Drosophila and mammalian models uncover a role for the myoblast fusion gene *TANC1* in rhabdomyosarcoma

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Rhabdomyosarcoma (RMS) is a malignancy of muscle myoblasts, which fail to exit the cell cycle, resist terminal differentiation, and are blocked from fusing into syncytial skeletal muscle. In some patients, RMS is caused by a translocation that generates the fusion oncoprotein PAX-FOXO1, but the underlying RMS pathogenetic mechanisms that impede differentiation and promote neoplastic transformation remain unclear. Using a *Drosophila* model of PAX-FOXO1–mediated transformation, we show here that mutation in the myoblast fusion gene rolling pebbles (*rols*) dominantly suppresses PAX-FOXO1 lethality. Further analysis indicated that PAX-FOXO1 expression caused upregulation of *rols*, which suggests that Rols acts downstream of PAX-FOXO1. In mammalian myoblasts, gene silencing of *Tanc1*, an ortholog of *rols*, revealed that it is essential for myoblast fusion, but is dispensable for terminal differentiation. Misexpression of PAX-FOXO1 in myoblasts upregulated *Tanc1* and blocked differentiation, whereas subsequent reduction of *Tanc1* expression to native levels by RNAi restored both fusion and differentiation. Furthermore, decreasing human *TANC1* gene expression caused RMS cancer cells to lose their neoplastic state, undergo fusion, and form differentiated syncytial muscle. Taken together, these findings identify misregulated myoblast fusion caused by ectopic TANC1 expression as a RMS neoplasia mechanism and suggest fusion molecules as candidates for targeted RMS therapy.

Introduction

Whereas most solid adult malignancies are epithelial carcinomas, solid childhood malignancies are often mesenchymal sarcomas. Soft tissue sarcomas account for 10% of all new pediatric malignancies, 50% of which are skeletal muscle–lineage rhabdomyosarcomas (RMS) (1, 2). Children with high-risk RMS endure a 3-year event-free survival rate of only 20% (3), emphasizing the need to uncover the molecular underpinnings of RMS neoplasia.

PAX-FOXO1–positive RMS (clinically termed alveolar RMS) is notoriously aggressive (4). The PAX-FOXO1 transcription factor (also known as PAX-FKHR) is generated by chromosomal translocations that fuse a *PAX* gene (*PAX3* on chromosome 2 or *PAX7* on chromosome 1) to *FOXO1* on chromosome 13. Since the PAX3 and PAX7 transcription factors influence skeletal muscle development, PAX3/7 gene targets are postulated to underlie PAX-FOXO1 neoplasia (5).

To dissect PAX3/7-FOXO1 pathobiology, we have generated *Drosophila* PAX-FOXO1 models (6), since PAX molecules show striking evolutionary conservation (refs. 6–8 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI59877DS1), and muscle development is similar between *Drosophila* and vertebrates. Misexpression of PAX3/7-FOXO1 in differentiating muscle tissue, an expression profile similar to a PAX3-FOXO1 tumorigenic mouse model (9), causes myoblast fusion defects that result in larval lethality. Although tumorigenesis is not observed, misfused PAX3/7-FOXO1 myogenic cells act aggressively, demonstrating an ability to infiltrate nonmuscle tissue compartments (6). Since these phenotypes are amenable to unbiased genetic modifier screening, we have been isolating new PAX-FOXO1 suppressors and enhancers (R.L. Galindo, unpublished observations). These modifiers, such as the myoblast fusion gene rolling pebbles (*rols*) reported here, are providing insight into the mechanisms underlying PAX-FOXO1 pathogenicity.

Results and Discussion

For these studies, we focused on a *Drosophila* chromosomal deletion, *Df(3L)vin5*, that dominantly suppresses PAX7-FOXO1–induced lethality (Supplemental Figure 2A). Human PAX7 demonstrates slightly higher sequence identity to *Drosophila* PAX3/7 than does human PAX3 and was therefore used here in flies. *Df(3L)vin5* deletes segments 68A2–69A1 on chromosome 3, which includes the muscle-patterning gene *rols*, located at 68F1. *rols* encodes an essential adaptor molecule that links the Kirre transmembrane receptor with the machinery that drives myoblast cell-cell fusion and syncytial muscle formation; therefore, *rols* expression in the somatic mesoderm temporally coincides precisely with embryonic myoblast fusion (10–12). However, we found by mRNA expression profiling that *rols* is misexpressed in *PAX7-FOXO1* larval muscle: it was reported as absent on control microarrays ($n = 3$), and expressed 3.6-fold above background on PAX7-FOXO1 arrays ($n = 3$) (R.L. Galindo, unpublished observations). Thus, we hypothesized that heterozygous deletion of the *rols* locus might account for *Df(3L)vin5*-mediated PAX7-FOXO1 suppression and that *rols* might act as a PAX7-FOXO1 target gene.

Of the 2 alternative transcripts expressed from the *rols* locus, only one of which is expressed in myoblasts (10–12); expression of the second is restricted to endodermal/ectodermal precursors. We tested 2 *rols* homozygous-lethal, P-element insertion loss-of-function alleles, *P1027* and *P1729*, for suppression of PAX7-FOXO1. Of these 2 alleles, only the *P1729* insertion disrupts expression of

Conflict of interest: The authors have declared that no conflict of interest exists.
Citation for this article: *J Clin Invest.* 2012;122(1):403–407. doi:10.1172/JCI59877.

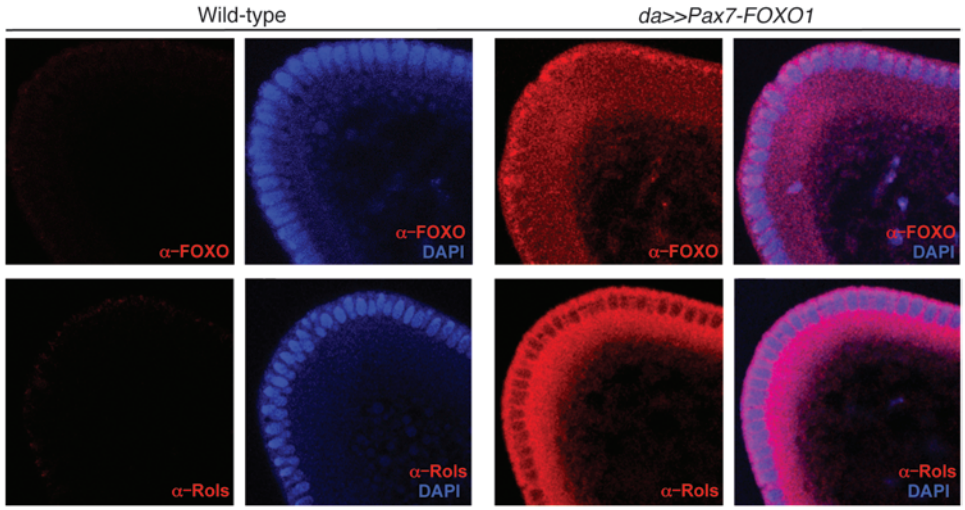

Figure 1
PAX7-FOXO1 induces Rols misexpression in *Drosophila*. Whole-mount wild-type and *daughterless-Gal*, *UAS-PAX7-FOXO1* (*da>>PAX7-FOXO1*) blastoderm embryos (anterior pole) were stained with anti–PAX7-FOXO1 and anti-Rols (red), and nuclei were stained with DAPI (blue). Wild-type embryos displayed no PAX7-FOXO1 or Rols protein expression; *daughterless-Gal*, *UAS-PAX7-FOXO1* embryos showed diffuse PAX7-FOXO1 (transcription factor) and Rols (cytoplasmic adaptor molecule) expression: whereas the latter was excluded from the nucleus, the former was found within both cytoplasm and nuclei. Original magnification, ×800.

the myoblast *rols* transcript (10–12) (myoblast expression of rols is unperturbed in *P1027*); accordingly, only the $rols^{P1729}$ allele suppressed PAX7-FOXO1–induced lethality and muscle pathogenicity (Supplemental Figure 2, A and B).

To investigate whether *rols* acts as a downstream PAX-FOXO1 target, we used the *daughterless-Gal4* transgene (commonly used in *Drosophila* to direct strong expression of UAS-transgenes in all cells throughout development) to drive ubiquitous embryonic expression of *UAS-PAX7-FOXO1* and probed for Rols misexpression. Since native Rols expression initiates at embryonic stage 11 (10–12), we focused only on embryos stage 10 or earlier. Diffuse expression of PAX7-FOXO1 and Rols was observed in blastoderm (stage 4–5) embryos (Figure 1), which consist of uncommitted precursor cells, and expression persisted in all examined cells — including nonmyogenic ectodermal and endodermal cells — of gastrulated (stage 9–10) embryos (Supplemental Figure 3, A–D). Taken together, these *Drosophila* studies revealed that *rols* acts as a PAX7-FOXO1 downstream target gene, direct or indirect, and as a bona fide genetic effector.

To extend our studies from *Drosophila* to mammals, we first questioned whether *rols* myoblast fusion activity is evolutionarily conserved. In mammals, 2 orthologs of *rols* are present, tetratricopeptide-repeat, ankyrin-repeat, coiled-coil–containing protein 1(*Tanc1*) and *Tanc2*, neither of which has been studied in muscle. Since only *Tanc1* is expressed in somites when myoblast fusion occurs (12), we hypothesized that *Tanc1* is the functional ortholog. We turned to mouse C2C12 cultured myoblasts, which, when switched from growth to differentiation medium (DM), differentiate and fuse to form syncytial myotubes. Quantitative RT-PCR (qRT-PCR) confirmed that *Tanc1* was expressed in C2C12 cells (Figure 2A) and that relative expression levels decreased 40% as differentiation proceeded (Supplemental Figure 4).

We next used shRNAs to establish that *Tanc1* activity is essential for myotube formation. We tested 2 separate constructs, A6 and A10, individually and in combination and used qRT-PCR to confirm mRNA knockdown (Figure 2A). *Tanc1* silencing potently blocked the formation of syncytial myotubes in a dose-dependent manner (Figure 2, B, D, and E). *Tanc1*-silenced cells, however, still transitioned from round precursors to spindle-shaped cells (Figure 2, D and E), suggestive of successful myocyte differentiation. Immunofluorescence for muscle-specific myosin heavy chain (MHC; a marker of terminal differentiation) confirmed that *Tanc1* shRNA–treated myoblasts successfully switched to a differentiated state (Figure 2, C and E). Thus, the mechanisms that drive cell-cell fusion uncouple from the myogenic signals that induce myocyte terminal differentiation.

Similar to the *rols*/PAX7-FOXO1 genetic interaction in *Drosophila*, *Tanc1* was critical for PAX-FOXO1 pathogenicity in mammalian myoblasts. We used retroviral-mediated gene transfer to generate stable PAX3-FOXO1–expressing C2C12 myoblasts, as PAX3-FOXO1 is the more common RMS chimera and the form most often profiled in mammalian cells, and confirmed that PAX3-FOXO1 protein misexpression levels were comparable to human PAX3-FOXO1 RMS cultured cells (specifically, RMS-13 cells; Supplemental Figure 5; also shown is *Drosophila* larval PAX7-FOXO1 expression). We found that PAX3-FOXO1 induced overexpression of *Tanc1* (Figure 3A) and that, like RMS cells, PAX3-FOXO1 myoblasts demonstrated neoplasia-related phenotypes, including impaired myogenic differentiation and an inability to form myotubes (Figure 3, B and C, and Supplemental Figure 6A). However, reducing *Tanc1* expression back to normal levels markedly suppressed PAX3-FOXO1 pathogenicity, as transient transfection of *Tanc1* shRNA into PAX3-FOXO1 myoblasts restored both differentiation and fusion potential (Figure 3, B and C). It is worth noting that in PAX3-FOXO1 cells, the A6 hairpin alone demonstrated the most effective rescue. Immunoblot analysis confirmed that PAX3-FOXO1 protein levels remained unchanged (Supplemental Figure 6B). Thus, *Tanc1* is a critical PAX3-FOXO1 downstream effector.

To determine whether Tanc1 overexpression itself perturbs myoblast fusion and/or differentiation, we again used retroviral-mediated gene transfer to generate C2C12 cells that constitutively overexpress *Tanc1* (Supplemental Figure 7A). Like PAX3-FOXO1

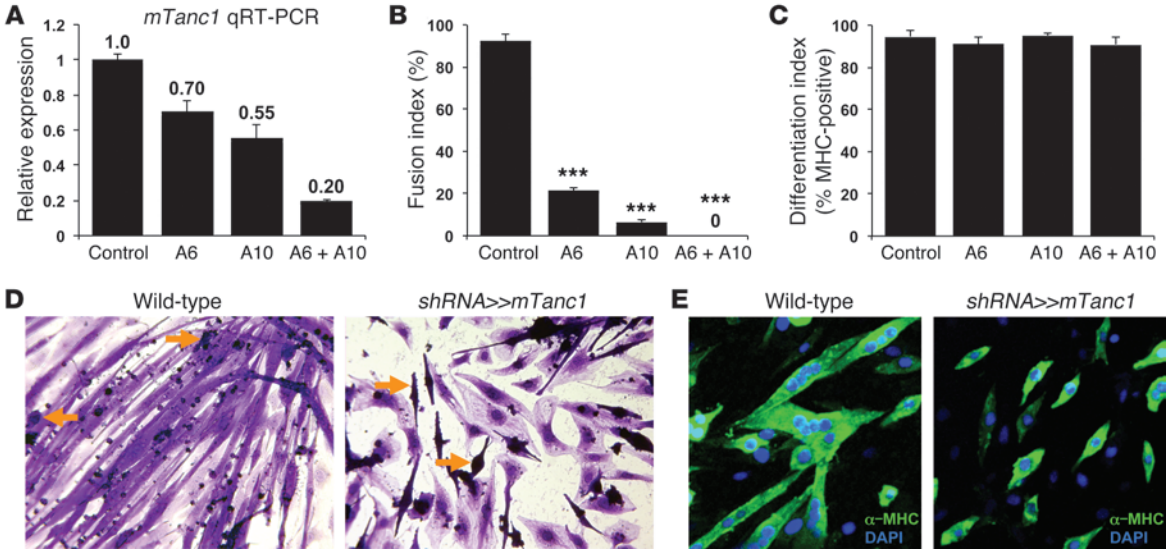

Figure 2

Tanc1 is essential for myoblast fusion, but not for myogenic differentiation. (**A**) qRT-PCR confirmed shRNA-mediated silencing of mouse *Tanc1* (*mTanc1*). After 72 hours of transient transfection, constructs A6 and A10 were tested individually and in combination and compared with control cells transfected with a GFP hairpin. *Tanc1* mRNA levels were normalized to β-actin, which did not change over the course of differentiation. Mean values are shown above bars. (**B**) *Tanc1* silencing blocked myoblast fusion. Fusion indices were calculated (see Methods) for cells transfected with the *Tanc1* hairpins or the control *GFP* hairpin. (**C**) *Tanc1*-silenced myoblasts differentiated into MHC-positive myocytes. Differentiation indices were calculated for C2C12 cells treated with the *Tanc1* hairpins or the *GFP* hairpin. (**D**) Crystal violet stain of wild-type C2C12 cells and fusion-defective *Tanc1*-silenced cells. Arrows denote residual dye particles. (**E**) Unfused *Tanc1*-silenced C2C12 cells differentiated into MHC-positive myocytes. Cells were stained with MF-20 antibody and DAPI. For all transfections, a total of 10 μg DNA was used. Original magnification, ×400 (**D** and **E**). ***$P < 0.001$ vs. control.

cells, Tanc1-infected myoblasts were severely crippled with regard to myoblast fusion potential; however, unlike PAX3-FOXO1 cells, Tanc1 myoblasts differentiated normally into myocytes (Supplemental Figure 7B). These finding again show that fusion potential can be uncoupled from myocyte terminal differentiation.

We next turned to RMS-13 cells to establish that human *TANC1* influences RMS. RMS-13 cells, similar to RMS myoblasts in vivo, exhibited little to no expression of MHC or fusion potential (Figure 3, E and F, and Supplemental Figure 8A). Treatment of RMS-13 cells with shRNA — the A6 and A10 hairpins, which target both mouse *Tanc1* and human *TANC1* — again reduced the relative expression of *TANC1* mRNA as well as TANC1 protein steady-state levels, whereas PAX3-FOXO1 protein levels remained unchanged (Figure 3D and Supplemental Figure 8, A and B). When switched to DM, *TANC1*-silenced RMS-13 myoblasts transitioned from polygonal myoblasts to spindled cells (Supplemental Figure 8C), suggestive of differentiation into myocytes. Immunofluorescence confirmed that *TANC1*-silenced RMS-13 cells differentiated and fused to form MHC-positive syncytia (Figure 3, E and F). Consistent with these findings, *TANC1*-silenced RMS-13 cells showed markedly diminished oncogenicity, as demonstrated by decreased anchorage-independent growth and colony formation on soft agar (Supplemental Figure 8D).

Finally, immunohistochemistry showed that TANC1 protein was strongly expressed in PAX3-FOXO1 RMS tumors ($n = 5$; Figure 3H) compared with control childhood skeletal muscle tissue ($n = 3$; Figure 3G). These findings highlight the notion that *TANC1* misexpression can be used to mark PAX-FOXO1 RMS tumor cells.

RMS model systems conveniently promote insights into not only neoplasia, but also muscle development. Although ultrastructural studies suggest that myoblast fusion biology is conserved (13–17), few of the *Drosophila* fusigenic genes have been identified as essential in mammals (13, 18–20), and none of these were from the founder subfamily. As the name implies, founder myoblasts are seminal to *Drosophila* myogenesis, uniquely dictating the location and physiology of each individual muscle (21). With *rols* and *Tanc1*, we have now shown that founder gene function is conserved in mammals and, furthermore, participates in human disease. How founder gene activity influences other forms of neuromuscular disease now becomes an intriguing issue.

Regarding RMS, we conclude that: (a) *Tanc1* is essential for mammalian myoblast fusion, but is dispensable for the myogenic differentiation of wild-type cells; (b) PAX-FOXO1 signaling drives *Tanc1* overexpression; (c) reducing *rols*/*Tanc1*/*TANC1* activity suppresses gain-of-function PAX-FOXO1 pathogenicity in multiple independent model systems, highlighting *TANC1* as a critical PAX-FOXO1 downstream effector; and (d) *TANC1* activity and altered myoblast fusion mechanistically contribute to PAX-FOXO1 RMS.

Genetic screening in a *Drosophila* model and loss-of-function/gain-of-function studies in mammalian platforms have collaboratively uncovered a PAX-FOXO1→TANC1 neoplasia axis, a finding we believe to be novel. Our results also argue that the relationship between myogenesis transcription factor (e.g., MyoD) signaling and myoblast fusion genes is intricate. In the presence of altered fusion potential, both *Drosophila* (10–12) and mammalian myoblasts (present study) transition to differentiated myocytes, which suggests that later aspects of myogenesis signaling must uncouple from the *TANC1* fusigenic pathway. Yet correcting PAX-FOXO1–mediated overexpression of *rols*/*TANC1* rescued PAX-FOXO1–induced differentiation and arrest. These results intimate that correction of the TANC1 fusigenic axis feeds back

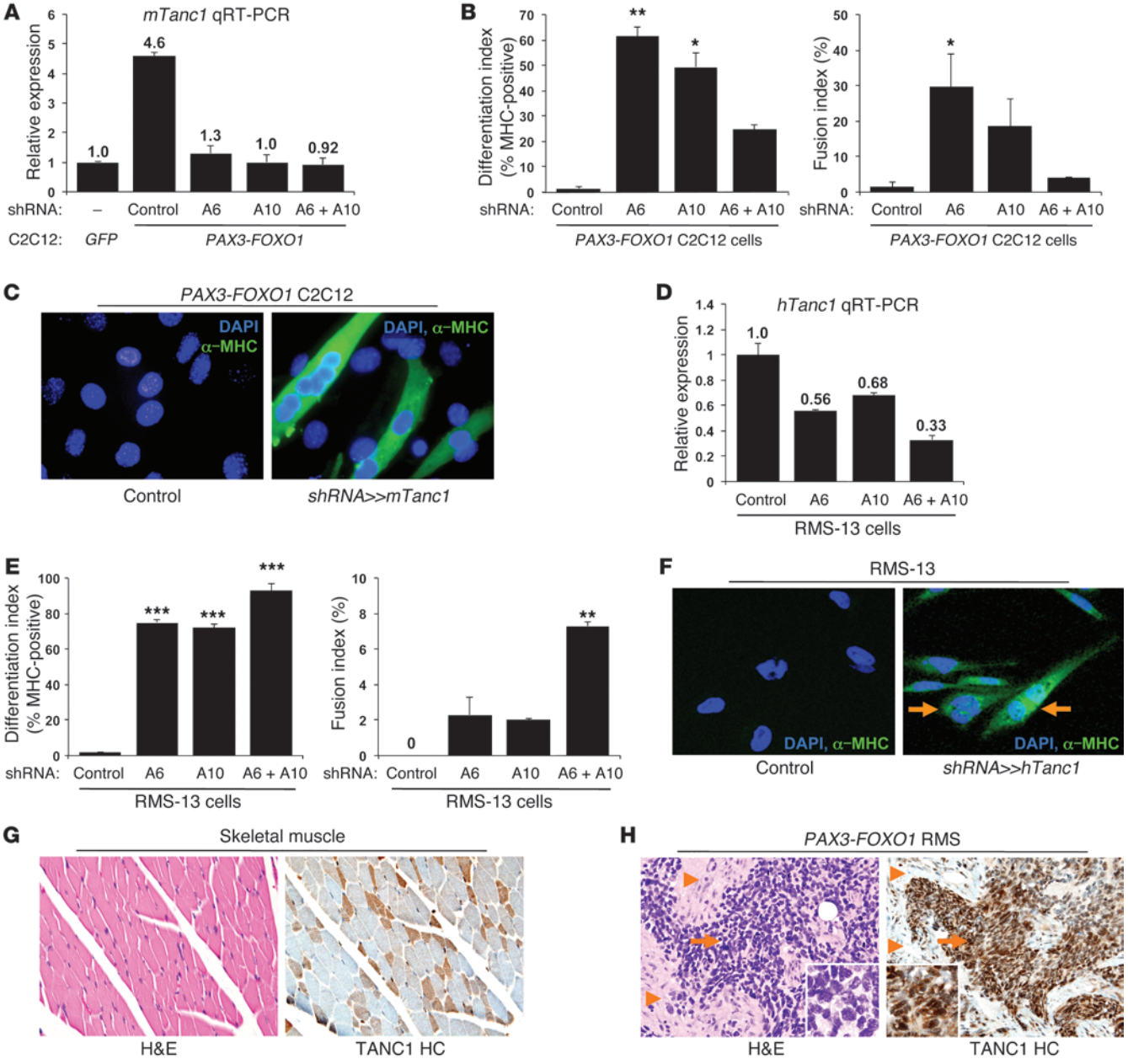

Figure 3

TANC1 silencing rescues differentiation arrest and failed fusion of PAX3-FOXO1 cells and marks RMS tumor cells. (**A**–**C**) *Tanc1* silencing in PAX3-FOXO1–infected mouse C2C12 cells. (**A**) *Tanc1* shRNA treatment reduced Tanc1 expression to levels approximating those of GFP-infected control cells. Cells were treated with A6, A10, or A6 and A10 combined. (**B**) *Tanc1*-silenced cells differentiated and fused into MHC-positive myotubes. Differentiation and fusion indices were calculated as in Methods. (**C**) *Tanc1*-silenced cells showed restored differentiation and fusion. (**D**–**F**) *TANC1* silencing in human PAX3-FOXO1 RMS-13 cells. (**D**) *TANC1* shRNA reduced TANC1 expression compared with cells treated with GFP shRNA. (**E** and **F**) *TANC1* silencing restored differentiation and fusion potential. (**G**) TANC1 immunohistochemistry in pediatric skeletal muscle. Scattered myofibers were positive for TANC1 protein. (**H**) TANC1 immunohistochemistry strongly highlighted PAX3-FOXO1 RMS. Malignant rhabdomyoblasts (arrows) were strongly positive for TANC1, whereas interwoven fibrovascular stroma (arrowheads) was negative. Arrows denote MHC-positive syncytial tissue. Original magnification, ×400 (**C** and **F**–**H**); ×1,000 (**H**, insets). In **A** and **D**, mean values are shown above bars. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control.

to and rescues PAX-FOXO1–mediated misregulation of myogenic signaling, raising fascinating questions regarding the mechanisms by which this occurs. Our observation that PAX3-FOXO1 protein levels remained unchanged in *TANC1*-silenced cells argues that rescue does not originate from decreased expression of PAX3-FOXO1 from the PAX3 promoter. Thus, we speculate that rescue occurs epistatically downstream of PAX3-FOXO1.

Interestingly, myoblasts were remarkably sensitive to modest variations in *TANC1* expression levels. A relatively wide range of rescue penetrance was observed for the 2 *TANC1* shRNA hairpins and com-

bined cocktail, even though the relative expression levels of mouse *Tanc1* or human *TANC1*, when treated by the various shRNAs, differed by 38% and 35%, respectively. We note, however, that the relative levels of *Tanc1* gene expression decreased only 40% over the normal course of differentiation. Thus, we favor the notion that a relatively precise requirement for appropriate *TANC1* expression is biologically relevant for both myoblast fusion and RMS pathobiology.

Reprogramming neoplastic precursor cells to undergo terminal differentiation — commonly referred to as differentiation therapy — is of particular clinical interest, as it is often substantially less toxic than general chemotherapeutic agents (e.g., use of retinoic acid in acute promyelocytic leukemia). Conceptually, targeting the myoblast fusion pathway may represent a new avenue for PAX-FOXO1 RMS differentiation therapy, although whether an equivalent TANC1 axis participates in PAX-FOXO1–negative (i.e., embryonal) RMS remains a provocative question. Of note, Yang and colleagues have demonstrated that forced inhibition of MyoD in embryonal RMS cells prompts terminal differentiation (22). Therefore, RMS in general appears to be a clinically ripe candidate for differentiation therapy.

Methods

Further information can be found in Supplemental Methods.

Genetics and expression profiling. In a screen for PAX7-FOXO1 suppressors, the *UAS-PAX7-FOXO1* and muscle-specific *Myosin Heavy Chain-Gal4* transgenes were used, and rescue of lethality was assessed as previously described (6). The *daughterless-Gal4*, *Df(3L)vin5*, *rols*^{P1027}, and *rols*^{P1729} stocks were obtained from the Bloomington *Drosophila* Stock Center. For microarray analysis, mRNA was extracted from *UAS-PAX7-FOXO1; Myosin Heavy Chain-Gal4* by the TRIzol reagent (Invitrogen) and control larvae, profiled on Affymetrix *Drosophila* Genome 2.0 chips, and analyzed with GenePattern software. Raw data were deposited into the ArrayExpress database (accession no. E-MTAB-839).

Cell culture, transfections, and cell lines. C2C12 cells (ATCC) were grown in DMEM (Sigma-Aldrich) with 20% FBS (Atlas Biolabs). For differentiation, DMEM was supplemented with 2% horse serum (Sigma-Aldrich). RMS-13 cells (ATCC) were cultured in RPMI 1640 (Sigma-Aldrich) with 10% FBS.

All shRNAs were obtained from Open Biosystems. Transfections were carried out by electroporation (320 V, 960 μF; Bio-Rad Gene Pulser II). Cells were trypsinized using Trypsin/EDTA after reaching 70% confluency, resuspended in Opti-MEM media, and electroporated. 1% formalin crystal violet solution was used for staining.

PAX3-FOXO1 and eGFP stable cell lines were generated using the pBabe vector (Addgene) and the pCK packaging vector (Roche-Applied Science); Tanc1 overexpression and control lines were generated using pDsRed2-C1 vector (Clonetech) and pCK; virus was generated with 6 μg vector, 6 μg pCK, and 36 μl Fugene mixture added to 70% confluent 293T cells, then harvested after 72 hours. C2C12 cells were infected with virus with polybrene and selected with 15 μg/ml blasticidine for 2 days, then 10 μg/ml for 5 days.

Statistics. Data represent mean ± SEM. Significance of differences was determined by unpaired 2-tailed Student's *t* test. A *P* value less than 0.05 was considered significant.

Study approval. Studies with human tumors and skeletal muscle were used without any identifying patient information, and were deemed exempt from IRB review and approval.

Acknowledgments

We are grateful to Thomas Gerald and Linh Tran for excellent technical support and Kristine Leos and Shama Khokhar for IHC support. We thank Mark Hatley, Rhonda Bassel-Duby, Diego Castrillon, and Eric Olson for manuscript review. This work was supported by the UTSW President's Research Council, Children's Cancer Fund (Dallas, Texas, USA), Burroughs Wellcome Career Award for Medical Scientists, and Alex's Lemonade Stand Foundation "A" Award.

Received for publication July 8, 2011, and accepted in revised form November 2, 2011.

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