EWS-FLI1 degradation is mediated by one lysine residue

Proteasomal degradation of the EWS-FLI1 fusion protein is regulated by a single lysine residue

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Abstract
E-26 transformation specific (ETS) proteins are transcription factors directing gene expression through their conserved DNA-binding domain. They are implicated as truncated forms or interchromosomal rearrangements in a variety of tumors including Ewing sarcoma, a pediatric tumor of the bone. Tumor cells express the chimeric oncoprotein EWS-FLI1 from a specific t(22;11)(q24;12) translocation. EWS-FLI1 harbors a strong transactivation domain from EWSR1 and the DNA-binding ETS domain of FLI1 in the C-terminal part of the protein. Even though Ewing cells are crucially dependent on continuous expression of EWS-FLI1, its regulation of turnover has not been characterized in detail.

Here, we identify the EWS-FLI1 protein as a substrate of the ubiquitin proteasome system with a characteristic poly-ubiquitination pattern. Using a Global Protein Stability approach, we determined the half life of EWS-FLI1 to lie between 2h-4h whereas full length EWSR1 and FLI1 were more stable. By mass spectrometry, we identified two ubiquitin acceptor lysine residues of which only mutation of K380 in the ETS domain of the FLI1 part abolished EWS-FLI1 ubiquitination and stabilized the protein posttranslationally. Expression of this highly stable mutant protein in Ewing cells, while simultaneously depleting the endogenous wild type protein, differentially modulates two subgroups of target genes to be either EWS-FLI1 protein dependent or turnover dependent. The majority of target genes is in an unaltered state and cannot be further activated.

Our study provides novel insights into EWS-FLI1 turnover, a critical pathway in Ewing sarcoma pathogenesis and lies new grounds to develop novel therapeutic strategies in Ewing sarcoma.

Introduction
E-26 transforming specific (ETS) family members are strong activators or repressors of transcription with a highly conserved ETS domain (1-3). ETS transcription factors (TFs) bind most commonly in complexes to a GGA core region in order to mediate gene expression (4,5). Their main biological functions include regulation of differentiation, lineage determination of the hematopoietic system and control of angiogenesis (6,7). Most of the ETS family members have oncogenic potential, since truncated or overexpressed ETS proteins have been linked to several cancer entities (8-11). ERG or ETV1 are...
EWS-FLI1 degradation is mediated by one lysine residue frequently fused to the TMPRSS2 promoter in prostate cancer, whereas ETV1 and ETV6 are implicated in leukemia (12,13). Like other aberrant fusion proteins, they act as drivers of uncontrolled cell growth and survival (14,15). However, most TFs do not harbor an enzymatic pocket and are therefore difficult to target directly. Novel strategies which uncover vulnerable sites in TFs are urgently needed to develop novel targeted therapies (16).

Ewing sarcoma is a rare pediatric bone and soft tissue tumor with an aggressive behavior and prevalence to metastasize (17,18). Its main genetic abnormalities are EWS–ETS rearrangements, among them most commonly the EWS gene on chromosome 22 fused to FLI1 on chromosome 11 which results in expression of the chimeric transcription factor EWS-FLI1 (19-21). Continuous expression of the fusion protein is crucial for tumor formation, progression and maintenance (22,23) and its downregulation inhibits proliferation and reduces tumor cell growth (24-26). EWS-FLI1 is thought to function mainly as modulator to activate and repress a wide range of target genes, but also to regulate splicing processes or being a component of large interaction networks (27- 31). However, inhibition of a single downstream target gene has not been proven effective yet for Ewing sarcoma therapy.

The turnover of most intracellular proteins is mediated via the ubiquitin proteasome system (UPS) which triggers protein degradation (32). Several ETS proteins can be poly-ubiquitinated by different E3 ligases and subsequently degraded by the proteasome (33-36). Considering their high conservation, proteasomal degradation is likely to be the main mechanism of turnover for most ETS family members. Most interestingly, truncated ERG and ETV1 are lacking the N-terminal E3 binding domain which results in a delayed turnover of aberrant ETS proteins (34, 36). Here, we focus specifically on the turnover of the fusion protein EWS-FLI1 which only harbors the ETS and the C-terminal domain from FLI1. The impact on the turnover of this domain fused to N-terminal region of EWSR1, has not been investigated yet. Hence, EWS-FLI1 proteasomal turnover represents a so far uncharacterized mechanism in Ewing sarcoma tumorigenesis, even though EWS-FLI1 degradation has already been linked to the lysosomal pathway (37). Moreover, the exact lysine acceptor residues for poly-ubiquitination have not yet been identified for any wild type or truncated ETS protein.

In the present study, we demonstrate that EWS-FLI1 is predominantly a proteasomal substrate with an unexpectedly high turnover rate mediated by poly-ubiquitination at a single lysine residue. Surprisingly, expression of this highly stable mutant protein in Ewing cells specifically induced subgroups of high-level expressed or turnover sensitive target genes, while the majority of target genes are unaltered.

Hence, our study provides novel insights into EWS-FLI1 turnover suggesting that deflecting its stability could contribute to new therapeutic concepts in Ewing sarcoma.

Results

EWS-FLI1 turnover is proteasome dependent. Since EWS-FLI1 expression is crucial for tumor cell survival (24-26), we were interested to analyze degradation and turnover rate of the fusion protein. To address this, we first used a panel of six different inhibitors targeting proteasomal, lysosomal and autophagosomal protein degradation pathways. Incubation with 20µM of the various inhibitors resulted in a 2-fold upregulation of EWS-FLI1 protein levels only upon MG-132 and Bortezomib treatment, both inhibitors of the chymotrypsin-like proteasome activity (Fig. 1A). We next confirmed this result by selectively inhibiting the proteasomal and lysosomal degradation pathways with two compounds in three different Ewing sarcoma cell lines. Incubation with 20µM of the proteasome inhibitor MG-132 resulted in stabilization of endogenous EWS-FLI1 fusion protein by at least 2-fold while this was not the case for the lysosomal inhibitor chloroquine (Fig. 1B). P27 and LAMP1 were used as positive controls and showed upregulation after treatment as expected. In addition, stabilization of the fusion protein in Ewing cells by MG-132 increased over a 2h-8h treatment period in a time- dependent manner (Fig. 1C). Hence, degradation of EWS-FLI1 is primarily proteasome dependent under steady-state conditions. However, incubation with 20µM cycloheximide (CHX) had only limited reducing effect on the endogenous fusion protein in the Ewing cells over the same 8h time period (Fig. 1D).
Next, we investigated whether the fusion protein is ubiquitinated. To this end, 3xflag-EWS-FLI1 was co-expressed with HA-ubiquitin in HEK293T cells and immunoprecipitated after 48h using anti-flag antibody. Western blotting with an anti-HA antibody revealed at least four distinct ubiquitin bands for EWS-FLI1 which could not be increased by prior treatment with MG132 (Fig. 1E), suggesting a constant ubiquitination of the fusion protein. We next co-expressed 3xflag-EWS-FLI1 with wild type HA-ubiquitin or ubiquitin mutants which are deficient for K48- and K63-linked chains. Immunoprecipitation of the ubiquitinated 3xflag-EWS-FLI1 revealed mainly a decrease of the ubiquitination pattern using K48R HA-ubiquitin (Fig. 1F) indicating that this is the major, but not only, linked ubiquitin chains of the fusion protein.

To further validate the fusion protein turnover and to overcome the limitations of classical CHX treatment to determine protein half lives, we next used the Global Protein Stability (GPS) approach (38) (Fig. 2A). Briefly, HEK293T cells were transduced with a reporter construct DsRed-IRES-EGFP in which EGFP is fused to EWS-FLI1. The ratio EGFP/DsRed was determined by FACS and represents a measure for protein stability. Known degron motifs with distinct half lives were used as internal standards and allowed to estimate the half life of EWS-FLI1 to be between 1h and 4h (Fig. 2B). Incubation with MG-132 shifted the ratio closer to 4h, confirming that EWS-FLI1 is a substrate of the proteasome system with a high turnover rate. Additionally, we treated cells with the nuclear export inhibitor leptomycin B (LMB) for 4h and 8h. Treatment with 50nM LMB stabilized EWS-FLI1 in a time-dependent manner (Fig. 2C) indicating that the proteasomal degradation mainly occurs in the cytosol. Similar results were obtained in transiently or stably transduced Ewing cells (Fig. 2D-E), suggesting that the high turnover of EWS-FLI1 is mostly independent of the cellular background. Interestingly, incubation with 20µM CHX again displayed a limited effect on the EGFP/DsRed ratio of EWS-FLI1 reporter construct (Fig. 2B,2E) indicating that classical CHX barely reflects the steady-state turnover rate of the EWS-FLI1 protein and possibly other proteins.

Taken together, our results indicate that EWS-FLI1 is a poly-ubiquitinated protein with an unexpectedly high turnover rate in Ewing sarcoma cells.

EWS-FLI1 turnover depends on one critical lysine residue. To better understand fusion protein turnover, we aimed to identify the lysine residue(s) that are important for degradation. To this, we purified large amounts of 3xflag-EWS-FLI1 or a dominant-negative R386N mutant (39,40) from HEK293T cells. The inactive mutant was included as the inability to bind DNA increases the possibility for ubiquitination. Both samples were then enzymatically digested and subjected to mass spectrometry to identify peptide shifts due to covalently attached ubiquitin. This identified two peptides with the characteristic glycine-glycine shift including residues K298 and K380 (Fig. 3A, supplementary S1A). To determine whether both sites are important for fusion protein turnover, we mutated them individually to arginine. After co-expressing the mutant EWS-FLI1 with HA-ubiquitin and immunoprecipitation from A673 Ewing cells, we found that the K298R mutant was still ubiquitinated comparable to the wild type, while the K380R lost this characteristic pattern (Fig. 3B). In addition, transient expression of the mutants in both A673 and SKNMC Ewing cells revealed that the K380R and the double mutant K298/380R were 2.5-fold, 3.5-fold respectively, more stable than wild type or the K298R mutant (Fig. 3C-D). As wild type EWS-FLI1, also the K380 mutant was still localized in the nucleus (Fig. 3E). We then investigated the role of both lysines in proteasomal turnover by GPS. The half life of the K298R was comparable to wild type EWS-FLI1 while K380R shifted close to 24h (Fig. 3F). Wild type EWS-FLI1 and the K298R could be stabilized by an additional incubation with MG-132, however the EGFP/DsRed ratio of the K380R mutant did not change upon treatment (Fig. 3G). Hence, our findings suggest that K380 is the major site required to regulate EWS-FLI1 protein turnover by the proteasome.

EWS-FLI1 fusion protein and wild type FLI1 share a unique site for turnover. To investigate whether turnover by K380 is unique to the fusion protein, we subsequently compared stability and ubiquitination pattern to the full length proteins EWSR1 and FLI1. Surprisingly, both wild type proteins were more stable with half
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lives of >4h (FLI1) and close to 24h (EWSR1) (Fig. 4A). While FLI1 could be stabilized by incubation with MG-132 (or destabilized by CHX), EWSR1 did not change its already long half life by these treatments (Fig. 4B). As FLI1 was clearly poly-ubiquitinated similar to the fusion protein, EWSR1 showed strong mono- and di-ubiquitination (Fig 4C). Next, we mutated the previously identified lysine residue to arginine (FLI1: K334). By GPS, the K334R also increased its half life towards 24h (Fig. 4D). Additionally, mass spectrometry of purified 3xflag-FLI1 revealed glycine-glycine shifts for peptides containing lysines K172 and K252 (peptide spectra in supplementary S1B-C). Whereas K172 is located in the N-terminal part and therefore not present in the fusion protein, K252 corresponds to the EWS-FLI1 K298 residue. Single arginine mutants of both residues display EGFP/DsRed ratios comparable to wild type FLI1 (Fig. 4D, dotted lines) indicating that both fusion and full length protein turnover is regulated by one single lysine residue. Indeed, incubation of FLI1 and K334R with MG-132 only shifted the wild type, but not the K334R mutant ratio (data not shown).

Interestingly, the K334/K380 residue is conserved within most ETS family members (Fig. 4E). Together, our findings indicate that, despite differences in turnover, stability of both EWS-FLI1 and wild type FLI1 is regulated by the same critical conserved residue in the ETS domain.

EWS-FLI1 target genes expression is subdivided into three major groups. Modulation of EWS-FLI1 target gene signature is one of the best studied mechanisms in Ewing sarcoma. EWS-FLI1 is activating a wide range of target genes like NKX2.2, NR0B1 or IGF1 (30,31,41-43), but has also repressive functions as shown for IGFBP3, PHLDA1 and LOX (44-46). Consequently, this is thought to be critical for oncogenic properties as depletion of the fusion protein results in growth inhibition and cellular senescence (24,47). To better understand the effect of EWS-FLI1 stability on the target gene signature, we established inducible exchange cell lines which were capable to knock down the endogenous EWS-FLI1 while simultaneously expressing 3xflag-EWS-FLI1 or the K380R mutant. To this, we first sorted cells expressing these flag tagged overexpression constructs by flow cytometry for the same low EGFP expression (the selection marker of the pInducer21 vector) to obtain a homogeneously expressing population (Fig. 5A). We then subsequently transduced each of these clonal pools with two different shRNA constructs against the 3’UTR of EWS-FLI1 (Fig. 5B). Upon doxycycline induction for 48h, endogenous EWS-FLI1 was exchanged with ectopic wild type or mutant 3xflag-EWS-FLI1 versions (Fig. 5C). As observed before, the K380R mutant stabilized the protein posttranslationally by at least four-fold (Fig. 5C) while EWS-FLI1 mRNA levels were comparable as shown for shEF#2 (Fig. 5D, not shown for shEF#1 based cell lines). We then extracted total RNA for microarray expression profiling and analyzed the EWS-FLI1 target genes signature for their differential expression upon stabilized fusion protein (supplementary S2). A total of 497 probe sets were downregulated by at least 1.5-fold due to EWS-FLI1 depletion from which 250 could be rescued to at least normal levels by induction of EWS-FLI1 wild type (Fig. 6A-B). Only if the EWS-FLI1 target genes were modulated and recovered in the paired comparison of EWS-FLI1 induction and knockdown at each 24h and 48h, they were considered as rescued. This pattern was similar for repressed target genes as 333 probe sets were at least 1.5-fold upregulated from which 164 could be again repressed by ectopic EWS-FLI1 (Fig. 6A, 6C). However, we were most interested in the target genes which were further modulated with stabilized protein levels. Surprisingly, the large majority of these genes appeared to be unmodified and displayed no difference in target gene expression between wild type and mutant EWS-FLI1. However, two subgroups were modulated differentially by the mutated fusion protein. One subgroup seems to be protein sensitive as 15 individual genes displayed higher RNA levels by at least 1.5-fold upon expression of stabilized EWS-FLI1 protein. From the other subgroup, 29 rescued candidates were fusion protein turnover sensitive and could only partly be modulated by the mutant in comparison to wild type EWS-FLI1 (Fig. 6A-B, list supplementary S2). Among the protein sensitive candidates, IGF1 and long non-coding RNA EWSAT1 have been already characterized (41,48) while most other target genes have not been implicated in EWS-FLI1 oncogenesis. Selected target genes from the protein dependent and unmodified group were validated by quantitative RT-PCR level for two
different shEWS-FLI1 sequences (Fig. 6D, shown for shEF#2). Even though the exact mechanism of EWS-FLI1 gene repression is not fully understood, the pattern of target gene modulation resembled that of activated target genes. The majority of repressed genes were unmodified, but also here two subgroups differentially modulated repression upon expression of stabilized protein levels (Fig. 6C, 6E, list supplementary S2).

Taken together, our results indicate that 82% of activated, and 93% of repressed target genes have unaltered levels and cannot be further increased by higher EWS-FLI1 protein levels. Only subsets of the target gene signature are differentially modulated implying that stability of EWS-FLI1 directs these important subgroup or primarily modulates other mechanisms.

Discussion
Here, we demonstrated that EWS-FLI1 is a polyubiquitinated substrate that is primarily degraded by the proteasome. The high turnover of the fusion protein is controlled by one critical lysine residue located in the conserved ETS domain. By exchanging the wild type EWS-FLI1 with its turnover deficient mutant, we could show that protein stabilization differentially modulates two subsets of target genes whose expression could either be enhanced (protein dependent) or only partially rescued (turnover dependent). However, the majority of target genes are in an unmodified state and independent of protein stabilization.

Attachment of ubiquitins to a substrate triggers a variety of outcomes including degradation and signaling depending on the ubiquitin acceptor site and ubiquitin chain linkage (32). Here, we identified the fusion protein EWS-FLI1 as a substrate of the proteasome system in a time and dose dependent manner. The previously suggested lysosomal degradation route (37) possibly contributes to turnover, but appears less relevant compared to the proteasomal pathway under steady state conditions.

During the last decades, a variety of different technologies have been developed to dissect and understand ubiquitin mediated processes (49). GPS profiling was initially established to identify novel proteasomal or cullin-RING ligase substrates (38,50,51). Thus, we applied this approach to analyze EWS-FLI1 turnover. Displaying the EGFP/DsRed ratio for the fusion protein and known standard reporter constructs, we confirmed EWS-FLI1 turnover as proteasome dependent and mapped the half life to be between 1h-4h. As most potent transcription factors, also EWS-FLI1 displays a fast turnover (52,53). Using Leptomycin B, we could show that EWS-FLI1 is indeed exported out of the nucleus for degradation suggesting that the fusion protein tightly keeps balance of its cytosolic proteolysis. The apparent exclusive nuclear localization might therefore be due to its rapid cytosolic degradation.

However, incubation of cells with the translation synthesis inhibitor CHX barely reflected the high turnover observed in the GPS under steady-state conditions. Using this assay, others already suggested EWS-FLI1 as a stable protein (37). In contrast, by using antisense oligodeoxynucleotides (26) and RNAi approaches, it is known that the fusion protein can be efficiently downregulated within a short 24h time frame which would not be possible with a highly stable protein. The discrepancy between the GPS/RNAi data and CHX treatment therefore remains. However, complete inhibition of protein synthesis by CHX also affects other components of the ubiquitin system such as E2 conjugating enzymes or E3 ligases. Possibly, if a required E3 ligase is unstable and immediately depleted, EWS-FLI1 turnover might be prolonged as seen here with classical CHX treatment.

While EWS-FLI1 has been shown to be posttranslationaly modified (54-56), ubiquitination remained unknown. Here, we demonstrated that the fusion protein is polyubiquitinated with a clear and distinct pattern which supports the concept of a constant turnover. To better understand how the ubiquitinated fusion protein is degraded, we used affinity purified EWS-FLI1 as a substrate of the proteasome system in a time and dose dependent manner. The previously suggested lysosomal degradation route (37) possibly contributes to turnover, but appears less relevant compared to the proteasomal pathway under steady state conditions.

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shifts mainly for K48-, but also K63-linked ubiquitins (data not shown) confirming our findings that K48-linked chains are highly abundant, but not the exclusive EWS-FLI1 ubiquitination pattern. Other ubiquitin linked chains or mixed chains might also be relevant.

Further, mutation of this residue may also inhibit other modifications such as sumoylation. Acetylation of this residue has not been detected in contrast to the K298 site (55).

The comparison of EWS-FLI1 with its full length proteins revealed that the polyubiquitin pattern is similar to FLI1. Indeed, mutation of the corresponding lysine residue in the ETS domain posttranslationally stabilizes both proteins. As the K380/K334 residue is highly conserved among most ETS family members, it might contribute to proteasomal degradation in other ETS proteins and their fusion proteins. Most interestingly, EWS-FLI1 displays a higher turnover compared to its full length proteins EWSR1 and FLI1. In contrast, the ETS proteins ERG or ETV1 which are fused to the TMPRSS2 promoter in prostate cancer stabilize the protein and confer a physiological advantage to cancer cells (33,34,36). The truncated protein versions display increased stability due to loss of the N-terminal E3 ligase motif. However, EWS-FLI1 is not a truncated version of FLI1, instead the N-terminal domain is dominated by EWSR1 with a stronger transactivation domain. If the turnover and subsequent E3 ligase binding of ETS members is conserved, EWS-FLI1 might possibly interact with E3 ligases via its EWSR1 domain giving rise to a new regulatory mechanism. However, the basic concept that truncated oncogenic TFs are more stable than their wild type counterparts (36) might not apply for fusion proteins consisting of two unrelated protein domains. Whether this is indeed a general paradigm or rather specific for EWSR1 related fusion proteins needs to be further elucidated.

We then deciphered the role of EWS-FLI1 turnover in transcriptional regulation of target genes. To investigate the influence of stabilized EWS-FLI1 on target gene expression, we established an inducible Ewing cell system allowing to deplete endogenous EWS-FLI1 protein while simultaneously expressing the wild type fusion protein or a turnover deficient mutant. This system largely circumvents interference with endogenous fusion protein, enabling us to study the dynamic behavior of posttranslationally stabilized EWS-FLI1 protein on transcriptional regulation. Surprisingly, around 85% of both activated and repressed target genes are in an unaltered state including well characterized target genes such as NR0B1 or STEAP1 (30,57).

Even though our EWS-FLI1 wild type overexpression constructs are around 2-fold above the endogenous EWS-FLI1 levels, it is possible that co-factors expressed at endogenous levels or interacting proteins are limiting further modulation. The knowledge of the EWS-FLI1 interactome is continuously expanding (28,29), however, it still remains unsolved which co-factors or interacting proteins are most important or influence the fusion protein in a context dependent matter. Nevertheless, longer induction of wild type or mutated ectopic EWS-FLI1 up to 96h induced cell death irrespective of the construct (data not shown).

Beside the majority of unmodified target genes, two other subgroups display a different behavior. 15 activated target genes are higher expressed upon stabilized EWS-FLI1 protein levels. This group includes protein-coding genes such as IGF1 (41) or LEMD1 and RNA-coding genes such as EWSAT1 (48). Interestingly, EWSAT1 was also observed to be elevated upon higher EWS-FLI1 expression in a different experimental setting (48) suggesting that our approach reflects a global pattern. Further, 29 activated target genes are less expressed upon stabilized EWS-FLI1. None of these turnover sensitive target genes have yet been characterized even though few interesting candidates as ALK or TP63 appeared.

Most interestingly, the activity of TFs can be influenced by their own turnover as shown for estrogen receptor α or c-MYC (58,59). It was recently shown that the proteasomal turnover of c-myc is required for full activity. Lysine mutated c-MYC failed to induce tumorigenesis as inhibitory complexes could not be removed during target gene activation (58) while other TFs are independent of ongoing degradation for their transcriptional activity (60). Surprisingly, EWS-FLI1 induced distinct behavior in three subgroups of target genes. Speculatively, the two small subgroups of EWS-FLI1 protein sensitive and turnover sensitive target genes might be of greater importance and their dynamics might trigger different response pathways in Ewing sarcoma.
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oncogenicity. Even though it is not yet clear how these subgroups are defined at their regulatory regions, possible driver target genes might be found in these two subgroups. Alternatively, since we see most target gene levels unaltered, oncogenic properties might depend also on additional mechanisms such as interacting proteins and/or influence on the splicing machinery.

Taken together, turnover regulation of the major oncogenic driver EWS-FLI1 represents an important regulatory mechanism in Ewing sarcoma pathogenesis. Interference with fusion protein degradation might not only be a novel therapeutic strategy in Ewing sarcoma treatment, but might also be applicable to other ETS based fusion proteins.

Experimental procedure

Cell lines and reagents- HEK293T cells were cultured in DMEM (Sigma Aldrich, Buchs, Switzerland) supplemented with 10% FBS (Sigma Aldrich), 2mmol/L glutamine (BioConcept, Allschwil, Switzerland) and 100U/ml penicillin/streptomycin (ThermoFisher Scientific AG, Reinach, Switzerland) at 37°C in 5% CO2. Ewing sarcoma cell lines A673, SKNMC and TC71 were cultured in RPMI as described above. Additionally, dishes were pre-coated with 0.2% gelatine (Sigma Aldrich). All cell lines have been tested mycoplasma negative. The following reagents were used: Bortezomib (Selleckchem, Houston, TX, USA), Chloroquine, cycloheximide, DMSO, doxycycline, E64, leptomycin B (all Sigma Aldrich), LY294002, MG-132 (Millipore, Billerica, MA, USA) and 3-Methyladenine (ApexBio, Houston, TX, USA).

Plasmids and cloning- The coding sequence for human EWS-FLI1, FLI1 and EWS, were subcloned into the NotI site of pCMV-3xflag vector (Sigma Aldrich). For Global Protein Stability, the coding sequences of DsRed-IRES-EGFP or d24-EGFP or d4-EGFP (addgene #41941, #41944, #41943, #41942) were cloned into the EcoR1 site of pR-EF1 (Cellecta Inc., Mountain View, CA, USA). All cDNAs (EWS-FLI1, FLI1, EWS and mutants) were inserted into the BSTB1 site at the 3’ end of DsRed-IRES-EGFP.

3xflag-EWS-FLI1 cDNA was cloned into the SpeI-BamH1 sites of pInducer21 ORF (Addgene #46948). The pRSIT-U6Tet-shRNA-PGKTetRep-2A-GFP-2A-puro vector with shRNAs against EWS-FLI1 was purchased from Cellecta Inc. with the following target sequences: shEF#1 5’ ATAGAGGTGGGAAGCTTATAA 3’ (previously described in (31)) and shEF#2 5’ CGTCATGTTCGTTTGTAGAT 3’ (designed against the C-terminal FLI1 part according to RefSeq# NM_002017.4).

Cloning was performed by In-Fusion cloning HD (Clontech Laboratories Inc., Mountain View, CA, USA) according to manufacturer’s protocol. All mutations were introduced using site-directed mutagenesis. Detailed information of plasmids, cloning and mutagenesis primers can be found in supplementary table ST1. All clonings were verified by sequencing.

Transient transfection- For HEK293T cells, DMEM complete medium was mixed with PolyethyleneimineMax (Polyscience, Cham, Switzerland) and plasmids for 15min and added to the cells for 48h. For Ewing sarcoma cells, JetPrime (Polyplus Transfections, Illkirch, France) reagent was used according to manufacturer’s instruction in antibiotics-free RPMI medium.

Viral production and transduction- HEK293T cells were transfected with cDNA vectors and pMDL, pREV, and pVSV with JetPrime according to manufacturer’s instruction in antibiotics-free DMEM medium. Medium was replaced 24h after transfection and virus was harvested after additional 48h. Viral supernatant was cleared by centrifugation, filtered and concentrated if necessary (Amicon® Ultra 15 mL, Millipore). Ewing sarcoma cells were infected with the viral supernatant supplemented with 10µg/ml polybrene (Sigma Aldrich) for 16-18h.

Global Protein Stability Assay and flow cytometry- Lentivirus from HEK293T transfected with pR-EF1-DsRed-IRES-d1/d4/d24/EGFP-cDNA, pMDL, pREV and pVSV was used to transduce HEK293T or Ewing sarcoma cells as described to about 5-10% of DsRed positive cells. Cells were harvested 72h after transduction, incubated with cycloheximide, leptomycin B or MG-132 for 4-8h as indicated and analyzed by FACS. Cells were resuspended in PBS and filtered.
EWS-FLI1 degradation is mediated by one lysine residue using a 40μm cell strainer (BD Becton, Dickinson and Company, Franklin Lakes, NJ, USA). FACS analysis was performed on a FACS Canto™ II Cytometry system (BD) and data were analyzed by FlowJo software (Treestar Inc., Ashland, OR, USA). At least 50000 cells were recorded for each experiment. Cell sorting was done with a FACS AriaTM III Cytometry system (BD).

Cell lysis, western blotting and antibodies- Cells were harvested and lysed in standard lysis buffer (50mM Tris/HCl, 150mM NaCl, 50mM NaF, 5mM Na2P2O7, 1mM Na3VO4 and 10mM β-glycerophosphate, 1% TritonX with protease inhibitor cocktail, Complete Mini® (Sigma Aldrich)). Lysates were sonicated and cleared by centrifugation. Proteins were separated by 4%-12% or 10% BisTris NuPAGE pre-cast gels (ThermoFisher Scientific AG) and transferred to nitrocellulose membranes (GE Healthcare). Membranes were blocked with 5% milk powder in 0.2% PBS-Tween and primary antibodies were incubated over night at 4°C followed by 2h of HRP-linked secondary antibody at room temperature. Proteins were detected by chemiluminescence using Amersham ECL detection reagent (GE Healthcare, Glattbrugg, Switzerland) or SuperSignal™ Western blotting reagent (ThermoFisher Scientific AG). Quantification of blots was performed by using ImageJ (version 1.46r). The following commercial antibodies were used: anti-Actin (dilution 1:1000, Cell Signaling), anti-Flag (clone M2, 1:1000, Sigma Aldrich), anti-FLI1 (1:1000, MyBiosource LLC, San Diego, CA, USA), anti-GAPDH (1:1000, Cell Signaling), anti-HA (1:1000, Millipore), anti-LAMP1 (1:500, DSHB, Iowa City, Iowa, USA), anti-p27 (1:1000, Cell Signaling and 1:200 ThermoFisher Scientific AG) and anti-Tubulin (1:40000 Sigma Aldrich).

Immunoprecipitation of ubiquitinated proteins- Cells were transfected for 48h and treated with 10μg MG-132 prior to lysis in ubiquitin lysis buffer (2% SDS, 150mM NaCl, 10mM Tris/HCl, 2mM Na2VO4, 50mM NaF with Complete Mini Protease inhibitor cocktail), boiled for 10min and sonicated. Lysates were diluted 1:10 in dilution buffer (150mM NaCl, 10mM Tris/HCl, 2mM EDTA, 1% Triton X), incubated for 30min at 4°C and cleared by centrifugation for 30min at maximum speed. Immunoprecipitation was performed using anti-Flag antibody (Sigma Aldrich) coupled to Dynabeads ProteinG (ThermoFisher Scientific AG). Lysates were incubated for 1h at 4°C, washed three times, eluted at room temperature using 3xflag peptide (Sigma Aldrich) and prepared for western blot analysis. Extended description for detection of EWS-FLI1 ubiquitination sites by mass spectrometry can be found in supplementary S1.

Immunofluorescence and microscopy- A673 cells were seeded on cover slides for 24h and transiently transfected with flag tagged plasmids for additional 48h. After fixing with 4% PFA (Carl Roth, Arlesheim, Switzerland), cells were permeabilized and stained with anti-Flag antibody (1:300, Sigma Aldrich) in 4% horse serum (Sigma Aldrich) and 0.1% PBS-TritonX over night. Fluorescent secondary antibody (Alexa-488 anti-mouse, Sigma Aldrich) in PBS with 4% horse serum was applied for 1h. Cover slides were fixed on objective glass with DAPI Vectashield® mounting medium (Vector laboratories Inc., Burlingame, CA, USA) and analyzed by an Axioskop 2 MOT Plus (Carl Zeiss Microscopy LLC, Thornwood, NY, USA).

Quantitative PCR and microarray analysis- Total RNA was extracted from Ewing sarcoma cells using Qiagen RNeasy Kit (Qiagen Instruments AG, Hombrechtikon, Switzerland). cDNA synthesis was carried out using High-Capacity Reverse Transcription Kit (Applied Biosystems by ThermoFisher Scientific AG). Quantitative PCR was performed using TaqMan gene expression master mix (ThermoFisher Scientific AG) and assays on demand (Applied Biosystems by ThermoFisher Scientific AG). A complete list can be found in supplementary table ST1. Replicate values were pooled and represented as geometric mean values with a 95% confidence interval. Microarray expression analysis with total RNA was performed using GeneChip® Human Gene 2.0 ST Array (Atlas Biolabs, Berlin, Germany). Data are accessible under GSE81018.

Microarray data analysis- The raw microarray data were processed and normalized using the RMA algorithm (61). Differential expression was computed using the Bioconductor package limma providing a moderated t-test that is adapted to low number of replicates (62).
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Differential expression results were filtered based on fold-change and p-values. All computations were done using R/Bioconductor. Extended description for target gene grouping can be found in supplementary S2.

FOOTNOTES

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Author's contributions: MG, BS, FN designed the study and wrote the paper. MG and FP designed, performed and analyzed the experiments. LL performed and analyzed the mass spectrometry experiments. LH provided help with flow cytometry and sorted stable cell lines. All authors reviewed the results and approved the final version of the manuscript.
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References

1. Hollenhorst, P. C., McIntosh, L. P., and Graves, B. J. (2011) Genomic and biochemical insights into the specificity of ETS transcription factors. *Annu Rev Biochem* 80, 437-471
2. Laudet, V., Hanni, C., Stehelin, D., and Duterque-Coquillaud, M. (1999) Molecular phylogeny of the ETS gene family. *Oncogene* 18, 1351-1359
3. Wasylyk, B., Hahn, S. L., and Giovane, A. (1993) The Ets family of transcription factors. *Eur J Biochem* 211, 7-18
4. Li, R., Pei, H., and Watson, D. K. (2000) Regulation of Ets function by protein - protein interactions. *Oncogene* 19, 6514-6523
5. Nye, J. A., Petersen, J. M., Gunther, C. V., Jonsen, M. D., and Graves, B. J. (1992) Interaction of murine ets-1 with GGA-binding sites establishes the ETS domain as a new DNA-binding motif. *Genes Dev* 6, 975-990
6. Lelievre, E., Lionneton, F., Soncin, F., and Vandenbunder, B. (2001) The Ets family contains transcriptional activators and repressors involved in angiogenesis. *Int J Biochem Cell Biol* 33, 391-407
7. Sharrocks, A. D. (2001) The ETS-domain transcription factor family. *Nat Rev Mol Cell Biol* 2, 827-837
8. Davidson, B., Reich, R., Goldberg, I., Gotlieb, W. H., Kopolovic, J., Berner, A., Ben-Baruch, G., Bryne, M., and Nesland, J. M. (2001) Ets-1 messenger RNA expression is a novel marker of poor survival in ovarian carcinoma. *Clin Cancer Res* 7, 551-557
9. Golub, T. R., Barker, G. F., Bohlander, S. K., Hiebert, S. W., Ward, D. C., Bray-Ward, P., Morgan, E., Raimondi, S. C., Rowley, J. D., and Gilliland, D. G. (1995) Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukemia. *Proc Natl Acad Sci USA* 92, 4917-4921
10. Petrovics, G., Liu, A., Shaheduzzaman, S., Furusato, B., Sun, C., Chen, Y., Nau, M., Ravindranath, L., Chen, Y., Dobi, A., Srikanthan, V., Sesterhenn, I. A., McLeod, D. G., Vahey, M., Moul, J. W., and Srivastava, S. (2005) Frequent overexpression of ETS-related gene-1 (ERG1) in prostate cancer transcriptome. *Oncogene* 24, 3847-3852
11. Tomlins, S. A., Rhodes, D. R., Perner, S., Dhanasekaran, S. M., Mehra, R., Sun, X. W., Varambally, S., Cao, X., Tchinda, J., Kuefer, R., Lee, C., Montie, J. E., Shah, R. B., Pienta, K. J., Rubin, M. A., and Chinnaiyan, A. M. (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* 310, 644-648
12. Kumar-Sinha, C., Tomlins, S. A., and Chinnaiyan, A. M. (2008) Recurrent gene fusions in prostate cancer. *Nat Rev Cancer* 8, 497-511
13. Seth, A., and Watson, D. K. (2005) ETS transcription factors and their emerging roles in human cancer. *Eur J Cancer* 41, 2462-2478
14. Mertens, F., Antonescu, C. R., Hohenberger, P., Ladanyi, M., Modena, P., D’Incalci, M., Casali, P. G., Aglietta, M., and Alvegard, T. (2009) Translocation-related sarcomas. *Semin Oncol* 36, 312-323
15. Mittelman, F., Johansson, B., and Mertens, F. (2007) The impact of translocations and gene fusions on cancer causation. *Nat Rev Cancer* 7, 233-245
16. Hagenbuchner, J., and Ausserlechner, M. J. (2015) Targeting transcription factors by small compounds-Current strategies and future implications. *Biochem Pharmacol*
17. Bernstein, M., Kovan, H., Paulussen, M., Randall, R. L., Schuck, A., Teot, L. A., and Juergens, H. (2006) Ewing’s sarcoma family of tumors: Current management. *Oncologist* 11, 503-519
18. Eslashvili, N., Goodman, M., and Marcus, R. B., Jr. (2008) Changes in incidence and survival of Ewing sarcoma patients over the past 3 decades: Surveillance Epidemiology and End Results data. *J Pediatr Hematol Oncol* 30, 425-430
EWS-FLI1 degradation is mediated by one lysine residue

19. Delattre, O., Zucman, J., Plougastel, B., Desmaze, C., Melot, T., Peter, M., Kovar, H., Joubert, I., de Jong, P., Rouleau, G., and et al. (1992) Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature 359, 162-165

20. May, W. A., Gishizky, M. L., Lessnick, S. L., Lunsford, L. B., Lewis, B. C., Delattre, O., Zucman, J., Thomas, G., and Denny, C. T. (1993) Ewing sarcoma 11;22 translocation produces a chimeric transcription factor that requires the DNA-binding domain encoded by FLI1 for transformation. Proc Natl Acad Sci U S A 90, 5752-5756

21. May, W. A., Lessnick, S. L., Braun, B. S., Klemz, M., Lewis, B. C., Lunsford, L. B., Hromas, R., and Denny, C. T. (1993) The Ewing's sarcoma EWS-FLI-1 fusion gene encodes a more potent transcriptional activator and is a more powerful transforming gene than FLI-1. Mol Cell Biol 13, 7393-7398

22. Bailly, R. A., Bosselut, R., Zucman, J., Cormier, F., Delattre, O., Roussel, M., Thomas, G., and Ghysdael, J. (1994) DNA-binding and transcriptional activation properties of the EWS-FLI-1 fusion protein resulting from the t(11;22) translocation in Ewing sarcoma. Mol Cell Biol 14, 3230-3241

23. Braun, B. S., Frieden, R., Lessnick, S. L., May, W. A., and Denny, C. T. (1995) Identification of target genes for the Ewing's sarcoma EWS/FLI fusion protein by representational difference analysis. Mol Cell Biol 15, 4623-4630

24. Kovar, H., Aryee, D. N., Jug, G., Henockl, C., Schenper, M., Delattre, O., Thomas, G., and Gadner, H. (1996) EWS/FLI-1 antagonists induce growth inhibition of Ewing tumor cells in vitro. Cell Growth Differ 7, 429-437

25. Tanaka, K., Iwakuma, T., Harimaya, K., Sato, H., and Iwamoto, Y. (1997) EWS-Fli1 antisense oligodeoxynucleotide inhibits proliferation of human Ewing's sarcoma and primitive neuroectodermal tumor cells. J Clin Invest 99, 239-247

26. Toretsky, J. A., Connell, Y., Neckers, L., and Bhat, N. K. (1997) Inhibition of EWS-FLI-1 fusion protein with antisense oligodeoxynucleotides. J Neurooncol 31, 9-16

27. Petermann, R., Mossier, B. M., Aryee, D. N., Khazak, V., Golemis, E. A., and Kovar, H. (1998) Oncogenic EWS-Fli1 interacts with hsRPB7, a subunit of human RNA polymerase II. Oncogene 17, 603-610

28. Selvananathan, S. P., Graham, G. T., Erkizan, H. V., Dirksen, U., Natarajan, T. G., Dakic, A., Yu, S., Liu, X., Paulsen, M. T., Ljungman, M. E., Wu, C. H., Lawlor, E. R., Uren, A., and Toretsky, J. A. (2015) Oncogenic fusion protein EWS-FLI1 is a network hub that regulates alternative splicing. Proc Natl Acad Sci U S A 112, E1307-1316

29. Toretsky, J. A., Erkizan, V., Levenson, A., Abaan, O. D., Parvin, J. D., Cripe, T. P., Rice, A. M., Lee, S. B., and Uren, A. (2006) Oncoprotein EWS-Fli1 activity is enhanced by RNA helicase A. Cancer Res 66, 5574-5581

30. Kinsey, M., Smith, R., and Lessnick, S. L. (2006) NR0B1 is required for the oncogenic phenotype mediated by EWS/FLI in Ewing's sarcoma. Mol Cancer Res 4, 851-859

31. Smith, R., Owen, L. A., Trem, D. J., Wong, J. S., Whangbo, J. S., Golub, T. R., and Lessnick, S. L. (2006) Expression profiling of EWS/FLI identifies NKX2.2 as a critical target gene in Ewing's sarcoma. Cancer Cell 9, 405-416

32. Hershko, A., and Ciechanover, A. (1998) The ubiquitin system. Annu Rev Biochem 67, 425-479

33. An, J., Ren, S., Murphy, S. J., Dalangood, S., Chang, C., Pang, X., Cui, Y., Wang, L., Pan, Y., Zhang, X., Zhu, Y., Wang, C., Halling, G. C., Cheng, L., Sukow, W. R., Karnes, R. J., Vasmatzis, G., Zhang, Q., Zhang, J., Cheville, J. C., Yan, J., Sun, Y., and Huang, H. (2015) Truncated ERG Oncoproteins from TMPRSS2-ERG Fusions Are Resistant to SPOP-Mediated Proteasome Degradation. Mol Cell 59, 904-916

34. Gan, W., Dai, X., Lunardi, A., Li, Z., Inuzuka, H., Liu, P., Varmeh, S., Zhang, J., Cheng, L., Sun, Y., Asara, J. M., Beck, A. H., Huang, J., Pandolfi, P. P., and Wei, W. (2015) SPOP Promotes
EWS-FLI1 degradation is mediated by one lysine residue

Ubiquitination and Degradation of the ERG Oncoprotein to Suppress Prostate Cancer Progression. Mol Cell 59, 917-930

35. Ji, Z., Degenrny, C., Vintonenko, N., Deheuninck, J., Foveau, B., Leroy, C., Coll, J., Tulasne, D., Baert, J. L., and Fafeur, V. (2007) Regulation of the Ets-1 transcription factor by sumoylation and ubiquitinylation. Oncogene 26, 395-406

36. Vetari, A. C., Leong, K. G., Newton, K., Yee, C., O'Rourke, K., Liu, J., Phu, L., Vij, R., Ferrando, R., Couto, S. S., Mohan, S., Pandita, A., Hongo, J. A., Arnott, D., Wertz, I. E., Gao, W. Q., French, D. M., and Dixit, V. M. (2011) COP1 is a tumour suppressor that causes degradation of ETS transcription factors. Nature 474, 403-406

37. Elzi, D. J., Song, M., Hakala, K., Weintraub, S. T., and Shioi, Y. (2014) Proteomic Analysis of the EWS-Fli-1 Interactome Reveals the Role of the Lysosome in EWS-Fli-1 Turnover. J Proteome Res 13, 3783-3791

38. Yen, H. C., Xu, Q., Chou, D. M., Zhao, Z., and Elledge, S. J. (2008) Global protein stability profiling in mammalian cells. Science 322, 918-923

39. Liang, H., Mao, X., Olejniczak, E. T., Nettlesheim, D. G., Yu, L., Meadows, R. P., Thompson, C. B., and Fesik, S. W. (1994) Solution structure of the ets domain of Fli-1 when bound to DNA. Nat Struct Biol 1, 871-875

40. Welford, S. M., Hebert, S. P., Deneen, B., Arvand, A., and Denny, C. T. (2001) DNA binding domain-independent pathways are involved in EWS/FLI1-mediated oncogenesis. J Biol Chem 276, 41977-41984

41. Riggi, N., Suva, M. L., Suva, D., Cironi, L., Provero, P., Terrier, S., Joseph, J. M., Stehle, J. C., Baumer, K., Kindler, V., and Stamenkovic, I. (2008) EWS-FLI1 expression triggers a Ewing's sarcoma initiation program in primary human mesenchymal stem cells. Cancer Res 68, 2176-2185

42. Kinsey, M., Smith, R., Iyer, A. K., McCabe, E. R., and Lessnick, S. L. (2009) EWS/FLI and its downstream target NR0B1 interact directly to modulate transcription and oncogenesis in Ewing's sarcoma. Cancer Res 69, 9047-9055

43. Tirado, O. M., Mateo-Lozano, S., Villar, J., Dettin, L. E., Llor, A., Gallego, S., Ban, J., Kovar, H., and Notario, V. (2006) Caveolin-1 (CAV1) is a target of EWS/FLI-1 and a key determinant of the oncogenic phenotype and tumorigenicity of Ewing's sarcoma cells. Cancer Res 66, 9937-9947

44. Boro, A., Preter, K., Rechfeld, F., Thalhammer, V., Oesch, S., Wachtel, M., Schafer, B. W., and Niggli, F. K. (2012) Small-molecule screen identifies modulators of EWS/FLI1 target gene expression and cell survival in Ewing's sarcoma. Int J Cancer 131, 2153-2164

45. Prieur, A., Tirode, F., Cohen, P., and Delattre, O. (2004) EWS/Fli-1 silencing and gene profiling of Ewing cells reveal downstream oncogenic pathways and a crucial role for repression of insulin-like growth factor binding protein 3. Mol Cell Biol 24, 7275-7283

46. Sankar, S., Bell, R., Stephens, B., Zhuo, R., Sharma, S., Bearss, D. J., and Lessnick, S. L. (2013) Mechanism and relevance of EWS/FLI-mediated transcriptional repression in Ewing sarcoma. Oncogene 32, 5089-5100

47. Matsunobu, T., Tanaka, K., Nakamura, T., Nakatani, F., Sakimura, R., Hanada, M., Li, X., Okada, T., Oda, Y., Tsuneyoshi, M., and Iwamoto, Y. (2006) The possible role of EWS-Fli1 in evasion of senescence in Ewing family tumors. Cancer Res 66, 803-811

48. Marques Howarth, M., Simpson, D., Ngok, S. P., Nieves, B., Chen, R., Siprashvili, Z., Vaka, D., Breese, M. R., Crompton, B. D., Alexe, G., Hawkins, D. S., Jacobson, D., Brunner, A. L., West, R., Mora, J., Stegmaier, K., Khavari, P., and Sweet-Cordero, E. A. (2014) Long noncoding RNA EWSAT1-mediated gene repression facilitates Ewing sarcoma oncogenesis. J Clin Invest 124, 5275-5290

49. Williamson, A., Werner, A., and Rape, M. (2013) The Colossus of ubiquitylation: decrypting a cellular code. Mol Cell 49, 591-600
EWS-FLI1 degradation is mediated by one lysine residue

50. Emanuele, M. J., Elia, A. E., Xu, Q., Thoma, C. R., Izhar, L., Leng, Y., Guo, A., Chen, Y. N., Rush, J., Hsu, P. W., Yen, H. C., and Elledge, S. J. (2011) Global identification of modular cullin-RING ligase substrates. Cell 147, 459-474

51. Yen, H. C., and Elledge, S. J. (2008) Identification of SCF ubiquitin ligase substrates by global protein stability profiling. Science 322, 923-929

52. Kodakek, T., Sikder, D., and Nalley, K. (2006) Keeping transcriptional activators under control. Cell 127, 261-264

53. Muratani, M., and Tansey, W. P. (2003) How the ubiquitin-proteasome system controls transcription. Nat Rev Mol Cell Biol 4, 192-201

54. Bachmaier, R., Aryee, D. N., Jug, G., Kauer, M., Kreppel, M., Lee, K. A., and Kovar, H. (2009) O-GlcNAcylation is involved in the transcriptional activity of EWS-FLI1 in Ewing’s sarcoma. Oncogene 28, 1280-1284

55. Schlottmann, S., Erkizan, H. V., Barber-Rotenberg, J. S., Knights, C., Cheema, A., Uren, A., Avantaggiati, M. L., and Toretsky, J. A. (2012) Acetylation Increases EWS-FLI1 DNA Binding and Transcriptional Activity. Front Oncol 2, 107

56. Klevernic, I. V., Morton, S., Davis, R. J., and Cohen, P. (2009) Phosphorylation of Ewing’s sarcoma protein (EWS) and EWS-FLI1 in response to DNA damage. Biochem J 418, 625-634

57. Grunewald, T. G., Diebold, I., Esposito, I., Plehm, S., Hauer, K., Thiel, U., da Silva-Buttkus, P., Neff, F., Unland, R., Muller-Tidow, C., Zobywalski, C., Lohrig, K., Lewandrowski, U., Sickmann, A., Prazeres da Costa, O., Gorlach, A., Cossarizza, A., Butt, E., Richter, G. H., and Burdach, S. (2012) STEAP1 is associated with the invasive and oxidative stress phenotype of Ewing tumors. Mol Cancer Res 10, 52-65

58. Jaenicke, L. A., von Eyss, B., Carstensen, A., Wolf, E., Xu, W., Greifenberg, A. K., Geyer, M., Eilers, M., and Popov, N. (2016) Ubiquitin-Dependent Turnover of MYC Antagonizes MYC/PAF1C Complex Accumulation to Drive Transcriptional Elongation. Mol Cell 61, 54-67

59. Reid, G., Hubner, M. R., Metivier, R., Brand, H., Denger, S., Manu, D., Beaudouin, J., Ellenberg, J., and Gannon, F. (2003) Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. Mol Cell 11, 695-707

60. Yao, J., Munson, K. M., Webb, W. W., and Lis, J. T. (2006) Dynamics of heat shock factor association with native gene loci in living cells. Nature 442, 1050-1053

61. Irizarry, R. A., Hobbs, B., Collin, F., Beazer-Barclay, Y. D., Antonellis, K. J., Scherf, U., and Speed, T. P. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4, 249-264

62. Gentleman, R. (2005) Bioinformatics and computational biology solutions using R and Bioconductor, Springer Science+Business Media, New York
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FIGURE LEGENDS

Figure 1: EWS-FLI1 protein turnover is proteasome dependent. (A) Western blot analysis of EWS-FLI1 protein levels. A673 cells were treated with 20µM of indicated compounds for 8h, EWS-FLI1 protein levels were detected with anti-FLI1 antibody. Quantification represents ratio of FLI1 over GAPDH compared to DMSO control. (B) EWS-FLI1 turnover in various Ewing sarcoma cell lines. A673, SKNMC and TC71 cells were treated with 20µM MG-132 or chloroquine (CQ) for 10h (A673), 8h (SKNMC) and 9h (TC71), and immunoblotted with anti-FLI1 antibody. Quantification represents ratio of FLI1 over tubulin compared to DMSO control. (C) EWS-FLI1 stabilizes in a time-dependent manner. Western blot of A673 cells treated with 10µM MG-132 for indicated time points. Three independent experiments were quantified and are represented in the scatter blot with n=3 (2h-8h) or n=5 (DMSO), error bars as SD. (D) Half-life of endogenous EWS-FLI1 protein. Western blot of A673 cells treated with 20µg/ml CHX for indicated hours. Quantification of three independent experiments with n=3 (2h-8h) or n=5 (DMSO), error bars as SD. (E) EWS-FLI1 is ubiquitinated. 3xflag-EWS-FLI1 and HA-ubiquitin were co-expressed for 48h in HEK293T cells and incubated with 10µM MG-132 for indicated hours. After immunoprecipitation of EWS-FLI1 with anti-Flag, ubiquitination was visualized by anti-HA antibody. (F) EWS-FLI1 ubiquitination consists of K48-linked ubiquitin chains. 3xflag-EWS-FLI1 and wild-type or mutant HA-ubiquitins were co-expressed for 48h in HEK293T cells, immunoprecipitated and ubiquitination was visualized by anti-HA antibody.

Figure 2: The EWS-FLI1 protein displays a high turnover. (A) Scheme illustrating GPS approach in which a reporter construct of DsRed-IRES-EGFP is fused at the C-terminus of EGFP to a protein of interest. The EGFP/DsRed ratio is determined by FACS and represents a measure for protein stability. (B) EWS-FLI1 turnover measured by GPS. HEK293T were transduced for 72h with a reporter construct fused to EWS-FLI1 or degron (d) motifs with half lives of 1h (d1h), 4h (d4h) and 24h (d24h) and analyzed by FACS. The reporter construct fused to EWS-FLI1 was additionally incubated with 20µg/ml CHX for 4h or (C) with 50µM Leptomycin B (LMB) for 4h and 8h (dotted line). (D) EWS-FLI1 stability in Ewing cell line A673. GPS analysis of reporter constructs fused to EWS-FLI1 or standard degron motifs 72h after transduction. (E) A673 cells stably transfected with reporter-EWS-FLI1 construct were sorted and incubated with DMSO, 20µM MG-132 or 20µg/ml CHX for 8h.

Figure 3: Mass spectrometry identifies K380 residue as the main ubiquitination site. (A) Two lysine residues were identified to be ubiquitinated by mass spectrometry. Peptide spectra with glycine-glycine shift for residue K380 (peptide spectra for the K298 site in supplementary S1A). (B) Mutation of lysine residue K380 prevents EWS-FLI1 ubiquitination. 3xflag-EWS-FLI1, K298R and K380R mutants were co-expressed with HA-ubiquitin for 48h in A673 cells, purified ubiquitinated EWS-FLI1 was analyzed using anti-HA antibody by western blotting. (C) Mutated K380 residue stabilizes EWS-FLI1 protein. 3xflag-EWS-FLI1, single and double mutants were transiently overexpressed for 48h in A673 and SKNMC cells and analyzed with an anti-Flag antibody by western blot. (D) Scatter blot for quantification for n=3 independent experiments, error bars as SD. (E) EWS-FLI1 mutant show nuclear localization. 3xflag-EWS-FLI1 and K380R single mutant were transiently expressed for 48h in A673 cells. Cells were fixed, stained with anti-Flag antibody and used for immunofluorescence (40x magnification). (F) Mutation of K380 stabilizes EWS-FLI1 posttranslationally. HEK293T cells were transduced for 72h with reporter constructs of EWS-FLI1, mutants K298R and K380R and standards. EGFP/DsRed ratios were analyzed by FACS, (G) after additional incubation with DMSO or 20µM MG-132 for 8h.

Figure 4: Regulation of proteasomal turnover is conserved between EWS-FLI1 and FLI1. (A) EWS-FLI1 displays fastest turnover. GPS analysis of EWS-FLI1, EWSR1 and FLI1 reporter constructs and standards transduced into HEK293T cells and analyzed after 72h by FACS. (B) Full length
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proteins EWSR1 and FLI1 were additionally treated with 20µM MG-132 or 20µg/ml CHX for 8h. (C) Immunoprecipitation of EWS-FLI1, EWSR1 and FLI1 proteins. 3xflag tagged versions were co-expressed with HA-ubiquitin for 48h and stabilized for additional 5h with 20µM MG-132. After immunoprecipitation of tag proteins, ubiquitin pattern was analyzed by anti-HA antibody. (D) Mutation of conserved residue K334R stabilizes FLI1 in GPS analysis. HEK293T cells were transduced for 72h with reporter constructs of FLI1, mutants K172R, K252R, K334R and standards. EGFP/DsRed ratio was analyzed by FACS. (E) Turnover dependent lysine residue is conserved within most ETS family members. ClustalW alignment of conserved ETS domain sequence, K334/K380 residue is marked in red.

Figure 5: Generation of exchange Ewing cell lines. (A) EGFP (selection marker) sorting of pInducer21 vector transduced cells. A673 cells with 3xflag, 3xflag-EWS-FLI1 or 3xflag-K380R mutant were sorted for same low EGFP expression. After sorting, all cell pools were analyzed by FACS for EGFP and plotted for corresponding mean fluorescent intensity (MFI). (B) ShEF sequences shRNA against the 3’ UTR of EWS-FLI1 are sufficient to downregulate EWS-FLI1 protein. Western blot of A673 cells with shEF constructs #1 and #2 after incubation with 0.1ng/µl doxycycline for 48h. (C) Inducible EWS-FLI1 exchange cell lines. Double transduced A673 cells were incubated with 0.1ng/µl doxycycline for 48h. Endogenous and exogenous EWS-FLI1 protein levels were analyzed by western blotting using anti-FLI1 antibody, (D) and mRNA levels were analyzed by quantitative RT-PCR, with n=4 and error bars represent 95% confidence intervals.

Figure 6: Differential regulation of target gene expression by EWS-FLI1 protein levels. (A) Analysis of microarray expression data of A673 exchange cell lines reveals three subgroups of target gene regulation. Pie chart distribution for EWS-FLI1 activated (upper) and repressed (lower) target genes and their differential modulation of stabilized over wild type EWS-FLI1. (B-C) Heat map of activated-rescued (B) and repressed-rescued (C) target gene signature. The comparison of stabilized over wild type EWS-FLI1 target genes resembled in three distinct groups. (D-E) Validation of selected activated (D) or repressed (E) target genes by quantitative RT-PCR based on RNA extracted from shEF#2 exchange cell lines, with n=4 and error bars represent 95% confidence intervals.
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Proteasomal degradation of the EWS-FLI1 fusion protein is regulated by a single lysine residue

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