Increased survival and cell cycle progression pathways are required for EWS/FLI1-induced malignant transformation

Ewing sarcoma (ES) is the second most frequent childhood bone cancer driven by the EWS/FLI1 (EF) fusion protein. Genetically defined ES models are needed to understand how EF expression changes bone precursor cell differentiation, how ES arises and through which mechanisms of inhibition it can be targeted. We used mesenchymal Prx1-directed conditional EF expression in mice to study bone development and to establish a reliable sarcoma model. EF expression arrested early chondrocyte and osteoblast differentiation due to changed signaling pathways such as hedgehog, WNT or growth factor signaling. Mesenchymal stem cells (MSCs) expressing EF showed high self-renewal capacity and maintained an undifferentiated state despite high apoptosis. Blocking apoptosis through enforced BCL2 family member expression in MSCs promoted efficient and rapid sarcoma formation when transplanted to immunocompromised mice. Mechanistically, high BCL2 family member and CDK4, but low P53 and INK4A protein expression synergized in Ewing-like sarcoma development. Functionally, knockdown of Mcl1 or Cdk4 or their combined pharmacologic inhibition resulted in growth arrest and apoptosis in both established human ES cell lines and EF-transformed mouse MSCs. Combinatorial targeting of survival and cell cycle progression pathways could counteract this aggressive childhood cancer.

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expression caused embryonic lethality, moderate EF expression in two transgenic founder lines was associated with mild, but consistent limb shortening with normal life span without ES appearance. Compound cross with p53-deficient mice (Prx1Cre-mediated p53 deletion) synergized in osteosarcoma formation. Furthermore, data from six independent laboratories presented 16 different Cre-mediated transgenic approaches to generate an Ewing Sarcoma mouse, which failed owing to high apoptosis and toxicity upon EF expression in multiple cell types.

We aimed to better model the cellular origin of ES and we used Prx1Cre (EFPrx1) mice, which facilitated EF expression to early mesenchymal progenitors. EF expression caused a bone differentiation arrest owing to changed developmental signaling. As a result, severe malformations of the skull, facial bones, sternum and limbs and early perinatal death occurred. EF-immortalized MSC-like cells isolated from EFPrx1 limbs displayed high levels of apoptosis but failed to form ES. However, when cells were transduced by retrovirus to express BCL2 family members, they formed efficiently sarcomas in NSG mice. Sarcomagenesis was accompanied by upregulation of the CDK4/cyclin D1/pRB axis, and reduced INK4A and P53 expression accelerating cell cycle and survival. Depletion of Mcl1 or Cdk4 in a panel of EF-dependent cell lines led to inhibition of cell proliferation and enhanced apoptosis. Combined blockade of MCL1 and CDK4 with pharmacologic inhibitors caused cell cycle arrest and apoptosis in EF-transformed cells.

Results

Skeletal defects in EFPrx1 mice are due to blocked bone differentiation. We limited EF expression to early mesenchymal progenitors using Prx1Cre (EFPrx1) to better model the cellular origin of ES. To regulate EF expression in target tissues we used an EF expression cassette flanked by a floxed STOP cassette knocked into the Rosa26 locus. Compound mice with conditional mesenchymal Prx1Cre recombinase expression (Figure 1a) were generated. Prx1Cre-mediated recombination was reported from E9.5 onwards in bone-forming cells. Prx1 promoter-driven EF expression was shown to be tolerated, which resulted in shortened limbs upon transgenic expression, but detailed bone development was not analyzed. Here, we aimed to express EF during early development, as cases of congenital ES in newborns exist. EF expression caused developmental abnormalities such as endochondral bone formation arrest, starting at E10.5 up to postnatal day P1 (Figure 1). EF-expressing embryos or newborns displayed gross malformations in limb development that became apparent at E12.5. The phenotype was more prominent from anterior-to posterior and at later developmental stages (Figure 1b; Supplementary Figure 1a–c). Malformations in rib cage architecture and in the craniofacial area most likely led to death of EFPrx1 newborn mice within the first 24 h postnatal owing to breathing and nutrition problems. Spine development remained normal (Supplementary Figure 1d). Alcian Blue/Alizarin Red staining, which stains cartilage in blue and bone elements in red, and skeletal analysis displayed severe reduction in calcified deposition (red elements) of long bones in EFPrx1 versus wild-type (wt) limb bones at E16.5. Furthermore, we observed a distorted sternum with deranged ossification center, causing an open, malformed rib cage. Calvaria bones lacked significant calcification, leading to skull malformations (Figure 1b). Mutant mice were born at normal Mendelian ratios (n = 174 EFPrx1 versus n = 161 wt controls were analyzed; Figure 1e). Detailed histological analysis of E13.5 and E18.5 embryos revealed two different phenotypes in limbs. All mutant embryos analyzed showed condensed cartilaginous elements instead of long bone formation. Although ~90% of mutants displayed polydactyly, only ~10% EF embryos lacked digits or long bone elements (Figure 1d; Supplementary Figure 1e). Van Kossa/Alcian Blue staining of E13.5 and E18.5 embryos revealed blocked mesenchymal differentiation in EFPrx1 mice at the prehypertrophic chondrocyte stage. In line, all mutant embryos showed absence of hypertrophic and mature chondrocytes or osteoblasts as confirmed by RNA in situ hybridization for bone lineage markers. Interestingly, although early markers of the chondrocyte lineage (Sox9, Collagen 2a1, Col2a1) were expressed in EFPrx1 limbs, markers of hypertrophic chondrocytes and osteoblast differentiation (Indian hedgehog, Ihh; Collagen10, Col10a1; Runx2 and Osterix, Osx) were largely absent in long bone areas, but weakly expressed in polydactyly digits. In rare cases, EF embryos lacked both digits and limbs, best visible by condensed Collagen 2a1-expressing cartilage elements at late development (Figure 1d; Supplementary Figure 3a). Similarly, osteoblast markers Col1a1 and Osc were absent in limbs isolated from EFPrx1 embryos, but expressed in wt limbs (not shown). Immunoblotting analysis confirmed significant EF expression in limb elements (Figure 1e). Efficient deletion of the STOP cassette and expression of EF was confirmed by genomic or real-time PCR (Supplementary Figure 2).

Figure 1 EF expression blocks bone differentiation causing skeletal defects in EFPrx1 mice. (a) Scheme of the ROSA26-lox-STOP-lox-HA-HEWS/FLI1 transgenic mouse. (b) Representative picture of wt and EFPrx1. E16.5 embryo displays malformed limbs as indicated by black dashed circle. Skeletal preparations and analysis with Alcian Blue/Alizarin Red staining, which stains cartilage in blue and bone elements in red, and skeletal analysis displayed severe reduction in calcified deposition (red elements) of long bones in EFPrx1 versus wild-type (wt) limb bones at E16.5. (c) Histology analysis of E13.5 and E18.5 embryos stained by H&E or Van Kossa/Alcian Blue, which stains cartilage in blue and bone elements in red, indicated that there was no significant hypertrophic region present in EFPrx1 embryos in contrast to wt limbs. RNA in situ hybridization of E13.5 limbs confirmed an arrest in chondrocyte and osteoblast differentiation. For all embryos consecutive sections were performed. At least three different independent embryos for every single staining and for each time point were analyzed. Black circles indicate the magnified region showing in the lower row. Detection of EF expression at the protein level at E16.5 using HA-tag antibody displayed significant transgenic protein product in limbs from EF mice. HSC-70 was used as a loading control. Fibro GFP or CRE: fibroblasts isolated from EF mice lentiviral-transduced with a construct containing GFP or the CRE recombinase was used as controls. Numbers of analyzed embryo/mice are indicated corresponding images.
Impairment of bone development pathways in EF<sup>Prx1</sup> limbs. EF<sup>+</sup> embryos at different embryonic stages (E14.5, E16.5 and P1) showed a severe reduction of mature chondrocytes in mutant limbs, as measured by dimethylmethylene blue staining, which reflects sulphated glycosaminoglycan content (Figure 2a; Supplementary Figure 3b). The WNT/β-catenin pathway has a pivotal role in chondrocyte and osteoblast differentiation in cooperation with BMP/SMAD and...
TGF-β signaling.\textsuperscript{17–22} We analyzed by immunostaining key marker proteins (β-CATENIN, SMAD1/5, pSMAD4, SMAD7, DLX5, SOX9, RUNX2 and OSTERIX) along bone differentiation markers. Automated quantification of immunostaining served as reliable source to quantify levels.\textsuperscript{23} RUNX2, OSTERIX, DLX5, pSMAD4, SMAD1/5, SMAD7 and β-CATENIN were found diminished at all embryonic stages in EF\textsuperscript{Ptx1} transgenic limbs (Figures 2a and b; Supplementary

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**Figure 2** Blocked bone signaling pathways upon EF expression. (a and b) Dimethylmethylene blue (DMMB) as a chondrocyte marker showed marked reduction of Glycosaminoglycan in mutant embryos. IHC staining analysis on consecutive embryo sections of E14.5 and postnatal day P1 mice showed that EF expression in limb mesenchyme promotes downregulation of TGF-β signaling pathway. Black circles indicating the magnified region showing in the next column. (b) Quantification of IHC signals from (a). At least three different independent embryos for every single staining and for each time point were carried out. Quantitation was done by (ANOVA) one-way analysis of variance. * \(P < 0.5\), ** \(P < 0.05\) and *** \(P < 0.005\)
Figure 4), whereas SMAD7 expression at birth was comparable with wt expression levels. SOX9 expression for prehypertrophic chondrocyte differentiation, controlling secretion of extracellular matrix molecules like collagen and proteoglycans\(^{24}\) was normal during early development. DLX5 as a TGF-β-induced protein was downregulated in mutant limbs compared with controls (Figures 2a and b; Supplementary Figure 4).

**Figure 3.** EF expression induces apoptosis and leads to diminished cell cycle progression. (a) HA-tag staining was performed to detect the EF translocation product. The rate of proliferation was comparable to wt based on Ki67 proliferation marker staining. TUNEL staining displayed highly elevated apoptosis at P1. (b) P21 (CDKN1A) protein level was upregulated. Prominent nuclear staining of P21 at E14.5, decreased at E16.5 and P1. cyclin D1 expression was decreased during development till P1. Black circles indicating the magnified region showing in the next column. (c) Quantification of IHC from part A and B. At least three different independent embryos of wt or mutant for every single staining and for each time point were analyzed. Experimental staining results were evaluated using (ANOVA) one-way analysis of variance. Insets in separate panel highlight cellular details. *\(P < 0.5\), **\(P < 0.05\) and ***\(P < 0.005\)
High level of apoptosis in limb elements of EF-expressing newborns. Next, we investigated EF expression and highest expression was seen at E14.5 and E16.5, whereas its expression diminished at P1 (Figures 3a–c; Supplementary Figure 5). The proliferation rate of EF-mutant chondrocytes was not significantly changed, as measured by Ki67 staining. However, TUNEL staining showed marked rates of apoptosis at P1 in mutant but not wt limbs (Figures 3a–c; Supplementary Figure 5). Of note, nuclear P21 (CDKN1A), a known senescence marker often lost or mutated in human cancer, was induced at E14.5, but suppressed from E16.5 on in EF-mutant limbs. Moreover, cyclin D1 expression was slightly decreased throughout development of EFPrx1 transgenic limbs, whereas its expression was diminished at birth (Figures 3a–c; Supplementary Figure 5).

**EF-expressing MSCs are immortal and fail to differentiate.** To study primary cells of malformed bone elements, 12 different primary EFPrx1 MSC-like cell cultures (MSC) from individual P1 EFPrx1+ (EFPrx1+MSC) or six wt limbs (wtMSC) as controls were propagated (Figure 4a; Supplementary Figure 6a–c). We applied specific differentiation protocols for osteoblasts, chondrocytes or adipocytes. WtMSCs displayed prominent differentiation into all three lineages, but EFPrx1+MSCs failed to differentiate (Figure 4a). Transgene expression of EF at the RNA- and protein level in EFPrx1+MSC cultures was verified and cells expressed CD90 (Thy-1.1; wtMSCL (passage 3) or 12 EF Prx1MSCL at early (passage 8) or late (passage 55) culture time as measured by propidium iodide (PI) staining.

Ihh PCR results were normalized to Gapdh in independent differentiation experiments is shown (right). (Materials and Methods to obtain osteocytes (stained with Alizarin red), chondrocytes (Van Kossa staining) and adipocytes (Oil red O staining). An example from three independent P1 EF Prx1 (EFPrx1MSCL) or six wt limbs (wtMSCL) were continuously passaged (Figure 4a; Supplementary Figure 6d; P53 protein level was also similar, not shown). WtMSCs displayed growth arrest after ~6–8 passages with senescence-like features such as PML* immunostaining (not shown). In contrast, mutant EF+MSC were continuously passaged (>50 passages). We did not observe senescence in EFPrx1+MSC and cells remained diploid even at passage 55. Cell cycle analysis of multiple different primary EFPrx1+MSC lines at early and late passage numbers (passage 8 and 55) displayed a large fraction (>40%) of cells at sub-G1 at early passage, but high rate of apoptosis was lost at late passage. Apoptosis loss was associated with a higher number of cells that remained in G2/M phase compared with wtMSCs (Figure 4d).

Figure 4: EFPrx1+ mesenchymal stem cells like cells (EFPrx1+MSC) are immortal, but display high apoptosis and fail to differentiate. (a) MSCs were isolated from P1 mouse long bone cartilaginous elements of wt or EFPrx1+ newborns (left). A representative primary cell line from low passage 3–4 in case of wtMSC or passage 8–10 in case of EFPrx1+MSC line is shown. WtMSCs were able to differentiate along mesenchymal lineages, whereas EFPrx1+MSC failed to differentiate. Differentiation was induced as detailed in Materials and Methods to obtain osteocytes (stained with Alizarin red), chondrocytes (Van Kossa staining) and adipocytes (Oil red O staining). An example from three independent differentiation experiments is shown (right). (b) Western blot analysis (n = 2, each) of HA-tag and qRT-PCR (n = 6, each) proved significant EF transgene expression. The EFPrx1+MSC displayed high surface expression of CD90 (Thy-1.1; n = 3, each), a MSC marker, compared with wtMSCs. (c) EFPrx1+MSC has blunted TGF-β signaling as measured by reduced expression levels of Tgfbr1, Tgfbr2 and Dlk5. Similarly, osteogenic markers like Oss, Sccd and Oss were expressed to lower levels, but enhanced hedgehog signaling was found as evident by higher Ihh, Smo and Gli1 expression using qRT-PCR. Error bars represent S.E.M. of six individual measurements. All qRT-PCR results were normalized to Gdpdh. For wt or mutant embryos at least three different independent experiments were carried out. (d) Representative cell cycle analysis of six wtMSC (passage 3) or 12 EFPrx1+MSC at early (passage 8) or late (passage 55) culture time as measured by propidium iodide (PI) staining. Caspase-3 expression was induced in EFPrx1+MSC as measured by qRT-PCR compared with wtMSCs levels causing high apoptosis. *P < 0.5, **P < 0.05 and ***P < 0.005.
Inhibition of apoptosis promotes Ewing sarcoma
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Figure captions:

(a) Immunofluorescence images of wt and EF prx1 cells at P1 and P2.

(b) Western blot analysis of HA, EWS/FLI1, and β-ACTIN proteins in wtMSCL and EF prx1 MSCL.

(c) mRNA expression levels of Tgfbr1, Tgfbr2, Gli1, Ihh, Smo, Dlx5, Runx2, Ostx, Sox9, and Osc in wtMSCL and EF prx1 MSCL.

(d) Flow cytometry analysis of cell cycle distribution and Caspase-3 mRNA expression in wtMSCL and EF prx1 MSCL.
sarcoma morphology (Figure 5c; Supplementary Figure 7c). All sarcomas showed uniform small round cells with prominent nucleoli surrounded by small cytoplasm. Next we tested sarcoma cells for two established ES markers: periodic acid schiff (PAS) and neuronal-specific enolase (NSE) immunostaining confirmed ES-like staining pattern (Figure 5c; Supplementary Figure 7c).

**MCL1 transduction resembles a close human ES gene expression profile.** To determine the similarity in gene expression of the transplant model with human ES we performed RNA-seq analysis. Data were compared with a published ES signature (Kauer data set). A more optimal gene expression profile for EFPrx1MSCL+Mcl1 sarcomas was prominent, when comparing relative expression levels of these signature genes in wtMSCL, EFPrx1MSCL, EFPrx1MSCL+Bcl2, EFPrx1MSCL+Mcl1 (Figures 5d and e) or EFPrx1MSCL+Bcl-xL (data not shown) to human data. This suggests that transformation with MCL1 renders EFPrx1MSCL more similar to ES. We next examined whether on a transcriptomic level ES correlates better with the EFPrx1MSCL+Mcl1 transplant model than other human sarcomas using a comprehensive set of microarray data for different human sarcomas. From this data set normalized gene expression for each sarcoma was correlated with the log2 fold change of EFPrx1MSCL+Mcl1 versus wtMSCL. Although overall correlations to other sarcomas were low, the highest positive correlation (red bars) was seen for ES. This result suggests that among the tested sarcomas gene expression changes in the EFPrx1MSCL+Mcl1 as compared with wtMSCL is overall most similar to ES (Figure 5e). To determine functional categories of gene regulation we performed gene set enrichment analysis. Most prominently, we found different gene sets relating to cell cycle to be enriched in genes upregulated in the comparison EFPrx1MSCL+Mcl1 versus wtMSCL cells (Figure 5f), whereas the p53 signaling pathway was downregulated.

Tunnel and PI staining showed less tunnel-positive cells and more proliferation rate in EFPrx1MSCL+Mcl1 cell population, indicating the crucial role of Mcl1 for bypassing apoptosis in EF-expressing cells (Figure 5g; Supplementary Figure 7d). We noted similar amounts of immunostaining for key markers such as CC-3, Ki67, B-CATENIN, CYCLIN D1, CDK4/6 expression (Supplementary Figure 7g) in the compared groups upon quantification of the IHC as shown in Figure 5h. Western blot analysis displayed in average among 8 EFPrx1MSCL+Mcl1 tumors compared with two EFPrx1MSCL+Mcl1 or two EFPrx1MSCL expressing cells similar protein levels of CC-3 and reduced INK4A or P53 levels as quantified by densitometry (Supplementary Figure 7h). RB expression was lower, but pS780-RB expression was higher and CDK6 expression was also higher. Furthermore, sarcoma cells isolated from EFPrx1MSCL+Mcl1 tumors and recultivated displayed changed expression, most notable for upregulated p53, MCL1 and CDK6 expression. Interestingly, we observed a severe reduction of P19ARF and P53 expression in the majority of eight analyzed tumors of the EFPrx1MSCL+Mcl1 group, whereas P19ARF expression was lower in six tumors. In line, we also found a significant reduction of P16Ink4A in both EFPrx1MSCL+Mcl1 cell lines and in the majority of EFPrx1MSCL+Mcl1 tumors. P19ARF and P16Ink4A were expressed lower in EFPrx1MSCL-Mcl1 tumor-derived cells and P53 expression was significantly enhanced in EFPrx1MSCL or even expressed/activated higher upon MCL1 transduction before transplant (Figure 5h). Total RB expression levels were slightly reduced (Supplementary Figure 7e).

**Blocked survival and proliferation arrest ES cell growth and induces apoptosis.** CDK4 activity is required for cell cycle progression and it was shown that Mcl1 knockdown prolonged early G1 phase associated with decreased CDK4 expression. Upon siRNA-mediated knockdown of Mcl1 or Cdk4 in Ewing-like mouse as well as in ES cell lines TC252 and SK-N-MC we could demonstrate that both genes are important for ES cell growth and survival (Figure 6). Mechanistically, we observed a slightly lower expression of pS780-RB after downregulation of Mcl1 or Cdk4, whereas total RB levels were increased, indicating that Mcl1 and Cdk4 downregulation resulted in blocked proliferation (Figure 6a, Supplementary Figure 7i). Tumorigenic potential of siRNA-treated cells was assessed by colony-forming assay. The amount of Mcl1 or Cdk4 correlated with the number of colonies, indicating that Mcl1 and Cdk4 are required for efficient transformation. A siRNA-positive control targeting EF in EFPrx1MSCL+Mcl1 cells confirmed proliferation was EF-dependent (Figures 6a and b). Cell cycle analysis revealed that less Mcl1 or Cdk4 led to a prolonged G0/G1 and

**Figure 5.** EFPrx1MSCL give rise to sarcomas upon enhanced expression of anti-apoptotic BCL2 family members. (a) ES cell lines SK-N-MC, TC71, TC252 and A673 expressed prominent level of MCL1, BCL2 and BCL-xL. Colorectal carcinoma cell lines HT29, HCT116, SW620 and LS174T were used as positive control. CDK4 expression was higher than in the control human prostate epithelial cell line RWPE-1. (b) Four different EFPrx1MSCL and four different wtMSCL were transduced by retrovirus with either GFP-vector, Bcl2-IRES-eGFP, Bcl-xL-IRES-eGFP or Mcl1-IRES-eGFP. A total of 12 primary cell transductions for each cell line were xenografted (n = 12, each). All Bcl2, Bcl-xL and Mcl1-expressing EFPrx1MSCL cell lines formed reproducibly sarcomas with full penetration as quantified in the right graph. None of the controls like the wtMSCL with or without BCL2 family member transduction or GFP-transduced EFPrx1MSCL formed any sarcoma up to 6 months post-transplant. (c) Xenografted sarcomas were positive for periodic acid Schiff (PAS) and neuronal-specific enolase (NSE) staining. SK-N-MC xenograft served as positive control. (d) RNA-seq analysis (from left to right) of wtMSCL, EFPrx1MSCL, two sarcoma samples from EFPrx1MSCL+Bcl2 and EFPrx1MSCL+Mcl1 revealed that samples become more similar to the ES gene expression signature (Kauer data set). (e) Clustering analysis showed the best matching of EFPrx1MSCL+Mcl1 tumor genes to human ES signatures. (f) Gene enrichment analysis (GSEA) exhibited a noticeable upregulation of cell cycle and a downregulation of p53 pathway in mouse EFPrx1MSCL+Mcl1 tumors versus wtMSCL. (g) EFPrx1MSCL at passage 30 served as controls. Two representative sarcoma lines EFPrx1MSCL+Mcl1 were established each that displayed an increase of GFP expression and a decrease in TUNEL cells after sarcomas formed, whereas PI staining displayed more proliferation in EFPrx1MSCL+Mcl1 and EFPrx1MSCL+Mcl1 tumor-derived cells. (h) Proliferative sarcoma cells were also positive for nuclear B-CATENIN, cyclin D1, CDK6 and CDK4, whereas cleaved caspase-3 (CC-3) was downregulated. All shown staining images are a representative of independent IHC analyses of n = 12 isolated tumors per group. (i) Western blot of two EFPrx1MSCL, two EFPrx1MSCL+Mcl1, eight EFPrx1MSCL+Mcl1 sarcomas and two EFPrx1MSCL+Mcl1 tumor-derived cells showed an overall reduced level of caspase-3 (Pro CC-3), CC-3, P16INK4A, P19ARF, P53 and higher amount of pS780-RB protein level upon enforced MCL1 expression in EFPrx1MSCL and associated tumor samples. Representative blots of three independent analyses are shown.
shortened S and G2/M phases (Figure 6c). To evaluate results of siRNA-mediated downregulation of Cdk4 and to translate findings to pharmacologic targeting, we treated EF-dependent cells with Palbociclib, an FDA approved dual CDK4/6 inhibitor. Palbociclib (IC₅₀ = ~1 μM in human ES cell lines and IC₅₀ = ~2 μM in mouse EF-dependent cell lines)
exposure led to reduced pS780-RB and increased total RB, whereas MCL1 levels were slightly decreased. Only a combination of CDK4/6 with the pan BCL2 family inhibitor Obatoclax resulted in significant growth inhibition of ES cells, whereas a BCL2 family inhibitor unable to target MCL1 (ABT-737) was inefficient to block proliferation (Figures 6d and e; Supplementary Figure 7j). Western blot analysis of treated human cell lines with Obatoclax and ABT-737 showed higher CC-3 intensity in cells treated with Obatoclax. The ES cell line T252 showed higher expression of CC-3 after treatment with ABT-737 (Supplementary Figure 8b).

Discussion

Childhood cancer studies are greatly hampered by small study cohorts and an animal model to study ES is needed for
preclinical drug testing and mechanistic insights into disease processes. We aimed to better model the cellular origin of ES through the use of Prx1Cre-driven EF expression. Moreover, we developed new primary cell models to study EF expression consequences on core cancer pathways such as apoptosis and cell cycle progression or bone developmental signaling pathways.\textsuperscript{25-27,33-35} We conclude that EF expression blocks mesenchymal differentiation and it induces apoptosis. This cell death in the developing mesenchyme caused severe malformations in bone development with death in newborn owing to severe bone differentiation abnormalities, making a transplant model necessary. Therefore, we established a new in vivo transplant system to serve for drug testing studies and for mechanistic insights into key pathways driving sarcomagenesis.

ES cases in babies were reported\textsuperscript{15,16} and therefore, our transgenic EF expression during limb differentiation could illuminate sarcoma formation in patients. We established multiple primary cell lines that were transformed to form sarcomas in vivo upon enforced BCL2 family member expression. We illuminated sarcoma formation through analysis and quantitation of key proteins involved in cancer progression. We found that our genetically engineered mouse model resembled sarcomas with close ES gene expression profile\textsuperscript{48} as determined by RNA-seq analysis, which could be useful to search for therapeutic intervention strategies. We found upregulated MCL1, CYCLIN D1, CDK4, but downregulated INK4A or P53 protein levels as prerequisites for ES proliferation and survival in line with similar findings in ES patients.\textsuperscript{36,37}

We observed an anterior-to-posterior differentiation defect, which could follow the wave of MSC dissemination in mammals that originate from neural crest. We noted deregulated WNT; HEDGEHOG, BMP and TGF-\beta pathways\textsuperscript{17-21} that culminated into skeletal development defects. Polydactyly was associated with elevated hedgehog signaling and GlI1 expression known to be EF-dependent.\textsuperscript{38} Analysis of chondrogenesis and osteogenesis markers revealed an arrest of endochondral bone development. Inhibition of Runx2, TgfbrI or TgfbrII could be due to reported repression by EF.\textsuperscript{39,40} Consequently, mature osteoblast markers lacked and TGF-\beta/ BMP signaling was diminished. Normally, cartilage-derived \beta-catenin signaling promotes chondrocyte maturation and it is involved in ossification. BMP and TGF-\beta signaling are required for osteoblast and chondrocyte differentiation.\textsuperscript{17,20,21,41} Bone invasion and osteolysis in ES are also associated with enhanced \beta-catenin signaling.\textsuperscript{32,43} We observed downregulated BMP, TGF-\beta and \beta-catenin signaling at late development in EF\textsuperscript{10} embryos, but high \beta-CATENIN expression was seen when sarcomas formed.

EF\textsuperscript{Prx1}MSCL remained diploid despite high passages, but transformation did not occur. Interestingly, EF\textsuperscript{Prx1}MSCL were immortal, but wtMSCL underwent senescence at low passage. Furthermore, despite contradictory literature, EF\textsuperscript{Prx1}MSCL failed to differentiate, whereas wtMSCL differentiated normal.\textsuperscript{52-54} EF-induced apoptosis was prominent in line with literature that identified \textit{Procaspase 3} to be a direct target gene of EF.\textsuperscript{11} Higher expression of \textit{CASPASE-3} could be seen in liver and spleen of transgenic mice expressing EF.\textsuperscript{10} Introduction of EF in human mesenchymal progenitor can also induce prominently apoptosis.\textsuperscript{44}

Enforced BCL2 family member expression was efficient to promote sarcomagenesis of transplanted EF\textsuperscript{Prx1}MSCL. MCL1 resulted in highest similarity of tumor gene expression to human ES. Although survival is a core cancer pathway, the high protein expression of BCL2 family members in ES was so far neglected. Currently, we cannot rule out that a possible post-translation mechanism for high BCL2 family member protein expression could be responsible during Ewing sarcomagenesis. We conclude that overcoming high CASPASE-3-induced apoptosis will be important for transformation. Similarly, cell cycle deregulation in ES is also a consequence of transformation. A direct correlation between EF expression and the amount of cyclin D1 expression was shown\textsuperscript{33,34,45} and c-myc upregulation correlates with the Ki67 proliferation marker expression.\textsuperscript{46} We observed low expression of INK4A and/or P53 genes in the majority of transplanted tumors from the MCL1 or BCL2 expressing cohort. Collectively, these results indicate a significant ES selection pressure for escape of apoptosis. Low expression of CDKN2A and/or P53 correlates with decreased apoptosis, or in case of CDKN2A loss also with enhanced self-renewal capacity of MSC.\textsuperscript{36} INK4A proteins are frequently lost owing to homozygous deletion or p53 mutations, both prominent in patients with ES.\textsuperscript{37,47} Enhanced survival in collaboration with decreased pS780-RB expression and increased CDK4 levels could also boost transformation. It was shown that MCL1 and cell cycle progression are interconnected and more MCL1 expression accelerated the cancer cell cycle progression.\textsuperscript{30,31} Here, we found that MCL1 knockdown led to a G1 cell cycle arrest by decreasing CYCLIN D1, CDK4/6 and by increasing P27 expression.\textsuperscript{30,31} Enforced survival led to increased CDK4 expression and pRB phosphorylation. Knockdown of \textit{Cdk4} or \textit{Mcl1} in a panel of EF-dependent cell lines led to inhibition of cell proliferation and reduced colony formation. Targeting BCL2 family members display ed higher efficacy with Obatoclax versus ABT-737 treatment, but combination of Obatoclax with Palbociclib was most effective. Our findings extend and confirm also recent results that describe the requirement of ES cells for CYCLIN D1-CDK4/6 function.\textsuperscript{35}

In summary, our findings propose that enhanced survival and proliferation with blocked mesenchymal differentiation are three prerequisites for Ewing Sarcoma formation. Our study displays mechanistic insights how the high apoptosis induced through EF-induced \textit{Caspase-3} mRNA and protein expression can be overcome to promote sarcomas. We could show that pharmacologic inhibitors of CDK4/6 and BCL2 family members are more effective when combined as targeted inhibitors. Thus, new targeting approaches could be tried to counteract this metastasizing disease in a more efficient way.

\textbf{Materials and Methods}

\textbf{Animal models and ethical approval.} Transgenic Rosa26-EF\textsubscript{10} were crossed to Prx1Cre mice.\textsuperscript{3,11} To isolate embryos vaginal plug checks were made, mice were separated and noon was counted as E0.5. Mice were kept under SBF conditions and animal experiments were approved (license number: BMWF-66009/0281-I/3b/2012). Immunocompromised NOD.Cg-Pkdcsclid2gtm1WjirSjJ (NSG) recipients were purchased (Harlan, United Kingdom).

\textbf{Cell culture, gene knockdown assay and inhibitor treatment.} Cells were cultivated for inhibitor Palbociclib (PD-032991) HCl in a dose–response
cellular viability assay, incubated for 24 h (Selleckchem, Houston, TX, USA) with doses: 0, 0.3, 0.5, 1, 2, 5 and 7 μM followed by viability counting using trypan blue. For inhibitor treatment viability values, IC_{50} was determined for human 48 h and for murine tumor cell lines up to 2 μM. Treated cells were harvested for Western blot after 24 h treatment. For time-course assays, cells were seeded in tissue culture for each hybridization.

For each individual gene were purchased and transfected as instructed (GE after 24 h treatment. For time-course assays, cells were seeded in tissue culture embedded in paraffin. Sections were processed prior to staining with specific transcription of 1 μm template DNA with RNA DIG labeling kit (Roche Diagnostics Gmbh, Mannheim, Germany) according to manufacturer. In Silico hybridizations were performed on serial paraffin sections as described. 48 For each probe and time point, embryo sections were located for staining on one slide to allow for comparison.

Quantitative immunostaining. Four present formalin-fixed embryos were embedded in paraffin. Sections were processed prior to staining with specific antibodies (see Supplementary Table S2). Quantification of stained embryos was performed with one-way Anova test with Histo Quest analysis software (Tissue Gnostics GmbH, Vienna, Austria).

Bioinformatic analysis. Illumina kits for strand-specific library preparation was used for RNA-seq and RNA was quantified using Qubit 2.0 Fluorometric Quantitation system (Life Technologies, Waltham, MA, USA). For comparison with human expression data orthops were mapped using the bioRaMI package (PMID:19617889). Gene set enrichment analysis was performed with java command line program (gsea2-2.0.13.jar) using the ‘preranked’ method from the Broad institute (PMID:16199517) and gene sets from MSigDb (http://software, broadinstitute.org/gsea/msigdb). Heatmap in Figure 5d: Mouse orthologs for genes in gene signature from was selected. Row-wise scaled, mean normalized expression levels for the four sample groups are shown. Genes in the heatmap are ordered by expression difference (ES tumor – ES control) and control levels. The heatmap is colored by expression levels for the four sample groups.

Statistical analysis. All results are presented as means ± S.E.M. Data were considered statistically significant as described in each panel. All analyses were performed using Graph Pad Prism software (Graph Pad Software, La Jolla, CA, USA).

Further description for Bioinformatic analysis, tissue culture procedures, IHC and statistical staining, FACS, qRT-PCR, primer table, specific antibody usage, transplant protocol and western blotting details please see Supplementary Data.

Conflict of Interest
The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)