displaying plasticity similar to MSCs (Suva, Riggi et al. 2009). Very recently, a transgenic model of ES was established in zebrafish (Leacock, Basse et al. 2012). The expression of human EWS-FLI1 in *Danio rerio* resulted in tumors with ES-like histology and allowed for the identification of an EWS-FLI1 gene expression signature in zebrafish which disclosed substantial conservation of EWS-FLI1 targets between fish and human (Leacock, Basse et al. 2012). Taken together, these findings point to a mesenchymal cellular origin of ES although further research is necessary to confirm this.

	DNA-binding domain	Cancer	Translocation	Frequency
	CHOP	Myxoid liposarcoma	t(12;16)(q13;p11)	
	ERG	Acute myeloid leukemia	t(16;21)(p11;q22)	
FUS/TLS	ATF-1	Angiomatoid fibrous histiocytoma	t(12;16)(q13;p11)	
	CREB312/BBF2H7	Low-grade fibromyxoid sarcoma	t(7;16)(q33;p11)	
	ERG	Ewing tumors	t(16;21)(p11;q22)	<1%
	FLI1	Ewing tumors	t(11;22)(q24;q12	85%
	ERG	Ewing tumors	t(21;22)(q22;q12)	10%
	ETV1	Ewing tumors	t(7;22)(q22;q12)	<1%
	ETV4/E1AF	Ewing tumors	t(17;22)(q12;q12)	<1%
	FEV	Ewing tumors	t(2;22)(q33;q12)	<1%
	CHOP	Myxoid liposarcoma	t(12;22)(q13;q12)	
EWSR1	ATF-1	Malignant melanoma of soft parts/ soft tissue clear cell sarcoma	t(12;22)(q13;q12)	
	WT1	Desmoplastic small round cell tumor	t(11;22)(q13;q12)	
	ZSG	Small round cell sarcoma	t(1;22)(p36.1;q12)	
	POU5F1 (OCT3/4)	Undifferentiated bone sarcoma	t(6;22)(p21;q12)	
	CHN/TEC	Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12)	
	CIZ/NMP4	Acute leukemia	t(12;22)(p13;q12)	
TAF15	CHN/TEC	Extraskeletal myxoid chondrosarcoma	t(9;17)(q22;q11.2)	
	CIZ/NMP4	Acute leukemia	t(12;17)(p13;q11)	

Table 1. Fusion genes of FUS/TLS, EWS and TAF15 in human cancer. Relative frequencies of gene fusions in ES as indicated. (modified from Law and Cann, 2006)

1.2.2 The fusion proteins of Ewing sarcoma

The predominant genetic feature of 85% of ESs is a chromosomal translocation, t(11;22)(q24;q12), which fuses two genes, EWSR1 and Friend leukemia integration 1 (FLI1) (Turc-Carel, Aurias et al. 1988; Delattre, Zucman et al. 1992). Of the remaining cases approximately 10-15% are characterised by the fusion of EWS and ERG and 1-5% express EWS-ETV-1, EWS-E1AF, EWS-FEV or FUS-ERG (Sorensen, Lessnick et al. 1994; Kovar 1998; Law, Cann et al. 2006; Riggi, Cironi et al. 2007; Mackintosh, Madoz-Gurpide et al. 2010) (table 1). EWS belongs to the FET family of RNA-binding proteins and has already been described in detail (see section 1.1). The FLI1 gene locus was first identified as the integration site of Friend murine leukemia virus (Ben-David, Giddens et al. 1991). FLI1 is a member of the ETS family of transcription factors which activate their target genes by binding to purine-rich GGA(A/T) DNA sequences through their conserved ETS domains in cooperation with other transcription factors and co-factors (Wasylyk, Hahn et al. 1993; Oikawa and Yamada 2003). The amino-terminus of FLI1 consists of one pointed domain that mediates protein-protein interactions and one FLI1 specific transactivation domain with a helix-loop-helix structure (Rao, Ohno et al. 1993). The carboxy-terminal domain includes the ETS DNA binding domain and a second, weaker transactivation domain (Rao, Ohno et al. 1993)(see also figure 8). The amino- and carboxy-terminal transactivation domains achieve their maximal transactivation potential through intramolecular interaction (Rao, Ohno et al. 1993). Depending on the cellular context and the respective promoter FLI1 can act as a transcriptional

repressor or activator, however, more genes containing the consensus FLI1 binding site are up-regulated (Rao, Ohno et al. 1993). In contrast, subsequent studies of the EWS-FLI1 fusion protein revealed a predominant negative regulatory function of the FLI1 carboxy-terminal transactivation domain (Arvand, Welford et al. 2001). FLI1 is expressed in hematopoietic cell lineages and vascular endothelial cells (Oikawa and Yamada 2003). Additionally, it is involved in erythroid, myelomonocytic and NK cell development (Masuya, Moussa et al. 2005) and acts as a key regulator of megakaryocytic differentiation (Jackers, Szalai et al. 2004). The target genes of FLI1 include oncogenes, tumor suppressor genes and genes with functions in apoptosis, differentiation, angiogenesis and invasion (Sharrocks 2001; Oikawa and Yamada 2003; Asano, Markiewicz et al. 2009).

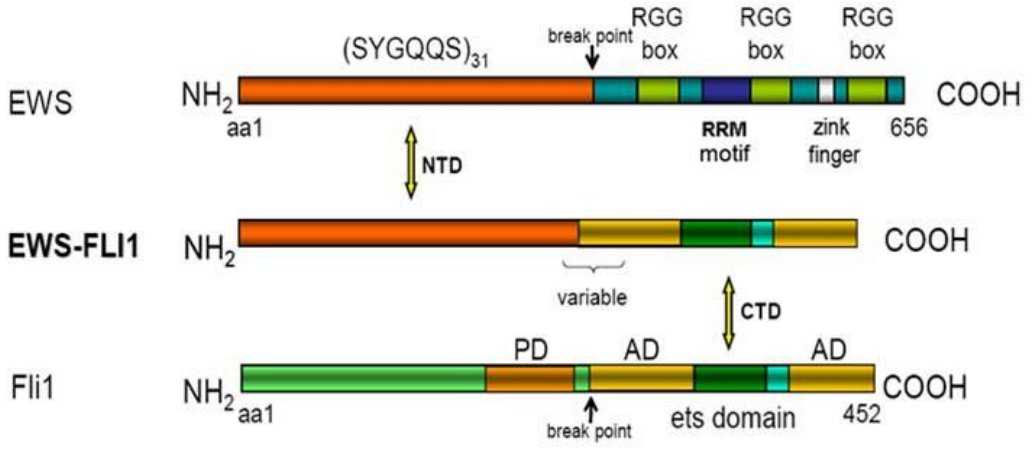


Figure 8. Structure of the EWS-FLI1 fusion protein. The amino-terminal domain of EWS is fused to the carboxy-terminal domain of FLI1. Due to variable break point positions in both genes the resulting protein has a variable fusion region and length. FLI1 protein structure: PD - amino-terminal pointed domain; AD - FLI1 specific activation domain; ets-domain - carboxy-terminal ETS-domain. For detailed depiction of EWS protein structure see figure 3. (figure modified from Bachmaier 2007)

The predominant and best characterized genetic fusion of a FET protein and an ETS transcription factor in ES is EWS-FLI1 (Delattre, Zucman et al. 1992; Zucman, Delattre et al. 1992). The fusion gene is under control of the EWS promoter on der(22), the reciprocal translocation product, FLI1-EWS, is not expressed and is even occasionally lost from ES cells (Bernstein, Kovar et al. 2006). Several different in frame fusion genes have been described resulting from variant breakpoint positions, the most prevalent being type I between EWS exon 7 and FLI1 exon 6 and type II between EWS exon 7 and FLI1 exon 5 (Delattre, Zucman et al. 1992; Zucman, Melot et al.

1993; Zoubek, Dockhorn-Dworniczak et al. 1996; de Alava, Kawai et al. 1998) (figure 8). It has been observed that the transcription factor partners seem to be linked to the specific tumor type while the EWS or FUS parts are functionally interchangeable and show no such association (Zinszner, Albalat et al. 1994; Aman, Panagopoulos et al. 1996; Li, Watford et al. 2007). The transformation capability of EWS-ETS fusions varies in different cellular backgrounds: NIH3T3 and bone marrow-derived mesenchymal progenitor cells were transformed by EWS-FLI1 in contrast to human or rat primary fibroblasts, mouse embryonic stem cells and embryonic fibroblasts [(Thompson, Teitell et al. 1999; Torchia, Jaishankar et al. 2003; Zwerner, Guimbellot et al. 2003; Riggi, Cironi et al. 2005) for review see also (Kovar 2005)]. Additionally, EWS-ERG was shown to induce neoplasia from lineage-committed haematopoietic cells (Codrington, Pannell et al. 2005).

1.2.2.1 Transcriptional functions of ES fusion proteins

EWS-FLI1 is a potent transcriptional modulator with unique properties which corresponds to its predominantly nuclear localization (Yang, Chansky et al. 2000). Although EWS-FLI1 was found to be able to dimerize and also weakly interact with germline EWS it binds to DNA as monomer (Spahn, Siligan et al. 2003) and in collaboration with additional factors, such as Fos-Jun (AP-1) or RHA (Kim, Denny et al. 2006; Toretsky, Erkizan et al. 2006). The generation of gene expression profiles from ES samples and ES cell lines enabled the definition of EWS-FLI1-specific signatures using mesenchymal progenitor cells as reference (Tirode, Laud-Duval et al. 2007; Kauer, Ban et al. 2009). Kauer and colleagues found the majority of EWS-FLI1 up-regulated genes to relate to cell cycle and cell proliferation whereas genes with functions in cell differentiation and morphogenesis were generally down-regulated which reflects the characteristic undifferentiated phenotype of ES (Kauer, Ban et al. 2009). This corresponds well with the finding that silencing of EWS-FLI1 restored plasticity of ES cells towards adipogenic, neuronal and osteogenic differentiation (Tirode, Laud-Duval et al. 2007) whereas ectopic expression of EWS-FLI1 in mesenchymal stem cells blocked differentiation potential and induced the initial steps towards ES development [(Riggi, Cironi et al. 2005; Riggi, Suva et al. 2008) (for a summary of validated direct targets of EWS-FLI1 see also table 2)]. Furthermore, promoter regions of EWS-FLI1 activated genes have been found to be enriched for E2F, NRF1 and NFY binding motifs whereas a

EWS-FLI1 activated genes	Consequences of target suppression	References
Id2	Not known	(Fukuma, Okita et al. 2003)
GLI1	Reduced anchorage independent growth	(Beauchamp, Bulut et al. 2009; Joo, Christensen et al. 2009)
VEGF	Decreased osteolysis	(Fuchs, Inwards et al. 2004; Guan, Zhou et al. 2009)
STYXL1	Not known	(Siligan, Ban et al. 2005)
PLD2	Inhibition of PDGF BB signalling	(Kikuchi, Murakami et al. 2007)
PTPL1	Reduced growth, increased chemosensitivity	(Abaan, Levenson et al. 2005)
CAV1	Reduced anchorage independent growth, reduced tumorigenicity	(Tirado, Mateo-Lozano et al. 2006)
GSTM4	Abrogation of oncogenic transformation, increased chemosensitivity	(Luo, Gangwal et al. 2009)
NR0B1	Abrogation of oncogenic transformation	(Kinsey, Smith et al. 2006; Mendiola, Carrillo et al. 2006; Kinsey, Smith et al. 2009)
EZH2	Reduced anchorage independent growth, reduced tumorigenicity	(Richter, Plehm et al. 2009)
AURKA, AURKB	Not known	(Wakahara, Ohno et al. 2008)
Tenascin C	Not known	(Watanabe, Nishimori et al. 2003)
EWS-FLI1 repressed genes	**Consequences of target restoration**	**References**
TGFBR2	Loss of tumorigenicity	(Hahm, Cho et al. 1999)
CDKN1A	Inhibition of cell growth	(Nakatani, Tanaka et al. 2003)
IGFBP3	Inhibition of cell growth and motility	(Prieur, Tirode et al. 2004; Benini, Zuntini et al. 2006)
FOXO1	Not known	(Yang, Hu et al. 2010)
DKK1	Decreased tumorigenicity	(Miyagawa, Okita et al. 2009; Navarro, Agra et al. 2010)

Table 2. Validated direct EWS-FLI1 target genes (modified from Kovar, 2011)

variety of DNA sequence motifs was found in repressed genes. This can be interpreted as suggestive for a less complex transcriptory regulation upstream of EWS-FLI1 up-regulated genes and points to a more direct role of EWS-FLI1 in gene activation than in gene repression (Kauer, Ban et al. 2009). Indeed, EWS-FLI1 has been previously identified as an activating transcription factor *in vitro* (May, Lessnick et al. 1993; Bailly, Bosselut et al. 1994). However, the mechanism of target selection of oncogenic ETS fusion proteins is poorly understood. EWS-FLI1 was found to bind specifically to GGAA microsatellite sequences that are significantly enriched in EWS-FLI1 regulated genes such as NR0B1 and GSTM4 thus suggesting a mechanism to direct fusion protein activity (Gangwal, Sankar et al. 2008; Luo, Gangwal et al. 2009). Correspondingly, ChIP-Seq revealed GGAA microsatellites and *bona fide* ETS binding site containing regions as EWS-FLI1 binding regions in ES cells (Guillon, Tirode et al. 2009). Interestingly, it was suggested that the lack of GGAA microsatellites in murine genes might be the reason for persisting problems in the establishment of genetically engineered mouse models of ES, since the expression of EWS-FLI1 would not up-regulate critical genes required for oncogenic transformation, e.g. murine Nr0b1

and Cav1 (Kinsey, Smith et al. 2006; Tirado, Mateo-Lozano et al. 2006; Gangwal, Sankar et al. 2008).

1.2.2.2 Post-transcriptional functions of ES fusion proteins

Besides its relatively well studied function as aberrant transcriptional regulator, EWS-FLI1 has been implicated in post-transcriptional processing, too. Functional dissection studies of EWS-FLI1 in NIH3T3 transformation assays first suggested that the minimal transforming and the minimal transcriptional activation domains can be separated from each other (Lessnick, Braun et al. 1995). Subsequently, the fusion protein was shown to retain residual transforming activity after destruction of the DNA-binding domain by point mutations or deletions (Jaishankar, Zhang et al. 1999; Welford, Hebert et al. 2001). In addition, EWS-FLI1 was found to achieve an antiapoptotic effect through interference with CBP-dependent transcriptional activity of RXR which appears to be independent of DNA binding (Ramakrishnan, Fujimura et al. 2004).

The amino-terminal domains of the ES fusion proteins have been demonstrated to interact with the same RNA processing factors as their germline counterparts thus potentially interfering with the respective functions (Yang, Embree et al. 1998; Zhang, Paley et al. 1998; Knoop and Baker 2000; Yang, Chansky et al. 2000; Chansky, Hu et al. 2001; Knoop and Baker 2001). EWS-FLI1 and FUS/TLS-ERG inhibit YB-1 mediated splicing as they fail to recruit the splicing factor to RNAPII (Chansky, Hu et al. 2001). Accordingly, EWS and FUS/TLS were shown to recruit SR splicing factors to hyperphosphorylated RNAPII thereby coupling RNA transcription to splicing. EWS-FLI1 as well as FUS/TLS fusions interferes with this function as they retain the ability to associate with RNAPII but fail to recruit SR proteins (Yang, Embree et al. 1998; Yang, Chansky et al. 2000). The splicing factor U1C, which is important in early stages of spliceosome formation, associates with EWS as well as with EWS-FLI1 and was shown to repress EWS-FLI1-mediated transactivation. This illustrates the connecting function of EWS/EWS-FLI1 between transcriptional and post-transcriptional processes (Knoop and Baker 2000). Furthermore, EWS-FLI1, but not EWS, was shown to interfere with hnRNP A1-dependent 5' splice site selection *in vivo* which was found to coincide with the transforming activity of the fusion protein (Knoop and Baker 2001). Quite recently, EWS-FLI1 was found to influence splicing of the protooncogene cyclin D1 (CCND1) favouring the oncogenic splice isoform D1b over D1a by decreasing the elongation rate of RNAPII (Sanchez, Bittencourt et al. 2008). This indicates that the fusion protein's role in post-transcriptional processing may not be regarded as transcription independent.

As already mentioned before, the effects of FET fusion proteins depends on the cellular background. Expression of EWS-FLI1 induced p53-dependent cell cycle arrest in primary human fibroblasts (Lessnick, Dacwag et al. 2002) and most other cell types were not found permissive to EWS-FLI1 expression (for review see (Kovar 2005)). This can be explained by the triggering of the p53 checkpoint through the oncogenic stress imposed by fusion transcript expression (Ban, Bennani-Baiti et al. 2008). ES circumvent this by modulating p53 activity either directly (Li, Tanaka et al. 2010) or via interference with the tumor suppressive NOTCH signalling pathway (Ban, Bennani-Baiti et al. 2008). Additionally, there is evidence for EWS-FLI1-mediated interference with other principal tumor suppressor pathways since knockdown of EWS-FLI1 resulted in hypophosphorylation and functional activation of pRb-1 thus evoking a senescence-like phenotype in ES cells (Hu, Zielinska-Kwiatkowska et al. 2008).

1.2.3 Prognostic factors and novel therapeutic approaches

ES is the second most frequent primary bone malignancy affecting mostly children and adolescents during their second decade of life. Since its first description in 1921 (Ewing 1921) efforts have been taken to elucidate the mechanisms of this disease in order to fuel prognostic and therapeutic progress and thus improve the outcome for the patients.

1.2.3.1 Prognosis

To date, a number of factors with prognostic value have been identified for ES. Besides responsiveness to chemotherapy (Jurgens, Exner et al. 1988), tumor volume and location (Gehan, Nesbit et al. 1981; Glaubiger, Makuch et al. 1981; Jurgens, Exner et al. 1988; Koscielniak, Jurgens et al. 1992; Abudu, Davies et al. 1999) the structure of the fusion transcript has been considered a major determinant of prognosis (de Alava, Kawai et al. 1998; Lin, Brody et al. 1999). At least 12 alternative in-frame fusions of EWS and FLI1 resulting from different genomic breakpoints have been clinically documented (Zucman, Melot et al. 1993). The two most frequently observed fusion types between EWS exon 7 and FLI1 exon 6 (type I) and EWS exon 7 and FLI1 exon 5 (type II) represent about 60% and 25%, respectively, of EWS-FLI1 fusions (Zucman, Melot et al. 1993; Zoubek, Dockhorn-Dworniczak et al. 1996; de Alava, Kawai et al. 1998). EWS-FLI1 type I was suggested to be transactivationally less active resulting in a less aggressive tumor phenotype (Lin, Brody et al. 1999). However, this could not be confirmed by the prospective evaluation of fusion transcript structure in 565 patients which abrogated the

status of the fusion gene as independent prognostic marker due to the lack of statistical significance (Le Deley, Delattre et al. 2010).

Furthermore, the presence of metastasis has proven to be a most unfavourable prognostic feature (Cangir, Vietti et al. 1990; Paulussen, Ahrens et al. 1998; Cotterill, Ahrens et al. 2000). About 25% of patients are diagnosed initially with metastatic disease (Bernstein, Kovar et al. 2006). The worst chances of survival are associated with bone or bone marrow metastases (≤20%) as opposed to pulmonary metastases, which have a slightly better outcome (about 30% survival)(Paulussen, Ahrens et al. 1998). In comparison, localised ES results in relapse-free survival of about 70% (Potratz, Dirksen et al. 2012). However, despite improved therapeutic measures, about 30% of patients experience relapse, either locally, distantly or combined which is tantamount to dismal prognosis (Bernstein, Kovar et al. 2006). The risk for relapse is significantly higher for patients with primary metastatic disease (Cotterill, Ahrens et al. 2000) and the chance for long-term survival after relapse is reduced to 20-25% with timing and type of recurrence being crucial prognostic determinants (Rodriguez-Galindo, Billups et al. 2002). The worst prognosis is associated with early (after less than two years) or combined local and distal recurrence (Shankar, Pinkerton et al. 1999; Rodriguez-Galindo, Billups et al. 2002). Recently, gene expression signatures were defined that discriminate between patients with fatal etiopathology or more favourable prognosis allowing for the identification of patients with ES nonresponsive to current treatments (Scotlandi, Remondini et al. 2009).

Moreover, a number of molecular prognostic factors have been defined so far. The before mentioned gain of 1q was shown to have predictive value adverse overall and event-free survival (Hattinger, Potschger et al. 2002). Loss of 16q was found to be closely associated with disseminated disease (Hattinger, Potschger et al. 2002). However, the most predominant chromosomal aberrations present in 50-60% of ESs, gain of chromosome 8 and chromosome 12, have never manifested prognostic value in clinical studies (Hattinger, Rumpler et al. 1999). The transcription factor p53 is one of the most prominent tumor suppressors disrupted in various malignancies (Sherr and McCormick 2002). Still, p53 mutations are relatively rare in ES and can be found in about 10% of cases (Huang, Illei et al. 2005). Nevertheless, alterations in p53 specify a subset of ES with markedly poor clinical outcome (de Alava, Antonescu et al. 2000) which is also the case for homozygous deletions of the CDKN2A locus ($p16^{INK4}$/$p14^{ARF}$)(Tsuchiya, Sekine et al. 2000) or a combination of the two aberrations (Huang, Illei et al. 2005). Additionally, 20% overall survival at 2 and 5 years for patients with overexpression of p53 was opposed by 85% 2-year survival and 71% 5-year survival of patients with normal p53 status (Abudu, Mangham et al. 1999). The relative rarity of p53 aberrations in ES suggests that they are not essential for primary

oncogenesis but may - as second hit-alterations - define a tumor subset with particularly aggressive behaviour, poor chemoresponse and thus dismal prognosis (Abudu, Mangham et al. 1999). Low expression levels of $p27^{KIP1}$, as can be found in about 76% of ESs, were shown to be associated with poor event-free survival (Matsunobu, Tanaka et al. 2004). Forced expression of $p27^{KIP1}$ reduced phosphorylated pRb as well as cell growth and promoted apoptosis in ES cells (Matsunobu, Tanaka et al. 2004). Malfunction of the transcription factor c-Myc is often implicated in cancer development and in fact, expression of c-Myc is strongly induced by EWS-FLI1 which leads to an activation of CDK4 and Id2 transcription (Dauphinot, De Oliveira et al. 2001). Furthermore, the G1 cyclin-CDK complex inhibitor $p57^{KIP2}$ (Dauphinot, De Oliveira et al. 2001) and the tumor suppressor $p21^{WAF1}$ (Maitra, Roberts et al. 2001) were found to be downregulated in ESs, the prognostic significance of this is however unclear.

1.2.3.2 Treatment

Current treatment of ES involves primary chemotherapy which is followed by local therapy, comprising either surgery or radiotherapy or both (Dunst, Sauer et al. 1991), and adjuvant chemotherapy (Bernstein, Kovar et al. 2006). Planning of the optimal local therapy requires an interdisciplinary team of experienced experts since local treatment should be individually adapted depending on the site and size of the tumor (Bernstein, Kovar et al. 2006). For patients with recurrent disease there is no established treatment procedure. Usually, a combination of multiagent chemotherapy and local control measures is administered (Jurgens, Exner et al. 1988; Paulussen, Ahrens et al. 1998; Paulussen, Ahrens et al. 1998; Rodriguez-Galindo, Billups et al. 2002). However, Chemotherapy options depend on the patient's prior treatment and are limited by potential impairment of organ function (Bernstein, Kovar et al. 2006).

Alternative therapeutic strategies that counteract the neoplastic process have been proposed and are being tested in preclinical models [for review see (Potratz, Jurgens et al. 2012)]. The novel approaches can be subdivided aiming at one of six possible mechanisms of carcinogenesis (Olsen, Tarantolo et al. 2002): First, disruption of chimeric transcription factor activity; second, prevention of post-translational modifications stimulating cellular growth; third, recovering function of tumor suppressors; fourth, inhibition of angiogenesis; fifth, induction of apoptotic pathways and sixth, introduction of toxic gene products.

Since the EWS-ETS fusion genes are unique to ES cells they pose potentially ideal therapeutic targets. Several approaches to disrupt EWS-FLI1 function have been reported including antisense methods (Ouchida, Ohno et al. 1995; Kovar, Aryee et al. 1996; Tanaka, Iwakuma et al. 1997;

Toretsky, Connell et al. 1997; Matsumoto, Tanaka et al. 2001; Asami, Chin et al. 2008), short hairpin RNA (shRNA) (Nozawa, Ohno et al. 2005; Garcia-Aragoncillo, Carrillo et al. 2008; Herrero-Martin, Osuna et al. 2009) and small interfering RNA (siRNA) (Chansky, Barahmand-Pour et al. 2004; Prieur, Tirode et al. 2004; Takigami, Ohno et al. 2011) which lead to increased apoptosis, reduced invasiveness and growth inhibition *in vitro* and in xenografted tumors. Two challenges remain to be solved regarding these approaches: First, efficient delivery of the reagents to the tumor site (Lambert, Bertrand et al. 2000; Hu-Lieskovan, Heidel et al. 2005; Toub, Bertrand et al. 2006; Elhamess, Bertrand et al. 2009) and second, possible long term side effects (Grimm, Streetz et al. 2006).

Furthermore, a number of different approaches to disrupt EWS-FLI1 function have been suggested. For instance, small pharmacological compounds have been found to either interfere with the expression of EWS-FLI1 downstream targets (Mateo-Lozano, Gokhale et al. 2006; Stegmaier, Wong et al. 2007; Grohar, Woldemichael et al. 2011) or block one of its protein-protein interactions (Erkizan, Kong et al. 2009) thereby reducing xenograft growth [for review see also (Metallo 2010)]. For instance, high-throughput screens identified mithramycin and cytosine arabinoside (ARA-C) as inhibitors of ES growth either by modulating EWS-FLI1 activity or fusion protein abundance, respectively (Stegmaier, Wong et al. 2007; Grohar, Woldemichael et al. 2011). Rapamycin was found to significantly delay tumor development *in vivo* (Mateo-Lozano, Gokhale et al. 2006) and the S-enantiomer of YK-4-279 was shown to specifically inhibit the interaction of EWS-FLI1 with RHA thus inducing apoptosis in ES cells (Erkizan, Kong et al. 2009; Barber-Rotenberg, Selvanathan et al. 2012).

Another example is the inhibition of the above mentioned O-linked-β-N-acetylglucosaminylation of EWS-FLI1 which was shown to interfere with the transactivation of the downstream target Id2 (Bachmaier, Aryee et al. 2009). With further investigations to better understand the physiological mechanisms behind this process it constitutes a probable basis for novel therapies against ES.

Promising results have been achieved with the MDM2 antagonist Nutlin-3a (Pishas, Al-Ejeh et al. 2011; Sonnemann, Palani et al. 2011). Since p53 mutations are relatively rare in ES the activation of wild-type p53 function could be a promising therapeutic strategy. The E3 ubiquitin ligase MDM2 negatively regulates the activity and protein levels of the tumor suppressor p53. Nutlin-3a blocks the interaction of MDM2 and p53 thus stabilizing the tumor suppressor protein which induces p53-dependent apoptosis.

Additionally, blockage of the autocrine IGF-1/IGF-1R loop by antagonistic antibodies has also been shown to inhibit ES cell growth *in vivo* (Scotlandi, Benini et al. 1998; Toretsky and Gorlick 2010). However, Garofalo and colleagues found that insulin receptor (IR)-A causes an intrinsic and adaptive resistance to anti-IGF-1R drugs suggesting that tumors with a low IGF-1R: IR-A ratio might not respond well to IGF-1R-directed therapies (Garofalo, Manara et al. 2011).

Another therapeutic alternative is the blockage of tumor vessel formation. Vascular endothelial growth factors (VEGFs) are key regulators of angiogenesis and vasculogenesis and have been found to be overexpressed in various tumors which seems to be correlated with adverse prognosis (Ferrara 2004). Blocking of VEGF receptor 2 with the antibody DC101 disrupted VEGF165 signalling thus reducing tumor vessel density and tumor growth (Zhou, Bolontrade et al. 2007). Furthermore, EWS-FLI1 and VEGF165 were shown to increase receptor activator NF-κB ligand (RANKL) promoter activity thereby contributing to the osteolytic process induced by ES cells. Thus, ES-associated osteolysis could possibly be reduced by DC101 treatment (Guan, Zhou et al. 2009).

Deregulation of miRNA expression, too, has been implicated in cancer development (Calin and Croce 2006; Thomson, Newman et al. 2006; Ma and Weinberg 2008; Sassen, Miska et al. 2008; Garzon, Calin et al. 2009). In fact, EWS-FLI1 and miR-145 were only recently identified to form a feedback loop where the fusion protein represses expression of the miRNA to which it is itself a target (Riggi, Suva et al. 2010; Ban, Jug et al. 2011). Consequently, targeting miRNAs which affect fusion protein activity might provide another avenue to ES therapy.

A critical determinant for solid tumor growth is adaptation to hypoxia, which modulates many aspects of tumor biology and response to therapy (Wilson and Hay 2011). In ES, hypoxia was found to transiently enhance EWS-FLI1 expression (Aryee, Niedan et al. 2010) thus offering new possibilities for innovative therapeutic methods.

Finally, Ordonez et al. have also suggested immunotherapeutic approaches involving both, specific antibodies and/or cellular therapy mediated by natural killer (NK) cells, as an optional future treatment for ES patients (Ordonez, Osuna et al. 2009). ES cells have proven to possess some unique immunologic features, such as complete or partial absence of class I and class II HLA which is mainly associated with advanced tumor stages (Berghuis, de Hooge et al. 2009). Moreover, although ES cells are specifically resistant to the cytolytic death-receptor pathway, they retain sensitivity to the perforin/granzyme pathway (de Hooge, Berghuis et al. 2007). However, further studies are required to gain insight into the connection between ES development and the immune system before immunotherapy for ES becomes feasible.

2. Hypothesis and Aims of the Study

Generally, only one allele of EWS (or FUS/TLS) is rearranged with an ETS transcription factor in ES resulting in co-expression of the fusion protein and germline EWS. So far, there is only one known case of ES where the expression of endogenous EWS was lost from the cancer cells (Kovar, Jug et al. 2001). Furthermore, EWS is co-expressed and has been found to interact with its oncogenic derivative (Spahn, Siligan et al. 2003). Based on this finding in addition to the current knowledge of FET protein function (as summarized in section 1.1) the following hypothesis was established:

EWS plays a role in mRNA transcription and processing and EWS-FLI1 interferes with this normal EWS function in a competitive or dominant negative manner. This interference is thought to contribute to the oncogenic activity of EWS-FLI1.

To get a better understanding of the functional interaction or competition between EWS and EWS-FLI1 and to define its impact on mRNA transcription and processing in the ET background, we aimed at the characterisation of a EWS-specific transcriptomic signature. To this end, two independent model systems were established from two different ES cell lines: the before mentioned unique ET cell line lacking endogenous EWS was studied upon stable restoration and re-knockdown of EWS expression (EWS-restoration model or EWS-RM), and an ET cell line with stable EWS knockdown was analyzed upon inducible RNAi-mediated EWS-FLI1 suppression (EWS-knockdown model or EWS-KDM). These systems provided the means to study the influence of modulated EWS expression on genome wide transcript expression and exon composition. The EWS-RM was the main focus of the study, while the EWS-KDM was designed to serve validation purposes. Affymetrix HuEx-1.0stv2 exon array analysis of both models yielded a pangenomic picture of alternatively/aberrantly spliced/transcribed RNAs. Validation of microarray data concerning the influence of EWS on exon skipping was achieved by RT-qPCR. Differentially expressed genes depending on the presence of EWS were examined by 5’ RACE PCR to exclude the possibility of EWS dependent differential transcription start site selection as a potential cause of altered transcript stability to account for the observed differential RNA expression effect.

3. Results

3.1 Experimental system and cellular models: EWS-restoration model and EWS-knockdown model

To explore the influence of EWS on the ES transcriptome and the EWS-FLI1 signature an experimental strategy was developed tailored for the investigation of a possible effect of EWS on genome wide transcript expression and exon composition (figure 9). This strategy was based on two different ES cell lines, STA-ET-7.2 and A673, which were transformed in order to enable for the modulation of EWS and EWS-FLI1 expression. The resulting cellular model systems were termed EWS-reconstitution (EWS-RM) and EWS-knockdown model (EWS-KDM), respectively (figure 10.A and 10.B). Overall gene expression and exon composition were studied on Affymetrix Hu-Ex1.0 stv2 arrays and validated by RT-qPCR and 5'-RACE PCR.

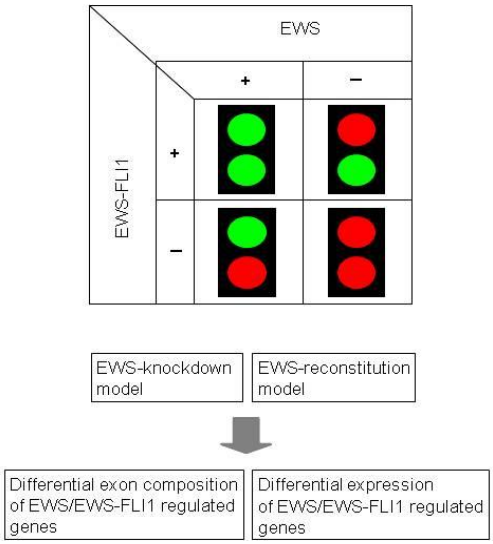


Figure 9. Experimental system. Miniature traffic lights indicate modulations of EWS and EWS-FLI1

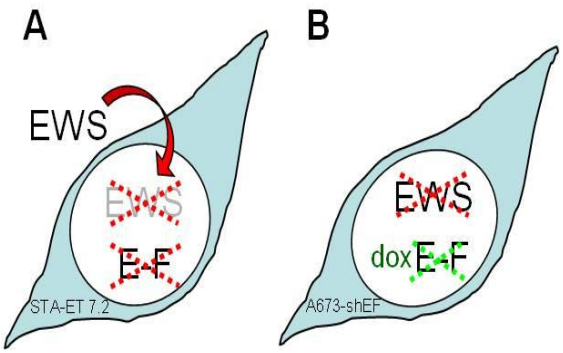


Figure 10. Cellular models. **(A)** EWS-restoration model (EWS-RM) established in the STA-ET-7.2 ES cell line; lack of endogenous EWS expression was compensated by reconstitution of stable EWS expression; re-knockdown of EWS and knockdown of EWS-FLI1 was achieved by transient transfection of shRNA-expression vectors. **(B)** EWS-knockdown model (EWS-KDM) based on the A673-shEF cell line; clones with disrupted EWS expression were obtained from stable transfections with shRNA-expression vectors; knockdown of EWS-FLI1 was induced by Doxicyclin.

The predominant cellular model of this study, the EWS-RM, was based on a unique ES cell line, STA-ET-7.2, that was first described by Kovar and co-workers (Kovar, Jug et al. 2001). In this cell line, the rearranged chromosome has been duplicated while the intact chromosome 22 was lost.

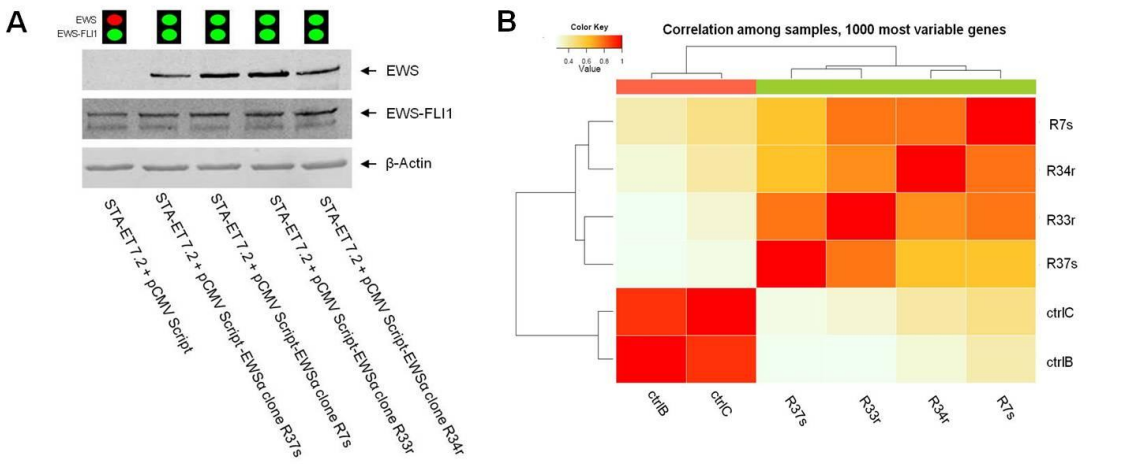


Figure 11. EWS-restoration model. (A) Immunoblot analysis of four stable clones of STA-ET-7.2 cells with restored EWS expression (R37s, R7s, R33r and R34r) compared to a polyclonal culture of STA-ET-7.2 cells transfected with the empty vector (pCMV Script) as a control (ctrlB and ctrlC). Approximately physiological protein levels of EWS were detected. **(B)** Hierarchial clustering of the six samples derived from the STA-ET 7.2 reconstitution system shows high within-group correlation between clones (+EWS) and control samples (-EWS).

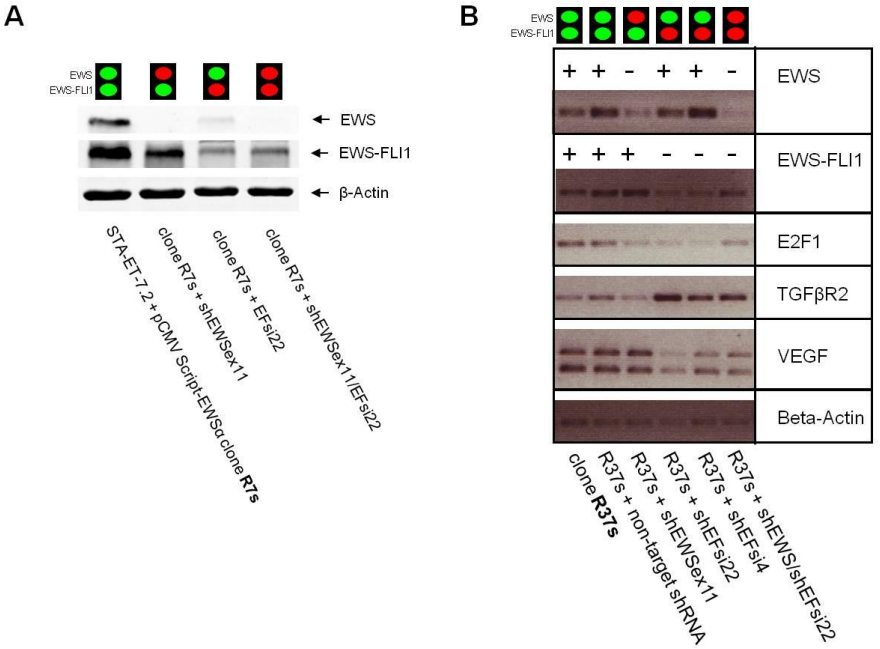


Figure 12. Re-knockdown of EWS from the EWS restoration model. (A) Immunoblot analysis of protein levels in clone R7s upon knockdown of EWS and/or EWS-FLI1 as indicated. Silencing efficiency of shEWSex11 was extremely high with low off-target effects on EWS-FLI1 protein levels. Silencing of EWS-FLI1 generally caused a reduction of EWS protein levels, which was however less pronounced in other experiments. **(B)** Semi-quantitative PCR analysis of clone R37s including three validated EWS-FLI1 target genes after knockdown of EWS and/or EWS-FLI1 as indicated. E2F1 and VEGF are EWS-FLI1-activated, TGFβR2 is an EWS-FLI1-repressed gene. EWS and EWS-FLI1 RNA levels are significantly reduced upon introduction of the indicated shRNA-constructs. TGFβR2 and VEGF exhibit expression patterns corresponding to the respective EWS-FLI1 expression status. E2F1, however, seems to be reduced upon EWS silencing while additional knockdown of EWS-FLI1 effects an increase in E2F1 RNA levels.

Thus, only the oncogenic fusion protein, EWS-FLI1, is expressed while endogenous expression of EWS is abolished. Reconstitution of EWS expression was achieved by transfection and selection of stable clones carrying the pCMV-Script-EWSα vector. Four clones (R7s, R37s, R33r and R34r) with approximately physiological levels of EWS expression were selected for further analysis (figure 11.A). Polyclonal cultures of STA-ET-7.2 cells stably transfected with the empty pCMV Script vector served as negative controls for this model (ctrlB and ctrlC). The hierarchial clustering shown in figure 11.B depicts the high within-group correlation between the four selected clones and the two control samples.

As an internal control, shEWSex11, a shRNA targeting the 5'region of EWS, was designed to enable for re-knockdown of EWS. Additionally, two already established shRNAs targeting EWS-FLI1, shEFsi22 and shEFsi4 (Kovar, Ban et al. 2003), were used to modulate fusion gene expression within this system. Knockdown of EWS and EWS-FLI1 was confirmed by Immunoblot analysis (figure 12.A) and semi-quantitative RT-PCR of validated EWS-FLI1 target genes (figure 12.B). Knockdown efficiency on the protein level was monitored by Immunoblot analysis for all examined samples. A slight reduction of EWS protein levels upon transfection with shRNA targeting EWS-FLI1 could be observed in all experiments and was interpreted to result from stress induced changes in protein synthesis due to oncogene deprivation. However, the comparably low level of EWS protein upon silencing of EWS-FLI1 observed in clone R7s (figure 12.A) was singular to the displayed experiment. Semi-quantitative RT-PCR was used to verify knockdown efficiency on RNA level for EWS and EWS-FLI1 directly, and downstream of EWS-FLI1 by additionally testing for three validated targets of the fusion gene – E2F1, VEGF (both activated by EWS-FLI1) and TGFβR2 (repressed by EWS-FLI1). A significant reduction of EWS and/or EWS-FLI1 RNA levels was detected upon transfection with the corresponding shRNA-expressing vectors (figure 12.B). Two EWS-FLI1 downstream targets, VEGF and TGFβR2, exhibited the expected expression patterns. However, E2F1 mRNA levels seemed to be increased upon double-knockdown of EWS and EWS-FLI1, while silencing of EWS alone reduced the expression of E2F1, which was observed in all examined clones (figure 12.B)

The second cellular model, the EWS-KDM, was designed as a validation system for the data obtained from the EWS-RM. It was established in the ES cell line A673-shEF containing a Doxicyclin-inducible shRNA targeting EWS-FLI1, that was originally obtained from the group of Javier Alonso, Unidad de Tumores Solidos Infantiles, Instituto de Salud Carlos III, Majahonda, Spain (Carrillo, Garcia-Aragoncillo et al. 2007). The original ES cell line, A673, co-expresses EWS and EWS-FLI1 in contrast to the STA-ET-7.2 cell line. From this cell line two stable clones (KD7s

and KD10s) with knockdown of EWS were obtained. Knockdown efficiency was again confirmed by Western blot analysis (figure 13). A673 cells stably expressing a shRNA targeting GFP were used as control for A673-shEF cells, and A673-shEF cells stably transfected with either empty pSUPER.retro.puro vector or a non-target shRNA were used as controls for the stable transfectants gained from this system.

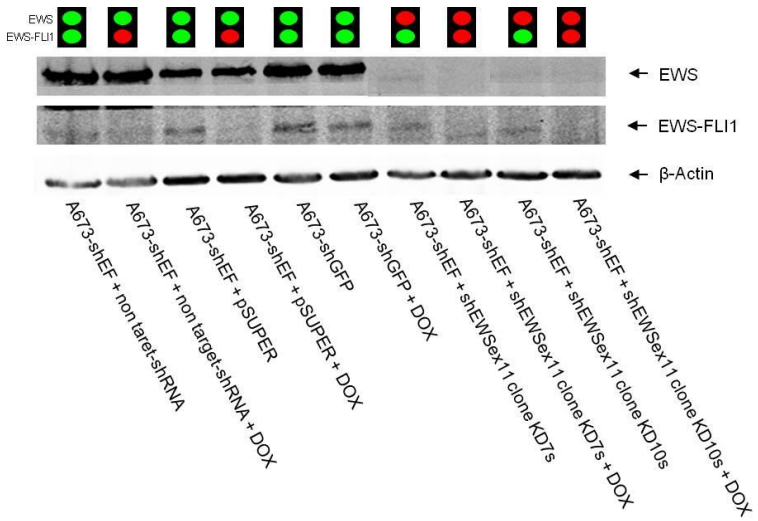


Figure 13. EWS-knockdown model. Two stable clones with disrupted EWS expression (KD7s and KD10s) were analysed on Immunoblot upon Doxicyclin-induced knockdown of EWS-FLI1 as indicated. A673 cells stably expressing a shGFP and A673-shEF cells transfected with non target-shRNA and empty vector (pSUPER. retro.puro), respectively, served as controls.

3.2 Phenotypic effects of EWS

Expression of EWS-FLI1 is the main characteristic feature and indispensable driver of sustained ES cell growth (Kovar, Zoubek et al. 1994). RNAi-mediated suppression of EWS-FLI1 expression results in growth inhibition, reduced viability and loss of tumorigenicity of ES cells (Ouchida, Ohno et al. 1995; Kovar, Aryee et al. 1996; Toretsky, Connell et al. 1997; Kovar, Ban et al. 2003). After the finding that germline EWS is dispensable for ES growth (Kovar, Jug et al. 2001), we were interested in the physiological consequences of EWS expression in STA-ET-7.2 cells. For this purpose, proliferation assays and colony formation assays were performed to monitor the effects of EWS under adherent as well as anchorage independent conditions.

3.2.1 Proliferation Assays

To determine whether EWS affects the growth of ES cells we compared the growth rates of STA-ET-7.2 cells transfected with empty pCMV Script vector as a control and three clones stably expressing EWS (R7s, R37s and R33r) for a period of 96 hours. No significant growth advantage could be detected for cells expressing EWS in adherent culture (figure 14) which recapitulates the observations previously published by Kovar and colleagues (Kovar, Jug et al. 2001).

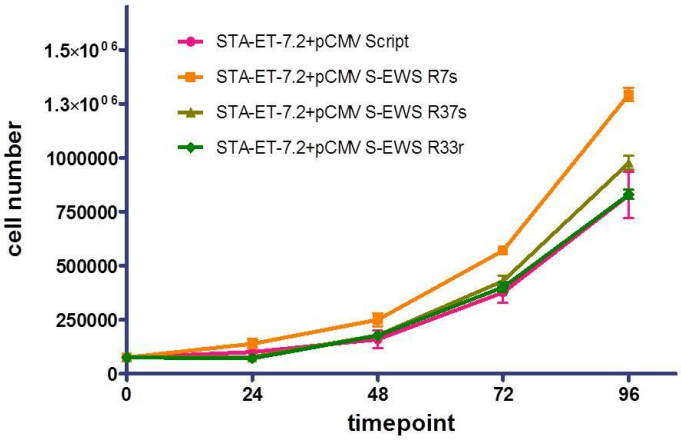


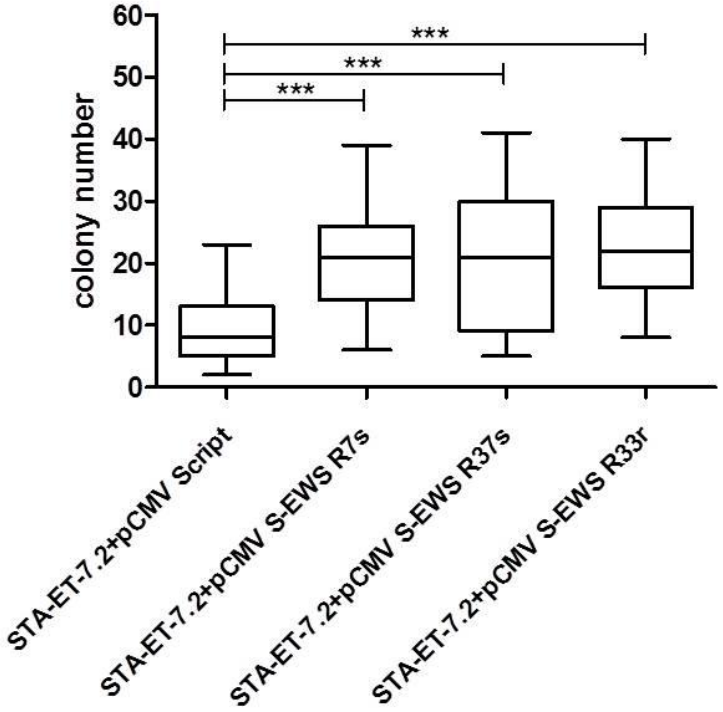
Figure 14. EWS expression does not affect anchorage dependent growth of STA-ET-7.2 cells. Growth rates of 3 EWS-expressing STA-ET-7.2 clones (R7s, R37s, R33r) were compared to control cells transfected with empty vector (pCMV Script) in 24 hour-intervals over a time span of four days. EWS conferred no detectable advantage for anchorage-dependent growth to STA-ET-7.2 cells. Cell numbers were counted using a Bürker-Türk chamber; standard deviations of three independent experiments, each performed in triplicate.

3.2.2 Colony Formation Assays

Additionally, we tested whether EWS has an effect on anchorage-independent growth of ES cells. STA-ET-7.2 cells stably transfected with empty vector were compared to three stable clones with restored EWS expression (R7s, R37s and R33r). A statistically highly significant effect could be observed with EWS-expressing cells forming almost thrice as many colonies than the control (figure 15.A and B). This effect could be reversed upon re-knockdown of EWS. All three clones were transiently transfected with either non-target shRNA or shRNA targeting EWS. A significant reversal of the growth advantage conveyed by EWS was detected as can be seen exemplary for clone R37s (figure 15.C and D).

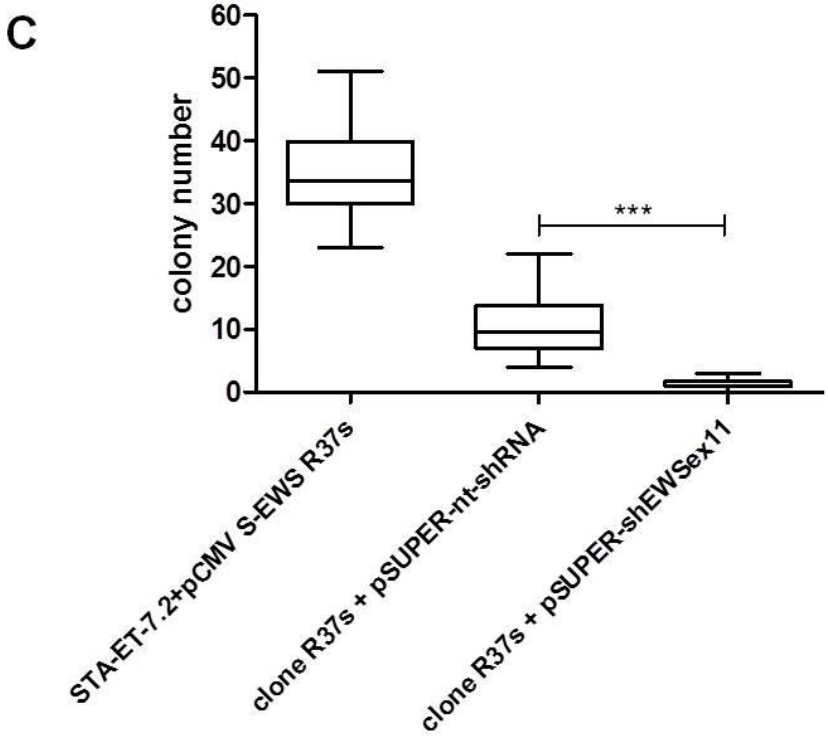
Taken together, the results from the functional assays are consistent with the suggested unique, non-redundant functions of FET proteins as compared to the housekeeping role that was attributed to this protein family in earlier studies, since they seem to be involved in specific processes, such as conveyance of anchorage-independent growth, rather than in the general maintenance of cellular functions.

A


colony number
60
50
40
30
20
10
0
***
***
***
STA-ET-7.2+pCMV Script
STA-ET-7.2+pCMV S-EWS R7s
STA-ET-7.2+pCMV S-EWS R37s
STA-ET-7.2+pCMV S-EWS R33r

B


STA-ET 7.2 + pCMV Script
STA-ET 7.2 + pCMV Script-EWS R7s
STA-ET7.2 + pCMV Script-EWS R37s
STA-ET7.2 + pCMV Script-EWS R33r



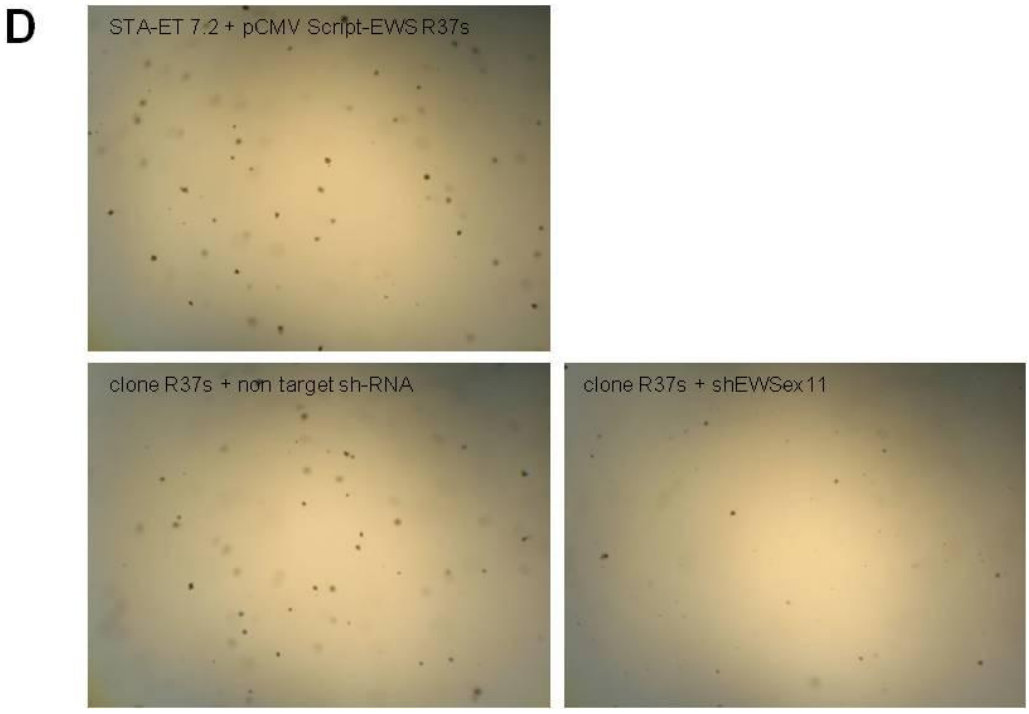


Figure 15. EWS expression affects anchorage independent growth in soft agar colony formation assays. (A) Restoration of EWS expression effected a significant increase of clonogenicity of STA-ET-7.2 cells as could also be observed in the microscope **(B)**. Re-knockdown of reconstituted EWS leads to a reversion of the observed effect **(C)** and **(D)**. Colonies were counted 17-20 days after seeding. **(A)(C)** Standard deviations resulting from three independent experiments, each performed in triplicate; statistical relevance was assessed involving the unpaired t-test. **(B)(D)** pictures taken at 50x magnification.

3.3 Microarray analyses

In order to acquire a global picture of the EWS- and EWS-FLI1-effect on transcriptional and post-transcriptional regulation, microarray analysis was performed. All experiments involving samples obtained from both cellular model systems (see table 3 for a list of samples) were hybridized on Affymetrix Hu-Ex1.0 stv2 chips. Figure 16 depicts the basic layout of these microarrays: With approximately four probes per exon and 40 probes per gene on average, this microarray platform enables two complementary levels of analysis—gene expression as well as exon composition. Hence, using this microarray platform, the analysis can be focused either on whole transcripts or on single exons. These two analyses can be merged into a "splicing" analysis where exons that deviate in their expression from whole-transcript expression are detected thus allowing for the detection of different isoforms of specific genes. Additionally, the Affymetrix Hu-Ex1.0 stv2 microarrays provide the possibility to analyse expression levels of whole transcripts by summarizing multiple probes on different exons into an expression value of all transcripts from the same gene. The data analyses are described in detail in the materials and methods section 5.2.7. "Exon graphs" were chosen to visualize our data retrieved from microarray analysis (see figure 17 for an explanatory schematic representation).

EWS-restoration model	
STA-ET-7.2 + pCMV Script	*Clone R37s + pSUPER-nt shRNA*
STA-ET-7.2 + pSUPER-nt shRNA	*Clone R37s + pSUPER-shEWSex11*
STA-ET-7.2 + pSUPER-shEFsi4	*Clone R37s + pSUPER-shEFsi4*
	Clone R37s + pSUPER-shEWSex11/pSUPER-shEFsi22
STA-ET-7.2 + pCMV Script-EWSα clone R7s	*Clone R33r + pSUPER-nt shRNA*
STA-ET-7.2 + pCMV Script-EWSα clone R37s	*Clone R33r + pSUPER-shEWSex11*
STA-ET-7.2 + pCMV Script-EWSα clone R33r	*Clone R33r + pSUPER-shEFsi4*
	Clone R33r + pSUPER-shEWSex11/ pSUPER-shEFsi22
EWS-knockdown model	
A673-shGFP	A673-shEF + pSUPER-shEWSex11 clone KD7s
A673-shGFP + DOX	A673-shEF + pSUPER-shEWSex11 clone KD7s + DOX
A673-shEF + pSUPER.retro.puro	A673-shEF + pSUPER-shEWSex11 clone KD10s
A673-shEF + pSUPER.retro.puro + DOX	A673-shEF + pSUPER-shEWSex11 clone KD10s + DOX
A673-shEF + pSUPER-nt shRNA	
A673-shEF + pSUPER-nt shRNA + DOX	

Table 3. Microarray samples. RNA samples obtained from stable clones as well as transient transfections (italics) were subjected to microarray analysis on Hu-Ex1.0 stv2 chips. +DOX indicates Doxicyclin-induced knockdown of EWS-FLI1 in the A673-shEF ES cell line; nt shRNA – non target shRNA; shEWSex11 – shRNA targeting EWS; shEFsi4/shEFsi22 – shRNAs targeting EWS-FLI1.

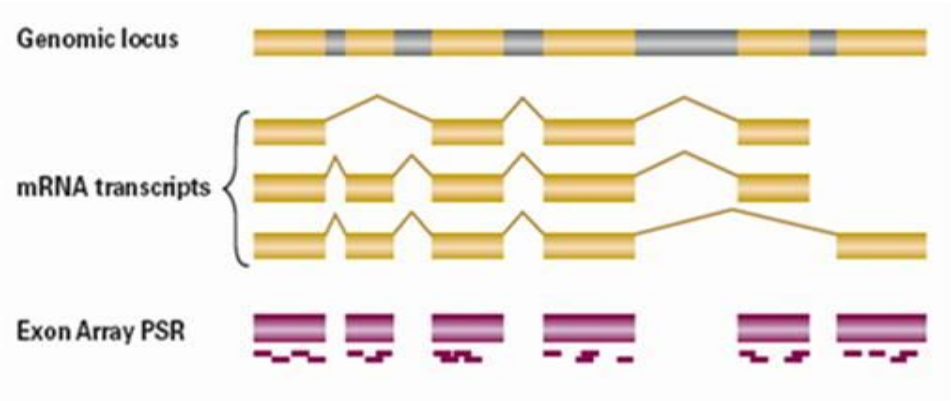


Figure 16. Scheme of microarray analysis. Approximately four exon-centric probes per exon constitute one probe set; one gene is defined by forty specific probes on average. PSR – probe selection region. Diagram by Affymetrix, Santa Clara, CA.

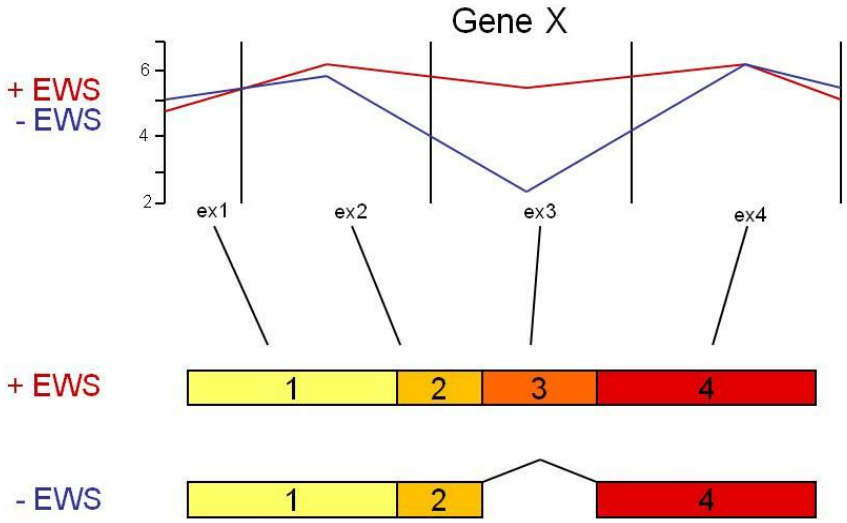


Figure 17. Schematic representation of an exon graph. In the upper panel, a basic graph with four sections representing exons 1-4 of gene X can be seen. The red and blue line depict relative expression levels of these exons with or without expression of EWS, respectively. The resulting alternative transcripts are schematically represented in the lower part of the figure.

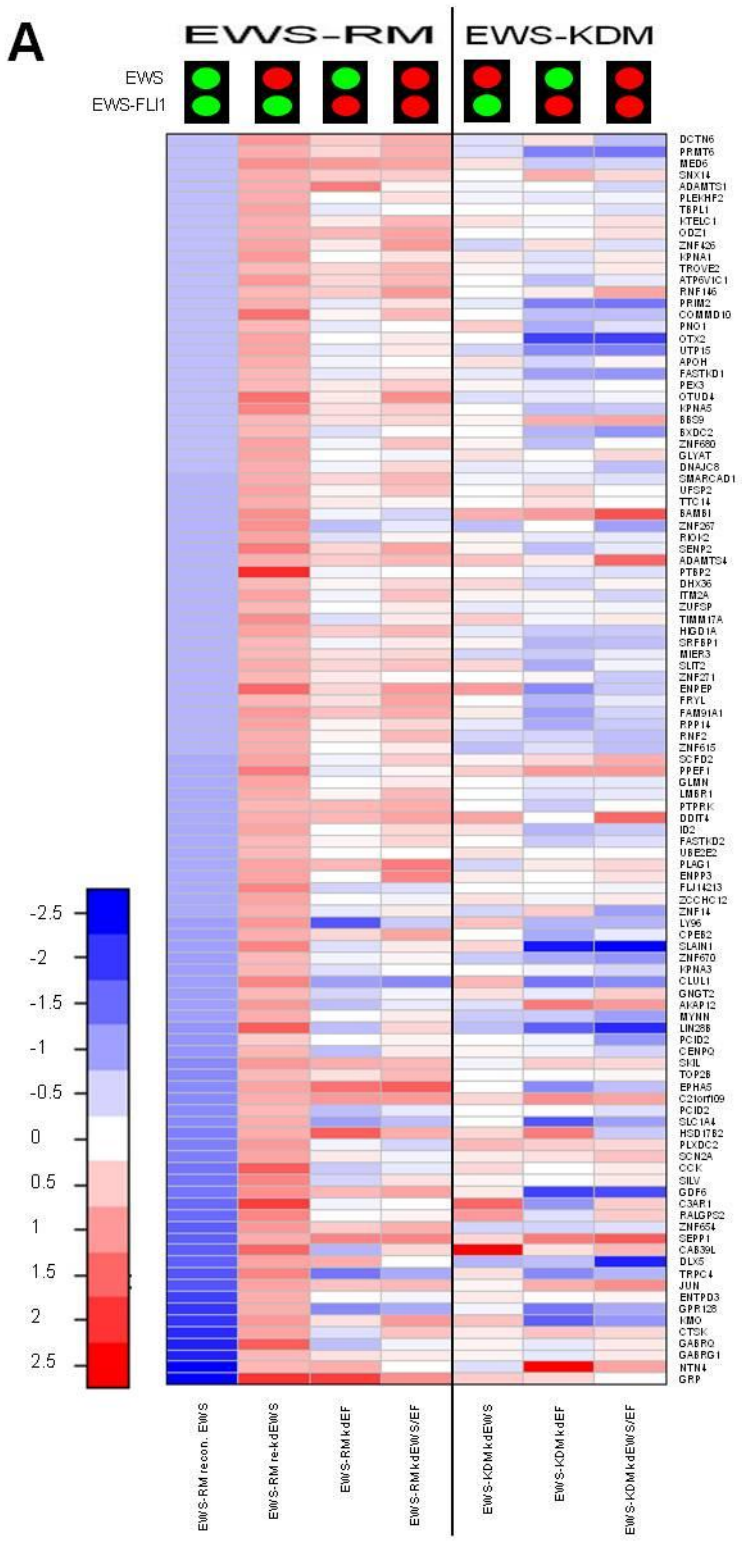
Utilizing the Affymetrix Hu-Ex1.0 stv2 microarray platform the following analyses were performed: First, analysis of expression level changes of whole transcripts in dependence of EWS and/or EWS-FLI1. Second, analysis of EWS and/or EWS-FLI1-dependent differential exon composition.

3.3.1 Analysis of whole transcript expression

In total, we found 689 genes up- and 694 genes down-regulated upon restored EWS expression in STA-ET-7.2 cells with a fold change of at least 1.5 fold for a p-value cutoff of <0.3. Re-knockdown of EWS from the EWS-RM effected negative expression changes in 320 genes and increased expression in 673 genes (see figure 18.A and B, columns 1-4, for top ranking examples). The effect of restored EWS expression could be reversed in 32 of the EWS-activated and in 117 of the EWS-repressed genes. Hence, for approximately 150 genes the gene expression changes induced by reconstitution of EWS expression could be rescued by the silencing of ectopically expressed EWS in this model system. This confirms the EWS-specificity of the observed effects. The effect of EWS-FLI1 silencing on the selected genes from the EWS-RM did not show a clear tendency of activation or repression (figure 18.A and B, column 3). Nevertheless, certain genes, such as CAB39L, DDIT4, BAMBI, UCP2 and LGR5, exhibited EWS- and EWS-FLI1-dependent expression patterns matching one of two possible models of functional cooperation between EWS and EWS-FLI1 (see also figure 19 and the last paragraph of this section).

Subsequently, we aimed for validation of these results by analysis of the EWS-KDM. However, the data overlap between the two cellular systems was very small. No significant effect of EWS in the EWS-KDM could be detected for genes selected from the EWS-RM (figure 18.A and B, columns 5-7; see also figure 21 and section 3.3.1.1).

From the total transcript expression data two subsets of genes were defined which in their EWS and EWS-FLI1 dependent expression patterns matched with one of two alternative models of functional relationship between EWS and EWS-FLI1. Genes such as C3AR1 and LGR5 are repressed respectively induced by EWS while EWS-FLI1 influences their expression to the opposite effect. Thus, they conform to a “functional competition model” in which EWS and EWS-FLI1 both directly influence the expression of target genes in opposite directions (figure 19.A). The expression patterns of genes like SULF1 and PLCXD1 on the other hand were found to correspond to a “functional interference model”, where EWS-FLI1 interferes with the gene regulatory function of EWS (figure 19.B).


A
EWS-RM
EWS-KDM
EWS
EWS-FLI1
-2.5
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
2.5
EWS-RM recon. EWS
EWS-RM re-kdEWS
EWS-RM kdEF
EWS-RM kdEWS/EF
EWS-KDM kdEWS
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EWS-KDM kdEWS/EF

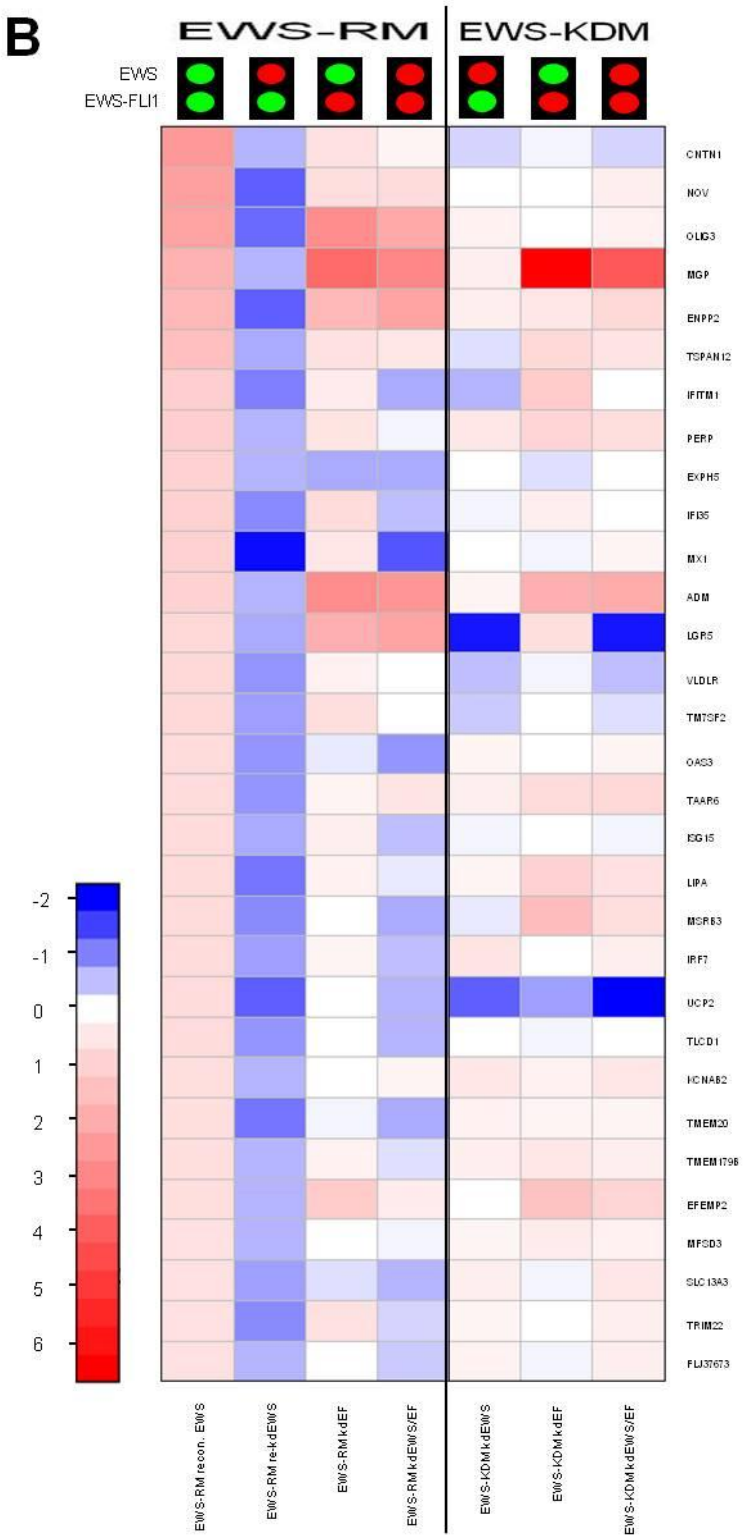


Figure 18. Heat maps of EWS de-regulated genes. Genes displaying an effect of EWS on gene expression in the EWS-RM were selected (logFC > 1.5, adjusted p < 0.3). **(A)** EWS-repressed genes **(B)** EWS-activated genes. The selected genes show an opposite effect of EWS reconstitution and re-knockdown in STA-ET-7.2 cells (columns 1 and 2). While the effect of EWS-FLI1 silencing on the expression levels of the selected genes showed no clear tendency (column 3), additional knockdown of EWS turned the effect in the same direction as single-knockdown of EWS from the EWS-RM (column 4). However, only some genes selected from the EWS-RM for their EWS-dependent changes in expression showed the same tendencies in the EWS-KDM (columns 5-7).

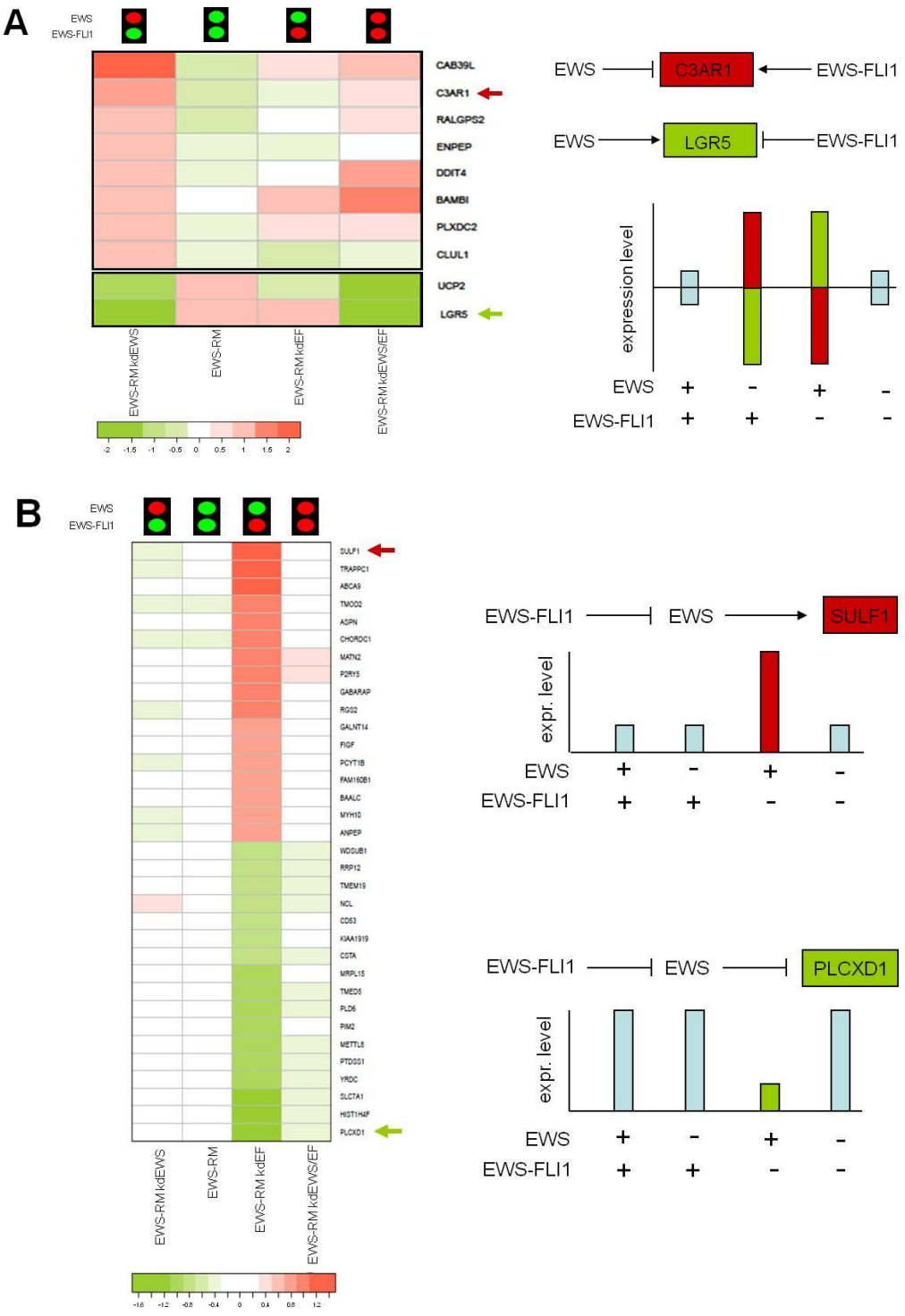


Figure 19. Functional relationship models of EWS and EWS-FLI1. (A) Functional competition model: EWS as well as EWS-FLI1 exert a direct effect on the target gene; top ranking examples for this model can be seen in the heat map on the left (adjusted $p < 0.3$). **(B)** Functional interference model: EWS-FLI1 does not affect expression of the target gene directly but interferes with the EWS-effect. Since EWS-FLI1 represses EWS function, effects on transcript expression can only be detected in case of EWS-FLI1 knockdown in the presence of EWS, while EWS-knockdown does not produce an alteration regarding expression levels (adjusted $p < 0.3$). x-axis – EWS/EWS-FLI1 status; y-axis – relative expression levels; light blue bars – expression level is not changed upon alteration of the EWS/EWS-FLI1 status as indicated; red/green bars – expression levels change along the indicated direction upon EWS/EWS-FLI1 status alterations.

3.3.1.1 Overlap between cellular models

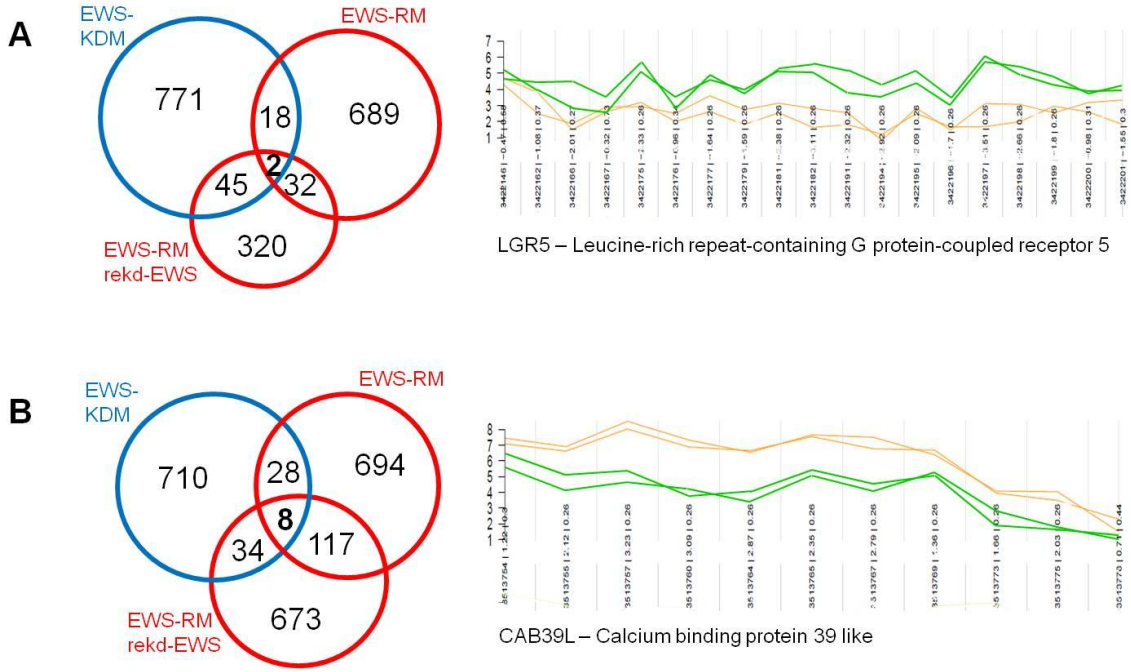
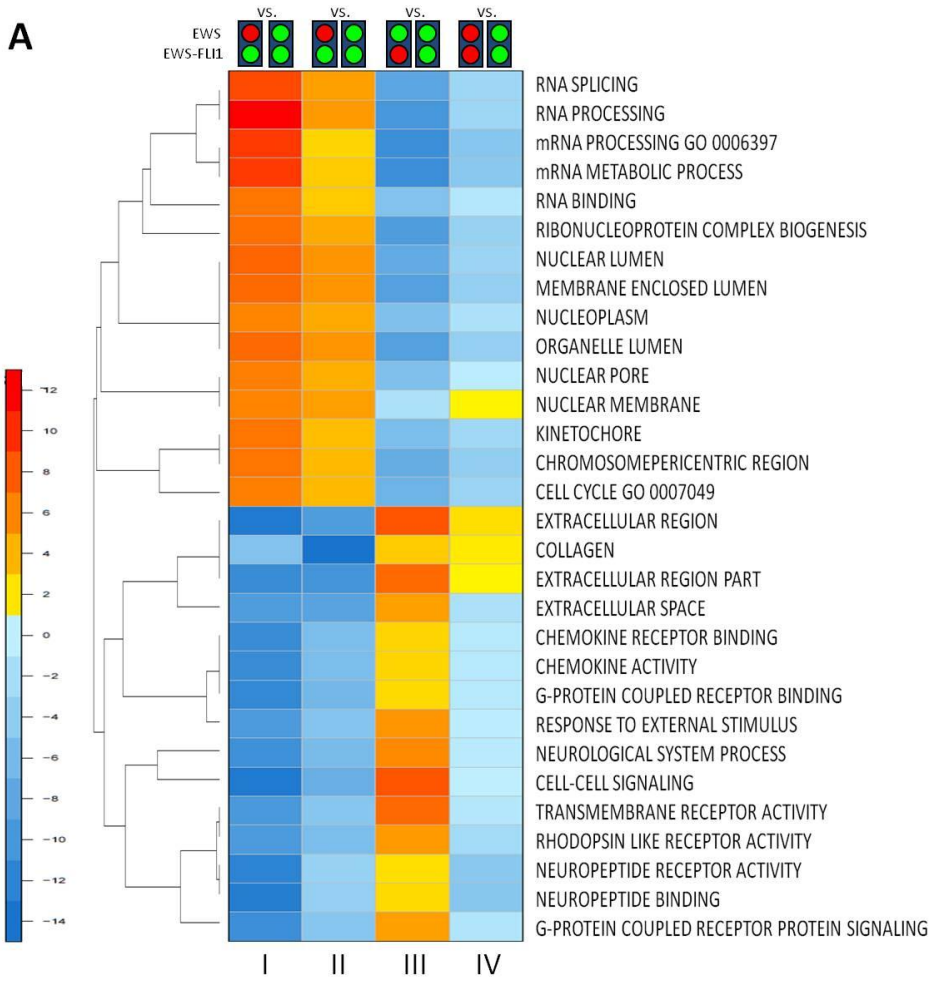


Figure 21. Overlap of data obtained from EWS-RM and EWS-KDM regarding transcript levels. (A) EWS-activated genes. Restoration of EWS expression in STA-ET-7.2 cells leads to elevated expression levels in 689 genes. Silencing of EWS leads to a reversion of the effect in about 5% of genes (32 genes). Knockdown of EWS expression in A673-shEF cells decreases expression levels of 771 genes, however, only 2 genes overlap with the data obtained from the EWS-RM, LGR5 and UCP2 (see figure 18.A). Genome graph from the re-knockdown of EWS in the EWS-RM for LGR5 can be seen on the right hand side. **(B)** EWS-repressed genes. 117 genes inversely correspond to restored respective re-silenced EWS-expression in STA-ET-7.2 cells but only 8 of these can also be found in data obtained from the EWS-KDM. Genome graph for one of the genes, CAB39L, from the re-knockdown of EWS in the EWS-RM can be seen on the right hand side. Green lines – gene expression levels in the presence of EWS (+EWS); orange lines – gene expression levels in the absence of EWS (-EWS); red circles ≙ EWS-RM (EWS-restoration model/STA-ET-7.2 cell line); blue circles ≙ EWS-KDM (EWS-knockdown model/A673-shEF cell line); exon fold change > 2; adjusted p < 0.3.

To ascertain the general relevance of our findings from the EWS-RM we aimed for validation involving the EWS-KDM based on the A673 ES cell line. The number of genes exhibiting significant changes in expression levels was comparable between knockdown of EWS from the EWS-KDM (1481 genes) and restoration of EWS in the EWS-RM (1383 genes). Surprisingly, the overlap between the two systems was relatively low (46 genes) and even decreased further when the re-knockdown of EWS in the EWS-RM was taken into account (10 genes: CAB39L, C3AR1, RALGPS2, ENPEP, DDIT4, BAMBI, PLXDC2, CLUL1, UCP2, LGR5) (figure 21). This result points to a strong inherent distinctness of the two cell lines which probably roots in their respective evolution with or without expression of EWS.

3.3.1.2 Functional annotation of top-ranking genes

Functional annotation of the top ranking EWS up- and down-regulated genes was performed by GSEA (gene set enrichment analysis) using the "pGSEA" package in the Bioconductor/R environment (Kim and Volsky 2005; Subramanian, Tamayo et al. 2005). Predominant among the EWS repressed genes in the EWS-RM were the functional terms "RNA processing", "RNA splicing" and "cell cycle" (figure 20.A). "Cell signaling" including G-protein coupled, neuropeptide and chemokine receptor signaling was most prevalent among functions connected with EWS activated genes. Notably, the observed reversion of the effect exerted by restored EWS expression on gene expression levels after re-knockdown of EWS was even more pronounced on the functional level: the effects of absent EWS expression are highly consistent whether the original STA-ET-7.2 cell line or re-silenced EWS expression is compared to samples with restored EWS (figure 20.A, columns I and II). EWS-FLI1 knockdown was observed to have an opposite effect on the top-ranking EWS-regulated GO terms (figure 20.A, column III). Interestingly, double-knockdown of EWS and EWS-FLI1 in the EWS-RM resulted in down regulation of the majority of GO-terms (figure 20.A, column IV).



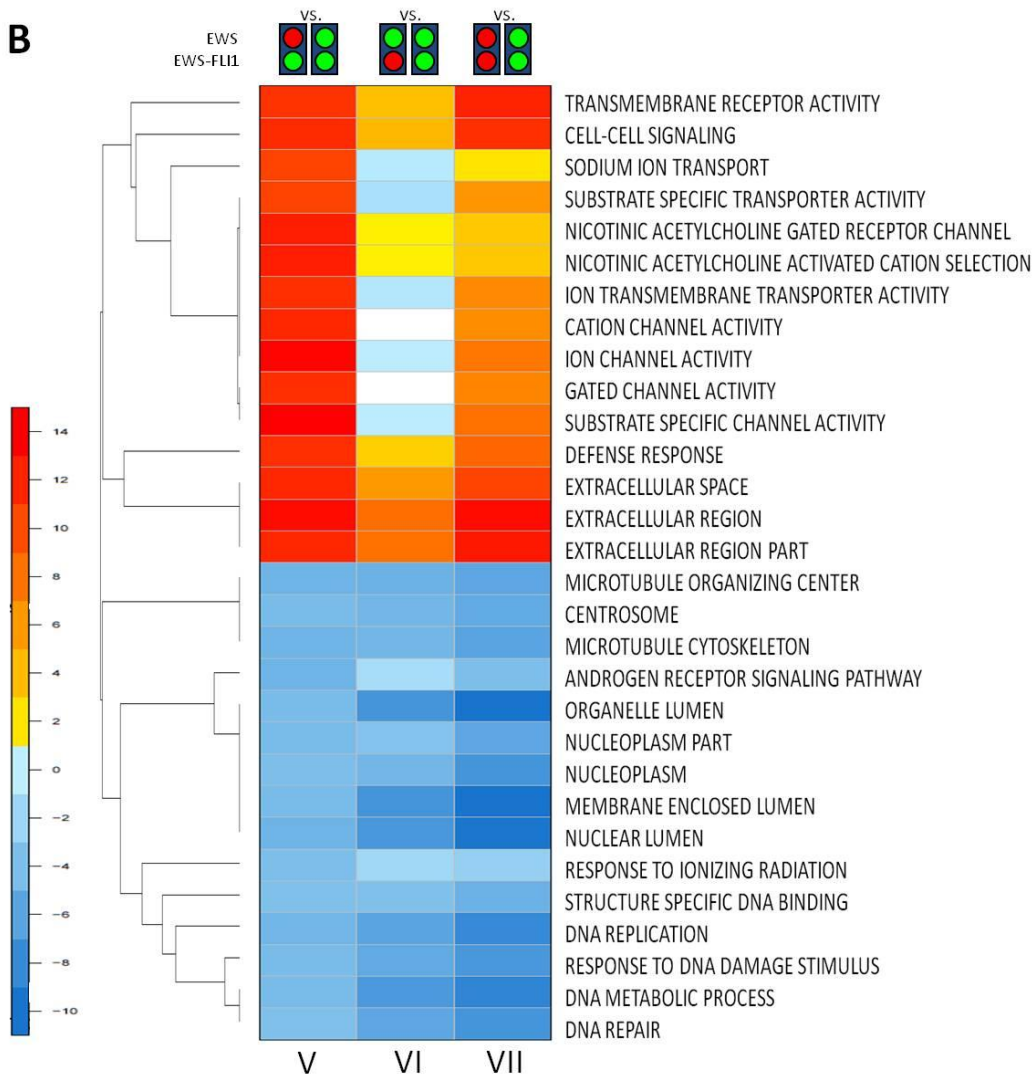


Figure 20. Gene set enrichment analysis (GSEA) identifies top enriched GO terms associated with EWS expression. (A) GSEA of the EWS-RM. Correlation of gene expression changes upon reconstituted/re-silenced EWS expression confirms EWS-specificity of the observed effect (columns I and II). On a functional level, EWS-FLI1 silencing has an opposite impact to absence of EWS while double-knockdown of EWS and EWS-FLI1 does not significantly affect the top-ranking EWS-regulated GO terms (columns III and IV). Column I – comparison of the original STA-ET-7.2 cell line lacking endogenous EWS to restored EWS expression; column II – re-knockdown of EWS vs. expression of EWS and EWS-FLI1 in STA-ET-7.2 cells; column III – silencing of EWS-FLI1 vs. expression of EWS and EWS-FLI1; column IV – double-knockdown of EWS/EWS-FLI1 compared to cells expressing EWS and EWS-FLI1. **(B)** GSEA of the EWS-KDM. Silencing of EWS-FLI1 has a similar effect compared to knockdown of EWS but is less pronounced for repressed genes. Double-knockdown is reminiscent of EWS-silencing although additional silencing of EWS-FLI1 attenuates the effect on suppressed GO terms. Column V – EWS knockdown compared to the original A673-shEF cell line expressing EWS and EWS-FLI1; column VI – EWS-FLI1 silencing vs. original A673-shEF cell line; column VII – EWS/EWS-FLI1 double-knockdown vs. original A673-shEF cell line.
Significant terms are clustered according to their similarity based on shared genes. EWS and EWS-FLI1 expression status is indicated by miniature traffic lights.

GSEA functional annotation analysis was also performed on EWS-affected genes from the EWS-KDM (figure 20.B). Similar to the results obtained on the gene level, there was no significant overlap between the two cellular models on a functional level. Prominent among the EWS-activated functional categories were terms connected with DNA metabolism and genotoxic stress response, such as “DNA replication”, “DNA repair” and “Response to DNA damage stimulus”. EWS-repressed genes from the EWS-KDM were predominantly involved in functions related to transmembrane transport. Compared to EWS silencing, knockdown of EWS-FLI1 affected the top-ranking EWS-repressed GO terms in the same direction but to a lesser extent (figure 20.B, columns V and VI). Furthermore, EWS-FLI1 knockdown in A673-shEF added to the effect of absent EWS expression (figure 20.B, column VII).

There is a fundamental difference in the evolution of the ES cell lines which form the basis for our two model systems: A673 evolved retaining expression of EWS while STA-ET-7.2 lost endogenous EWS expression early in its development. Thus, knockdown of EWS expression might activate stress response pathways in A673 cells in contrast to STA-ET-7.2 cells that have already adapted to the absence of EWS. Hence, the functional divergence between the two cellular models upon EWS silencing can be explained by the distinct evolution of the two utilized ES cell lines.

Taken together, these results suggest that loss of endogenous EWS expression from the STA-ET-7.2 cell line conforms to - if not increases – the functional effect of EWS-FLI1 on gene expression. Regarding the functional relationship between EWS and EWS-FLI1, it is noteworthy that a subset of genes with functions in cell signaling could not be rescued from EWS-FLI1 mediated repression by silencing of EWS-FLI1 when EWS was not expressed either (figure 20.A, column IV). Moreover, GSEA of EWS-regulated genes from the EWS-KDM supported the observed disparity between the two cellular model systems on the gene level (see section 3.3.1.1) also on a functional level.

3.3.1.3 Determination of transcription start sites by 5'-RACE-PCR

Based on the finding that EWS interacts with a number of transcription factors (see section 1.1.3.2) it was implied to be involved in various steps of transcription regulation. EWS has been demonstrated to be an associated factor of TFIID and RNAPII (Bertolotti, Melot et al. 1998). Thus, we wanted to test whether the effect of EWS on whole transcript levels might be due to EWS-mediated differential selection of transcription start sites ultimately resulting in altered transcript stability or transcription efficiency. For this purpose, we performed 5'-RACE (rapid amplification of cDNA ends) PCR on three of the top ranking genes: leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), calcium binding protein 39-like (CAB39L) and uncoupling protein 2 (UCP2) (figure 22). However, no remarkable differences concerning the transcription start sites of the examined genes could be observed. The obtained PCR products mapped in frame within the first 20 base pairs of the transcripts available at the UCSC (University of California Santa Cruz) Genome Browser. The maximal difference associated with EWS expression were a 12 base pair shorter transcript of LGR5 when EWS was expressed. However, we considered the observed minor differences concerning transcript start sites of the examined genes to be unlikely causing differences in transcript stability and thus in expression levels.

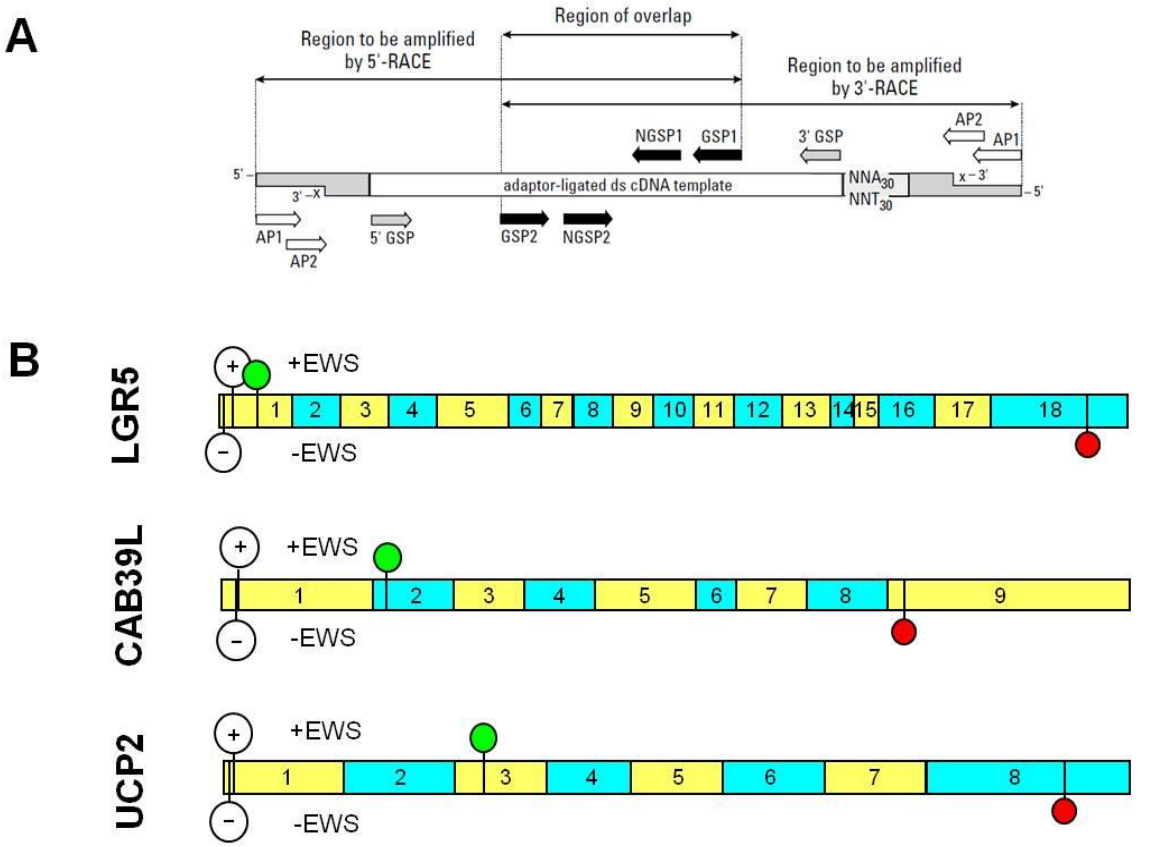


Figure 22. EWS does not affect transcription start site selection. (A) Schematic representation of the applied RACE-PCR technique. cDNA is ligated to a DNA adapter and subsequently amplified by PCR involving one adapter primer and one gene specific primer. In order to increase product specificity, nested PCR can be performed with the second adapter primer and a nested gene specific primer. After gel elution, PCR products were subjected to sequencing. **(B)** Three genes have been tested for differences in transcription start site depending on the presence/absence of EWS: LGR5, CAB39L and UCP2. All transcript start sites detected by 5'RACE PCR were mapped in frame within the first 20 base pairs of the sequences available at the UCSC Genome Browser for the respective genes. Differences in transcription start site depending on EWS expression ranged between 3 and 12 base pairs. AP1/2 – adaptor primer 1/2; GSP1/2 – gene specific primer 1/2; NGSP1/2 – nested gene specific primer 1/2; green lollipop – translation start site; red lollipop – translation termination site; (-)-lollipop – transcription start site detected in STA-ET-7.2 control (-EWS). (+)-lollipop – transcription start site detected in samples with restored EWS expression (+EWS).

3.3.2 Analysis of exon composition

As EWS has also been strongly implicated to play a role in alternative splicing (see section 1.1.3.3), we analysed the microarray data for variations in exon composition of expressed genes and found significant differences in both model systems depending on the presence/absence of EWS. However, the impact of EWS-FLI1 knockdown on the exon composition of expressed genes was remarkably strong thus obscuring a possible additional or even augmenting effect of EWS-silencing in the double-knockdown situation. Positive correlation of changed exons upon EWS-FLI1 knockdown and EWS/EWS-FLI1 double knockdown confirmed this (figure 23).

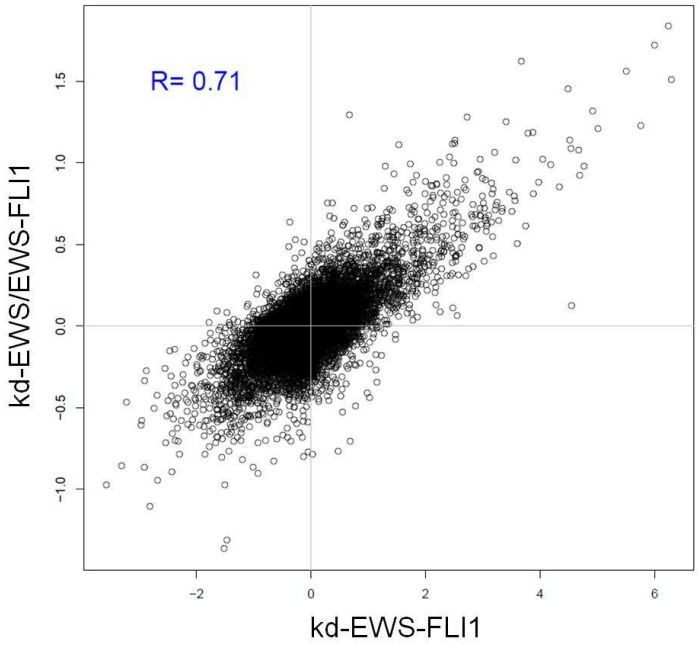


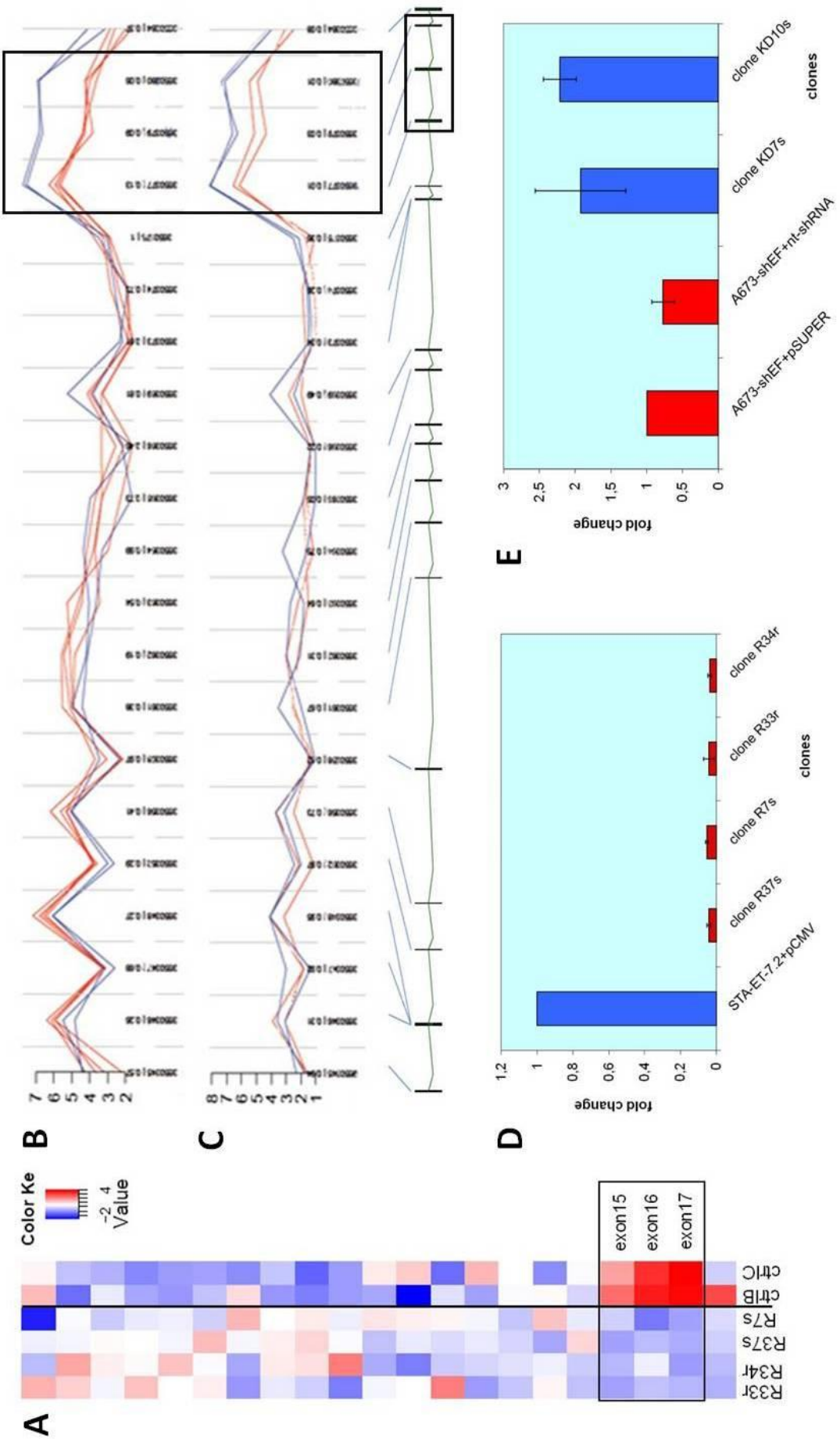
Figure 23. Correlation of exon expression between EWS-FLI1 silencing and double-knockdown of EWS and EWS-FLI1. The plot shows positive correlation implying that most exons that are modulated in one direction upon EWS-FLI1 knockdown show the same pattern when EWS is silenced additionally. Hence, the effect of disrupted EWS-expression does not alter or augment the exon expression results obtained from EWS-FLI knockdown. X-axis – mean logFC of Doxicyclin induced EWS-FLI1 knockdown in A673-shEF cells (+EWS/-EWS-FLI1) vs. untreated control A673-shEF cells (+EWS/+EWS-FLI1); Y-axis – mean logFC of Doxicyclin treated stable clones with silenced EWS expression (KD7s, KD10s) (-EWS/-EWS-FLI1) vs. control A673-shEF cells transfected with non-target-shRNA (+EWS/+EWS-FLI1).

3.3.2.1 Validation by RT-qPCR

Skipping of one or several centrally or terminally located exons was observed in 348 genes after restoration of EWS expression in STA-ET-7.2 cells and for approximately ten times as many (3523) genes for the EWS-KDM for a p-value cutoff of 0.3 and an exon level expression fold change of at least twofold. Validation of the results from the *in silico* analysis was achieved by RT-qPCR. For instance, exons 15, 16 and 17 of adenylate kinase 7 (AK7) were downregulated upon restoration of EWS expression (figure 24.A and B). This result was confirmed by RT-qPCR (figure 24.D) where a clear decrease of AK7 exons 15 - 17 levels was demonstrated for samples without EWS expression. The effect of lacking EWS expression could be confirmed by data obtained from the EWS-KDM (figure 24.C) which was validated by RT-qPCR as well (figure 24.E). Moreover, structural maintenance of chromosome 5 (SMC5) showed reduction of exon 8 upon restored EWS expression in STA-ET-7.2 cells (figure 25.A and B). However, this result was not supported by data obtained and validated from the stable EWS knockdowns in the EWS-KDM (figure 25.C, D and E). Another example, NOD-like receptor C5 (NLRC5) showed higher levels of exon 22 upon reconstituted EWS expression (figure 26.A and B) but like SMC5 displayed no effect after silencing of EWS from A673-shEF cells (figure 26.C). Again, this could be validated by RT-qPCR (figure 26.D and E).

Taken together, these results support our hypothesis of a EWS-mediated effect on post-transcriptional gene regulation. However, the observed substantial differences between the cellular models largely exclude a specific effect of EWS on the pattern of mRNA isoform expression in the ES background.

Figures 24, 25 and 26. Alterations in exon composition due to modulated EWS expression. **Figure 24. AK7:** exons 15, 16 and 17 are decreased upon restored EWS expression in STA-ET-7.2 cells; when EWS is silenced in A673-shEF cells, the expression levels of the same exons are increased. **Figure 25. SMC5:** expression levels of exon 8 are decreased after restoration of EWS expression in STA-ET-7.2 cells; however, no inversion of this effect can be observed after knockdown of EWS in A673-shEF cells. **Figure 26. NLRC5:** Exon 22 is expressed at higher levels in the presence of EWS in STA-ET-7.2 cells; disruption of EWS expression in A673-shEF cells exerts no detectable effect on exon composition of NLRC5.
(A) Heat maps. Relative expression levels of single exons of the respective genes for samples indicated at the bottom of the graph. **(B)(C)** Exon graphs. Red lines signify samples with EWS expression (+EWS); blue lines denote samples without EWS expression (-EWS): **(B)** EWS-RM - 4 clones with restored EWS expression (R37s, R7s, R33r, R34r) vs. two polyclonal control samples without EWS expression; **(C)** EWS-KDM – two clones with stable knockdown of EWS (KD7s, KD10s) vs. two controls (A673-shEF cells transfected with empty vector and non-target shRNA, respectively). **(D)(E)** Validation of microarray data by RT-qPCR; red bars indicate experiments with EWS (+EWS), blue bars represent experiments without EWS (-EWS); mRNA expression was normalized to GUSB; standard deviations resulting from at least two independent experiments, each performed in triplicate. **(D)** EWS-RM. Fold change was calculated in comparison to STA-ET-7.2 cells transfected with pCMV Script empty vector. **(E)** EWS-KDM. Fold change was inferred from A673-shEF cells transfected with pSUPER.retro.puro empty vector.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STA-ET-7.2+pCMV
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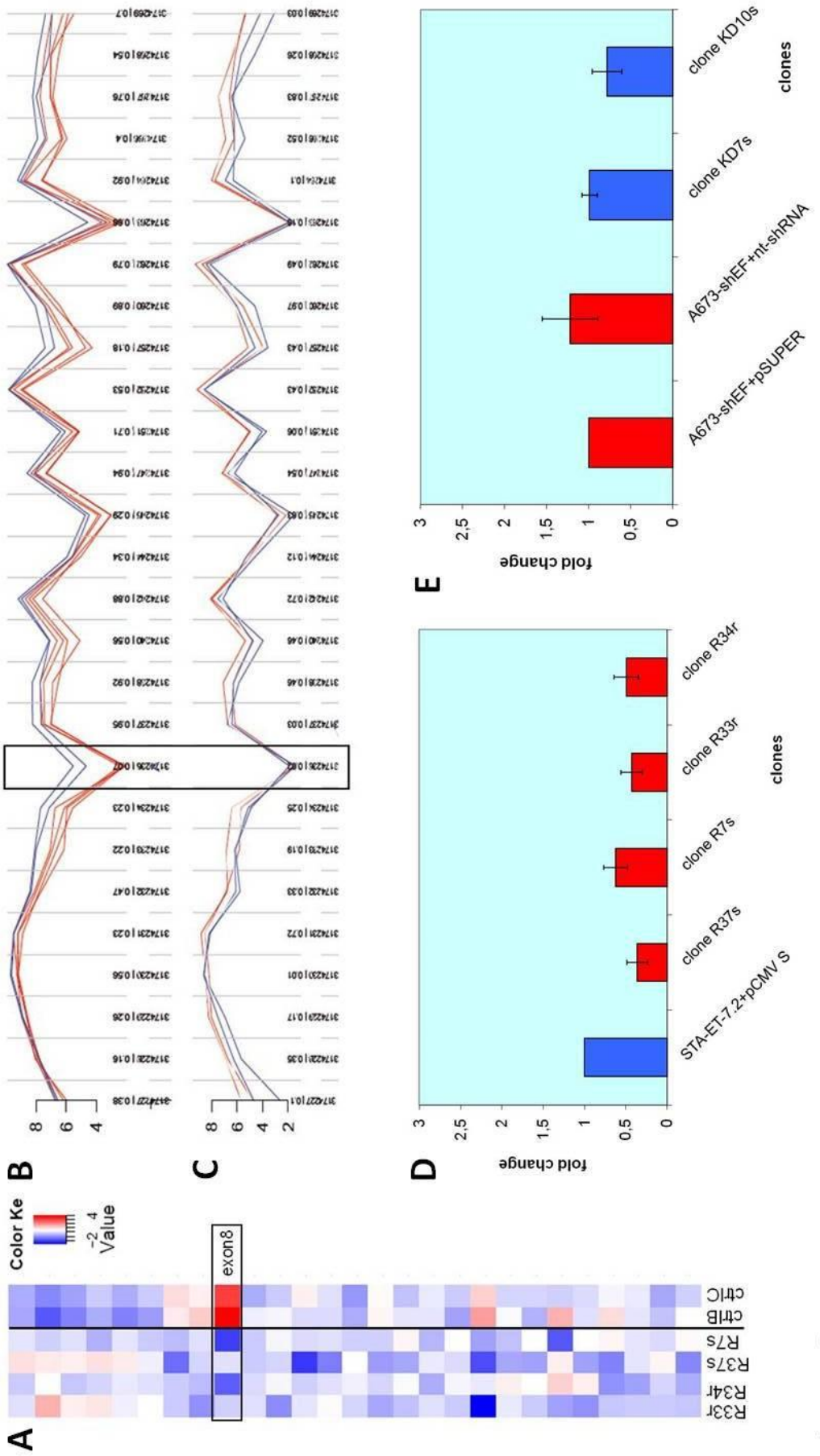
clone R34r
A673-shEF+pSUPER
A673-shEF+nt-shRNA
clone KD7s
clone KD10s

Figure 24.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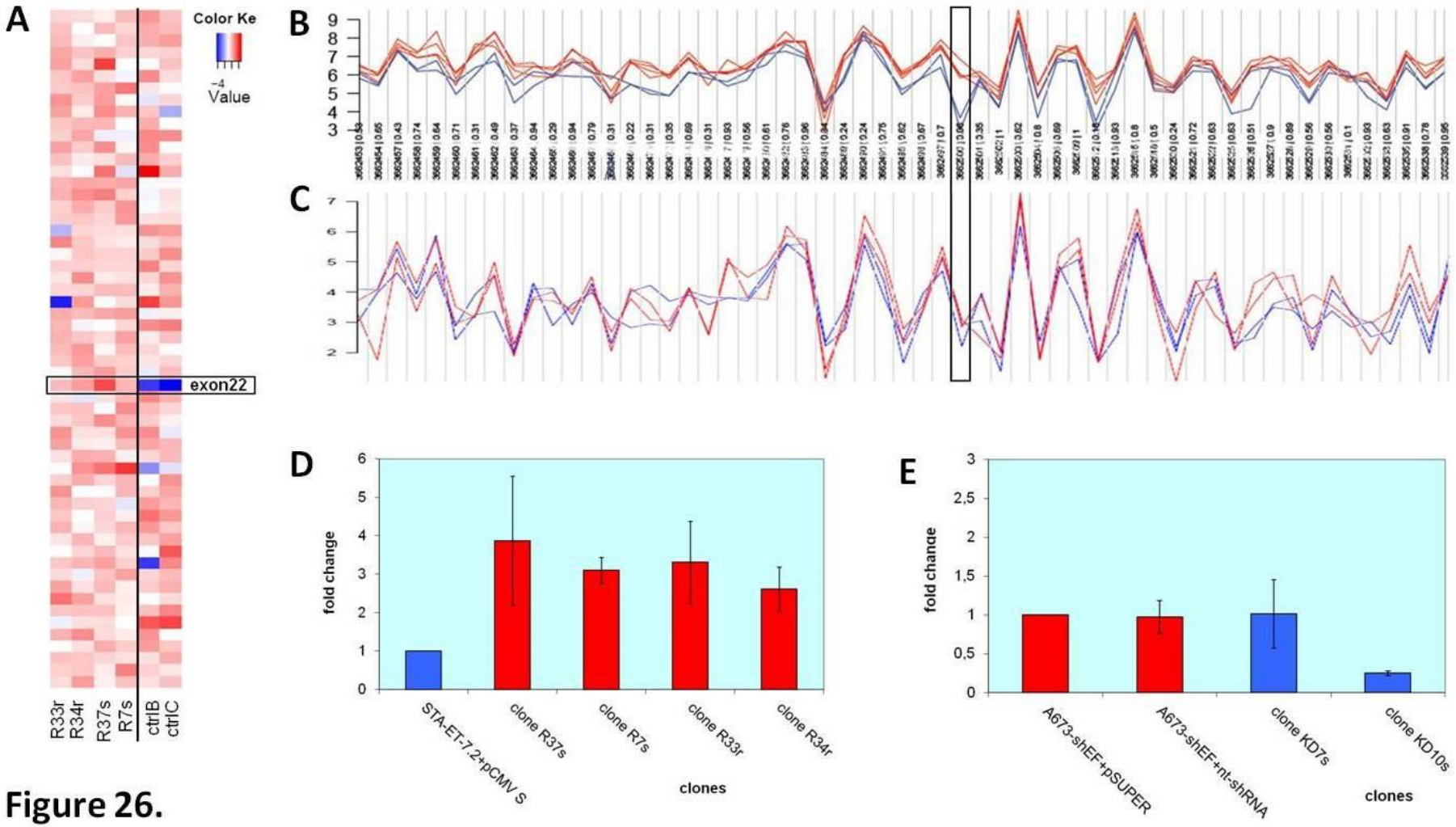
A673-shEF+nt-shRNA
A673-shEF+pSUPER

Figure 25.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clone R7s
clone R33r
clone R34r
clones
E
fold change
A673-shEF+pSUPER
A673-shEF+nt-shRNA
clone KD7s
clone KD10s
clones

Figure 26.

3.3.2.2 Overlap between cellular models

As already mentioned before, significant changes of exon composition upon restoration/knockdown of EWS could be detected in both of our cellular models. 348 genes changed in exon composition upon restoration of EWS expression in the EWS-RM while approximately ten times as many genes responded to knockdown of EWS from A673-shEF cells (fold change > 2, adjusted p < 0.3). While the overlap between the two data sets was extremely low (2 genes) it was even further reduced to zero when the re-knockdown of EWS from STA-ET-7.2 cells was considered as well (figure 27). In fact, the before mentioned gene AK7 was the only gene with consistently significant changes of transcript structure in STA-ET-7.2 and A673-shEF cells upon restored respective silenced EWS expression. Nevertheless, the effect observed for AK7 could not be rescued by re-knockdown of EWS in the EWS-RM. In this respect, SMC5 and NLRC5 have to be regarded as representative examples for the nature of our data concerning the effect of EWS on the splicing pattern in the ES background.

This result suggests an intrinsic divergence either of the different cellular backgrounds on which our models were based or the applied techniques of EWS/EWS-FLI1 gene expression modulation. Therefore, no general conclusion concerning an effect of EWS on mRNA-splicing in ES can be drawn from the obtained data.

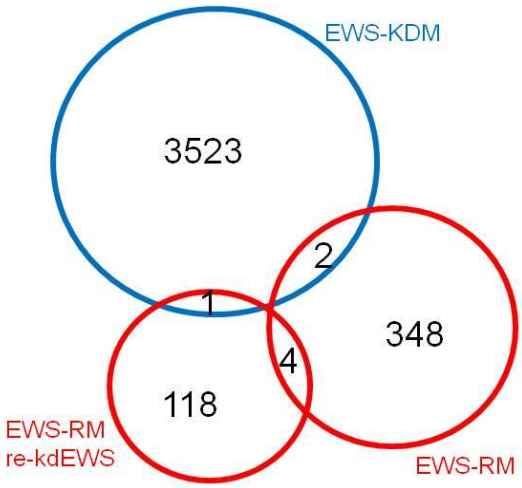


Figure 27. No overlap of differentially expressed exons across experimental systems. Of 348 genes affected in exon composition by reconstituted EWS expression in STA-ET-7.2 cells only 4 genes displayed reversion of the observed effect after re-knockdown of EWS. Additionally, of 3523 genes with altered exon composition upon silencing of EWS in A673-shEF cells only 3 genes were shared with the EWS-RM, none of which belonged to the 4 genes from the intersection. Red circles ≙ EWS-RM (EWS-restoration model/STA-ET-7.2 cell line); blue circles ≙ EWS-KDM (EWS-knockdown model/A673-shEF cell line); exon fold change > 2; adjusted p < 0.3.

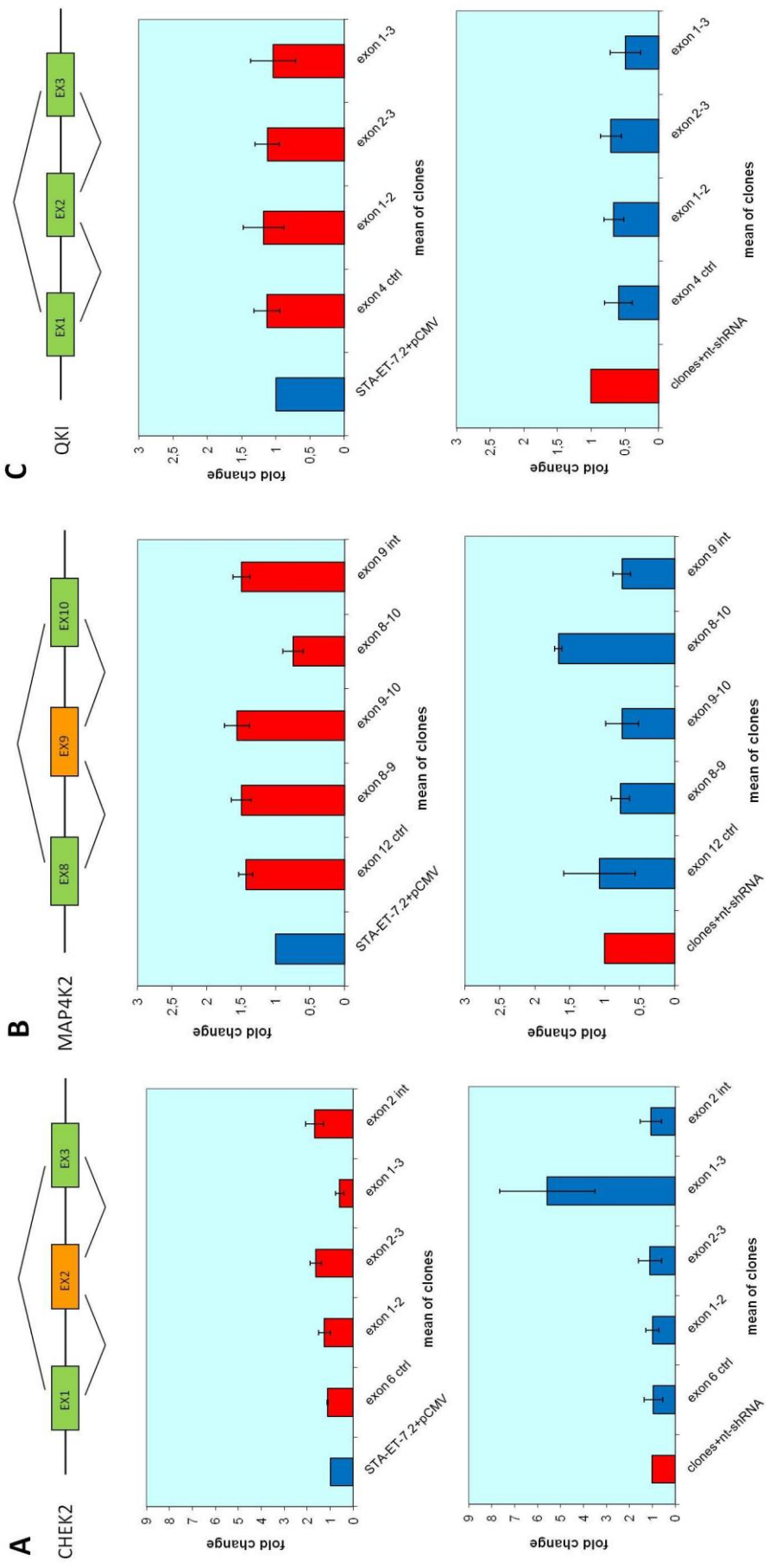
3.3.2.3 Applicability of data obtained from different cell lines

Recently published data adds to the evidence for a role of EWS in post-transcriptional gene regulation (Paronetto, Minana et al. 2011). Depletion of EWS was found to be causal for alternative splicing of genes involved in DNA repair and genotoxic stress signaling in the HeLa background. Paronetto and colleagues achieved transient silencing of EWS upon transfection of siRNA. The resulting samples were analysed on splicing sensitive microarrays involving exon-centric as well as exon-junction spanning probes. The applied microarray platform was designed to specifically investigate 1804 alternative splicing events in 482 genes which encode for proteins involved in RNA processing and cancer (Munoz, Perez Santangelo et al. 2009). Knockdown of EWS was reported to cause transcript level changes higher than 1.3-fold in 9 genes and 39 alternative splicing events in 31 genes. For their successive studies Paronetto and colleagues selected three genes from their microarray data for validation: CHEK2, MAP4K2 and ABL1. Knockdown of EWS produced higher levels of exon 2 skipping in the checkpoint kinase CHEK2 and increased skipping of exon 9 from the germinal center kinase MAP4K2 mRNA. Furthermore, knockdown of EWS was reported to affect 5'splice donor selection in exon 3 of the nonreceptor tyrosine kinase ABL1. As a control, the non-regulated exon 2 of the gene QKI was used.

Since we detected substantial differences regarding the effect of EWS on exon composition between two models established in different ES cell lines, we were eager to know to what extent data from HeLa cervical carcinoma cells could be applied to our own cellular models. To this end, we compared the results of Paronetto and colleagues for the genes CHEK2, MAP4K2 and ABL1 with our own data obtained from the EWS-RM. By *in silico* analysis of whole genome expression data on Hu-Ex1.0 stv2 arrays, we detected slight, non-significant changes in the respective exons of CHEK2 and MAP4K2 but not ABL1. This is most likely due to the applied Affymetrix Hu-Ex1.0 stv2 chips not being designed for the optimal detection of alterations regarding the splice donor/acceptor site. To confirm our *in silico* observation we performed SYBRgreen RT-qPCR on the samples from the EWS-RM. The results could be validated for CHEK2, MAP4K2 and the control QKI (figure 28.A, B and C). The PCR results obtained from ABL1 were not conclusive (data not shown). Accordingly, the data of Paronetto et al. is consistent with our finding that EWS affects transcript levels and exon composition. Resulting from fundamental differences concerning the experimental setup (one vs. two cellular models, transient silencing vs. reconstituted expression and stable silencing of EWS, microarrays covering a specific gene set involving exon and exon-junction probes vs. microarrays covering the whole genome by exon-

centric probes only) little overlap between the data of the two studies was expected. Nevertheless, EWS-dependent splice-isoforms from the HeLa system could be confirmed in the ES background.

Figure 28. Applicability of microarray data generated in the HeLa background to the ES background. All three genes identified by Paronetto and colleagues as being affected in exon composition by the absence of EWS were tested in our own model systems for general applicability of the reported results. Results for ABL1 were not conclusive and are not shown. The uppermost panels show schematic representations of the examined exons of interest. Middle panels exhibit SYBRgreen PCR results obtained from samples derived from STA-ET-7.2 cells with restored EWS expression. The lowermost panels represent results obtained after re-knockdown of EWS. For each examined exon or exon-exon-junction a mean of clones was calculated that was normalized to the respective control (first column of each panel: STA-ET-7.2 cells transfected with pCMV Script empty vector or STA-ET-7.2 clones with restored EWS expression transfected with non-target shRNA). Red bars indicate experiments with EWS expression (+EWS), blue bars represent experiments without EWS expression (-EWS); standard deviations result from two independent experiments, each performed in triplicate. **(A)** Increased levels of CHEK2 exon 2 were detected in cells with reconstituted EWS expression resulting in decreased PCR product from probes spanning exons 1-3. Silencing of EWS lead to enhanced skipping of exon 2 thus increasing PCR product yield due to smaller amplicon size. **(B)** Expression of EWS resulted in elevated levels of MAP4K2 exon 9, while knockdown of EWS lead to increased skipping of MAP4K2 exon 9. **(C)** QKI served as negative control in the experimental setting and exon composition was therefore not altered upon modulation of EWS expression. mRNA expression was normalized to β-Actin.


A
CHEK2
EX1
EX2
EX3
fold change
exon 2 int
exon 1-3
exon 2-3
exon 1-2
exon 6 ctrl
STA-ET-7.2+pCMV
mean of clones
clones+nt-shRNA
B
MAP4K2
EX8
EX9
EX10
exon 9 int
exon 8-10
exon 9-10
exon 8-9
exon 12 ctrl
C
QKI
exon 4 ctrl

4. Discussion

The oncogenic fusion protein EWS-FLI1 is specific to ES and has been shown to be the key determinant of ES oncogenesis and progression (Bailly, Bosselut et al. 1994; Chansky, Barahmand-Pour et al. 2004; Ordonez, Osuna et al. 2009; Kovar 2010) either by its role as potent transcriptional modulator (for references see table 2) or by various functions in post-transcriptional processing (Yang, Chansky et al. 2000; Chansky, Hu et al. 2001; Knoop and Baker 2001; Sanchez, Bittencourt et al. 2008). Additionally, it was proposed that EWS-FLI1 might as well interfere with other, transcription-independent tumor suppressor pathways (Hu, Zielinska-Kwiatkowska et al. 2008). However, the mechanisms behind EWS-FLI1 mediated carcinogenesis are only partially understood. Since EWS-FLI1 was found to associate with its germline counterpart EWS (Spahn, Siligan et al. 2003), it was suggested that this interaction interferes to some extent with normal EWS function. Hence, the project reported in this thesis is based on the hypothesis that the functional interference/interaction between EWS and EWS-FLI1 contributes to ES tumorigenesis.

To date, the molecular functions of FET family proteins in general, and of EWS in particular, are not very well defined. The work represented in this thesis deals with the role of EWS on transcription regulation and splicing in the ES background and the possible effects of a functional interference of EWS-FLI1. To this end, two different cellular models enabling for the modulated expression of EWS and/or EWS-FLI1 have been established: the EWS-RM based on the STA-ET-7.2 ES cell line and the EWS-KDM utilizing A673-shEF ES cells. Since the EWS-RM allowed for an internal validation by re-knockdown of reconstituted EWS expression it was selected as predominant cellular model. The EWS-KDM was intended to serve validation purposes for results gained from the EWS-RM. However, the overlap between the microarray datasets concerning either alternative exon composition or differential gene expression obtained from the two models was surprisingly small. GSEA of the top-ranking genes from both cellular models confirmed the observed disparity on the gene level also on a functional level. Variations in the evolution of the two ES cell lines with and without endogenous EWS, respectively, could very likely account for the observed differences. For instance, GO terms related to “stress response” were prominent in the EWS-KDM but not in the EWS-RM. Indeed, the activation of stress response pathways upon depletion of a multifunctional protein, such as EWS, is plausible in context of a cell line that is not adapted to the absence of this gene. Furthermore, different experimental backgrounds of the two model systems might also account for the lack of shared gene/ exon expression data. The functional relationship between EWS and its oncogenic

derivative is probably more complex than initially postulated, most likely involving additional factors. Thus, the comparison of two different “on/off”-systems, one featuring stable over-expression and transient re-knockdown (EWS-RM) the other endogenous expression and stable knockdown of EWS (EWS-KDM), both in combination with transient silencing of EWS-FLI1, may not be sufficient to recapitulate the interplay between EWS and EWS-FLI1.

Samples from both cellular models were subjected to microarray analysis employing Affymetrix Hu-Ex stv2 chips. Utilizing only exon-centric probes, this microarray platform allows for the analysis of two aspects of gene regulation: differential exon composition and expression level variations of whole transcripts. However, due to the absence of exon-junction spanning probes, this array platform is not suited for the detection of altered splice donor/acceptor sites. Nevertheless, the choice of the cheaper Affymetrix arrays allowed for the analysis of a greater number of samples on a whole genome scale in comparison to the analysis performed by Paronetto et al. which covered only a limited gene set (Paronetto, Minana et al. 2011).

The microarray data obtained from the cellular model systems was analysed regarding different aspects of EWS function and a possible involvement of EWS-FLI1. Concerning a combined effect of EWS and EWS-FLI1, the differentially expressed genes shared by the two model systems could be classified according to two different models of possible functional interference/interaction between EWS and the fusion protein: First, the “functional competition model” includes genes directly regulated by EWS and EWS-FLI1 but in opposite directions. Second, the “functional interference model” comprises genes whose expression is directly affected by EWS which is in turn inhibited by the fusion protein. Hence, the “functional interference model” is in line with the functional relationship between EWS and EWS-FLI1 which also represents the hypothetic basis of this study.

Concerning the EWS/EWS-FLI1-dependent regulation patterns associated with the “functional competition model”, our observations were supported by functional annotation of the involved genes. The top enriched GO terms identified by gene set enrichment analysis (GSEA) were highly reminiscent of previously suggested functions for EWS such as “RNA splicing” and “RNA processing” for EWS-repressed genes and “cell signalling” for EWS-activated genes. This strongly supports our hypothesis of an EWS-involvement in transcription regulation.

Furthermore, the analysis of the microarray data yielded valuable information concerning EWS-specific effects on gene expression and exon composition. LGR5, which is known to be a marker of adult stem cells (Haegebarth and Clevers 2009), is one of the genes described in the “functional competition model”. Hence, its activation was detected in presence of EWS expression and its repression upon absence of EWS expression as well as re-silencing of EWS in

the EWS-RM. These results were further supported by unpublished data from Elizabeth R. Lawlor's lab (University of Michigan, MI, USA) concerning a correlation of EWS and LGR5 expression in human neural crest stem cells. Accordingly, expression of LGR5 decreases with progressive differentiation, as well as EWS expression (personal communication). This observation is supported by the earlier results of Andersson et al. that expression of all FET proteins was decreased in differentiating human embryonic stem cells and during induced differentiation of neuroblastoma cells. Furthermore, it was reported that no expression of FET proteins could be detected in terminally differentiated melanocytes and cardiac muscle cells (Andersson, Stahlberg et al. 2008). Thus, our findings of EWS expression correlating with increased LGR5 expression are in line with previous observations of a positive effect of EWS on the expression of the stem cell markers HNF-4 (Araya, Hirota et al. 2003) and OCT-4 (Lee, Rhee et al. 2005) which very likely contributes to the maintenance of the undifferentiated state characteristic for ES cells. Together, our results further add to the evidence of EWS being involved in transcription regulation of genes. The molecular mechanism of this function, however, remains to be elucidated.

Various factors and mechanisms have been reported to account for transcriptional modulations such as alternative transcription start site selection (Sandelin, Carninci et al. 2007; Thorsen, Schepeler et al. 2011; Lenhard, Sandelin et al. 2012). Indeed, EWS has been proposed to partake in transcription start site recognition (Hume, Sasmono et al. 2008). Consequently, we wanted to test whether EWS-dependent differential selection of transcription start sites accounts for the detected differences in gene expression levels of single genes probably by causing variant transcript stability or transcription efficiency. However, results obtained from 5' RACE-PCR experiments did not support the idea of EWS-dependent differential transcription start site selection as a potential cause of altered transcript stability to account for the observed differential RNA expression effect for the selected candidate genes.

Significant differences in exon composition of genes were detected in each of the two applied cellular systems which could also be validated by RT-qPCR. However, almost no overlapping data could be obtained from the two different models. Additionally, the impact of EWS-FLI1 silencing on alternative exon composition was so strong that the effect of additional EWS knockdown could not be assessed. Thus, no general conclusion could be drawn concerning the combined involvement of EWS and EWS-FLI1 in alternative splicing in the ES background. The establishment of an improved cellular model enabling for fine-tuning adjustment of EWS and EWS-FLI1 expression as well as microarray analysis focusing on alternative splicing by involving exon-junction spanning probes might help to solve this question. Since very recently published

results attained from a HeLa model are strongly in favour of a role for EWS in alternative splicing (Paronetto, Minana et al. 2011), we cross-checked these data with our own cellular models. For two of the three tested genes of the Paronetto-study we could reproduce the reported results involving SYBRgreen RT-qPCR and the published primer sequences. However, the significance of the results was rather low due to high clonal variations. Nevertheless, any consistency between the two studies is noteworthy considering the fundamental differences regarding cellular background, experimental setup, applied microarray platform and analysis.

Taken together, the results concerning differential exon composition were not conclusive concerning the effect of EWS on RNA processing in the ES background. In case of this particular study, independent evolution of the applied ES cell lines may explain the divergence observed in data regarding exon composition: On the one hand, A673 cells retained EWS expression throughout tumorigenesis. Loss of EWS from STA-ET-7.2 tumor cells, on the other hand, most probably induced these cells to compensate for this deficit possibly by an expansion of EWS-FLI1 function. Nevertheless, it was shown that the cellular background exerts great influence on the results of a study. Similar observations have been reported for the interpretation of EWS-FLI1 target gene studies in the ES background (Kovar 2010).

In order to investigate EWS-specific effects on the ES cell phenotype, two different functional assays were performed: proliferation assays and soft agar colony formation assays. Reconstituted EWS expression in the EWS-RM conferred no advantage concerning anchorage-dependent growth in the proliferation assay setting which is supported by earlier findings of Kovar et al. (Kovar, Jug et al. 2001). Anchorage-independent growth, on the other hand, was significantly improved by restoration of EWS expression to STA-ET-7.2 cells as could be observed in soft agar colony formation assays. EWS-specificity was confirmed by the reversion of this effect upon re-knockdown of EWS from STA-ET-7.2 cells. It is generally known that tissue culture conditions involving anchorage-dependent growth do not recapitulate the physiologic conditions of human tissue. However, morphologic and histochemical analysis showed, that cells grown in soft agar colony formation assays exhibit the same characteristics as original tumor cells (Hamburger, Salmon et al. 1978). Hence, the results obtained from the soft agar colony formation assays stress the relevance of EWS expression in ES tumorigenesis by enhancing tumor cell growth.

In summary, the results described in this thesis are consistent with the current scientific evidence for an involvement of EWS in transcriptional and post-transcriptional regulation. The differences observed in two different cell line models emphasize the necessity to consider the cellular background for the interpretation of scientific results. Significant effects of EWS on exon

composition of transcripts were detected in both cellular model systems. However, the resulting data could not be merged to draw a general conclusion. From the overlapping data of the two cellular models concerning EWS-dependent transcriptional regulation two types of genes could be defined matching two different models of functional relationship between EWS and EWS-FLI1. This may point to a probably more subtle, indirect commitment of EWS in the splicing process compared to its role in transcription regulation. Taken together, this suggests a complex functional relationship between EWS and EWS-FLI1, probably involving additional factors, which cannot be reproduced by overexpression and/or silencing of EWS and/or EWS-FLI1 alone but requires an advanced system of refined expression modulation of involved factors.

5. Materials & Methods

5.1 Materials

5.1.1 Buffers

5.1.1.1 General

PBS

137mM NaCl
3mM KCl
6,5mM Na2HPO4
1,5mM KH2PO4

Dulbecco's PBS (DPBS) 1x (PAA, Pasching, Austria) without Ca and Mg was used for cell culture work

TBS

50mM Tris
150mM NaCl
pH 7,5

PBS-T and TBS-T

Add 0,1% Tween 20 to above mentioned PBS and TBS, respectively

5.1.1.2 SDS-PAGE buffers

2x Sample Buffer

20% (v/v) glycerol
6% ß-mercaptoethanol
3% SDS
125mM Tris-Cl pH 6,8
Bromphenol Blue

Laemmli Buffer

15,1g Tris
72g glycine
25ml 20 % SDS
per 1000ml

Transfer Buffer

14g glycine
3g Tris
20% methanol per 1000 ml

Ponceau Staining Solution (10x)

2g Ponceau S
30g trichloroacetic acid
30g 5-sulfosalicylic acid
per 100ml

5.1.1.3 Protein extraction buffers

Hunt Buffer

500mM NaCl
50mM Tris-Cl pH 8,0
1mM EDTA
0,5% NP-40
1 tablet Complete™ Mini Protease inhibitor cocktail per 10 ml (Roche Diagnostics, Mannheim, Germany)

5.1.2 Media

5.1.2.1 Bacterial media

Luria Broth (LB) Medium

1% NaCl
1% Tryptone (Oxoid, Hampshire, UK)
0,5% yeast extract (Oxoid, Hampshire, UK)
optional 2% bacteriological agar (Oxoid, Hampshire, UK) for plates
sterilize by autoclaving

5.1.2.2 Cell culture media

RPMI 1640, GlutaMAX™-I (GIBCO™, Life Technologies, Carlsbad, CA)

2g/L D-Glucose, 1mg/L Glutathione (reduced)

DMEM (Dulbecco's Modified Eagle Medium), high glucose, GlutaMAX™-I, pyruvate (GIBCO™, Life Technologies, Carlsbad, CA)

4.5g/L D-Glucose, 110mg/L Sodium Pyruvate.

OPTI-MEM I (GIBCO™, Life Technologies, Carlsbad, CA)

2xRPMI

2,086g RPMI 1640 Powder (GIBCO™, Life Technologies, Carlsbad, CA) and 0,2g $NaHCO_3$ were dissolved in 80ml ddH_2O. pH was set to 7,1-7,2 with 6M HCl and ddH_2O was added to a final volume of 100ml. 2xRPMI was filter sterilized with a 0,20 Micron disposable filter (Iwaki™, AGC, Chiyoda, Japan) and stored at 4°C until use.

5.1.3 Antibiotics

G418 Sulphate (50mg/ml) (PAA, Pasching, Austria), endconcentration 500µg/ml

Doxicyclin Hyclate (SIGMA-Aldrich, Saint Louis, MI), endconcentration 1µg/ml

Blasticidin (InvivoGen, San Diego, CA), endconcentration 2µg/ml

Zeocin (InvivoGen, San Diego, CA), endconcentration 50µg/ml

Puromycin dihydrochloride (SIGMA-Aldrich, Saint Louis, MI), endconcentration 1µg/ml or 3µg/ml

5.1.4 Enzymes

Restriction

All restriction endonucleases and corresponding buffers were purchased from New England Biolabs (NEB, Ipswich, MA) and used according to manufacturer's instructions:

- EcoRI
- XhoI
- BglII
- EcoRV-HF

Dephosphorylation

Calf intestine alkaline phosphatase (CIP) (NEB, Ipswich, MA)

Ligation

T4 DNA ligase (Promega, Madison, WI)

5.1.5 Antibodies

Immunoblot

Primary antibodies

- α-EWS: Rabbit Anti-EWS 139/2 (by C. Denny)
- α-FLI1: Rabbit Anti-FLI1 (PAb RB-9295-P) (Eubio, Vienna, Austria)
- α-β-Actin: MS mAb to beta Actin (mAbcam 8226)

Secondary antibodies

- α-rabbit: Goat Anti-Rabbit IgG (H+L) DyLight™ 800 conjugated (Thermo Fisher Scientific, Rockford, IL)
- α-mouse: Goat Anti-Mouse IgG (H+L) DyLight™ 800 conjugated (Thermo Fisher Scientific, Rockford, IL)

5.1.6 Plasmids

pCMV Script Vector (Agilent Technologies, Santa Clara, CA) is a mammalian expression vector, containing the cytomegalovirus (CMV) immediate early promoter, the kanamycin-resistance gene for bacterial selection and the neomycin-resistance gene for mammalian selection with G418 which is driven by the SV40 early promoter.

pSUPER.retro.puro (OligoEngine, Seattle, WA) is a mammalian expression vector that directs the intracellular synthesis of siRNA- or shRNA-like transcripts under control of the polymerase III H1-RNA gene promoter. Furthermore, the vector contains the puromycin and ampicillin-resistance gene, the 3'delta LTR and the 5' LTR.

pGEM-T Easy Vector System (Promega, Madison, WI) pGEM-T Easy is a linearized vector with a single terminal 3'-thymidine overhang at both ends. These T-overhangs prevent the vector from recircularization and facilitate cloning of PCR products.

pLKO.1-puro (SIGMA-Aldrich, Saint Louis, MI) is a lentiviral plasmid vector for intracellular expression of shRNA-transcripts. Ampicillin and puromycin antibiotic resistance allow for bacterial or mammalian selection.

5.1.7 shRNAs

non-target control

sense: TCCAACACGACACTCACTA

targeting EWS:

3'UTR: sense TGCATTGACTACCAGATTTAT

Exon 11: sense GTACAAGGATTAAATGACA

targeting EWS-FLI1

EFsi22: sense AGCTACGGGCAGCAGAGTTCACTGCTGGCCTAT

EFsi4: sense CACCCACGUGCCUUCACACTT

5.1.8 Primers

PCR

EWS	forward CAGCCAAGCTCCAAGTCAATATAG (Tm 61,4°C) reverse TCATGCCTCCACGATCAAAT (Tm 61,4°C)
EWS-FLI1	forward TCCTACAGCCAAGCTCCAAGTC (Tm 62,1°C) reverse ACTCCCCGTTGGTCCCCTCC (Tm 65,5°C)
β-Actin	forward GCCGGGAAATCGTGCGTG (Tm 60,5°C) reverse GGGTACATGGTGGTGCCG (Tm 60,5°C)
E2F1	forward TGCTGCTCTTCGCCACACCG (Tm 62°C) reverse ATGCGCCGCTTCTGCACCTT (Tm 62°C)
TGFbR2	forward TTGCTCACCTCCACAGTGATC (Tm 64°C) reverse GAGCCGTCTTCAGGAATCTTC (Tm 64°C)
VEGF	forward CCTCCGAAACCATGAACTTT (Tm 58°C) reverse AGAGATCTGGTTCCCGAAAC (Tm 60°C)

RT-qPCR

AK7	forward TGGGAGGAGTGGAATAAACG reverse CGTTGCAACATTCATTCAGG probe TGGAGGAAGTGAAAAGAGAAGAAAGAGAAT
SMC5	forward ATTTGGAGGCTCGAATCAA reverse GCCTGCTGAAGTTCCTCAA probe GAAAAGGCAACAGATATTAAGGAGGCATC
GUS	forward GAAAATATGTGGTTGGAGAGCTCATT reverse CCGAGTGAAGATCCCCTTTTT probe CCAGCACTCTCGTCGGTGACTGTTCA

RACE-PCR

AP1		CCATCCTAATACGACTCACTATAGGGC
AP2		ACTCACTATAGGGCTCGAGCGGC
		[sequences of AP1 and AP2 taken from Marathon cDNA Amplification kit manual (Clontech, Mountain View, CA)]
LGR5	GSP1	CTCTCCAGGTTTGCAGTTCCAGTT
	GSP2	GAGGCACCATTCAGAGTCAGTGTTC
	GSP3	AAGTCTCTAGGCTGTGGAGCCCATC
	GSP4	GGTGTATTTTGTTCAGGGCCAAGG
	GSP5	CAGAGCTTCTGTGGGTACGTGTCTT
	GSP6	TAGGTAGGAGGTGAAGACGCTGAGG
CAB39L	GSP1	CCCACAACGTAAGGCAATCTGTG
	GSP2	CTGCTTCTGTTGGGGGTTCTTTCT
	GSP3	TCCGAGTGCCTATCTGTCTTCTCAAG
	GSP4	GTAGACACATGGTGAGGTGCTGAAGA
	GSP5	AGCGTGGCAGAGAAAAGAGTTTCC
UCP2	GSP1	CATTGACGGTGCTTTGGTATCTCC
	GSP2	TGCTCAGAGCCCTTGGTGTAGAAC
	GSP3	CCCCTGACTTTCTCCTTGGATCTGT
	GSP4	AGCTTCTCTAAAGGTGTCCCGTTCTTC
	GSP5	GTGGGAGACGAAACACCTAATGGTC

SYBRgreen-qPCR

Primer sequences taken from supplementary material of Paronetto et al. (Paronetto, Minana et al. 2011)

CHEK2	ex6-F	GGTGCCTGTGGAGAGGTAAA
	ex6-R	GCCTCTCTTGCTGAACCAAT
	ex1-F	GTTTAGCGCCACTCTGCTG
	ex2-R	CGAGACATCACGACCGCGTG
	ex2-F	GACCAAGAACCTGAGGACCA
	ex3-R	TCATTCACACATTCAAGATTGG
	ex3-R	CATTCACACATTCGCGTGAG
	ex2-Fbis	CTCTGGGACACTGAGCTCCT
	ex2-Rbis	TGGTCCTCAGGTTCTTGGTC
MAP4K2	ex12-F	ACCCGTTCACGACTCAGC
	ex12-R	CTCCAGCTCACAGTCCTCAG
	ex9-F	AGCGCAAAGGTGGCTACAAT
	ex9-R	GGGTGCAGGTGGAACAGA
	ex8-9-F	TTGGGACTCCCTACTGGATG
	ex9-10-R	CATGAGCATCAGGGCCCTCATG
	ex8-F	CTGACTTTGGGGTGTCAGG
	ex8-10-R	CATGAGCATCAGGGCCCAGTAG

QKI	ex4-F	GGAGCAAAATAGAGGCAAGC
	ex4-R	TCTGAGCATCTTCCACAGTGA
	ex1-F	CCACCCCAGATTACCTGATG
	ex1-2-R	GCTAATTTCTTCGTCCAGCA
	ex1-3-R	CAAAATTAAACTTCGTCCAGCA
	ex2-F	GGAGTGCAGAATTGCCTGAT
	ex2-3-R	CCCAACAAAATTAAAATCTGGGTA
ABL1	ex2-F	CGGCCAGTAGCATCTGACTT
	ex2-R	GGTTGGGGTCATTTTCACTG
	ex3-F	ATCACAATGGGGAATGGTGT
	ex3-R	CTTGCCATCAGAAGCAGTGT
	ex3-4-R	TCGGAGGAGACGTAGACAC

5.1.9 Bacterial Strains

JM109 competent E.coli; genotype: endA1, recA1, gyrA96, thi, hsdR17 (rk–, mk+), relA1, supE44, Δ(lac-proAB), [F', traD36, proAB, laqIqZΔM15] (Promega, Madison, WI)

5.1.10 Cell lines

STA-ET-7.2: Ewing's Sarcoma cell line established by the laboratory of Heinrich Kovar, Children's Cancer Research Institute, Vienna, Austria. Normal chromosome 22 was lost while the chimeric chromosome was duplicated. Thus, endogenous expression of EWS was lost and only EWS-FLI1 type 2 is expressed (Kovar, Jug et al. 2001).

A673-shEF: Ewing's Sarcoma cell line established by the laboratory of Javier Alonso, Unidad de Tumores Solidos Infantiles, Instituto de Salud Carlos III, Majahonda, Spain. EWS-FLI1 type 1 is expressed. This cell line was stably transfected with a doxicyclin-inducible shRNA expression-construct targeting EWS-FLI1 type 1 (Carrillo, Garcia-Aragoncillo et al. 2007).

A673-shGFP: Ewing's Sarcoma cell line established by the laboratory of Javier Alonso, Unidad de Tumores Solidos Infantiles, Instituto de Salud Carlos III, Majahonda, Spain, as a negative control to the A673-shEF cell line. This cell line stably expresses a shRNA targeting GFP.

5.2 Methods

5.2.1 Plasmid Mini Prep

For plasmid mini preparations the Plasmid Mini Kit (QIAGEN, Hilden, Germany) was used according to the manufacturer's instructions.

5.2.2 Plasmid Maxi Prep

For endotoxin-free plasmid maxi preparations EndoFree Plasmid Maxi Kit (QIAGEN, Hilden, Germany) was used according to the manufacturer's instructions.

5.2.3 RNA extraction

For extraction of whole RNA from mammalian tissue culture cells RNeasy Mini Kit (QIAGEN, Hilden, Germany) was used according to the manufacturer's instructions.

5.2.4 Bacterial Transformation

JM109 competent bacteria were thawed on ice and incubated with the plasmid of interest for 10 minutes on ice. 200µl LB medium were added after heat shock (42°C for 1 minute) followed by incubation at 37°C for 20 minutes. Finally, bacteria were plated on LB plates containing 0,1mg/ml ampicillin and incubated over night at 37°C.

5.2.5 Cell Culture Techniques

5.2.5.1 General techniques

Cells were kept adherently in cell culture dishes (petridishes or flasks by Iwaki™, AGC, Chiyoda, Japan and SPL Lifesciences, Pocheon City, South Korea, respectively). STA-ET-7.2 cells were kept in RPMI 1640 + GlutaMAX™-I (GIBCO™, Life Technologies, Carlsbad, CA; see also section 5.1.2.2.1) and A673-shEF were kept in DMEM (Dulbecco's Modified Eagle Medium), high glucose + GlutaMAX™-I (GIBCO™, Life Technologies, Carlsbad, CA; see also section 5.1.2.2.2) with Blasticidin and Zeocin, respectively. Both media were supplied with 10% Fetal Bovine Serum (FBS) Gold (PAA, Pasching, Austria) and Penicillin/Streptomycin (100x) (PAA, Pasching, Austria). For STA-ET-7.2 cells cell culture dishes were coated with Fibronectin pure (Roche Diagnostics, Mannheim, Germany). To remove the cells from the cell culture dish they were washed once with DPBS (PAA, Pasching, Austria), treated for approximately 1-4 minutes with Accutase or Trypsin-EDTA (both PAA, Pasching, Austria) and taken up in the corresponding medium or DPBS.

5.2.5.2 Cell transfection and selection

Cells were seeded 24 hours prior to transfection at a density of approximately 30% in a 75ccm cell culture flask (SPL Lifesciences, Pocheon City, South Korea). The normal growth medium was aspirated, cells were washed once with OPTI-MEM and starved in OPTI-MEM for approximately 1 hour prior to transfection. In the meantime, transfection mixes A and B were prepared where mix A consisted of 625µl OPTI-MEM, 4µg DNA, and 20µl Plus™ Reagent (Invitrogen, Carlsbad, CA) and mix B contained 625µl OPTI-MEM and 30µl Lipofectamine™ LTX (Invitrogen, Carlsbad, CA). Both mixes were vortexed thoroughly and incubated for 15 minutes at RT. Mixes A and B were combined and again incubated at RT for 15 minutes after which 6 ml of OPTI-MEM were added. OPTI-MEM was aspirated and the cells were incubated with the transfection mix for 3,5 hours at 37°C/ 5% CO_2. The transfection mix was aspirated, 10 ml of growth medium without any antibiotics were added and the cells recovered for 24 hours at 37°C/ 5%CO_2. Then, growth medium was exchanged for selection medium containing the required antibiotics and incubated for 24 or 72 hours before proceeding with Soft Agar Assay or harvesting, respectively.

5.2.5.3 Proliferation Assay

75000 cells were seeded per well of a cell culture 6 well-plate (Iwaki™, AGC, Chiyoda, Japan) in triplicate. Cells were harvested after 24, 48, 72 and 96 hours, respectively, and counted with a Bürker-Türk chamber.

5.2.5.4 Soft Agar Assay

1.4% Difco™ Agar Noble (BD, Franklin Lakes, NJ) in ddH_2O was boiled in a microwave and equilibrated to 45-50°C in a waterbath. In the meanwhile, 2xRPMI and FBS were equilibrated to 37°C in a waterbath. For the underlayer 675µl 2xRPMI, 150µl FBS, 15µl Penicillin/Streptomycin (and other antibiotic if required) are mixed with 675µl 1,4% agar per well of a 6-well plate. Temperature of the agar mix must not be too low and the whole bottom of the well must be equally covered. Store underlayer in the incubator until the cell layer is ready (approximately 15 minutes). For the cell layer mix A (15000 cells in 2,5ml RPMI 1640) and mix B (1,25ml 2xRPMI, antibiotics and 1,25ml 1,4% agar) are prepared in separate tubes. Mix A and mix B are combined and pipetted onto underlayer. Leave in the laminar at RT to polymerize for 15 minutes and add upper layer

(same procedure as for the underlayer). Incubate at 37°C/ 5% CO_2 for 7-21 days and feed the cells every 3rd day by adding 2 drops of culture RPMI 1640.

5.2.6 Protein Analysis

5.2.6.1 Protein extracts

Crude extract

Cells were harvested and counted using a Z2 Coulter® Particle Count and Size Analyzer (Beckman Coulter™, Krefeld, Germany). After washing twice with PBS the cell pellet was dissolved in equal amounts of PBS and 2x Sample Buffer resulting in a concentration of 30000cells/µl. The extract was boiled at 96°C for 7 minutes and spun down at 13000rpm for 15 minutes.

Hunt extract

Cells were harvested, counted and washed as described above. The cell pellet was dissolved in Hunt buffer to a concentration of 60000cell/µl. The cells were disrupted by two cycles of drop-freeze in liquid nitrogen followed by thawing on RT and on ice, respectively. The extract was spun down at 13000rpm for 15 minutes, the supernatant was transferred to a fresh tube and diluted 1:1 with 2x Sample Buffer. Finally, the extract was boiled at 96°C for 7 minutes and spun down at 13000rpm for 15 minutes.

5.2.6.2 SDS-PAGE and Immunoblotting

For immunoblot analysis, total protein extracts were resolved by SDS-PAGE and processed for Western Blotting essentially as described by Laemmli (Laemmli 1970). Electrophoretic transfer of proteins from gel to nitrocellulose membranes was either done at 400mA for 1 hour under constant cooling in an ice box or at 22mA over night at 4°C. Filters were stained with PonceauS to check for equality of loading and transfer efficiency and blocked for 1 hour at RT with 1x blocking reagent (Roche Diagnostics, Mannheim, Germany). Incubation with primary antibody in 0,5x blocking reagent over night at 4°C was followed by incubation with secondary antibody in LI-COR Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 hour at RT. Signals were detected with a Odyssey® Imager (LI-COR Biosciences, Lincoln, NE).

5.2.7 Affymetrix Exon Array and Computational Analysis

Exon array (Hu-Ex1.0 stv2, Affymetrix, Santa Clara, CA) analysis was performed on total RNA in collaboration with the Gene Expression Profiling Unit of the Medical University Innsbruck. cRNA target synthesis and GeneChip® processing were performed according to standard protocols (Affymetrix, Inc., Santa Clara, CA). CEL files were analyzed in R statistical environment using Bioconductor packages (Gentleman, Carey et al. 2004). The "aroma-affymetrix" package was used to perform exon array specific analyses (Bengtsson, Simpson et al. 2008). Only "core" probesets were analyzed using the custom cdf provided by the aroma-affymetrix project (http://aroma-project.org/node/29). In the following analysis also "cross-hybridizing" probes and probesets were excluded (as indicated in the NetAffx Annotation Files available from affymetrix.com). The analysis pipeline followed essentially the vignette for FIRMA analysis available from: http://aroma-project.org/vignettes/FIRMA-HumanExonArrayAnalysis. In brief, CEL files were read into R, background corrected, summarized by the RMA algorithm and quantile normalized (Irizarry, Bolstad et al. 2003; Bengtsson, Simpson et al. 2008). Subsequently exon-level and transcript-level expression estimates were extracted. The FIRMA algorithm was applied to indentify exons that deviate from RMA estimates of the transcripts (Purdom, Simpson et al. 2008) and FRIMA scores were compared for each exon between experimental treatments using a moderated t-test from the R package limma (Wettenhall and Smyth 2004). Limma was also used to detect differences between experimental groups in whole transcript levels and in exon levels separately. To preclude erroneous results due to very low hybridization signals these probesets were filtered out before analysis (quantile < 0.4).

5.2.8 PCR Techniques

RT-PCR

Total RNA was transcribed into cDNA using random hexamer primers (Promega, Madison, WI) and M-MLV Reverse Transcriptase (Promega, Madison, WI) according to manufacturer's instructions. PCR reactions were set up with DyNAzyme™ II DNA Polymerase (Finnzymes, Thermo Fisher Scientific, Rockford, IL) and run in a Dyad Disciple Thermal Cycler (Bio-Rad, Hercules, CA).

RACE-PCR

Total RNA was transcribed into cDNA and ligated to a DNA-adaptor using the Marathon cDNA Amplification Kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. 5'RACE-PCR reactions were performed in a Dyad Disciple Thermal Cycler (Bio-Rad, Hercules, CA) involving the Advantage 2 Polymerase Mix (Clontech, Mountain View, CA).

SYBRgreen PCR

For quantitative SYBRgreen PCRs the Maxima® SYBR Green/ ROX qPCR Master Mix (2x) (Fermentas, Thermo Fisher Scientific, Rockford, IL) was used according to manufacturer's instructions. The reactions were run in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and analysed with the corresponding software.

RT-qPCR

Total RNA was transcribed into cDNA as described in 2.8.1. Quantitative RT-PCR reactions were set up using TaqMan® Gene Expression Master Mix (2x) (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions and run in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and analysed with the corresponding software.

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It is now 04:56 a.m. I have worked the last four years on this project and it took me almost six months to write up this thesis. Finally, one more question remains to be solved:

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It is now 06:04 a.m. – enough time sentimentally spent! I should try and get some sleep...

Vienna, September 2012

Danksagung

Es ist jetzt 04:56 Uhr morgens. Die letzten vier Jahre habe ich mit der Arbeit an diesem Projekt verbracht und es hat fast sechs Monate gedauert, diese Doktorarbeit zu schreiben. Schlussendlich gibt es noch eine ungelöste Frage:

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Ich danke euch, Max und Ulli, für all eure Hilfe und Unterstützung! Vier Eltern sind mindestens doppelt so gut wie zwei!

Außerdem bedanke ich mich bei meinen Geschwistern, Barbara, Katharina und Paul, und dem Rest meiner sehr großen Familie – es ist für eine Genetikerin immer interessant, mit was für verschiedenen Menschen man doch verwandt sein kann.

Und schließlich möchte ich mich noch bei Helene, meiner allerersten Lehrerin, und allen Lehrern, die ich jemals hatte, bedanken. Ich habe es immer geliebt zu lernen und die meisten von ihnen haben mich dabei gefördert (und der Rest konnte es mir zumindest nicht austreiben).

Niemand hat je gesagt, dass es einfach werden würde und das war es auch nicht. Das ist nur fair. Trotzdem würde ich das um Nichts in der Welt versäumt haben wollen!

Es ist jetzt 06:04 Uhr morgens – genug Sentimentalitäten für diese Tageszeit! Ich sollte doch noch etwas Schlaf bekommen…

Wien-Ottakring, September 2012

Curriculum Vitae

Last name:	Riedmann
First names:	Lucia Theresia
Date of birth:	30.08.1981
Place of birth:	Oberstdorf, BRD
Address:	Lorenz Mandl-Gasse 30/18, 1160 Vienna
Citizenship:	Austria
Languages:	German (mother tongue)
	English (fluent)
	French (basic)
	Russian (basic)

Practical Experience

May 2008 – October 2012	PhD position at the Children's Cancer Research Institute (CCRI), St. Anna Kinderkrebsforschung, Vienna, Austria Project: "The Effect of EWS and its Oncogenic Derivative EWS-FLI1 on Transcriptional and Post-Transcriptional Gene Regulation in Ewing Sarcoma" Supervisor: Prof. Dr. Heinrich Kovar
October 2005 – January 2007	Diploma position at the Department of Chromosome Biology, University of Vienna, Vienna, Austria Project: "Generation of *C. elegans* Transgenic Lines Expressing Variants of LAP-tagged PROM-1 for Cytological and Biochemical Analysis" Supervisor: Prof. Dr. Josef Loidl
August 2005 – September 2005	Trainee position at the Department of Cell and Molecular Biology (CMB), Medical Nobel Institute, Karolinska Institutet, Stockholm, Sweden Project: "Cloning of Plasmids for the Generation of ES Cells with Inducible NOTCH Signaling" Supervisor: Prof. Dr. Urban Lendahl

Education

Since March 2008	Dr.-Studium der Naturwissenschaften Genetik-Mikrobiologie
October 2006 – April 2008	Undergraduate Studies of International Economics, Economic University, Vienna, Austria
November 2007	Masters Degree with Excellent Success
October 2001 – November 2007	Undergraduate Studies Microbiology-Genetics, University of Vienna, Vienna, Austria Principal Subject: Cyto- and Developmental Genetics Elective Subjects: Biochemistry Molecular Genetics and Pathology
March – June 2001	Undergraduate Studies History of Arts, University of Vienna, Vienna, Austria
June 2000	A-levels with Special Emphasis on Arts with Excellent Success
September 1996 – June 2000	Secondary School, BORG Grieskirchen with Special Emphasis on Arts

Attended Meetings

October 2012	EMBO-ESF meeting, Pultusk, Poland Poster Presentation "The Effect of EWS on Transcriptional and Post-transcriptional Gene Regulation in Ewing's Sarcoma"
September 2011	3rd EMBO Meeting, Vienna, Austria
September 2010	ÖGMBT Annual Meeting, Vienna, Austria Poster Presentation "Evidence for an Effect of EWS on Transcript Exon Composition in Ewing's Sarcoma"
May 2010	Microsymposium on Small RNAs, Vienna, Austria
April 2010	AACR 101st Annual Meeting, Washington D.C., Maryland, USA Poster Presentation "Evidence for a Role of EWS in Alternative Splicing in Ewing's Sarcoma"
September 2009	ÖGMBT Annual Meeting, Innsbruck, Austria Poster Presentation "The Role of EWS and EWS-FLI1 in Alternative Splicing"
May 2009	Microsymposium on Small RNAs, Vienna, Austria
May 2008	Microsymposium on Small RNAs, Vienna, Austria

List of Publications

Kovar H, Alonso J, Aman P, Aryee DNT, Ban J, Burchill SA, Burdach S, De Alava E, Delattre O, Dirksen U, Fourtouna A, Fulda S, Helman LJ, Herrero-Martin D, Hogendoorn PC, Kontny U, Lawlor ER, Lessnick SL, Llombart-Bosch A, Metzler M, Moriggl R, Niedan S, Potratz J, Redini F, Richter GH, **Riedmann LT**, Rossig C, Schafer BW, Schwentner R, Scotlandi K, Sorensen PH, Staege MS, Tirode F, Toretsky J, Ventura S, Eggert A, Ladenstein R. "The First European Interdisciplinary Ewing Sarcoma Research Summit". (2012) Frontiers in Pediatric Oncology 2: 54. DOI 10.3389/fonc.2012.00054.

Herrero-Martin D, Fourtouna A, Niedan S, **Riedmann LT**, Schwentner R, Aryee DNT. "Factors Affecting EWS-FLI1 Activity in Ewing's Sarcoma". (2011) Sarcoma 352580. DOI 10.1155/2011/352580

Riedmann LT and Schwentner R. (2010) „miRNA, siRNA, piRNA and Argonautes: News in Small Matters". (2010) 7(2):133-139. DOI 11288 [pii]