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TBX2 blocks myogenesis and promotes proliferation in rhabdomyosarcoma cells

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Abstract

Rhabdomyosarcomas (RMS) are the most frequent soft tissue sarcomas in children that share many features of developing skeletal muscle. We have discovered that a T-box family member, TBX2, is highly up regulated in tumor cells of both major RMS subtypes. TBX2 is a repressor that is often over expressed in cancer cells and is thought to function in bypassing cell growth control, including repression of p14 and p21. The cell cycle regulator p21 is required for the terminal differentiation of skeletal muscle cells and is silenced in RMS cells. We have found that TBX2 interacts with the myogenic regulatory factors MyoD and myogenin and inhibits the activity of these factors. TBX2 is expressed in primary myoblasts and C2C12 cells, but is strongly down regulated upon differentiation. TBX2 recruits the histone deacetylase HDAC1 and is a potent inhibitor of the expression of muscle specific genes and the cell cycle regulators, p21 and p14. TBX2 promotes the proliferation of RMS cells and either depletions of TBX2 or dominant negative TBX2 up regulate p21 and muscle specific genes. Significantly, depletion or interference with TBX2 completely inhibits tumor growth in a xenograft assay, highlighting the oncogenic role of TBX2 in RMS cells. Thus, the data demonstrate that elevated expression of TBX2 contributes to the pathology of RMS cells by promoting proliferation and repressing differentiation specific gene expression. These results show that deregulated TBX2 serves as an oncogene in RMS, suggesting that TBX2 may serve as a new diagnostic marker or therapeutic target for RMS tumors.

Keywords

TBX2; p21; myogenin; MyoD; RMS

Introduction

Rhabdomyosarcoma (RMS) is a highly malignant cancer that is the most common form of soft tissue tumors in children. It is thought to arise as a consequence of myogenic precursors

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failing to differentiate into normal muscle[1]. The more common form of the disease is the embryonal subtype (ERMS), characterized by loss of heterozygosity at the 11p15 locus, a region which harbors insulin-like growth factor 2 (IGF2). Alveolar RMS (ARMS) is the more aggressive form of RMS which is characterized by t(2;13)(q35;q14) or t(1;13)(q36;q14) translocations. The translocations result in chimeric transcripts that fuse the 5′ portion of the paired box proteins 3 or 7 (PAX3 or PAX7), including an intact DNA binding domain, to the transactivation domain of a forkhead transcription factor (FKHR), creating novel PAX3-FKHR (t(2;13)(q35;q14)) or PAX7-FKHR (t(1;13)(q36;q14)) fusion proteins[2, 3].

During embryogenesis, skeletal muscle arises from the paraxial mesoderm where muscle progenitor cells express both PAX3 and PAX7. In normal myogenesis, myoblasts, which are single nuclei cells committed to the skeletal muscle fate, fuse to form multinucleated myofibers. During this process, the PAX genes are down regulated concomitant with an downregulation of the myogenic regulatory factors (MRFs), a group of four highly related bHLH transcription factors composed of Myf5, MyoD, Myf6, and myogenin, which are required for myogenesis[4]. Myf5 and MyoD function early in the commitment steps of myogenesis[5], Myf6 acts both early in myogenesis and later in myofiber formation[6] and myogenin is involved in the later stages of differentiation by promoting efficient myoblast fusion and the differentiation of mature skeletal muscle fibers[7, 8].

MyoD and myogenin are used as diagnostic markers for RMS as these myogenic regulatory factors are expressed in almost every RMS tumor including both major histological subtypes[9]. Several cell lines have been derived from RMS tumors and the cell lines exhibit many of the characteristics of RMS tumors. These lines include RD (ERMS), RD2 (ERMS), RH28 (ARMS) and RH30 (ARMS) cell lines. The RMS cell lines express Myf5, MyoD and myogenin, but the proteins appear non-functional[10]. When MRF responsive reporters are transfected into RD cells, little activity is detected[11, 12]. Ectopic expression of the MRFs does not rescue the block to differentiation[10].

TBX2 is a member of the T-box gene family of transcription factors which play a critical role in embryonic development. The T-box gene family contains the well known developmental regulator *brachyury* along with 18 different T-box genes with diverse regulatory functions in development and disease[13]. TBX2 and TBX3 have been shown to function as transcriptional repressors[14, 15]. Abnormal expression of TBX2 has been reported in several cancers including breast, pancreas and melanoma[16]. This evidence strongly suggests that TBX2 plays a role in tumorigenesis. TBX2 induces a downregulation of p14 ^{ARF}(human) or p19^{ARF}(murine) [17] and functions as a direct repressor of the cell cycle regulator cyclin-dependent kinase (Cdk) inhibitor p21$^{CIP1/WAF1}$ (*CDKN1A*), hence referred to as p21[18]. The suppression of both p21 and p14/19^{ARF} by TBX2 may provide a powerful and cooperative anti senescence signal to cancer cells. Expression of a dominant negative TBX2 up regulates p21 and induces replicative senescence[19]. TBX2 mediates repression at least in part by binding the histone deacetylase HDAC1 which serves to target HDAC1 to promoters[19]. TBX2 has also been shown to drive proliferation in both breast cancer[20] and melanoma cells[21].

The cell cycle regulator p21 is induced early in myoblast differentiation and functions to block cell cycle progression[22, 23]. p21 is regulated by MyoD in normal muscle cells[24] and the inactivation of this factor in RMS cells contributes to the silencing of p21 in RMS cells[12]. Induction of p21 in RMS cells is correlated with growth arrest in RMS cells[25–27] and the differentiation of RD cells[25].

We discovered TBX2 as an interaction factor with myogenin in RD cells (ERMS) and confirmed this interaction in RH30 cells (ARMS) as well. We have found that TBX2 is highly up regulated in both ERMS and ARMS subtypes of RMS and demonstrate that TBX2 is a repressor of myogenesis by binding to MyoD and myogenin and inhibiting their activity. We also show that TBX2 is a repressor of p21 and p14 in RMS cells. TBX2 recruits the histone deacetylase, HDAC1, to muscle specific promoters which leads to the deacetylation of histones at these promoters. Over expression of TBX2 in normal myoblasts strongly inhibits differentiation while depletion or interference of TBX2 in RMS cells decreases cell proliferation, inhibits clonal growth *in vitro* and prevents tumor growth *in vivo*.

Materials and Methods

Cell Culture

RD and SJRH30 (RH30) cells (ATCC) were grown in growth medium (Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum ((Hyclone)), according to standard protocols. RD2 and RH28 were obtained from Denis Guttridge, Ohio State University, and grown as described above. Mouse primary cell cultures were established from murine RMS tumors which were minced and digested in collagenase (10mg/ml) overnight at 4°C. U33915 and U37125 represent ERMS type tumors derived from the *Pax7CreER, Ptch1, p53* conditional model of spindle ERMS/UPS [28] and U48484 was derived from the *Myf6Cre, Pax3:Fkhr, p53* conditional mouse model of ARMS [29]. JW41 cells were isolated from an ERMS tumor from a $p53^{-/-}/c\text{-}fos^{-/-}$ mouse[30]. Proliferating C2C12 myoblasts (ATCC), HEK293 cells (ATCC) and 10T1/2 cells were grown in growth medium. To induce the differentiation of C2C12 cells, cells were grown to 100% confluence and the media switched to differentiation medium (DMEM supplemented with 2% horse serum ((Hyclone)). C2C12 cells were grown in differentiation medium for the number of days indicated.

Cloning

RNA from C2C12 cells was extracted with Trizol (Invitrogen) and used to generate cDNA through the use of the Superscript III First Strand Synthesis System for RT-PCR kit with oligo (dT) primers (Invitrogen). The resulting PCR product, using primers listed in Supplemental Table 1, was cloned using the pEF6/V5-His Topo TA Expression Kit (Invitrogen). The resulting mTBX2-V5 clone was confirmed by sequencing.

Co-immunoprecipitations

HEK293 cells were transiently transfected with plasmids expressing myogenin, MyoD and TBX2. EMSV-myogenin (provided by Diane Edmondson, The University of Texas Medical School at Houston), pEMCIIS (provided by Andrew Lassar, Harvard Medical School) and

mTBX2-V5 were used for expressing myogenin, MyoD and TBX2, respectively. Following the transfection, whole cell extracts were made in radio immunoprecipitation buffer (RIPA) as well as whole cell extracts for the endogenous co-immunoprecipitations. For all, 300 μg of extract was used for each immunoprecipitation with 2 μg of antibody. The antibodies used are listed in Supplemental Table 2. Following an overnight incubation, antibody:antigen complexes were collected with protein A agarose beads (Invitrogen). The beads were washed with RIPA buffer and resuspended in protein loading dye. Immunoprecipitated samples with appropriate controls were loaded onto SDS PAGE gels and transferred to PVDF membranes for western blot analysis. For each immunoprecipitation, the blot was probed with both the antibody against the reciprocal factor to test for the co-immunoprecipitation and with the antibody used for the immunoprecipitation to confirm that the IP was successful. All immunoprecipitations were performed at least twice to confirm the results.

Luciferase assays

10T1/2 cells were transfected with calcium phosphate according to standard protocols. The plasmids EMSV-myogenin, pEMCIIs and mTBX2-V5 were used for expressing myogenin, MyoD and TBX2, respectively. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). 10T1/2 cells were seeded at a density of 5×10^3 cell per well in 96 well plates and transfected with 0.4 ug of DNA. Transfections were normalized to Renilla luciferase. Transfections were performed in triplicate and all data sets were repeated at least twice.

Quantitative real time reverse transcriptase PCR (qRT-PCR)

RNA was isolated from cells by Trizol extractions (Invitrogen). Following treatment with DNase (Promega), 2 μg of total RNA was reversed transcribed with MultiScribe™ MuLV reverse transcriptase (Applied Biosystems). cDNA equivalent to 40 ng was used for quantitative polymerase chain reaction amplification (Applied Biosystems) with SYBR green PCR master mix (Applied Biosystems). Samples in which no reverse transcriptase was added (no RT) were included for each RNA sample. The relative levels of expression of genes were normalized according to those of *HPRT*. qRT-PCR data were calculated using the comparative Ct method (Applied Biosystems). Standard deviations from the mean of the [Δ] Ct values were calculated from three independent RNA samples. Primers used are listed in Supplemental Table 1. Where possible, intron spanning primers were used. All quantitative RT-PCR was performed in triplicate and three independent RNA samples were assayed for each time point. qRT-PCR gene expression data are shown using two formats. For measurements of relative gene expression (fold change), a fold change was calculated for each sample pair and then normalized to the fold change observed at *HPRT*. Samples were also compared against 18S to confirm the normalization.

Western Blot Analysis

Cell extracts were made by lysing PBS washed cell pellets in radio-immunoprecipitation assay buffer (RIPA) supplemented with protease inhibitors (Complete, Roche Diagnostics) and clear lysates were obtained by centrifugation. Protein concentrations were determined

by Bradford's assay (Bio-Rad) and 100 μg of protein was loaded on SDS-PAGE gels. Proteins were transferred onto a PVDF membrane using a tank blotter (Bio-Rad), membranes were blocked with 5% milk in 1X Tris buffered saline plus tween 20 (TBST) and then incubated with primary antibody overnight at 4°C. Membranes were then washed with 1X TBST and incubated with the corresponding secondary antibody. Membranes were again washed with 1X TBST, incubated with chemiluminescent substrate according to manufacturer's protocol (SuperSignal, Pierce) and visualized by autoradiography. The antibodies used are listed in Supplemental Table 2.

Chromatin immunoprecipitation assays

ChIP assays were performed and quantified as described previously[31] with the following modifications: 1×10^7 cells were used for each immunoprecipitation and protein A agarose beads (Invitrogen) were used to immunoprecipitate the antibody:antigen complexes. The antibodies used are listed in Supplemental Table 2. Primers used are listed in Supplemental Table 1. All data were normalized to gene desert regions of the Chr19 or IgH loci. The real time PCR was performed in triplicate. Values of [Δ][Δ] Ct were calculated using the following formula based on the comparative Ct method: Ct, template (antibody) − Ct, template (IgG) = [Δ] Ct. Fold enrichments ([Δ][Δ]Ct) were determined using the formula: $2^{-[\Delta]\,\mathrm{Ct.}}\,(\mathrm{experimental})/2^{-[\Delta]\,\mathrm{Ct}}$ (reference, Chr 19 or IgH). Standard error from the mean was calculated from replicate [Δ][Δ] Ct values from independent experiments. All ChIP assays shown represent at least three individual experiments.

HDAC inhibitors

Toxicity curves were generated for each drug before examining the effects on gene expression. Based on these initial studies, cells were grown to 100% confluence, switched to differentiation media and incubated with 100 nM trichostatin A (TSA, Sigma) or 5μM suberoylanilide hydroxamic acid (SAHA, SCBT) for 24 hours. All assays were performed at least twice to confirm results.

Immunohistochemistry

Cells were grown to confluency and differentiated for 4 days on cover slips prior to fixation with 4% paraformaldehyde. Cells were then incubated with goat serum and 1.0 % NP-40 for one hour and washed with PBS. Primary antibodies against myosin heavy chain were incubated for 2 hours at room temperature, washed with PBS and detected by Alexa Fluor-488 goat anti-mouse antibody (1:500, Invitrogen). Cell nuclei were stained by incubating with DAPI (1 uM, Invitrogen).

Proliferation Assay

To assay for proliferation by cell number, cells were seeded in a six well plate at 4×10^4 per well and harvested every two days for cell counts with a hemocytometer. Viability was determined by trypan blue staining. Counts were performed in duplicate and experiments repeated three times. To assay for proliferation by quantitation of newly synthesized DNA, a Click-iT EdU Cell Proliferation Assay kit (Life Technologies) was used according to manufacturer's instructions.

Cell motility Assays

For the scratch wound assays, cells were grown to 100% confluency and the cell monolayer scraped in a straight line with a p200 pipet tip. The debris was removed by washing the cells once with 1 ml of growth medium. Markings were created near the scratch to obtain the same field during the image acquisition. The dish was then placed in a CO_2 incubator at 37°C for 0–18 hours.

Soft agar assays were carried out in 60 mm dishes in which 2 ml of 0.7% Noble agar (USB) in growth medium was overlaid with 2 ml of 0.35% agar in growth medium containing the cells. Cells of each clone (5×10^4) were plated in triplicate. 1 ml of culture medium was added to the top of each plate every 5 days and cells were grown at 37°C for 20 days. Colonies were counted in five random fields using a dissecting microscope.

For the migration assay, cells were resuspended in 100 μl serum free DMEM medium and plated in triplicate into uncoated 8-mm transwell filter inserts (Corning) of 24-well plates covered with 200μl serum free DMEM medium. The bottom wells contained 750 μl of growth medium. After incubation of cells at 37°C for 9 hours, cells on the upper surface of the filter were removed with a cotton swab and cells on the bottom side were fixed in 100% methanol and stained with 0.5% crystal violet. Cells were counted in five random fields on microscopic images taken at 200× magnification.

Xenograft

For *in vivo* tumor formation, cells were harvested by trypsin treatment, washed with PBS and suspended in PBS. 2×10^6 cells were subcutaneously injected into the hind flanks of 10 week old female athymic nude mice (*Foxn1*^{nu}/*Foxn1*^{nu}, Jackson Laboratory). Six animals were used in each experimental group. Mice were monitored every other day and tumor dimensions were measured with electronic calipers. Tumor size was estimated by using the modified ellipsoid formula 1/2(length × width^2). All animal experiments were conducted according to procedures approved by the Institutional Animal Care and Use Committee at Southern Illinois University.

Statistics

Statistical comparisons were performed using unpaired two-tailed Student's *t* tests, with a probability value of <0.05 taken to indicate significance.

Results

TBX2 binds to myogenin and MyoD

To identify potential repressors of myogenesis, we identified protein interaction partners of myogenin in RD cells by an affinity purification mass spectrometry approach. Stable cell lines expressing N-TAP myogenin were selected, amplified, harvested for the PrA-based purification and co-enriching proteins were identified as we previously reported [32]. At a 0.1% false discovery rate, 66 proteins were found to co-enrich with N-TAP myogenin, which included the putative interacting protein TBX2. The interaction between myogenin and TBX2 was confirmed by a co-immunoprecipitation assay. HEK293 cells were

transfected with expression constructs for myogenin and TBX2 followed by immunoprecipitation for myogenin. The western blot was probed with antibodies against both TBX2 and myogenin to confirm the interaction and immunoprecipitation, respectively (Figure 1A). To determine if the interaction was specific to myogenin, we repeated the experiment with expression constructs for MyoD. We found that MyoD also interacts with TBX2, suggesting that the interaction is common to MyoD and myogenin (Figure 1B). To confirm the interaction in RMS cells, we also repeated the experiments with endogenous proteins in both RD and RH30 cells. We found that antibodies against myogenin (Figure 1C) or MyoD (Figure 1D) immunoprecipitated TBX2. The interaction was reciprocal as myogenin and MyoD could also be detected in immunoprecipitations for TBX2 in RH30 cells (Figure 1E).

TBX2 inhibits the activity of MyoD and myogenin

To determine how the interaction with TBX2 affects the activity of myogenin and MyoD, we first tested the effect of TBX2 on a muscle specific luciferase construct. The construct chosen contained a minimal promoter element of murine leiomodin 2 (Lmod2), a gene we have previously characterized as highly dependent on myogenin in vivo[33]. Consistent with previous data[3433], transfection of myogenin or MyoD activates this construct in 10T1/2 cells, a fibroblast cell line considered ‘poised’ to enter the myogenic fate. We found that co-transfection with TBX2 acted as a potent inhibitor of activation by myogenin and MyoD (Figure 1F). To confirm that the inhibition mediated by TBX2 was specific to the muscle specific reporter, we also tested the effect of TBX2 on the pGL3 (+) vector, which drives luciferase with the constitutive CMV promoter. We found that the transfection of TBX2 had no significant effect on the pGL3 (+) vector (Figure 1G). Thus, the effect observed was specific to the MRF driven activation of the muscle specific reporter. We then assayed for the effects of TBX2 on endogenous muscle specific genes using the 10T1/2 cell line, a fibroblast cell line which can be induced to express muscle specific genes upon transfection with the MRFs[34]. 10T1/2 cells were transfected with myogenin or MyoD in combination with TBX2 and gene expression changes were determined for troponin 1, type 2 (*Tnni2*), myosin light chain, phosphorylatable, fast skeletal muscle (*Mylpf*), actin (*Acta1*) and the p21 gene (*Cdkn1A*). Each of these myogenin dependent gene products are integral to muscle contraction. To confirm the transfections, gene expression of each of the transfected constructs was assayed by quantitative real time PCR (data not shown). We found that TBX2 acts as a potent inhibitor of endogenous MRF driven gene activation, including p21 (Figure 1H).

TBX2 is over expressed in both subtypes of RMS

Following our identification of TBX2 as a repressor of myogenin and MyoD, we next assayed for mRNA and protein expression of TBX2 in normal myoblasts and RMS cell lines representing both major tumor subtypes. Normal myoblasts are represented by C2C12 cells, an immortal murine cell line used as a model of myogenesis that is commonly used as a comparative cell line for RMS cells. We found that TBX2 transcript was robustly over expressed in RD and RH30 cells when compared to normal myoblasts (Figure 2A). The protein expression of TBX2 was detected with an anti-TBX2 antibody that recognizes both murine and human proteins. We found that TBX2 protein was over expressed in RD, RH30,

RD2, RH28 as well as in primary cells derived from mouse tumor models of RMS that represent both major tumor subtypes (Figure 2B). We also assayed for TBX2 expression in primary myoblasts isolated from neonatal mice and found that TBX2 was more highly expressed in these cells as compared to C2C12 cells (Figure 2C). TBX2 was weakly expressed in proliferating C2C12 cells and the expression was down regulated upon differentiation (Figure 2D).

TBX2 inhibits myogenesis

To understand the functional consequence of TBX2 overexpression in RMS cells, we first assayed for the function of TBX2 in normal muscle. To address this question, TBX2 was exogenously expressed in C2C12 cells (Figure 3A). Cells were differentiated and effects on muscle specific genes were assayed. We found that TBX2 repressed muscle specific genes dependent on MyoD and myogenin (Figure 3B). Results for *ACTA1* and *TNNI2* are shown. To assay for an inhibition of differentiation, cells were immunostained with antibodies against myosin heavy chain. Cells expressing exogenous TBX2 showed a reduced number of myosin heavy chain positive cells ($MyHC^+$) and less robust myofiber formation as quantitated by the number of nuclei present in individual $MyHC^+$ fibers (Figure 3C). We also assayed for p21, which is regulated by MyoD in muscle cells[12], and found that p21 was down regulated in cells expressing exogenous TBX2 (Figure 3D).

TBX2 inhibits differentiation in RMS cells

As the data from normal myoblasts suggested that TBX2 was a repressor of myogenesis, we next asked if TBX2 acted as an inhibitor of differentiation in RMS cells. First, TBX2 was depleted with three individual shRNA constructs in RH30 cells. We confirmed the depletion of TBX2 at the protein level (Figure 4A) and found that p21 was up regulated when TBX2 was depleted (Figure 4A). Moreover, the amount of TBX2 depletion generated by each construct corresponded to the amount of p21 upregulation (Figure 4A). We also assayed for the gene expression level of p21 and p14 and found that both p21 and p14 were up regulated when TBX2 was depleted (Figure 4B). To confirm our results, we also used a dominant negative construct of TBX2 (dnTBX2) which lacks the C-terminal domain of TBX2 necessary for transcriptional repression and interaction with HDAC1, but retains amino acids 1-301 of the T-box DNA binding activity of TBX2, thus blocking the function of endogenous TBX2[19]. We found that stable expression of CMV HA TBX2 1-301 (C. Goding, unpublished) in RH30 cells (Figure 4C) also led to an upregulation of p21 (Figure 4D and E) and p14 (Figure 4E). Finally, we assayed for expression of muscle specific genes in the presence of TBX2 depletion or dominant negative TBX2. We found that cells depleted for TBX2 showed an upregulation of muscle specific genes (Figure 4F) that was also observed in cells expressing dnTBX2 (Figure 4G). To determine if TBX2 regulated p21 in ERMS cells as well, we transiently transfected shRNA constructs against TBX2 and the dnTBX2 construct in RD cells. We found that either depletion or interference with TBX2 up regulated p21 in RD cells (Figure 4H).

TBX2 inhibits differentiation by recruiting HDAC1 to promoters

To investigate the mechanism by which TBX2 repressed gene expression when recruited by myogenin or MyoD, we performed chromatin immunoprecipitation (ChIP) assays for TBX2 and a class I histone deacetylase, HDAC1, to muscle specific genes and *CDKN1A* as TBX2 has been shown to recruit HDAC1 to the *CDKN1A* gene in melanoma cells[19]. ChIP assays were also used to assay for the acetylation status of the histones by examining the acetylation of lysine 9 and 18 on histone H3 (H3K9, 18^{ac}). We found that in C2C12 cells expressing exogenous TBX2, TBX2 and HDAC1 were bound to *Cdkn1A* and *Tnni2* promoters (Figure 5A). In C2C12 cells expressing vector, this binding was not observed (Figure 5A). The binding of TBX2 and HDAC1 was concomitant with a reduction in acetylation at the promoter (Figure 5A).

To determine if the recruitment of TBX2 to muscle specific genes was dependent on the MRFs, we depleted myogenin from C2C12 cells using shRNA constructs. Following selection of stable cell lines, the depletion was confirmed at the protein level (Figure 5B). ChIP experiments with antibodies against TBX2 were performed on C2C12 cell lines transfected with the scrambled control (scr) or shRNA against myogenin. We found that myogenin was required for the binding of TBX2 to the promoters of *Cdkn1a*, *Tnni2* and *Lmod2* (Figure 5C), suggesting that the interaction with the MRFs serves to stabilize TBX2 binding to the DNA.

To determine if TBX2 and HDAC1 bound to muscle specific promoters in RMS cells, the ChIP assays for TBX2, HDAC1 and H3K9, 18^{ac} were repeated in RD and RH30 cells which express high amounts of endogenous TBX2. We found that both TBX2 and HDAC1 bound to the *CDKN1A*, *TNNI2* and *LMOD2* promoters in both RD (Figure 5D) and RH30 (Figure 5E) cells and that low levels of histone H3 acetylation were observed at these promoters in the presence of TBX2 (Figure 5D and E). To confirm that the recruitment of HDAC1 was dependent on TBX2, we repeated the ChIP experiment for HDAC1 on RH30 cells depleted for TBX2 and found that the recruitment of HDAC1 was dependent on the presence of TBX2 (Figure 5F). To determine if HDACs were required for the repression mediated by TBX2, C2C12 cells expressing TBX2 or a vector control were treated with the class I and class II HDAC inhibitors TSA and SAHA while differentiating. We found that addition of either TSA or SAHA blocked the repression of muscle specific genes and p21 by TBX2 (Figure 5G).

TBX2 promotes proliferation in ARMS cells

As TBX2 has been shown to promote proliferation in other cancer cell types[20, 21], we tested for the effect of TBX2 on proliferation in ARMS cells. We assessed RH30 expressing dnTBX2 for proliferation and viability as assayed by cell counts for both total cells and non-viable cells. We found that interference with TBX2 significantly reduced cell proliferation and increased cell death (Figure 6A). RH30 cells depleted for TBX2 by shRNA constructs were also assayed for proliferation and we found that cell proliferation was again reduced in the presence of shTBX2 (data not shown). To confirm our results, we also quantitated newly synthesized DNA by measuring EdU incorporation and found that expression of dnTBX2 inhibited the synthesis of DNA (Figure 6B).

TBX2 promotes migration in ARMS cells

We were also interested in the effects of TBX2 on cell motility and migration in RMS cells. In melanoma cells, the depletion of TBX2 had no effect on cell motility in a scratch wound assay[21], but in breast cancer cells, TBX2 did promote cell mobility and migration[35]. Thus, we tested the mobility and migration of RH30 cells expressing dnTBX2. We found that these cells had reduced mobility as assayed by a scratch wound assay (Figure 6C). We also found that RH30 cells expressing dnTBX2 were significantly reduced in migration as measured by a transwell assay (Figure 6D). Thus, blocking the activity of TBX2 in ARMS cells inhibits both the motility and migration of these cells.

Interference with TBX2 inhibits tumor growth

Finally, TBX2 was assayed for effects on cell growth *in vitro* and *in vivo*. RH30 cells depleted for TBX2 were tested by a soft agar assay and we found that the depletion of TBX2 suppressed anchorage-independent growth (Figure 6E). RH30 cells expressing dnTBX2 were similarly assayed and we found that dnTBX2 also inhibited anchorage independent growth (Figure 6F). Finally, we tested whether disruption of TBX2 in RH30 cells could suppress tumor growth *in vivo*. To assay for the effects of the depletion of TBX2, 2×10^6 cells from RH30 cells with scrambled control or RH30 cells expressing shRNA against TBX2 were injected into the hind limbs of nude mice and the tumor size measured every two days. RH30 cells with scrambled control formed visible tumors within the first 2 weeks. In contrast, depletion of TBX2 led to a complete block of tumor growth (Figure 6G). Mice were sacrificed at 4 weeks and tumors resulting from the RH30 cells with scrambled control were dissected, measured and weighed. The overall tumor sizes from the individual animals were comparable (data not shown). To assay for the effects of dominant negative TBX2, we repeated the experiment described above with RH30 cells with vector control and RH30 cells expressing dnTBX2. Expression of dnTBX2 also completely suppressed tumor growth (Figure 6H). Again, the overall tumor sizes from the vector control were comparable (data not shown). Thus, our data show that TBX2 is an essential component of tumorigenesis *in vivo*.

Discussion

Understanding the factors that control the cell cycle in cancer cells is of paramount importance in understanding tumor progression. Here, we have shown that TBX2 is highly expressed in both major subtypes of rhabdomyosarcoma where it functions to promote proliferation and repress myogenesis by repressing p21 and muscle specific genes through an interaction with myogenin and MyoD. Our data indicate that TBX2 also functions in the early stages of normal myogenesis to inhibit premature differentiation and promote proliferation. The highest expression of TBX2 in normal myogenic cells was observed in primary myoblasts suggesting that indeed, TBX2 is part of the normal myogenic program. TBX2 expression is down regulated upon differentiation in C2C12 cells, consistent with the induction of differentiation. Intriguingly, the other TBX family member that has been shown to function as a repressor, TBX3, has also been shown to inhibit myogenesis when exogenously expressed in C2C12 cells[36].

Our data suggest that maintenance or upregulation of TBX2 may be one of the seminal events in the progression of an RMS tumor. TBX2 is a direct transcriptional target of PAX3 in the melanocyte lineage[37] and it will be important to determine if PAX3 and the PAX3/PAX7-FOXO1 fusion proteins that characterize ARMS tumors regulate TBX2. We show that TBX2 is required for anchorage independent growth *in vitro* and for tumor formation *in vivo*. While these experiments were performed in the ARMS subtype of RMS, it is likely that TBX2 will function similarly in the ERMS subtype. Confirming this hypothesis will be an important direction for our future studies.

The discovery that TBX2 functions as a repressor of p21 in RMS is highly significant. An understanding of the signaling pathways and factors that control p21 expression is essential for understanding RMS progression and for designing potential strategies to restore activation of this crucial cell cycle regulator, thus inhibiting tumor growth. TBX2 appears to function as a repressor through recruitment of HDAC1, which leads to a reduction in histone acetylation levels and promotes a closed chromatin state at genes such as *CDKN1A*. Previous work has shown that histone deacetylase (HDAC) inhibitors induce growth suppression and cell death in both subtypes of RMS and lead to an upregulation of p21 [38]. The HDAC inhibitor induced reactivation of p21 has been shown to act synergistically with small molecule inhibiters of PAX3/FOXO1 to suppress tumor growth [26]. Our work supports these studies and provides a novel molecular explanation for how HDAC inhibitors can lead to growth suppression and an upregulation of p21 expression in RMS cells through interference with the repression mediated by TBX2.

The interaction with the MRFs is intriguing, as it suggests that TBX2 interacts with bHLH proteins to block their activity. As bHLH proteins play a role in many cellular and developmental processes, it suggests that TBX2 may modulate the activity of bHLH proteins. In skeletal muscle, the interaction between TBX2 and the MRFs may serve an important biological role in inhibiting myogenesis in the early phases of muscle development. However, in RMS cells, the aberrant expression of TBX2 may at least partially account for the inactivity of the MRFs observed in RMS cells. Myogenin and MyoD are used as clinical markers for the diagnosis of RMS, but the MRFs do not function and ectopic expression of the MRFs cannot rescue the activity [39]. Thus, our data suggest that the presence of TBX2 in RMS cells may serve to block the activity of the MRFs. In the human *CDKN1A* promoter, consensus E-boxes flank the TBX2 consensus binding motif. The sequence of both E-boxes immediately flanking the TBX2 binding motif is CAGCTG, which is the preferred binding motif for MyoD and myogenin [40]. MyoD is known to be an activator of p21 in muscle cells [24]. We have found that TBX2 consensus binding sites are located in close proximity to E-boxes critical for MRF function in muscle specific genes as well. It will be an important future direction of our studies to understand how TBX2 and the MRFs contribute to the respective binding of these DNA elements. Understanding the detailed molecular mechanism of how TBX2 interferes with MRF activity will be essential in understanding normal muscle development and cancer progression.

Our data clearly suggest that TBX2 is of central importance in the pathology of RMS and may serve as a novel therapeutic target for RMS. Our work also highlights the likely importance of TBX2 in RMS progression. We show that depletion or interference of TBX2

completely inhibits tumor cell growth in ARMS cells, offering a novel target for this aggressive form of RMS. Unraveling the function and regulation of TBX2 in RMS cells will provide novel molecular insight into the pathology of RMS, particularly the regulation of p21, facilitating the design of improved therapeutic strategies for RMS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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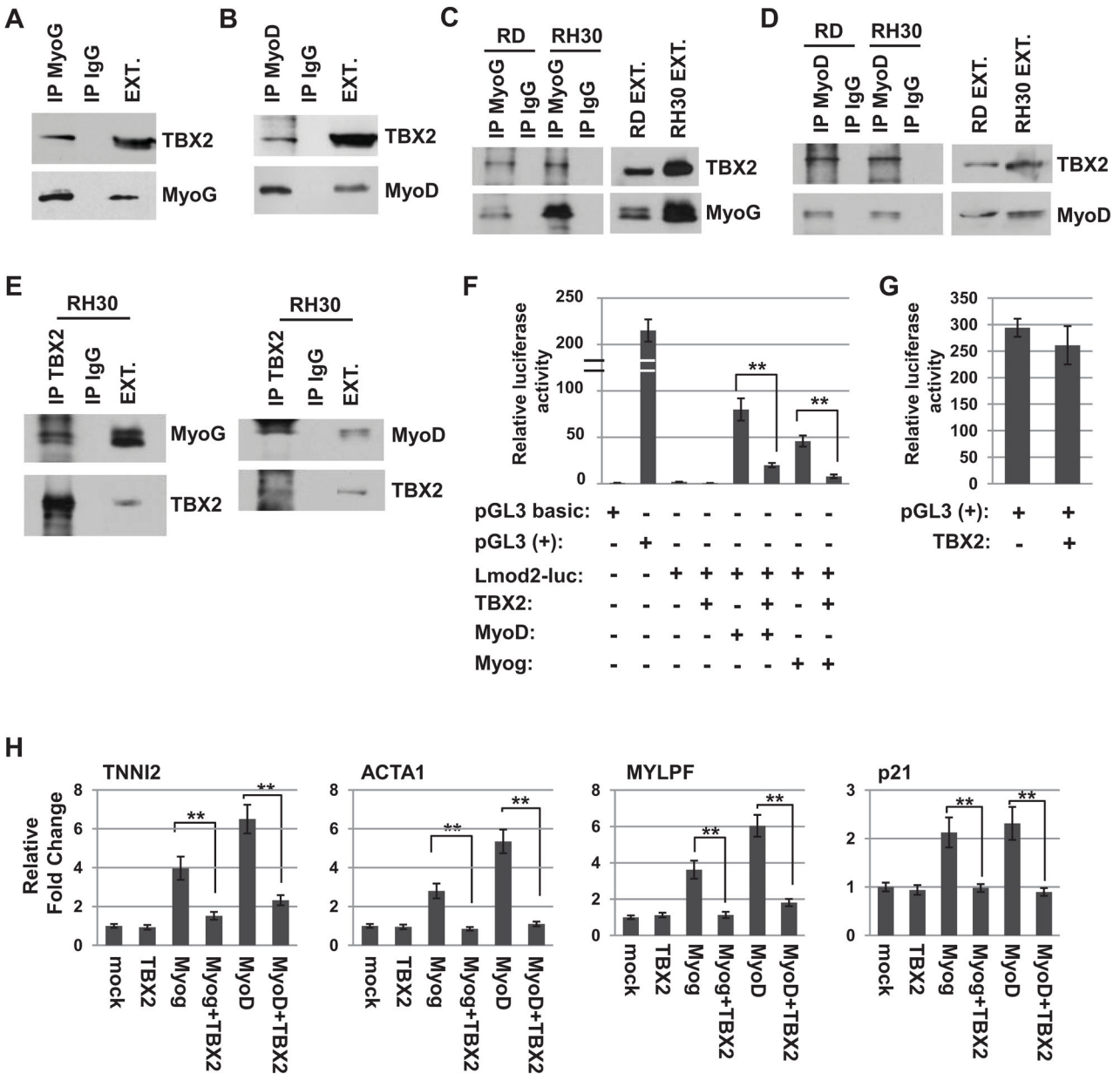

Figure 1.

TBX2 interacts with myogenin and MyoD and represses MRF activity. A. TBX2 interacts with myogenin. Expression constructs for myogenin and TBX2 were transfected into HEK293 cells, immunoprecipitated with antibodies against myogenin and detected with antibodies against myogenin and TBX2. Cell extract is labeled EXT. B. TBX2 interacts with MyoD. Experiment was performed as in A. with a MyoD expression construct and antibodies against MyoD. C. Endogenous TBX2 interacts with myogenin. Extracts from RD and RH30 cells were immunoprecipated with antibodies against myogenin and detected with antibodies against TBX2 and myogenin. D. Endogenous TBX2 interacts with MyoD. Experiment was performed as in C. except using antibodies against MyoD. E. The interaction is reciprocal. Extract from RH30 cells was immunoprecipitated with antibodies

against TBX2 and probed with antibodies against myogenin or MyoD and TBX2. F. TBX2 represses the activity of myogenin and MyoD on muscle specific luciferase reporter constructs. 10T1/2 cells were transfected with the indicated constructs. Values are represented with respect to a luciferase vector with no promoter (pGL3 basic). pGL3 (+) represents a luciferase vector with the constitutive CMV promoter. Lmod2-luc represents a luciferase vector with a ~300 bp Leiomodin 2 (*Lmod2*) promoter. Bars, SD. **P<0.001. G. TBX2 does not repress pGL3 (+). H. TBX2 represses endogenous MRF target gene expression. 10T1/2 cells were transfected with expression constructs for myogenin, MyoD and TBX2 as indicated and gene expression was determined by qRT-PCR for the indicated genes. Data were normalized to the expression of *Hprt*. Mock represents a vector transfection where the expression level was set to 1. Bars, SD. **P<0.001.

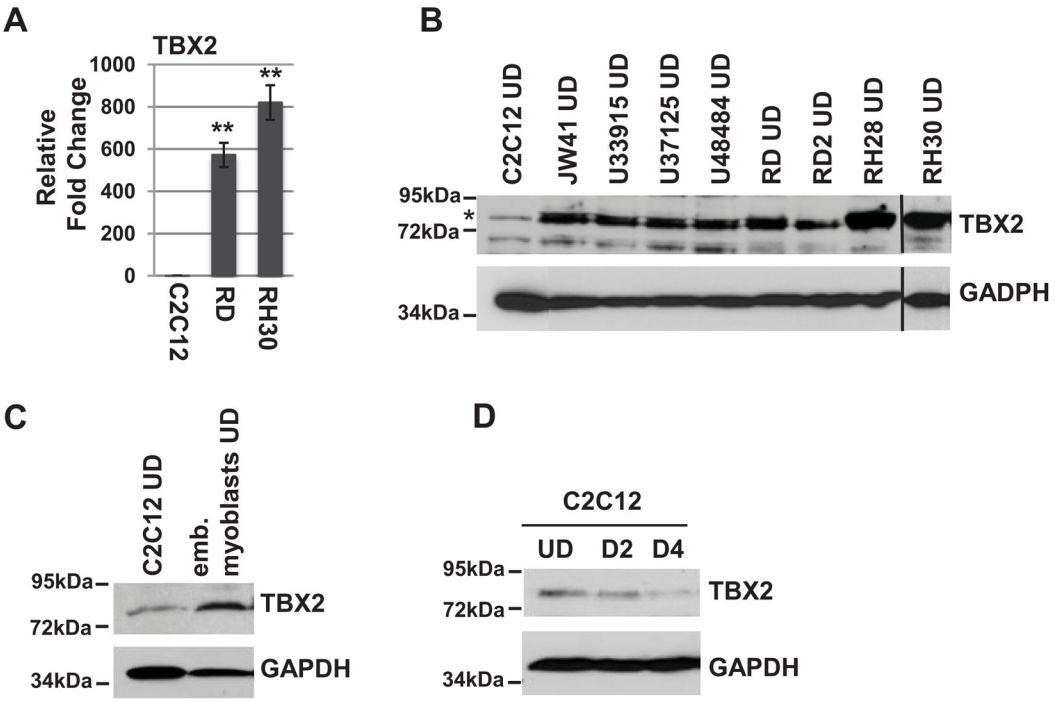

Figure 2.

TBX2 is over expressed in RMS cell lines. A. TBX2 mRNA is highly over expressed in RMS cells. qRT-PCR was used with primers specific to human and murine TBX2. Bars, SD. **P<0.001 compared to C2C12. B. TBX2 is over expressed at the protein level in RMS cells. RD (ERMS), RD2 (ERMS), RH28 (ARMS), RH30 (ARMS) are human RMS cell lines and JW41 (ERMS), U33915 (ERMS), U37125 (ERMS), U48484 (ARMS) are primary murine RMS cells. Western blot was probed with antibodies against TBX2 and GAPDH. UD indicates undifferentiated cells. Black lines indicate sister blots used to assay all samples. C2C12 samples were run on both blots and results were equivalent. Asterisk indicates the predicted size of TBX2. C. TBX2 is expressed in primary myoblasts. Blot was probed as in B. D. TBX2 is down regulated upon differentiation. C2C12 cells were assayed while proliferating (UD) and after 2 days (D2) and 4 days (D4) of differentiation.

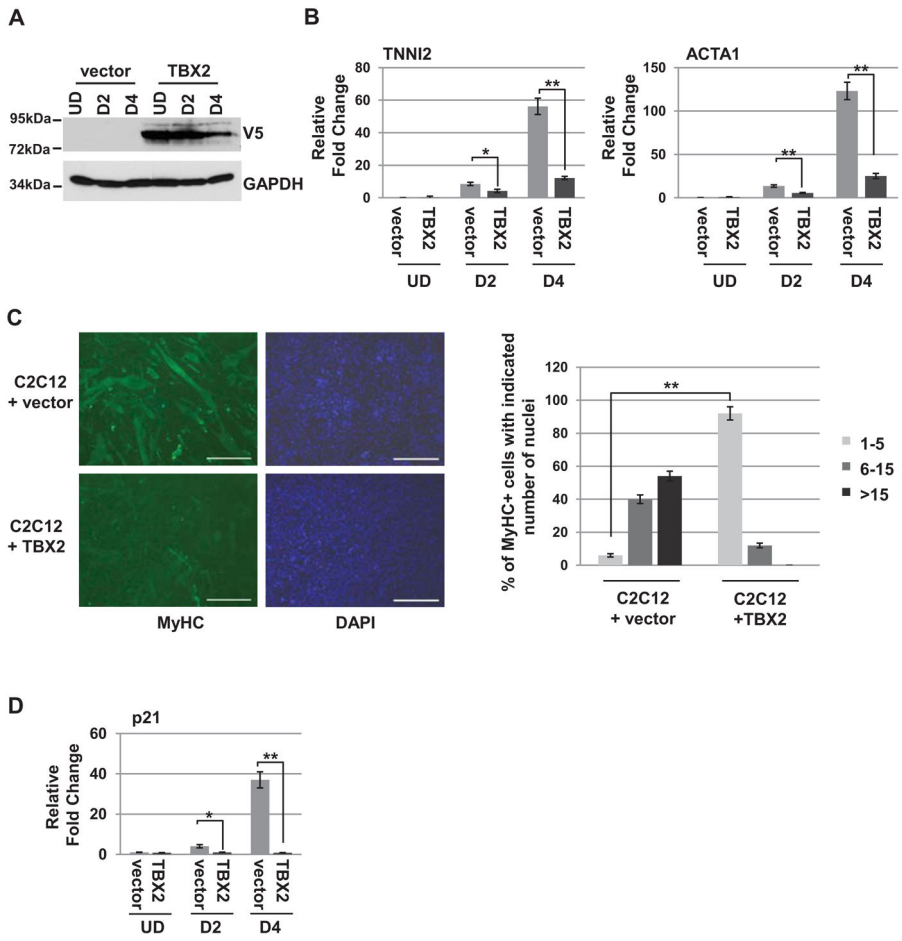

Figure 3.

TBX2 inhibits differentiation in muscle cells. A. Stable C2C12 cell lines over expressing TBX2-V5 or a vector control (vector) were confirmed by western blot with anti-V5 antibodies. Cells were assayed while proliferating (UD) and after 2 days (D2) and 4 days (D4) of differentiation. B. Muscle specific genes are down regulated in the presence of TBX2. Cell lines described in A. were assayed at UD, D2 and D4 for gene expression by qRT-PCR for the indicated genes. Bars, SD. *P<0.05, **P<0.001. C. TBX2 inhibits myosin heavy chain (MyHC) expression. Cell lines described in A. were immunostained with antibodies against MyHC and DAPI. Images were taken at 200X magnification and scale bars represent 10 μms. D. TBX2 represses p21. Cell lines described in A. were assayed as in B. for expression of p21.

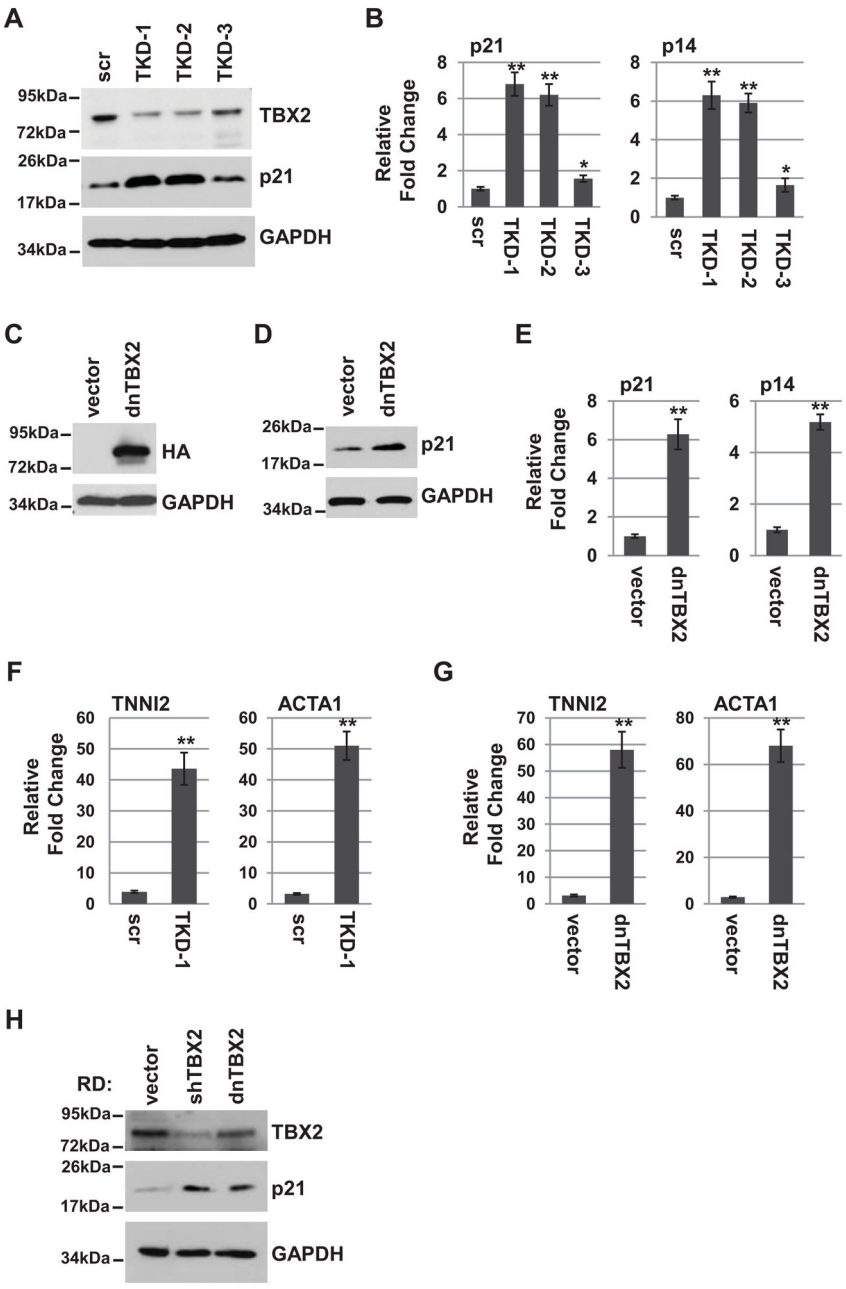

Figure 4.

TBX2 represses p21 and muscle specific genes in RMS cells. A. TBX2 depletion up regulates p21. Stable RH30 cell lines expressing shRNA constructs against TBX2 (TKD-1, 2 and 3) or the scrambled control (scr) were assayed by western blot with antibodies against TBX2 and p21. B. Gene expression was assayed on the cell lines shown in A. by qRT-PCR. Bars, SD. **P<0.001. C. Dominant negative TBX2 (dnTBX2) is expressed in RH30 cells. Western blot was probed with the indicated antibodies. D. dnTBX2 up regulates p21. Stable RH30 cell lines transfected with CMV HA TBX2 1-301 or vector control were selected with G418 and assayed with antibodies against p21. E. Gene expression was assayed on the cell lines shown in C. by qPCR with primers against p21 and p14. F. Depletion of TBX2 up

regulates muscle specific genes. Cell lines expressing shTBX2 were assayed for gene expression of the indicated genes by qPCR. G. Expression of dnTBX2 up regulates muscle specific genes. Cells described in C. were assayed as in F. G. TBX2 depletion up regulates p21 in RD cells. Transient transfection of the shRNA construct TKD-1 or the dnTBX2 construct were harvested for protein and the western blot probed with the indicated antibodies.

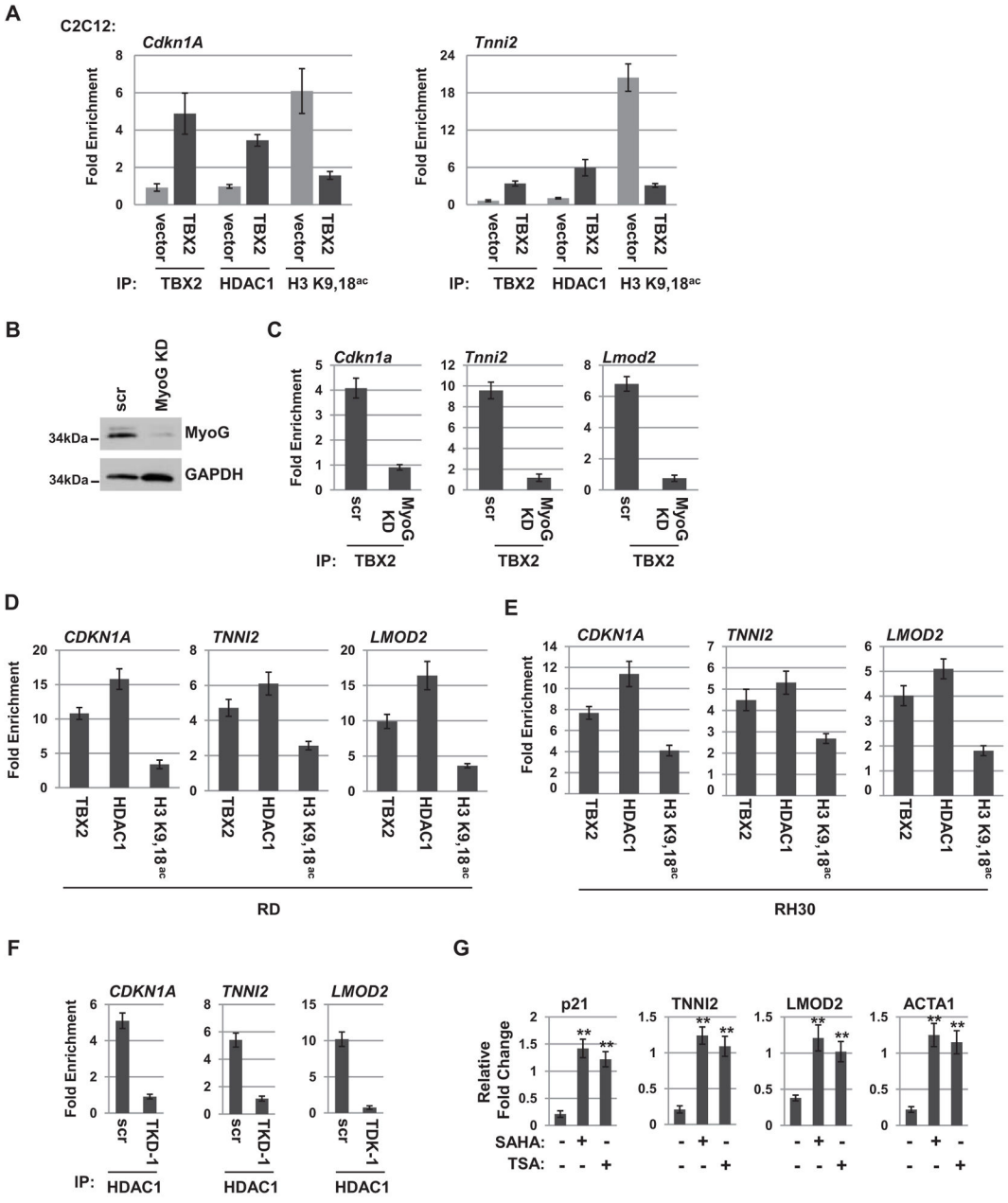

Figure 5.

TBX2 and HDAC1 bind to p21 and muscle specific promoters. A. ChIP assays were performed on stable C2C12 cell lines over expressing TBX2 or a vector control (vector) with antibodies against TBX2, HDAC1 and histone H3, lysine 9, 18^{ac} (H3 K9, 18^{ac}) and promoter specific primers. B. Myogenin is depleted by shRNA constructs. C. Myogenin is required for TBX2 recruitment to target genes. ChIP assays were performed with antibodies against TXB2 in the cell lines shown in B. D. ChIP assays were performed on RD cells as described in A. E. ChIP assays were performed on RH30 cells as described in A. F. ChIP assays were performed with antibodies against HDAC1 on RH30 cells transfected with shTBX2 (TKD-1) or the scrambled control (scr). G. C2C12 cells expressing TBX2 or vector

control were treated with TSA or SAHA for 24 hours while differentiating. Data are presented with respect to the vector control value, which was set to one.

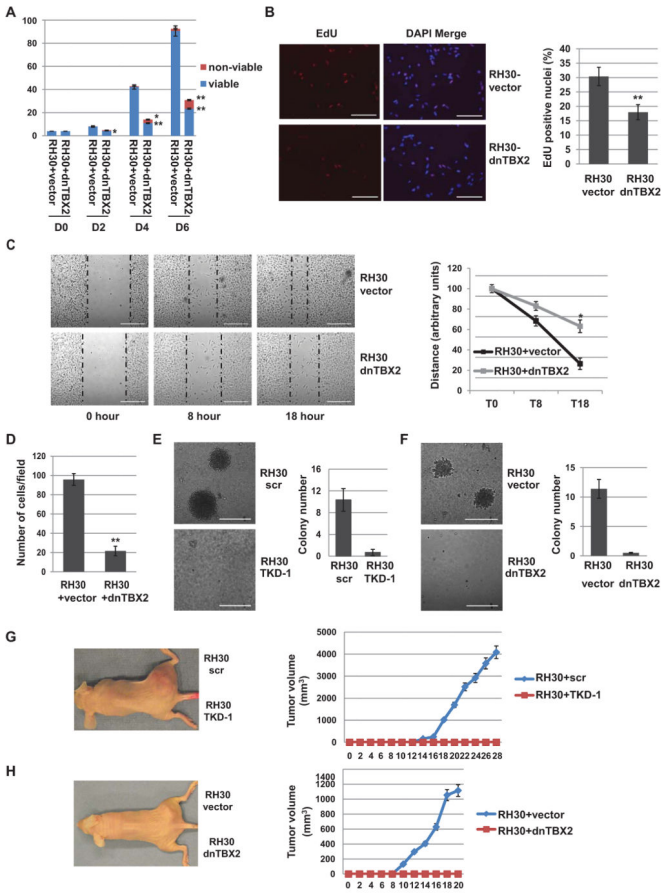

Figure 6.

Loss of TBX2 inhibits proliferation, anchorage independent growth and tumor formation of RMS cells. A. Interference with TBX2 inhibits the proliferation of RH30 cells. Cells expressing a dnTBX2 construct or vector control were seeded at equivalent densities and harvested for cell counts every two days. Error bars, SD. B. Proliferation of cells expressing dnTBX2 was also assayed by an EdU cell proliferation assay. C. dnTBX2 inhibits the migration of RH30 cells. Phase contrast images were taken immediately after wounding (0 h) or at indicated times. Left panel: images were taken at 100X magnification and scale bars represent 20 μms. Right panel: data were quantified with a statistical evaluation of the distance between the borders (dotted lines, left panel) in three independent assays. Error bars, SD. D. dnTBX2 inhibits migration of RH30 cells as shown by a transwell migration assay. The graph represents three independent assays. Error bars, SD. E. Depletion of TBX2 inhibits anchorage independent growth. RH30 cells expressing a shRNA construct against TBX2 (TKD-1) were used for soft agar colony assays. Images were taken at 100X magnification and scale bars represent 20 μms. F. Dominant negative TBX2 inhibits anchorage independent growth. RH30 cells expressing dnTBX2 were assayed as in E. G. Depletion of TBX2 suppresses tumor growth *in vivo*. Each animal was injected in the right flank with RH30-scr cells and in the left flank with RH30-TKD-1 cells. Tumor growth 4 weeks after s.c. injection of 2×10^6 RH30-scr or RH30-TKD-1 cells is shown. H. Dominant

negative TBX2 suppresses tumor growth *in vivo*. Each animal was injected as in G. Tumor growth 3 weeks after s.c. injection of 2×10^6 RH30-vector or RH30-dnTBX2 cells is shown.