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Author(s): Gizaw, Nebeyu Yosef; Kallio, Pauliina; Pungner, Tatjana; Gucciardo, Erika; Haglund, Caj; Böhling, Tom; Lehti, Kaisa; Sampo, Mika; Alitalo, Kari; Kivelä, Riikka

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PROX1 transcription factor controls rhabdomyosarcoma growth, stemness, myogenic properties and therapeutic targets

Nebeyu Yosef Gizaw^a, Pauliina Kallio^b, Tatjana Pungner^a, Erika Gucciardo^c, Caj Haglund^{b,d,e}, Tom Böhling^f, Kaisa Lehti^{c,g,h} , Mika Sampo^e, Kari Alitalo^{b,i,1} 

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Rhabdomyosarcoma (RMS) is an aggressive pediatric soft-tissue cancer with features of skeletal muscle. Because of poor survival of RMS patients and severe long-term side effects of RMS therapies, alternative RMS therapies are urgently needed. Here we show that the prospero-related homeobox 1 (PROX1) transcription factor is highly expressed in RMS tumors regardless of their cell type of origin. We demonstrate that PROX1 is needed for RMS cell clonogenicity, growth and tumor formation. PROX1 gene silencing repressed several myogenic and tumorigenic transcripts and transformed the RD cell transcriptome to resemble that of benign mesenchymal stem cells. Importantly, we found that fibroblast growth factor receptors (FGFR) mediated the growth effects of PROX1 in RMS. Because of receptor cross-compensation, paralog-specific FGFR inhibition did not mimic the effects of PROX1 silencing, whereas a pan-FGFR inhibitor ablated RMS cell proliferation and induced apoptosis. Our findings uncover the critical role of PROX1 in RMS and offer insights into the mechanisms that regulate RMS development and growth. As FGFR inhibitors have already been tested in clinical phase I/II trials in other cancer types, our findings provide an alternative option for RMS treatment.

sarcoma | cancer | PROX1 | FGFR | myogenesis

Rhabdomyosarcoma (RMS) is a highly aggressive soft-tissue sarcoma, which accounts for about 50% of childhood and 3% of adult soft-tissue sarcomas (1). Two main types of RMS are distinguished histologically: embryonal (ERMS), which represents approximately 60% of all RMS cases, and alveolar (ARMS), which accounts for about 25% (2). About 80% of ARMS tumors are associated with pathogenic chromosomal translocations that result in the expression of a PAX3-FOXO1 or PAX7-FOXO1 fusion protein and have poorer prognosis than ERMS (3). The remaining 20% of ARMS lacking these translocations are classified as fusion-negative tumors, whose outcomes are similar to those of ERMS. Thus, in the clinics, RMS is often classified as being either fusion-positive or fusion-negative (FP-RMS and FN-RMS) (4). Current RMS treatment includes chemotherapy, surgery, and/or radiation, which may induce severe side effects. Despite the multimodal therapy, the 5-y event-free survival rate of RMS patients with metastatic disease at diagnosis or relapse continues to be less than 30% (5), which emphasizes the urgent need to further uncover the molecular mechanisms regulating RMS development and growth.

RMS tumors have been suggested to arise from muscle stem cells (i.e., satellite cells) that fail to differentiate into mature skeletal muscle fibers (6–8). During myogenesis, the temporal expression of myogenic regulatory factors MYOD1, MYF5, MYF6, and myogenin drives satellite cell differentiation and a terminal cell-cycle exit (9). RMS cells express most of these factors but fail to execute terminal differentiation (10). It has also been suggested that RMS can arise from mesenchymal progenitor cells that reside in several nonmuscular tissues (11). Recently, animal models of FP-RMS and FN-RMS demonstrated that RMS can arise from both myogenic and nonmyogenic precursors, both of which acquire myogenic features during tumor development (5). Thus, identification of the different cell types that can give rise to RMS is currently among the most interesting and challenging questions to be answered (1). This open question further emphasizes the need to uncover the common molecular mechanisms that regulate the myogenic and oncogenic phenotypes in RMS, independently of the cell of origin.

Previous work has shown that the prospero-related homeobox 1 (PROX1) transcription factor is essential for satellite cell and myoblast differentiation and slow muscle fiber type characteristics in skeletal muscle (12, 13). In vertebrates, PROX1 is a highly conserved gene, which regulates lymphatic endothelial cell differentiation and the development of, e.g., eye, liver, and the heart (14). Because PROX1 has been shown to be involved in myoblast differentiation and in colorectal cancer (12, 15), we explored here if PROX1 is

Significance

Rhabdomyosarcoma is an aggressive pediatric soft-tissue cancer. Identification of RMS precursor cells and models has been a challenge due to unknown molecular mechanisms that drive the common proliferative myogenic phenotype. Our study revealed that the PROX1 transcription factor is essential for the growth and myogenic features of RMS, and its downregulation reverts RD cells to a phenotype resembling benign mesenchymal stem cells. Our findings demonstrate that the effects of PROX1 on RMS cell growth are mediated by FGFR, predominantly by FGFR1 and FGFR4, which were found to compensate for each other. This study places PROX1 as a major mediator of RMS characteristics and development and suggests that pan-FGFR inhibitors provide a promising option for the treatment of RMS.

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¹To whom correspondence may be addressed. Email: kari.alitalo@helsinki.fi or riikka.kivela@helsinki.fi.

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involved in the development and progression of RMS. Our findings demonstrate that PROX1 is essential for the myogenic phenotype and growth of RMS. The growth and viability features of the PROX1 silencing phenotype were recapitulated by silencing or chemical inhibition of fibroblast growth factor receptors (FGFRs), which we found to be regulated by PROX1.

Results

PROX1 Is Highly Expressed in Rhabdomyosarcoma. To gain insight into the PROX1 expression in RMS, we first analyzed tumor RNA expression profiles obtained from various sarcoma subtypes and patient cohorts using publicly available datasets deposited in Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>). Analysis of the RNA sequencing (RNAseq) data from primary patient samples showed that in 50% of the patients with FN-RMS ($n = 33/66$) and in almost all patients with FP-RMS ($n = 34/35$), PROX1 expression in the tumor is significantly higher than in healthy skeletal muscle (Fig. 1*A*) (GEO: GSE108022) (16). PROX1 expression was also significantly higher in both ERMS and ARMS mouse models than in healthy skeletal muscles (Fig. 1*B*) (GEO: GSE22520) (8, 17). Comparison between different sarcomas indicated that high PROX1 expression is specific to RMS, as we found only few Ewing sarcomas and synovial cell sarcomas with high PROX1 expression (Fig. 1*C*) (GEO: GSE2553) (18).

We studied PROX1 expression also in various RMS cell lines. The results showed that PROX1 mRNA and protein were more abundant in the ERMS (RD, RH36, and RMS-YM) and ARMS (KLHEL1 and RH30) cell lines than in human primary myoblasts (Fig. 1*D* and *E*). Next, we collected and analyzed 155 human RMS tumor samples from the Helsinki Biobank. Immunohistochemistry revealed high PROX1 expression in the majority of primary RMS tumor samples and RMS metastases ($n = 142/155$). In particular, intense nuclear PROX1 staining was found in almost all primary tumor samples ($n = 24/24$ ERMS and $n = 20/21$ ARMS) (Fig. 1*F* and *G*). Similarly, nuclear PROX1 staining was observed in the RD cells, which represent one of the most commonly used cell lines in RMS research (Fig. 1*F*).

PROX1 Is Required for RMS Cell Growth. To study the functional significance of PROX1 in RMS, we silenced PROX1 by using two independent lentiviral short hairpin RNAs (shRNAs) in the RD (FN-RMS) cell line and in the KLHEL1 (FP-RMS) cell line that we derived from a FP-RMS patient (Fig. 2*A* and *K*). Four days after lentiviral transduction, the PROX1-silenced RD and KLHEL1 cells had developed more rounded morphology that was distinct from the spindle shape observed in the cells transduced with the control vector (shSCR) (Fig. 2*B* and *L*). Analysis of 5-ethynyl-2'-deoxyuridine (EdU) incorporation showed significantly less proliferation in the PROX1-silenced RD and KLHEL1 cells than in the shSCR-transduced control cells (Fig. 2*C*, *D*, and *M*, *N*). The colony formation assay that was used to evaluate the stemness of the cells showed that the PROX1-silenced RD and KLHEL1 cells formed fewer and smaller colonies (Fig. 2*E*, *F*, and *O*, *P*). Further, in the 3D spheroid assay of the tumor cell self-renewal capacity in vitro (19), the PROX1-silenced RD and KLHEL1 cells formed significantly less rhabdospheres than the shSCR-transduced cells (Fig. 2*G*, *H*, and *Q*, *R*). Importantly, live cell imaging and analysis revealed that the growth of PROX1-silenced RD cells was completely inhibited and the growth of PROX1-silenced KLHEL1 was markedly decreased (Fig. 2*I* and *S* and *SI Appendix*, Fig. S1*A* and *B*). The PROX1-silenced RD and KLHEL1 cells also showed more apoptotic cells than their

shSCR-transduced controls (Fig. 2*J* and *T*). Furthermore, the reduced proliferation in PROX1-silenced RD and KLHEL1 cells was rescued by reintroduction of PROX1 expression with PROX1 overexpressing vector resistant to the shPROX1 construct (*SI Appendix*, Fig. S1*C–H*). These data demonstrated that PROX1 is essential for the proliferation, viability, and stemness of RMS cells, regardless of the tumor subtype.

PROX1 Is Essential for the Growth of RMS Tumor Xenografts.

Next, we assessed if PROX1 has tumor propagating potential in vivo using engraftment of control and PROX1-silenced RD (FN-RMS, silencing efficiency ~70%) and KLHEL1 (FP-RMS, silencing efficiency ~50%) cells into the left and right flanks of NOD/SCID/IL2rg female mice (*SI Appendix*, Fig. S2*A* and *B*). Serial tumor volume measurements showed significant growth inhibition in the PROX1-silenced tumors in comparison with the shSCR-transduced control tumors (Fig. 3*A*). The tumors from the PROX1-silenced cells were significantly smaller and weighted less than their respective controls (Fig. 3*B* and *C*). Hematoxylin and Eosin (H&E)-stained tumor sections derived from the PROX1-silenced RD and KLHEL1 cells showed reduced cell density compared to the control tumors (Fig. 3*D*, *H*, and *L* and *SI Appendix*, Fig. S2*C*, *G*, and *K*). Immunofluorescence for Ki67 and cleaved caspase 3 (CC3) revealed fewer proliferating cells (Fig. 3*E*, *I*, and *M* and *SI Appendix*, Fig. S2*D*, *H*, and *L*) and more numerous apoptotic cells (Fig. 3*F*, *J*, and *N* and *SI Appendix*, Fig. S2*E*, *I*, and *M*) in PROX1-silenced tumors. PROX1 RNA and immunohistochemistry (IHC) analysis confirmed lower PROX1 expression in shPROX1 derived tumors than in the control tumors during tumor development (Fig. 3*G*, *K*, and *O* and *SI Appendix*, Fig. S2*F*, *J* and *N*). These results demonstrated that PROX1 is essential for RMS tumor growth in vivo.

PROX1 Is a Master Regulator of the Myogenic and Malignant Transcriptome of RMS Cells.

To decipher the molecular basis of PROX1-dependent regulation of RMS tumor growth, we performed whole-genome RNAseq of four biological replicates of PROX1-silenced (75% silencing) and control RD cells. Principal component analysis (PCA) and hierarchical clustering of the RNAseq data showed that the PROX1-silenced and control cells formed two clearly distinct groups (Fig. 4*A* and *B*). Analysis of the differentially expressed genes (DEGs), using a statistical significance cutoff at false discovery rate (FDR) < 0.05 and biological significance cutoff at ≥ 1.5 -fold, showed 461 upregulated and 433 downregulated genes in the shPROX1 cells (*SI Appendix*, Tables S1 and S2) and 1,141 upregulated and 1,119 downregulated genes when using FDR < 0.05 and \log_2 fold-change ≤ -0.25 and ≥ 0.25 (Fig. 4*C*). Gene ontology (GO) analysis revealed that cell–cell adhesion, angiogenesis, and cell–matrix adhesion were among the most significantly enriched GO terms among the upregulated DEGs (Fig. 4*D*), and skeletal muscle contraction and muscle filament gliding were enriched among the downregulated DEGs (Fig. 4*E*). Interestingly, these GO terms have previously been shown to be among the top biological processes that shift, in an opposite manner, when nonmyogenic mesenchymal cells are driven into FP-RMS (20) and during FN-RMS tumor development from endothelial precursor cells (21). Intriguingly, Gene Set Enrichment Analysis (GSEA) revealed that the gene sets that are altered in mesenchymal stem cells after forced expression of the PAX3-FKHR(FOXO1) fusion gene to drive RMS tumorigenesis (20) were changed in an opposite direction by PROX1 silencing, i.e., shifting the RD cells toward a benign mesenchymal stem cell phenotype. This indicated that a common set of transcripts is regulated by PROX1 in RD cells

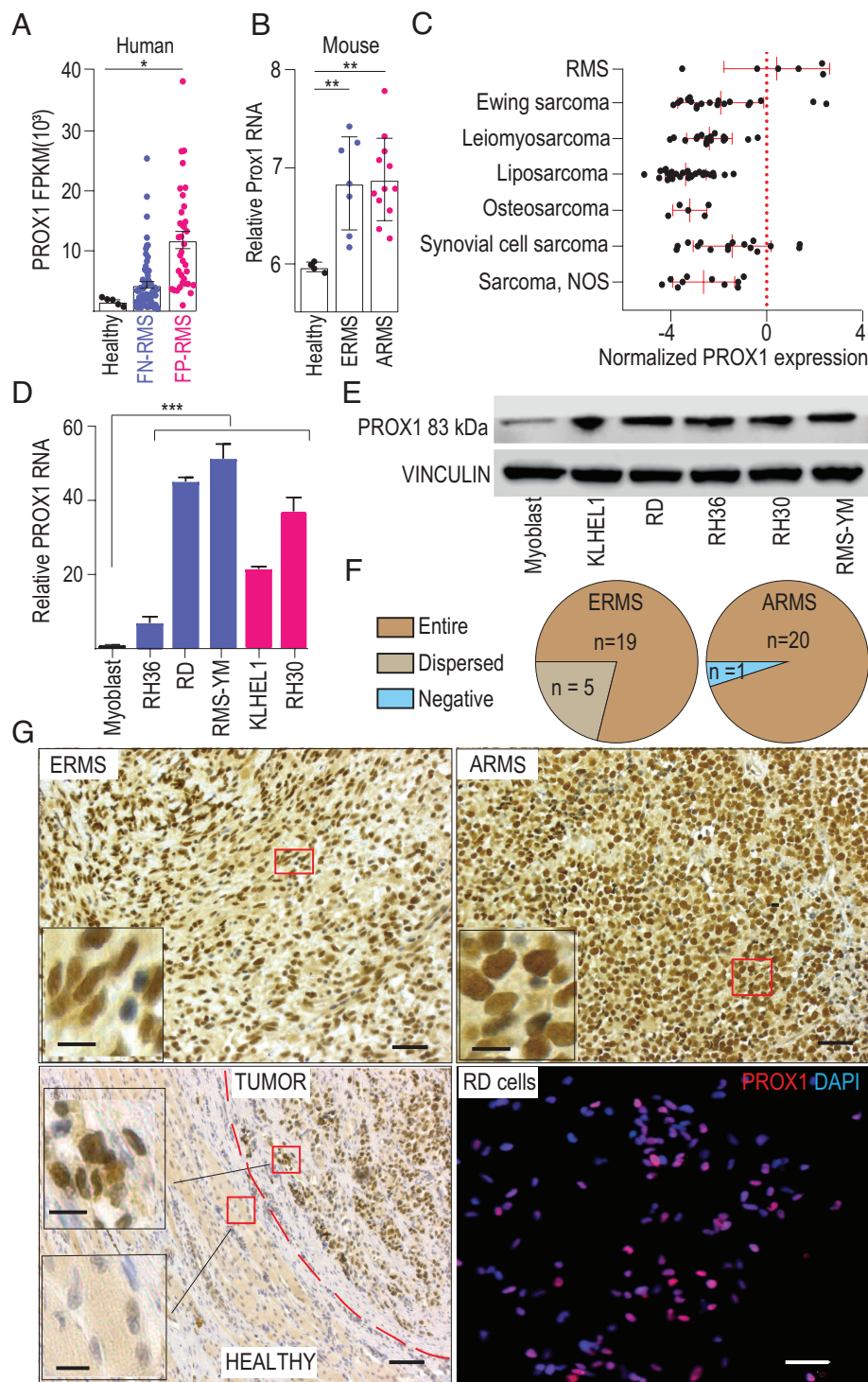


Fig. 1. High PROX1 expression in primary RMS tumors and metastases. (A) Normalized mRNA expression of PROX1 (FPKM) in healthy skeletal muscle (Healthy SKM) and in tumors from FN-RMS and FP-RMS RMS patients (GSE108022, FDR < 0.05). (B) mRNA expression of Prox1 in healthy mouse skeletal muscle and in ERMS and ARMS tumors (GSE22520, **FDR < 0.01). (C) Microarray data depicting PROX1 expression in various sarcomas (GSE2553). (D) Real-time qPCR analysis of PROX1 mRNA in FN-RMS (RD, RH36 and RMS-YM) and FP-RMS (KLHEL1 and RH 30) cell lines compared to healthy human myoblasts (*** $P < 0.001$). (E) Western blot analysis of PROX1 in RMS cell lines and in healthy human myoblasts. Vinculin was used as a loading control. (F) Classification of PROX1 expression pattern in the primary RMS tumor samples from the Helsinki Biobank. (G) Immunohistochemical staining of PROX1 in human tumor samples from the Helsinki Biobank. Both ARMS and ERMS patient samples show strong nuclear PROX1 expression. The *Bottom Left panel* shows the difference in PROX1 expression between the tumor and the adjacent healthy muscle. The dashed line marks the tumor boundary. In the *Lower Right panel*, RD cells are stained for PROX1 (magenta) and nuclei (blue). Scale bars: for full image 50 μm and for the magnified inset 10 μm . Data are presented as mean \pm SEM.

and by PAX3-FOXO1 fusion gene during RMS development (Fig. 4 *F* and *G*). GSEA also revealed that many myogenesis hallmark genes were highly downregulated in the PROX1-silenced cells (Fig. 4*H*). Several genes characteristic of the mesenchymal stem cell phenotype were found to be significantly induced by

PROX1 silencing (Fig. 4*I*), and genes regulating myogenesis and myogenic features were repressed (e.g., MYOG, MEF2C, MYH7, MYH8, MYH3, STAC3, MYL1, MYL6B, ENO3, TNNT1, TNNC1, TNNC2, PAX7, and ACTC1) (*SI Appendix, Table S1*). In line with the findings from the Incucyte experiments,

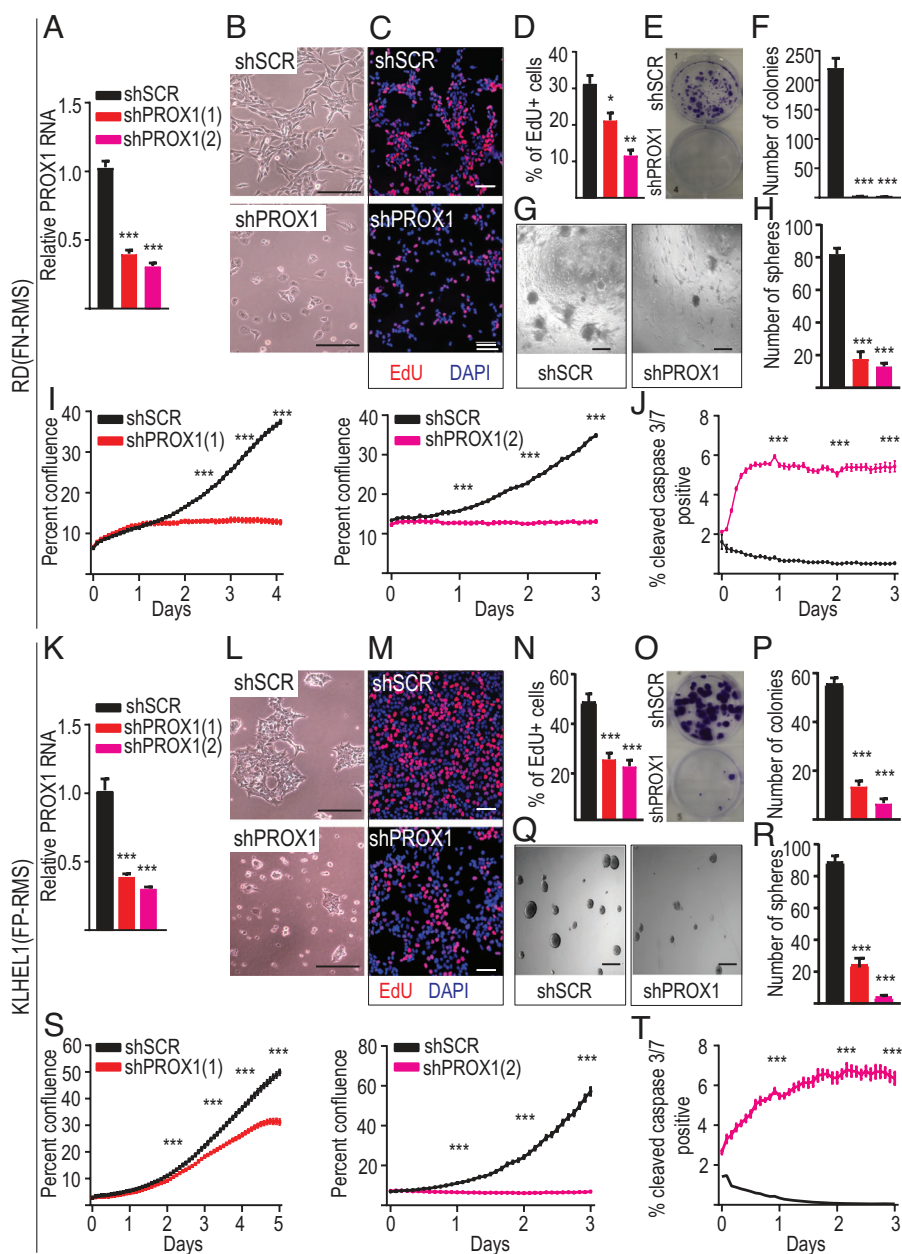


Fig. 2. PROX1 is required for RMS cell growth (A) qPCR analysis of PROX1 mRNA expression in RD cells after shSCR (control) and shPROX1 transduction. (B) Bright field images depicting morphological changes in RD cells at 4 days post transduction. Scale bars 100 μ m. (C, D) EdU incorporation and quantification of proliferating cells in PROX1 silenced and control RD cells. Scale bars 50 μ m. (E, F) Colony formation assay and quantification in RD cells. (G, H) Representative images and quantification showing rhabdospheres formed from shSCR and shPROX1 -treated RD cells at day 9. Scale bar 500 μ m. (I) Cell growth based on IncuCyte live cell imaging for stably expressing shSCR or shPROX1 RD cells using two different silencing constructs. (J) Caspase 3/7 activity was quantified in control and PROX1 silenced RD cells by a fluorescent reporter by IncuCyte. (K) qPCR analysis of PROX1 mRNA expression in KLHEL1 cells following shSCR (control) and shPROX1 transduction. (L) Bright field images depicting morphological changes in KLHEL1 cells at 4 days post transduction. Scale bars 100 μ m. (M, N) EdU incorporation and quantification of proliferating cells in PROX1 silenced and control KLHEL1 cells. Scale bars 50 μ m. (O, P) Colony formation assay and quantification in KLHEL1 cells. (Q, R) Representative images and quantification showing rhabdospheres formed from shSCR and shPROX1 -treated KLHEL1 cells at day 9. Scale bar 500 μ m. (S) Cell growth based on IncuCyte live cell imaging for stably expressing shSCR or shPROX1 KLHEL1 cells using two different silencing constructs. (T) Caspase 3/7 activity was quantified in control and PROX1 silenced KLHEL1 cells by a fluorescent reporter. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Data is presented as mean \pm SEM.

GSEA demonstrated upregulation of genes related to hallmarks of apoptosis (FDR < 0.001) and a trend for downregulation of cell cycle genes (FDR = 0.268) upon PROX1 silencing.

To further examine the phenotype of the PROX1-silenced cells, we stained the cells for the mesenchymal stem cell marker NT5E/CD73 (22). In line with the transcriptomic data, the CD73 protein was highly increased in the PROX1-silenced RD cells (Fig. 4 J and K). Similarly, the expression of NT5E/CD73 was increased more than eightfold and MYOG was decreased in the tumors

derived from PROX1 knockdown cells when compared to shSCR-transduced control (SI Appendix, Fig. S3A). PROX1 silencing in FP-RMS cells (RH30 and KLHEL1) decreased the expression of myogenic genes but did not increase the mesenchymal stem cell markers. This indicates that while PROX1 silencing commonly represses the myogenic gene signature, it reinduces the gene expression related to the cells of tumor origin, which likely differs between the three cell lines used by us (SI Appendix, Fig. S3 A and B). Acquisition of mesenchymal features is associated

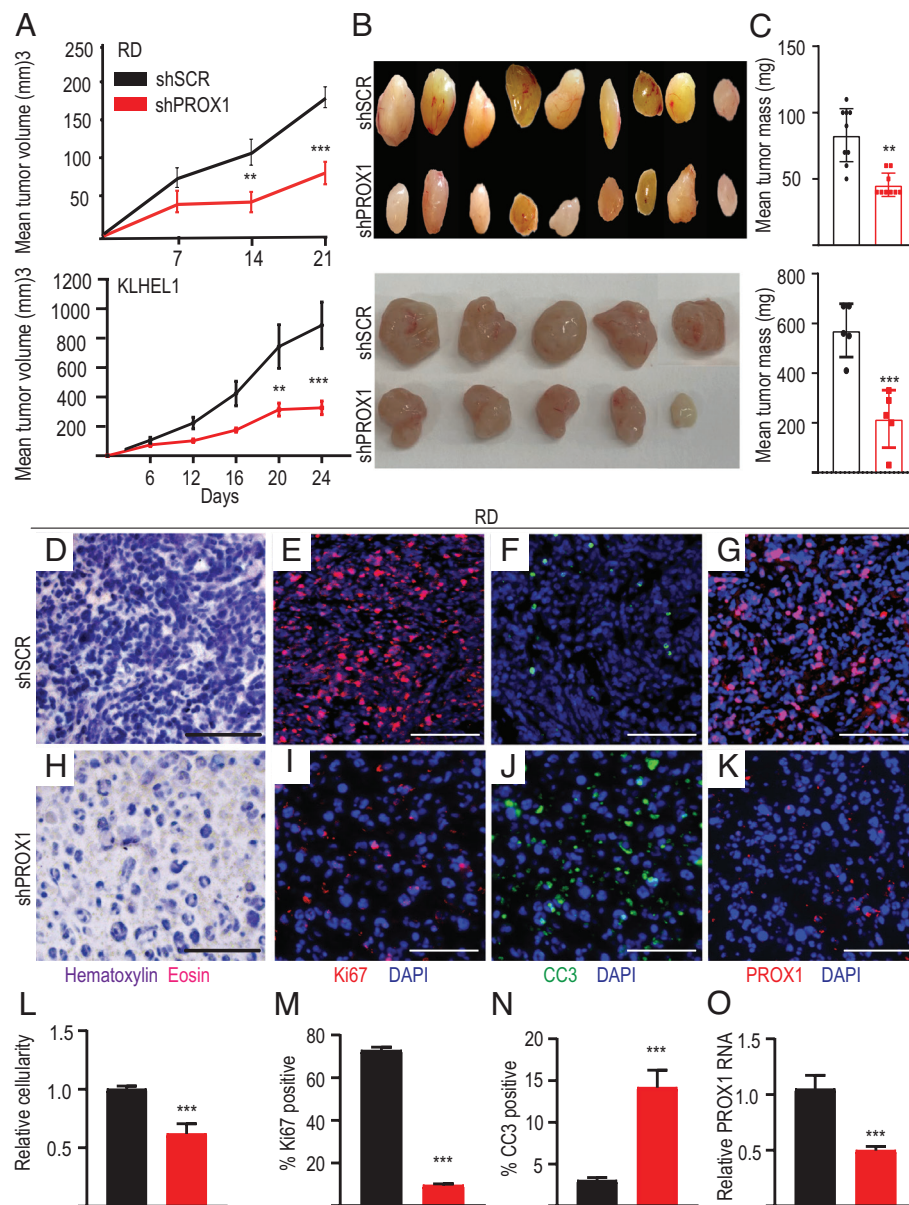


Fig. 3. PROX1 is required for the growth of RMS tumor xenografts. (A) Quantification of tumor growth based on tumor volume measurement (mm³) in RD (Top) and KLHEL1 (Bottom) xenografts (n = 9 for RD and n = 5 for KLHEL1 per group). (B) Representative images of shSCR and shPROX1 xenograft tumors derived from RD (Top) and KLHEL1 (Bottom) cells. (C) Tumor weight in shSCR or shPROX1 tumors at the end of the experiment (RD Top and KLHEL1 Bottom). (D–K) Histological characterization of the shSCR (D–G) and shPROX1 (H–K) RD xenograft tumors. Representative images of the H&E-stained tumor sections (D and H) and immunostained for Ki67 (red) and DAPI (blue) (E and I), cleaved caspase 3 (CC3) (13), and DAPI (blue) (F and J) and PROX1 (red) (G and K). (L) Quantification of nuclei count/tumor area. (M) Percentage of Ki67-positive cells within tumors. (N) Percentage of cleaved caspase 3 (CC3)-stained cells. (O) RNA expression of PROX1 in the excised tumors. *P < 0.05, **P < 0.01, and ***P < 0.001. Data are presented as mean ± SEM. Scale bar: 100 μm.

with increased invasiveness in many tumors. Thus, we investigated cell migration capacity of PROX1-silenced and control RMS cells using a wound healing assay. We found that the PROX1-silenced RMS cells migrated slightly less than the WT control cells, indicating that the acquired mesenchymal features do not promote migration in RD cells (SI Appendix, Fig. S3 C–F). These data suggest that PROX1 silencing reverts the RMS cell phenotype towards its origin by repressing the myogenic features and by reinducing characteristics of the original benign cell type.

FGFRs Regulate RMS Growth Downstream of PROX1. Elevated protein tyrosine kinase and serine/threonine kinase activities (PTK, STK, respectively) are involved in the oncogenic processes that lead to RMS by regulating cell proliferation and tumorigenesis

(23). Since PROX1 is a transcription factor, and thus not an easily druggable target, we next assessed if the tumorigenic effect of PROX1 is mediated through regulation of the RMS kinome by using nontargeted PTK and STK assays. Intriguingly, the kinase activity analysis showed that FGFR signaling, which is altered in both FN- and FP-RMS (16), was the most significant kinase pathway affected by PROX1 silencing (Fig. 5A). In line with this, the RNAseq results showed that FGFR4 and FGFR1 are highly expressed in RD cells and are repressed upon PROX1 silencing (Fig. 5B). A similar effect was observed when we silenced PROX1 in the FP-RMS cell lines KLHEL1 and RH30 (Fig. 5C and D). Analysis of RMS tumor mRNA data deposited in MediSapiens (n = 49 tumors, <https://medisapiens.com/>) revealed a significant correlation between PROX1 and FGFR4 expression (Fig. 5E).

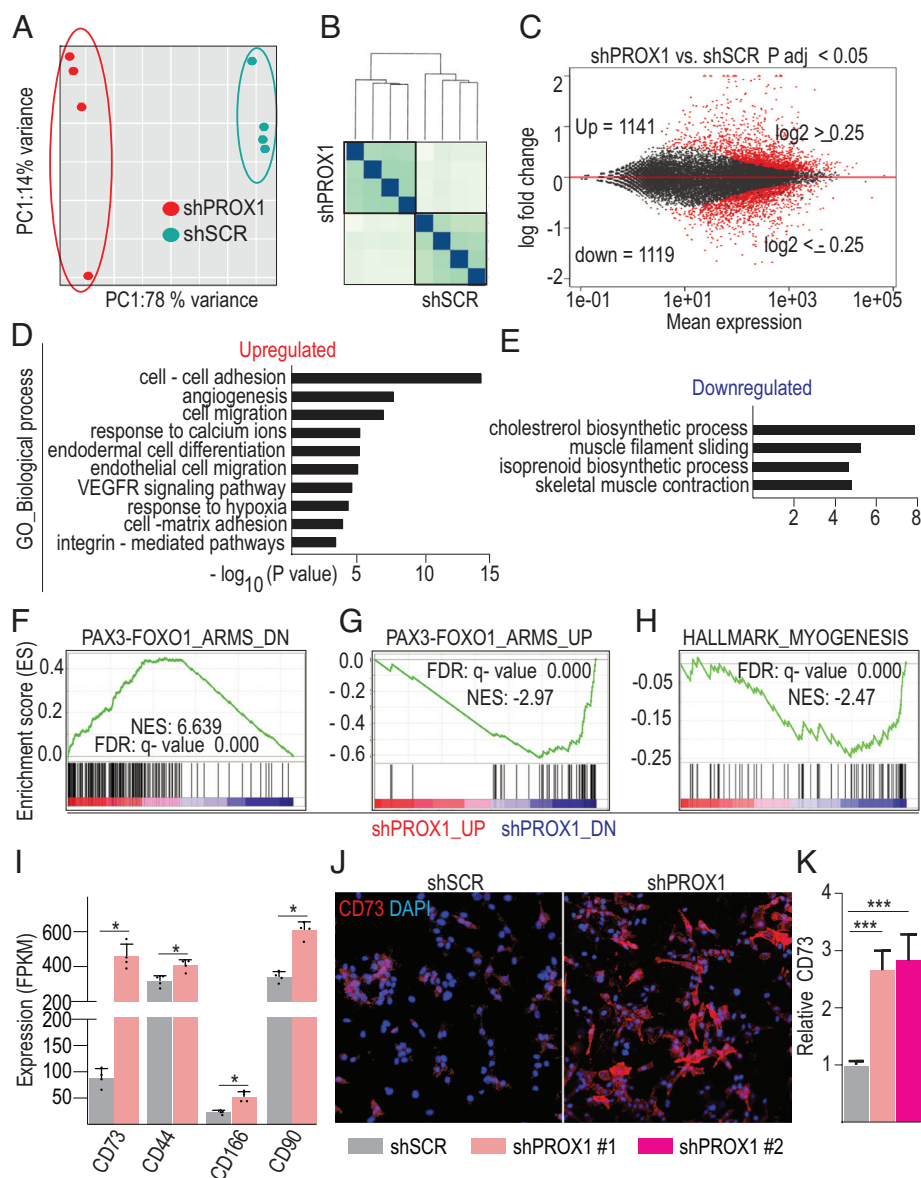


Fig. 4. PROX1 is a potent regulator of myogenic and malignant transcriptome in RMS cells. Global gene expression analysis of PROX1-silenced RD cells by RNAseq. (A) PCA of the variance between samples and (B) sample distance analysis of RNA-seq samples ($n = 4 + 4$). (C) MA plot showing the DEGs in red (FDR < 0.05, \log_2 fold change (FC) cutoff 0.25) in PROX1-silenced compared to control RD cells, including 1,141 upregulated and 1,119 downregulated genes. (D and E) GO analysis showing the most significant functional categories among the upregulated genes (D) and the downregulated genes (E) in PROX1-silenced cells. (F–H) GSEA plots for gene sets overlapping with PROX1-driven DEGs in RD cells. NES, normalized enrichment score. (I) Normalized expression values (FPKM) of mesenchymal stem cell marker genes in the control and PROX1-silenced RD cells. (*FDR < 0.05). (J) Immunofluorescence staining for CD73 (red) and nuclei (blue) in control and PROX1-silenced RD cells. (K) Quantification of CD73 fluorescence intensity in shRNA-treated RD cells (*** $P < 0.001$). Data are presented as mean \pm SEM.

These data indicate that PROX1 regulates FGFR signaling at gene expression and/or kinase activity level.

As FGFR4 has been shown to be highly expressed in RMS and to contribute to tumorigenesis by stimulating cell proliferation in ERMS (24), we first assessed if increasing FGFR4 expression would further increase tumor cell proliferation. Indeed, lentiviral overexpression of FGFR4 slightly but significantly enhanced the proliferation of RD cells (SI Appendix, Fig. S4 A–C). Next, we investigated whether the effect of PROX1 on proliferation is mediated by FGFR4. To examine if FGFR4 silencing would phenocopy the effects of PROX1 silencing, two independent shFGFR4 constructs were used (SI Appendix, Fig. S4D). The colony and rhabdosphere formation capacity of RD cells was significantly reduced in the FGFR4-deficient cells (SI Appendix, Fig. S4 E–H). However, the overall effect was not as striking as after PROX1 silencing. Since the PTK assay indicated that FGFR1 was the most affected

receptor tyrosine kinase, we next examined the role of FGFR1 in RMS proliferation and colony formation. Silencing of FGFR1 with two independent shRNA constructs in RD, KLHEL1, and RH30 cells also decreased colony formation (SI Appendix, Fig. S4 H and J).

Interestingly, we found that FGFR1 silencing markedly increased the expression of FGFR4 and vice versa in both RD and KLHEL1 cells, demonstrating a compensatory mechanism between these two receptors (Fig. 6 A–D). To examine this further, we evaluated the effect of inhibiting FGFR4 and FGFR1 separately, FGFR1 and 4 together, or all FGFRs on RMS cell growth using small molecules BLU9931 (FGFR4i), PD173074 (FGFR1i), and LY2874455 (pan-FGFRi). Intriguingly, the combined inhibition of both receptors (FGFR1 and FGFR4) decreased RD and KLHEL1 cell growth much more potently than inhibition of either receptor alone (Fig. 6E). Most importantly, inhibition of

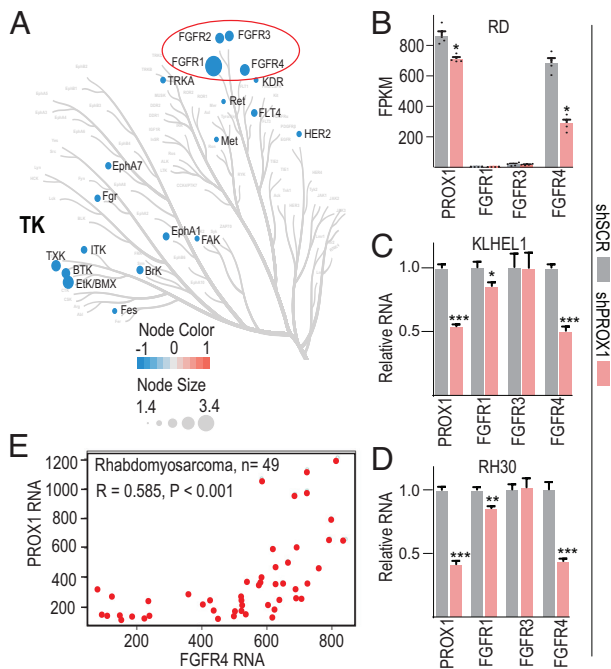


Fig. 5. PROX1 regulates RMS FGFR kinase activity and gene expression. (A) A phylogenetic kinome tree of the top 20 significantly altered tyrosine kinases in PROX1-silenced RD cells. The red circle shows the dominant FGFR cluster among the tyrosine kinases. The node size is based on a specificity score, 3.4 being the highest. Color scale depicts changes in kinase activity in PROX1-deficient vs. control cells (−1 blue strong decrease, 0 white no difference, and +1 red strong increase in kinase activity). (B) FGFR normalized expression values (FPKM) in control and PROX1-silenced RD cells. (*FDR < 0.05). (C and D) qPCR analysis of FGFR mRNA expression in PROX1-silenced vs. control KLHEL1 (C) and RH30 cells (D). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Data are presented as mean \pm SEM. (E) Correlation analysis of PROX1 and FGFR4 expression in RMS tumor RNA expression data from the Medisapiens database.

all FGFR receptors with pan-FGFRi completely inhibited cell growth and induced apoptosis in RMS cells (Fig. 6 E and F). Combined inhibition at lower doses also decreased colony formation significantly in all the studied cell lines (RD, KLHEL1, RH30), whereas single receptor inhibition did not have any effect (Fig. 6 G–H). We next used a high-throughput drug sensitivity and resistance testing (DSRT) (25) to compare the efficacy of the pan-FGFR inhibitor with the HDAC inhibitor entinostat, which was recently shown to be effective in both RMS cell culture and preclinical studies (26). The results showed that the pan-FGFR inhibitor had an equivalent effect on cell viability as entinostat in RD cells and even a stronger effect in KLHEL1 and RH30 cells (SI Appendix, Fig. S5 A–C). Interestingly, a combination of FGFR and HDAC inhibition produced an additive effect in all three cell lines (SI Appendix, Fig. S5 A–C). Analysis of PROX1 expression after 24-h treatment with pan-FGFRi showed that RMS cells responded by increasing PROX1 expression despite their complete growth inhibition (SI Appendix, Fig. S5D). This indicated that PROX1 cannot promote proliferation without FGFR signaling. However, the mesenchymal stem cell markers were not induced upon FGFR inhibition (SI Appendix, Fig. S5E), suggesting that FGFR signaling mediates the PROX1 effects on growth and proliferation but not on the cell differentiation characteristics.

To test if reintroducing FGFR4 expression into PROX1-silenced cells can rescue the proliferation and colony formation capacity of the cells, we overexpressed FGFR4 in PROX1-silenced RMS cells. The results demonstrated that overexpression of FGFR4, indeed, was able to enhance proliferation (Fig. 6 I–K) and partially rescue the colony formation capacity of the

PROX1-silenced cells (SI Appendix, Fig. S5 F and G). These data support our findings that FGFR signaling is a downstream effector of PROX1 regulating RMS growth and cell proliferation.

We also tested the in vivo effect of the pan-FGFR inhibitor on the growth of the RD and KLHEL1 xenografts. A previous report has shown that pan-FGFRi (LY2874455) at a dose of 3mg/kg twice a day can attenuate tumor growth in other FGFR-dependent cancers (27). Although at this dose of intragastric (i.g.) administration there was no effect on KLHEL1 tumors, i.g.-dosed pan-FGFRi significantly reduced RD tumor growth (SI Appendix, Fig. S6A). The pan-FGFR inhibitor-treated RD tumors were smaller and weighed less than the control tumors (SI Appendix, Fig. S6 B and C). They also exhibited reduced cell density in comparison with the vehicle-treated tumors (SI Appendix, Fig. S6 D, G and J) and had fewer Ki67⁺ proliferating cells (SI Appendix, Fig. S6 E, H and K). In line with the in vitro data, PROX1 expression was increased, despite the growth inhibition in the pan-FGFRi-treated RD vs. control tumors (SI Appendix, Fig. S6 F, I and L). These results indicate that the inhibition of tumor growth obtained by PROX1 silencing in RD cells can be recapitulated by inhibiting FGFR signaling.

Discussion

Our results demonstrate that the PROX1 transcription factor is an important regulator of the myogenic phenotype and tumorigenic properties of RMS. PROX1 was strongly expressed in almost all primary FN- and FP-RMS tumors and their metastases in a large biobank cohort. PROX1 silencing in RMS cells repressed hallmarks of myogenesis and decreased cell proliferation, clonogenic growth, and rhabdosphere formation. A similar effect was observed in vivo, where the growth of the tumors derived from PROX1-silenced cells was markedly impaired. Simultaneously with the loss of myogenic features, the expression of mesenchymal stem cell genes was highly increased in the PROX1-silenced RD cells. Our data indicate that PROX1 silencing inhibits RMS growth by reverting the tumor cells towards their cell of origin, whereby the cells lose markers of myogenic differentiation. Furthermore, we found that PROX1 controls the expression and activity of FGFRs, mainly FGFR1 and FGFR4, in RMS, and that inhibitors that act on both of these receptors repress the proliferation/stemness of the RMS cells.

During myoblast differentiation into myotubes, PROX1 expression increases to regulate the expression of other myogenic factors and components of the muscle contractile machinery (12, 13). In experimental RMS models, several myogenic regulatory factors are expressed in the tumors regardless of their myogenic or non-myogenic origin (7). Via promotion of chromatin acetylation, the PAX3–FOXO1 fusion gene has been shown to generate active super-enhancers to drive MYOD1 and MYCN, which then drive MYOG expression (28). However, PAX3–FOXO1 can activate myogenic determination and MYOG expression also independently of MYOD1 (29, 30). Our work here identifies PROX1 as one of the inducers of the myogenic transcription program and acquisition of myogenic features in RMS, similarly as demonstrated in healthy muscle (12, 13). This finding is also supported by a recent chemical genomics analysis, in which PROX1 was listed among the core regulatory transcription factors in RMS (31).

In nonmyogenic endothelial progenitor cells, PROX1 was among the most upregulated genes during FN-RMS development induced by activation of the hedgehog pathway using an activated form of Smo (SmoM2) (21). PROX1 was also significantly increased in an FP-RMS model induced by forced PAX3–FOXO1

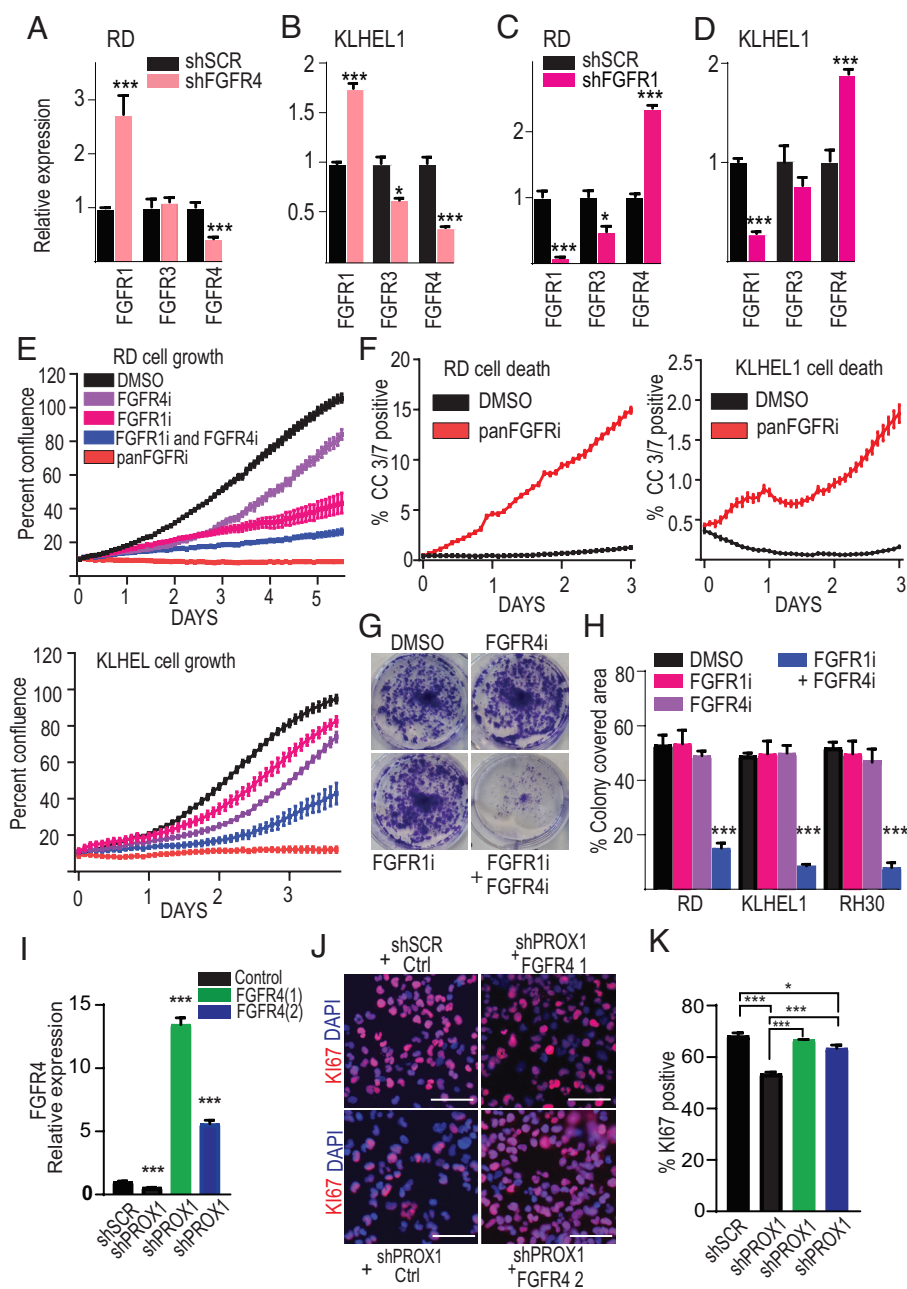


Fig. 6. FGFRs act together as downstream effectors of PROX1 regulated RMS growth. (A–D) FGFR gene expression analysis in FGFR4-silenced (A and B) and FGFR1-silenced (C and D) RD (A and C) and KLHEL1 cells (B and D). (E) Growth curves of RD (FN-RMS) (Top) and KLHEL1 (FP-RMS) cells (Bottom) treated with 5 $\mu\text{mol/L}$ PD173074 (FGFR1i) alone or Blu9931 (FGFR4i) alone or a combination of PD173074 and Blu9931 or LY2874455 (panFGFRi), and analyzed by IncuCyte live cell imaging. (F) Caspase 3/7 activity in dimethyl sulfoxide (DMSO) and panFGFRi-treated RD and KLHEL1 cells by a fluorescent reporter measured using IncuCyte. (G–H) Colony formation assay in RD, KLHEL1, and RH30 cells with the same inhibitors as in E at 2 $\mu\text{mol/L}$ concentration. (I) qPCR analysis of PROX1-silenced RD cells transfected with two independent FGFR4 overexpressing lentiviral vectors or a control vector. (J) Ki67 staining and (K) quantification of the percentage of Ki67-positive cells. Scale bar: 50 μm . * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Data are presented as mean \pm SEM.

expression in mesenchymal stem cells (20). In both of these RMS models, despite their different cell types of origin, GO analysis of the upregulated genes showed overrepresentation of muscle contraction-related genes, whereas cell adhesion was among the downregulated pathways. We obtained exactly opposite results when PROX1 was silenced in RD cells, demonstrating that PROX1 deficiency transforms the RD cell transcriptome to resemble that of benign mesenchymal stem cells. Thus, our results in combination with the analyses of the publicly available data sets indicate that PROX1 is needed for the muscle specification program in RMS, irrespective of the tumor cell origin. Moreover, our work suggests that PROX1 silencing in RMS not only reverts the muscle

phenotype and cell adhesion properties but allows the RMS cells to regain the characteristics of their cells of origin. This could provide further insights into the various myogenic and nonmyogenic cell lineages that can contribute to RMS tumorigenesis.

PROX1 expression has been associated with tumor progression and prognosis also in other cancer types (14). Previously, we have shown that PROX1 is important in the transition of benign colon adenomas to carcinomas and in the maintenance of cancer stem cell features in intestinal adenomas and colorectal cancer (15, 32). PROX1 is also essential for the clonogenic growth in colorectal cancer cells (33), and high PROX1 expression is correlated with a poor prognosis of rectal neuroendocrine tumors and esophageal

cancer patients (34, 35). As a vital transcription factor in many tissues, PROX1 is not an easily druggable target. Thus, we studied the PROX1 downstream signaling that could be targeted by using currently available small-molecule inhibitors. Our kinome and transcriptome data revealed that the expression and activity of FGFR1 and FGFR4 were dependent on PROX1 in RMS. Previously, PROX1 has been shown to regulate FGFR4 expression in colorectal cancer cells (33), FGF signaling in pancreatic beta-cells (36), and FGFR3 during lens fiber differentiation (37). Silencing either of the two FGF receptors alone only partially phenocopied the effects of PROX1 silencing, likely because of the observed reciprocal regulation between these two receptors. However, inhibition of both receptors by combining FGFR1 and FGFR4 small-molecule inhibitors or using the pan-FGFR inhibitor LY2874455 potently reduced the viability, proliferation, and stemness and increased apoptosis of RMS cells, an effect comparable to that of PROX1 silencing. Our finding that the pan-FGFR inhibitor LY2874455 inhibited RD but not KLHEL1 tumor growth in vivo warrants further investigation of the mechanisms of FGFR inhibition in FN-RMS vs. FP-RMS. We also compared the effects of LY2874455 and the HDAC inhibitor entinostat, which was recently shown to inhibit RMS cell growth both in vitro and in a preclinical mouse model (26). In all three studied RMS cell lines, panFGFRi was at least as potent as entinostat and had an additive effect when the two drugs were used in combination.

During embryonic development, FGFR4 is expressed starting in the myotomal precursors of skeletal muscle cells, and its overexpression and mutations have been shown to be important for RMS development (24, 38). Our current study shows that downstream of PROX1, the cross-regulated FGFR1 and FGFR4 act together in RMS. Simultaneous targeting of both receptors could thus provide an efficient strategy to treat RMS, and combinatorial treatment with panFGFRi and HDAC inhibitors together with chemotherapy could provide an even more effective therapy, which needs to be further tested in different preclinical RMS models. Inactivation of FGFRs by tyrosine kinase inhibitors (TKI) has achieved great success in tumor-targeted therapy with more than 80 clinical trials recruiting patients at the moment (39, 40). Resistance to FGFR-TKI and management of the side effects has become a concern since the current inhibitors are not fully specific for FGFRs. However, preclinical models suggest that combination regimens such as synergistic inhibition of FGFR inhibitors and mTOR or MAPK pathway inhibitors could bypass the resistance mechanisms (39).

In summary, our results combined with the analysis of the previously published data sets demonstrate a central role for PROX1 in the regulation of myogenic hallmarks and tumorigenic properties of RMS cells. We also discover FGFR signaling as a downstream mediator of the effects of PROX1 on RMS growth and show that inhibition of both FGFR1 and FGFR4 could provide

an effective therapeutic modality in RMS. Our findings should stimulate further studies on advanced treatment options for RMS, especially as several FGFR inhibitors are currently in clinical trials and some have already been approved for other cancer types.

Materials and Methods

The materials used in this study, including cell lines, reagents, antibodies, and sequences for shRNAs and RT-qPCR primers, are described in *SI Appendix*. The detailed description of methodologies for cell culture and transduction, bioinformatic analysis of human RMS datasets, RMS cell growth, migration, and apoptosis, tyrosine kinase and STK activity profiling, mouse tumor models, histology and immunohistochemistry, RNA extraction and quantitative real time PCR, protein extraction and Western blotting analysis, RNAseq and GSEA, drug sensitivity and resistance testing (DSRT), and statistical analyses are also included in the *SI Appendix* for *Materials and Methods*. The ethical committee of the Helsinki University Hospital approved the study, and written consent was obtained from the patient.

Data, Materials, and Software Availability. RNAseq data have been deposited in NCBI Gene Expression Omnibus (41) database under accession number GSE145171 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145171>).

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Author affiliations: ^aStem Cells and Metabolism Research Program, Research Programs Unit, Faculty of Medicine, University of Helsinki, Helsinki 00014, Finland; ^bTranslational Cancer Medicine Program and iCAN Digital Precision Cancer Medicine Flagship, Faculty of Medicine, University of Helsinki, Helsinki 00014, Finland; ^cIndividualized Drug Therapy Research Program, Research Programs Unit, Faculty of Medicine, University of Helsinki, Helsinki 00290, Finland; ^dDepartment of Surgery, University of Helsinki and Helsinki University Hospital, Helsinki 00014, Finland; ^eDepartment of Pathology, University of Helsinki and Helsinki University Hospital, Helsinki 00014, Finland; ^fDepartment of Pathology, University of Helsinki, Helsinki 00014, Finland; ^gDepartment of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Solna 17165, Sweden; ^hDepartment of Biomedical Laboratory Science, Norwegian University of Science and Technology (NTNU), Trondheim 7491, Norway; ⁱWihuri Research Institute, Helsinki 00290, Finland; and ^jFaculty of Sport and Health Sciences, University of Jyväskylä, Jyväskylä 40700, Finland

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