CRISPR screen identifies the NCOR/HDAC3 complex as a major suppressor of differentiation in rhabdomyosarcoma

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Dysregulated gene expression resulting from abnormal epigenetic alterations including histone acetylation and deacetylation has been demonstrated to play an important role in driving tumor growth and progression. However, the mechanisms by which specific histone deacetylases (HDACs) regulate differentiation in solid tumors remains unclear. Using pediatric rhabdomyosarcoma (RMS) as a paradigm to elucidate the mechanism blocking differentiation in solid tumors, we identified HDAC3 as a major suppressor of myogenic differentiation from a high-efficiency Clustered regularly interspaced short palindromic repeats (CRISPR)-based phenotypic screen of class I and II HDAC genes. Detailed characterization of the HDAC3-knockout phenotype in vitro and in vivo using a tamoxifen-inducible CRISPR targeting strategy demonstrated that HDAC3 deacetylase activity and the formation of a functional complex with nuclear receptor corepressors (NCORs) were critical in restricting differentiation in RMS. The NCOR/HDAC3 complex specifically functions by blocking myoblast determination protein 1 (MYOD1)-mediated activation of myogenic differentiation. Interestingly, there was also a transient up-regulation of growth-promoting genes upon initial HDAC3 targeting, revealing a unique cancer-specific response to the forced transition from a neoplastic state to terminal differentiation. Our study applied modifications of CRISPR/CRISPR-associated endonuclease 9 (Cas9) technology to interrogate the function of essential cancer genes and pathways and has provided insights into cancer cell adaptation in response to altered differentiation status. Because current pan-HDAC inhibitors have shown disappointing results in clinical trials of solid tumors, therapeutic targets specific to HDAC3 function represent a promising option for differentiation therapy in malignant tumors with dysregulated HDAC3 activity.

histone deacetylase | HDAC3 | NCOR | rhabdomyosarcoma | CRISPR

Abnormal epigenetic alterations play an important role in driving tumor growth and progression (1, 2). Histone deacetylases (HDACs), which are major epigenetic modifiers, are dysregulated in a significant subset of cancers (3, 4). Although pan-HDAC inhibitors have elicited promising therapeutic responses in some hematologic malignancies (1, 2, 5), limited therapeutic benefits have been reported in clinical trials for most solid tumors, including sarcomas (6). The inefficacy of HDAC inhibitors in solid tumors most likely results in part from their broad and unknown substrate range and their pleiotropic effects. Despite these early clinical failures, HDACs remain prominent therapeutic targets in cancers because of their ability to reprogram gene-expression networks. Improved understanding of the molecular mechanisms underlying specific HDAC function will lead to more effective drug and therapy designs.

Rhabdomyosarcoma (RMS), which consists of two major subtypes, embryonal (ERMS) and alveolar (ARMS), is the most common pediatric soft tissue malignancy. Although the two major subtypes are driven by distinct genetic alterations, both are characterized by a block in the myogenic differentiation program (7, 8). We have previously shown that treatment of RMS cells with HDAC inhibitors results in the suppression of tumor growth

through the induction of myogenic differentiation (9). However, the mechanism by which aberrant activity of specific HDAC(s) represses differentiation and contributes to the malignant transformation of RMS remains unclear.

Although recent advances in Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated endonuclease 9 (Cas9) genome-editing technology have facilitated the identification of essential tumor genes, detailed phenotypic and functional characterization of essential cancer genes with the current technology is limited by the inability to expand mutant tumor clones harboring essential gene mutations and by poor CRISPR targeting efficiency in pooled cells. In this study, we used modifications of CRISPR/Cas9 genome-editing technology, including high-efficiency phenotypic screens and inducible gene targeting, to interrogate the functions of essential cancer genes. These genomic tools were used to identify the underlying HDAC-mediated epigenetic mechanisms blocking differentiation of RMS tumor cells, which are essential for tumor progression.

Results

CRISPR-Mediated Knockout of HDAC3 Induces Myogenic Differentiation in RMS. To characterize the role of specific HDACs in regulating RMS tumor growth, we performed a CRISPR/Cas9-based phenotypic screen of class I and class II HDAC genes using human 381T ERMS cells (Fig. 1A and Fig. S1A). In contrast to single guide RNA (gRNA) CRISPR screens, the lentiviral phenotypic screen used dual gRNAs (DgRNA) targeted to each HDAC gene to increase overall targeting efficiency to 50–80% (Fig. 1B and Table S1). This strategy enabled direct analysis of phenotypic effects of pooled tumor cells without the need for stable selection or isolation of mutant clones.

CRISPR-mediated targeting of HDAC1, 2, 3, 4, and 6 significantly decreased tumor cell growth (Fig. 1C). Knockout of either HDAC3 or HDAC4 also resulted in distinct myogenic differentiation, as shown by the presence of morphologically multinucleated

Significance

Current histone deacetylase (HDAC) inhibitors have shown mixed results in the treatment of many cancer types. Our study has demonstrated significant antitumor phenotypes resulting from targeted disruption of HDAC3 and the NCOR complex with genome engineering technology. Our findings provide compelling evidence that the HDACs and their essential interacting factors remain key cancer therapeutic targets and that the next generation of selective HDAC inhibitors may improve survival of cancer patients.

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Fig. 1. CRISPR-based phenotypic screen of class I and II HDAC genes. (A) Schematic of the CRISPR phenotypic screen. (B, Upper) Depiction of the PCR-based method for detecting genomic DNA deletions between gRNAs as indicated by black arrows. Blue and green arrows represent primer pairs spanning each gRNA target site. (Lower) Evidence of HDAC class I and II gene targeting by PCR amplification of gDNA deletions in HDAC targeted cells. (C) Cell growth change by cell counts in 381T cells with CRISPR targeting of each class I and II HDAC gene, normalized to cells with safe-harbor control targeting 10 d after lentiviral transduction. (D and E) Immunofluorescence (IF) of MF20 in 381T cells with control safe-harbor (D) or HDAC3 CRISPR (E) targeting. (Scale bar: 50 μ m.) (F) Quantification of MF20 IF in targeted deletion of HDAC class I and II genes. (G) Quantification of MF20 IF in ERMS (RD and SMS-CTR) and ARMS (Rh3, Rh5, and Rh30) cell lines with HDAC3 CRISPR targeting. (H and I) IHC for HDAC3 in primary human skeletal muscle (H) and a representative RMS tumor section (I) (IHC magnification: 400 $\times$). (Scale bar: 500 μ m.) (Insets) Arrowheads point to nuclei. (Scale bar: 10 μ m.) (J) Summary of HDAC3 IHC from human RMS tumor samples. Error bars in C, F and G represent mean $\pm$ SD of three biological replicates. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

myotubes highlighted by myosin heavy chain (MF20)-positive immunostaining. However, the effect of *HDAC4* knockout was limited compared with the suppression of tumor cell growth (>90% reduction in growth) and the extent of differentiation (60–80% differentiated) exhibited by *HDAC3* knockout (Fig. 1D–F and Fig. S14). *HDAC3* targeting also induced myogenic differentiation to varying degrees in a panel of five additional RMS cell lines (RD, SMS-CTR, Rh3, Rh5, and Rh30) derived from both ERMS and ARMS subtypes (Fig. 1G and Fig. S18). In addition to single *HDAC* gene knockout, we targeted *HDAC1* and *HDAC2* simultaneously because they are known to have redundant functions (10). Double knockout of *HDAC1* and *HDAC2* resulted in no evidence of myogenic differentiation (Fig. S14), suggesting that *HDAC1* and *2* have a role in regulating ERMS proliferation but not myogenic differentiation pathways.

Immunohistochemistry (IHC) revealed that normal skeletal muscle lacked nuclear HDAC3 expression. By contrast, 22 of 23 primary ERMS samples and 20 of 24 primary ARMS samples

exhibited distinct nuclear expression of HDAC3 (Fig. 1H–J). Proliferating human myoblasts as well as all analyzed RMS cell lines also exhibited nuclear HDAC3 expression, suggesting that HDAC3 likely has important functions in undifferentiated myoblast-like cells (Fig. S1C).

The level of myogenic differentiation observed with *HDAC3* targeting was substantially higher than has been previously reported for treatment of RMS cells with pan-HDAC inhibitors (9). Because pan-HDAC inhibitors are unable to induce large-scale differentiation in RMS, we treated RMS cells with the HDAC3-selective inhibitor RGFP966 (Selleck Chemicals LLC) to determine if direct HDAC3 inhibition can induce the extent of myogenic differentiation observed with *HDAC3* knockout. Surprisingly, the treatment of RMS cells with RGFP966 resulted in only modest growth suppression (Fig. S2A–C) and myogenic differentiation (30–35%) (Fig. S2D–F), suggesting that current HDAC inhibitors lack the potency necessary to suppress growth and induce differentiation of RMS as a single agent.

Conditional *HDAC3* Knockout Arrests Tumor Growth and Induces Myogenic Differentiation of RMS Tumors in Vivo. To investigate the function of HDAC3 in RMS, we developed a tamoxifen-inducible Cas9-ERT2 PiggyBac transposon to control gene targeting temporally both in vitro and in vivo (Fig. 2A). Tamoxifen-induced *HDAC3* gene knockout in ERMS cells (Fig. 2B) validated the results from the CRISPR phenotypic screen by inducing growth arrest and concomitant myogenic differentiation in more than 75% of the treated cells (Fig. 2C and D and Fig. S3A–F). This high-efficiency inducible gene targeting of pooled RMS tumor cells significantly reduced overall HDAC3 protein levels without affecting the levels of other class I HDAC proteins (Fig. 2B and Fig. S3G). *HDAC3*-knockout cells exhibited up-regulated expression of key myogenic regulatory genes (Fig. S3H–K) with no increase in apoptosis (Fig. S3L), suggesting that the decrease in tumor cell growth was predominantly caused by terminal myogenic differentiation.

We validated the *HDAC3* loss-of-function effects in vivo by inducing *HDAC3* targeting in ERMS tumor xenografts. Immunocompromised NOD-SCID Il2rg^{−/−} (NSG) mice were injected on bilateral flanks with tamoxifen-inducible 381T ERMS cells targeting either *HDAC3* or a safe-harbor region of the genome (Chr 4: 58,110,237–58,110,808; GRCH38.p2) (Fig. 2A). After tumor development, the mice were treated with tamoxifen to induce targeted gene knockout in the respective tumors (Fig. 2E). In contrast to safe-harbor control xenografts, *HDAC3*-targeted xenografts showed at least 50% reduction in tumor growth 2 weeks after tamoxifen treatment (Fig. 2F and G). Histological analysis of *HDAC3*-knockout tumors revealed an increase in the presence of multinucleated cells resembling myotubes (Fig. 2H and I). Similarly, *HDAC3*-targeted xenografts demonstrated a significant decrease in the proliferation index (Ki67) (Fig. 2J and K) and an increased number of MF20⁺ differentiated cells (Fig. 2L and M) but no increase in apoptosis (cleaved caspase-3) (Fig. 2N and O).

The Nuclear Receptor Corepressor/HDAC3 Complex Blocks Myogenic Differentiation in RMS. Using mass spectrometry and coimmunoprecipitation (co-IP) assays on 381T ERMS cells, we identified nuclear receptor corepressors 1 (NCOR1) and 2 (NCOR2) as the top HDAC3-interacting factors with transcription factor-binding activity (Fig. S4). Nuclear expression of NCOR1 was also detected in myoblasts and all analyzed RMS cell lines (Fig. S5A–I). Although individual knockout of *NCOR1* and *NCOR2* resulted in limited myogenic differentiation, likely because of functional redundancy, double knockout of *NCOR1* and *NCOR2* in a panel of six RMS cell lines (RD, 381T, SMS-CTR, Rh3, Rh5, and Rh30) resulted in large-scale myogenic differentiation and reduction in cell growth, similar to that seen with *HDAC3* knockout (Fig. 3A–D and Fig. S5J–O). These results suggest that the NCOR/HDAC3 transcriptional repressor complex, not an alternative HDAC3-mediated process, is primarily responsible for restricting myogenic differentiation in RMS.

Fig. 2. Tamoxifen-inducible CRISPR targeting of *HDAC3* in vitro and in vivo. (A) Tamoxifen (Tam)-inducible CRISPR/Cas9 experimental design. (B) T7 endonuclease assay assessing targeting efficiency of each *HDAC3*-specific gRNA (Upper) and Western blot of *HDAC3* protein after tamoxifen induction (Lower). (C and D) Quantification of MF20 IF and cell growth by cell counts (C) and normalized to no TAM control (D) in *HDAC3*-targeted 381T cells with tamoxifen (10 d posttreatment) or without tamoxifen. Error bars the mean $\pm$ SD of three biological replicates. (E) PCR-based detection of targeted genomic deletions in three tumor xenografts per treatment group. (F) 381T xenograft tumors isolated from the safe-harbor control or *HDAC3*-inducible treatment groups. (G) Tumor growth change, normalized to day 0 of tamoxifen treatment. Error bars represent the mean $\pm$ SEM. (H–O) Histology of xenograft tumors by H&E (H and I) and IHC for Ki-67 (J and K), MF20 (L and M), and cleaved caspase-3 (CC3) (N and O). (Magnification for H&E and IHC: 200 $\times$.) (Scale bar: 500 μ m; Inset: 20 μ m.) *** P < 0.001, **** P < 0.0001.

We next performed structure–function analysis to determine whether the NCOR-interaction domain and/or the enzymatic activity of *HDAC3* were required for its function in RMS. Site-directed mutagenesis of *HDAC3* was used to produce two mutant proteins, one lacking two distinct NCOR-binding sites in the N terminus (Δ B12) (11) and the second with a Y298F point mutation in the catalytic site (Fig. 3E) (11). These mutant *HDAC3* overexpression constructs were made CRISPR/Cas9 resistant by introducing silent mutations in the two *HDAC3* gRNA target sites (Fig. 3E). Both *HDAC3* mutants exhibited loss of deacetylase activity (Fig. 3F) when overexpressed in *HDAC3*-knockout ERMS cells, as is consistent with previous studies demonstrating that the NCOR/*HDAC3* complex is required for *HDAC3* deacetylase activity (11, 12). However, only the Δ B12 *HDAC3* mutant failed to interact with the NCOR complex (Fig. 3G). Myogenic differentiation in *HDAC3*-targeted ERMS cells was fully rescued by the overexpression of

CRISPR/Cas9-resistant WT *HDAC3* (Fig. 3H–K and Fig. S5P). In contrast, overexpression of either the Δ B12 or the Y298F *HDAC3* mutant failed to restrict differentiation after *HDAC3* knockout, suggesting that both the NCOR/*HDAC3* complex and *HDAC3* deacetylase activity were critical for suppressing differentiation in RMS (Fig. 3L–O and Fig. S5P). Interestingly, the Y298F *HDAC3* mutant was also able to induce myogenic differentiation independently in ERMS cells, likely through dominant-negative competition with endogenous *HDAC3* (Fig. 3N and O and Fig. S5P).

The NCOR/*HDAC3* Complex Blocks Myoblast Determination Protein 1-Mediated Myogenic Differentiation in ERMS. To identify essential genes and pathways regulated by the NCOR/*HDAC3* complex, RNA sequencing was performed on ERMS cells 2, 4, and 10 d after tamoxifen-induced CRISPR-mediated *HDAC3* targeting. Based on Gene Ontology (GO) analysis, differentially expressed genes immediately after *HDAC3* knockout (day 4; 414 genes, P < 0.05) were enriched for receptor and signal transduction functions, whereas late-stage differentially expressed genes (day 10; 199 genes, P < 0.05) were enriched for cytoskeletal protein binding

Fig. 3. The NCOR complex interacts with *HDAC3* to suppress myogenic differentiation in ERMS. (A and B) MF20 IF images of 381T cells with safe-harbor control CRISPR targeting (A) and *NCOR1* and *NCOR2* double-gene targeting (B). (C) Summary of MF20 IF in 381T cells with *HDAC3*, *NCOR1*, and *NCOR2* CRISPR targeting. (D) Cell growth by cell counts normalized to cells with safe-harbor control targeting. (E) Schematics of CRISPR mutant *HDAC3* (*HDAC3*m) expression constructs. (F) Assay of *HDAC* deacetylase activity in *HDAC3*m mutants at day 4 after *HDAC3* knockout. (G) Western blot/co-IP assays assessing the interaction of *HDAC3*m mutants. (H–O) MF20 IF images and quantification data of *HDAC3* CRISPR-inducible 381T cells transduced with empty-vector control (H and I), WT *HDAC3*m (J and K), or *HDAC3*m mutants (L–O) with or without tamoxifen (Tam) treatment. Error bars in C and D represent the mean $\pm$ SD of three biological replicates. All cell-culture experiments (viral transduction or tamoxifen treatment) were analyzed 10 d posttreatment. (Scale bars in B and H: 50 μ m.) ** P < 0.01, **** P < 0.0001.

and striated muscle structural and contraction functions (Fig. 4 A–C and Table S2). This global gene-expression profile induced by *HDAC3* knockout in ERMS cells highlighted the clear transition from a neoplastic to fully differentiated muscle state.

Previous studies have shown that repression of myoblast termination protein 1 (MYOD1)-driven differentiation pathways results in a characteristic arrest of RMS cells in a myoblast-like proliferative phase of development, regardless of subtype or initiating genetic events (8, 13, 14). However, even though MYOD1 dysfunction was implicated in RMS more than two decades ago (15), the precise mechanism of MYOD1 transcriptional repression remains unclear. To determine whether the NCOR/HDAC3 complex is involved in the repression of MYOD1 target genes in ERMS, we first analyzed the promoter motif of the top 500 differentially expressed genes 4 days after *HDAC3* gene targeting and identified an enrichment for the E-box motif, the binding site for MYOD1, and the interacting factor T-cell factor 3 (TCF3) (also known as “E2A”) [$P < 9.00\text{E-}20$, false discovery rate (FDR) $q < 1.32\text{E-}17$]. By comparing the expression levels of 137 known MYOD1 target genes (14) at 2, 4, and 10 d after *HDAC3* targeting, we observed enrichment for select downstream MYOD1 target genes ($P < 0.0001$) 4 d and 10 d after *HDAC3* knockout (Fig. 4 D and E). Using ChIP studies, we further demonstrated binding of HDAC3 to the E-box-containing regulatory regions of MYOD1 target genes, but this interaction was abrogated upon MYOD1 knockout (Fig. 4F). The same E-box regulatory regions showed increased binding for acetylated H3K9, a histone mark for transcriptionally active promoters, upon *HDAC3* targeting (Fig.

4G). MYOD1 also was shown by co-IP to interact directly with E2A and the NCOR/HDAC3 complex in ERMS cells (Fig. S6).

We next investigated the importance of MYOD1 in regulating myogenesis in ERMS using a dual gene-targeting strategy to disrupt MYOD1 function in *HDAC3*-knockout ERMS cells. 381T ERMS cells were transduced with Cas9- and MYOD1-targeting DgRNA lentivirus to establish MYOD1-knockout cells. Three days later, the MYOD1-targeted cells were transduced with *HDAC3*-targeting DgRNA lentivirus to produce dual MYOD1/HDAC3-knockout cells. MYOD1 targeting completely blocked the ability of ERMS cells to differentiate after *HDAC3* knockout (Fig. 4H). Similar *HDAC3* dual gene targeting of E2A and of the known MYOD1 coactivators CREB-binding protein (CBP) and E1A binding protein p300 (EP300) also limited the ability of *HDAC3*-deficient RMS cells to undergo myogenic differentiation (Fig. 4H). Taken together, these findings show that the NCOR/HDAC3 complex interacts with MYOD1/E2A and associated cofactors to repress transcriptional activation of myogenic genes.

Loss of the Differentiation Block in ERMS Causes Transient Up-Regulation of Cell Growth Pathways.

In addition to the enrichment for MYOD1 target genes, pathway analysis of gene-expression changes in the early stage of *HDAC3* knockout (i.e., day 4) revealed a transient up-regulation of tumor growth-promoting signaling pathways, most of which were down-regulated upon myogenic differentiation by day 10. This cellular proliferation response was mediated by growth factors such as insulin-like growth factor 1 (IGF1), hepatocyte growth factor (HGF), and TGF β , as well as epithelial–mesenchymal transition and metastasis pathways (Fig. S4 and Table S3). We therefore performed a second CRISPR phenotypic screen in 381T ERMS cells to determine the function of 42 candidate genes with signaling or transcriptional activity linked to HDAC3 through expression-profiling or mass spectrometry studies (Fig. S5B). None of the candidate genes was able to prevent myogenic differentiation when targeted with *HDAC3* in a dual gene-targeting phenotypic screen. However, independent disruption of five of the genes [ephrin type-A receptor 5 (EPHA5), myogenic factor 5 (MYF5), forkhead box P2 (FOXP2), nitric oxide synthase 1 (NOS1), and AT-rich interactive domain-containing protein 1 (ARID1A)] significantly decreased tumor cell growth without inducing myogenic differentiation (Fig. S5C). ChIP assays demonstrated enrichment of the acetylated histone mark H3K9ac in the promoters of MYF5, FOXP2, NOS1, and EPHA5 (Fig. S5D), suggesting that up-regulation of these genes was likely caused by altered histone landscapes in the regulatory regions.

Discussion

In this study, we have identified HDAC3 as a major suppressor of myogenic differentiation in RMS from a high-efficiency CRISPR phenotypic screen of class I and class II HDAC genes and further characterized the *HDAC3* loss-of-function phenotype in vitro and in vivo using a tamoxifen-inducible CRISPR gene-targeting strategy. *HDAC3* knockout in RMS cells resulted in significant suppression of tumor growth through the activation of terminal myogenic differentiation. Interestingly, *HDAC4* knockout also increased myogenic differentiation in RMS cells to a limited extent, supporting the current model that class IIa HDACs, including HDAC4, function as scaffolding molecules to recruit HDAC3 to transcriptional target sites (16). Although HDAC1 and HDAC2 have been shown to have redundant functions in muscle development (10), disruption of HDAC1 and HDAC2 did not induce any significant effect on RMS cell differentiation. However, targeting either gene did reduce overall cell proliferation to a limited extent. Our findings indicate that abnormal HDAC3 activity, rather than HDACs 1 and 2, is essential for the repression of terminal myogenic differentiation in RMS cells.

The *HDAC3*-knockout phenotype was recapitulated by double targeting of both *NCOR1* and *NCOR2*, demonstrating that HDAC3 blocks myogenic differentiation in RMS through the NCOR/HDAC3 transcriptional repressor complex. The NCOR complex was originally identified as transcriptional corepressors of nuclear receptors,

Fig. 4. HDAC3 suppresses MYOD1/E2A-mediated myogenic differentiation in ERMS. (A) Heatmap analysis showing expression levels of differentially expressed genes 2, 4, and 10 d after tamoxifen-induced CRISPR *HDAC3* targeting. Expression values are derived from RNA sequencing analysis of two biological replicates. (B and C) GO analysis of top differentially expressed genes ($P < 0.05$) at days 4 (B) and 10 (C). (D) Heatmap showing expression patterns of the MYOD1-target gene set (137 genes) in 381T cells with *HDAC3* targeting at days 2, 4, and 10 after tamoxifen induction. RT-qPCR-validated genes are identified. (E) Expression of MYOD1 target genes (RT-qPCR) at days 4 and 10 normalized to day 2 after *HDAC3* gene targeting. (F and G) ChIP using antibody against HDAC3 in control and MYOD1-targeted cells (F) or antibody against acetylated H3K9 in control and *HDAC3*-targeted cells (G) 4 d after transduction. (H) Summary of MF20 IF in 381T cells with dual CRISPR targeting of MYF5, MYOD1, E2A, CBP, or EP300 before *HDAC3* targeting. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Fig. 5. HDAC3 targeting in ERMS cells results in transient up-regulation of growth-promoting genes. (A) Changes in gene expression of selected top differentially expressed genes at day 4 and 10 normalized to day 2 after tamoxifen-induced HDAC3 targeting. (B) Heatmap summarizing the cell growth from a CRISPR phenotypic screen targeting 42 candidate genes. Yellow: no effect; green: >50% decrease in cell growth. (C) Summary of cell growth by cell counts in RMS cells with targeted disruption of top candidate genes. Cell counts were normalized to control. Gray bars: genes identified through RNA-seq; black bars: control and *ARID1A* identified from mass spectrophotometry. Error bars represent the mean $\pm$ SD of three biological replicates. (D) Summary of ChIP assays showing fold enrichment of the acetyl histone mark (H3K9Ac) in the promoter region of each gene. Error bars in A, C, and D represent the mean $\pm$ SD of three technical replicates. ** $P < 0.01$, **** $P < 0.0001$.

e.g., thyroid hormone receptor and retinoid acid receptor (17). The association of HDAC3 and the NCOR complex has been shown to be essential for the regulation of circadian and metabolic physiology, muscle physiology, and genomic stability (3, 18, 19). HDAC3 binds to the NCOR complex through the deacetylase-activating domain (DAD), which activates HDAC3 deacetylase activity (11, 12). In agreement with these findings, we demonstrated through structure function analysis that the interaction of HDAC3 with the NCOR complex is required for functional deacetylase activity and NCOR/HDAC3-mediated repression of myogenic differentiation in RMS. Of note, the antitumor effects elicited by the treatment of RMS cells with the HDAC3-selective inhibitor RGFP966 were not as robust as the loss-of-function phenotype generated by CRISPR-mediated HDAC3 knockout. Although RGFP966 inhibits histone deacetylase activity, the substrate range and mechanism of action underlying RGFP966 remain unclear. Our findings suggest that screening drug candidates for NCOR/HDAC3 complex specificity rather than for deacetylase activity may improve the therapeutic efficacy of HDAC inhibitors for RMS and other solid tumors with dysregulated HDAC3 activity.

ERMS and ARMS share pathologic features of myogenic differentiation arrest characterized by defects in the MYOD1-mediated transcriptional program (8, 13–15). Previous studies in the ERMS RD cell line have shown that competitive binding of MYOD1 by inhibitory factors such as myosin and other altered E-box transcription factors, as well as repression of transcription by the Polycomb repressive complex 2 (PRC2), can contribute to blocking the differentiation of tumor cells (20, 21). However, the common mechanism blocking the differentiation in RMS remains unknown. Here we demonstrate that both ERMS and ARMS subtypes undergo extensive myogenic differentiation from CRISPR targeting of either *HDAC3* or combined *NCOR1* and *NCOR2* knockout. Nuclear HDAC3 is present in proliferating myoblasts as well as in RMS cells but is down-regulated in differentiated skeletal muscle, supporting a role for HDAC3 in undifferentiated myogenic cells. Furthermore, the NCOR/HDAC3 complex interacts with MYOD1 and associated cofactors to repress the transcriptional activation of myogenic genes. Although the initiating genetic events in ERMS and ARMS are unique, our findings suggest that the driving mechanisms of both RMS subtypes converge on the NCOR/HDAC3 complex as one of the common mechanisms for repressing MYOD1-mediated myogenic differentiation, in turn promoting uncontrolled proliferation of myoblast-like cells (Fig. 6). Although our data suggest that the NCOR/HDAC3 complex is critical to suppressing myogenic differentiation in RMS, the molecular pathways or genetic alterations leading to dysregulation of NCOR/HDAC3 activity in RMS remain to be elucidated.

The goal of differentiation therapy in cancer is to reactivate the endogenous differentiation program with concomitant loss of tumor cell phenotypes (22). We have demonstrated by phenotypic characterization and expression-profiling studies that targeted disruption of the NCOR/HDAC3 complex by CRISPR is sufficient to switch RMS cells from a neoplastic state to a terminally differentiated muscle state, indicating the feasibility and potential efficacy of differentiation therapy in RMS. Surprisingly, targeting *HDAC3* in ERMS cells also resulted in a transient but robust up-regulation of oncogenic genes and pathways including IGF, HGF, and MAPK signaling pathways before the completion of terminal differentiation (Fig. 6). In our CRISPR screen of 42 additional candidate genes linked to HDAC3 function, we have identified *FOXP2*, *EPHA5*, *NOS1*, or *ARID1A* as essential genes for RMS tumor growth. Recurrent mutations in *FOXP2* and *ARID1A* and a differential methylation pattern of *NOS1* have been shown in RMS (8, 23). These studies along with our loss-of-function studies indicate the important roles of these genes in RMS pathogenesis.

Fig. 6. (Upper) Proposed mechanism of NCOR/HDAC3-mediated transcriptional repression of myogenic differentiation in RMS. The NCOR/HDAC3 complex represses MYOD1/E2A-mediated transcription in RMS to block myogenic differentiation. Upon HDAC3 targeting, the MYOD1/E2A transcriptional-activating complex including CBP and P300 can initiate the myogenic program. (Lower) A transient up-regulation of tumor-promoting genes occurs before RMS tumor cells are completely switched to a terminal differentiation state.

Based on the hyperacetylated status of histone H3 in the promoter regions of *MYF5*, *FOXP2*, *EPHA5*, and *NOS1*, a subset of genes with oncogenic function likely undergoes transient activation upon HDAC3 loss of function. The transient up-regulation of tumor-promoting genes could represent either direct de-repression of the transcription block by the HDAC3 repressor complex or a secondary adaptive response as tumor cells transition from a neoplastic state to a terminally differentiated state. The implication of our findings for differentiation therapy is that incomplete induction of differentiation may not be sufficient to counteract continued growth of tumor cells because of the transient up-regulation of oncogenic genes and pathways (Fig. 6).

Our study demonstrated that genome-engineering technology could be used to induce large-scale differentiation of cancer cells where direct chemical inhibitors have proven ineffective. Knockout of the NCOR/HDAC3 complex revealed a significant vulnerability of RMS cells to targeted disruption of factors blocking differentiation. This effect was observed in both RMS subtypes (ARMS and ERMS), suggesting that there are common pathways restricting differentiation of tumor cells with similar tissue lineage regardless of initiating oncogenic events. Because pan-HDAC inhibitors have shown disappointing results in clinical trials of solid tumors, the development of new targeted therapies with increased selectivity to specific HDACs or interacting repressor complexes may improve treatment of certain cancer types in which current pan-HDAC inhibitors have previously failed.

Methods

Animal studies were approved by the University of Washington Subcommittee on Research Animal Care under protocol no. 4330-01_SC_v19. Archived paraffin tissue blocks for human RMS tumor samples were obtained under an approved human Internal Review Board protocol 14988 at Seattle Children's Hospital/University of Washington. Additional RMS tissue microarrays were obtained from US Biomax, Inc.

CRISPR/Cas9 Gene Targeting. Rapid single and multiplex gene knockout was accomplished by treating RMS cells with multiple lentiviral particles containing separate DgRNA-targeting and Cas9-expressing viruses. Lentiviral-transduced RMS cells were split into specific experiments 3 d postinfection and were analyzed 7 d later without antibiotic selection or isolation of clonal cells. Each DgRNA-targeting viral vector contained dual gRNAs targeting conserved exons of each gene. All DgRNAs were cloned into the CRISPR plasmids using a one-step Gibson reaction.

CRISPR/Cas9-inducible cells were created using PiggyBac transposition. Stable cell lines were integrated with an all-in-one construct containing DgRNAs and expressing an ERT2-Cas9-ERT2 fusion protein for tamoxifen-inducible gene targeting. Inducible gene targeting in vitro was accomplished

by treating ERMS cells for 3 d with 2 μ M 4-hydroxytamoxifen. For structure-function studies, HDAC3-inducible cells were transduced with constructs overexpressing either WT or mutant CRISPR/Cas9-resistant HDAC3 linked to a T2A-GFP cassette. Cells were sorted for GFP fluorescence, and then endogenous HDAC3 was targeted with tamoxifen treatment.

In vivo CRISPR-inducible tumor xenografts were created by injecting NSG immunocompromised mice with tamoxifen-inducible cells targeting either a safe-harbor region of the genome or HDAC3. After tumor development, the mice were treated with five injections of 100 mg/kg tamoxifen to induce in vivo gene targeting. Targeting efficiency was validated from both in vitro and in vivo experiments using either a T7 endonuclease assay to examine targeting efficiency at individual gRNA target sites or PCR to look for genomic deletions between both gRNAs.

Cellular Assays. RMS cell growth was quantified by direct cell counting. Analysis of RMS differentiation was determined with IHC by fixing treated cells with 2% paraformaldehyde followed by MF20 immunostaining. Stained cultures were imaged and quantified for the percent of MF20⁺ cells. An annexin V flow cytometry-based assay was used to analyze apoptosis. To assess HDAC3 deacetylase activity, a nuclear extract kit (Active Motif, catalog no. 40010) was used to obtain nuclear extracts from HDAC3-knockout 381T cells overexpressing the CRISPR-resistant HDAC3 mutants at 4 d post targeting. HDAC3 (WT or mutant) was immunoprecipitated from nuclear extracts, and HDAC deacetylase activity was quantified using a fluorescent HDAC activity assay kit (Active Motif, catalog no. 56200), which uses a short peptide substrate that contains an acetylated lysine residue that can be deacetylated by class I, IIb, and IV HDAC enzymes.

Co-IP. Co-IP of HDAC3 or NCOR1 was performed using the nuclear complex co-IP kit (Active Motif, catalog no. 54001). Two hundred micrograms of extract and 2 μ g of antibody were used. Dynabead protein G (Life Technologies, catalog no. 10003D) was used to capture the protein/antibody complex.

All quantitative RT-PCR (RT-qPCR) primer pairs and ChIP primer pairs are listed in Table S4.

Detailed experimental procedures are described in SI Methods.

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