Translin-associated Factor X Is Post-transcriptionally Regulated by Its Partner Protein TB-RBP, and Both Are Essential for Normal Cell Proliferation*

Shiceneng Yang‡, Yoon Shin Cho†, Vargheese M. Chennathukuzhi‡, Lara A. Underkoffler‡, Kathleen Looness‡, and Norman B. Hecht‡¶

From the ‡Center for Research on Reproduction and Women’s Health and the ¶Department of Pediatrics, Children’s Hospital of Philadelphia, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6142

To determine the functions of the DNA/RNA-binding protein TB-RBP in somatic cells, we examined cultured primary mouse embryonic fibroblasts (MEFs) derived from TB-RBP-deficient mice. The TB-RBP-deficient MEFs exhibit a reduced growth rate compared with MEFs from littermates. Reintroduction of TB-RBP remedies this defect. A partner protein of TB-RBP, Translin-associated factor X (TRAX), was absent in TB-RBP-deficient MEFs, despite normal TRAX mRNA levels. TRAX is dependent upon the presence of TB-RBP and is removed from null MEFs following ubiquitination. Re-introduction of TB-RBP, but not TB-RBP lacking an oligomerization domain, into null MEFs stabilized TRAX protein without changing TRAX mRNA levels. The coordinated expression of TB-RBP and TRAX is also seen in synchronized cells, where the amount of TRAX protein but not TRAX RNA closely parallels TB-RBP levels throughout the cell cycle. In transgenic mice overexpressing TRAX in testis, total TB-RBP and TRAX levels are constant with reductions of endogenous TRAX compensating for exogenous TRAX. Using RNA interference, reductions of either TB-RBP or TRAX (without affecting TB-RBP) slow cell growth rates. We conclude that TRAX is post-transcriptionally stabilized by TB-RBP and both proteins are needed for normal cell proliferation.

The human protein Translin and its mouse orthologue, testis-brain RNA-binding protein (TB-RBP),¹ are single-stranded DNA- and RNA-binding proteins with proposed functions in chromosomal translocations in lymphoid cells and mRNA transport and storage in brain and testis (1–4). TB-RBP is a highly conserved protein with mouse and human proteins differing in 3 of 228 amino acids (5, 6). In in vitro assays, TB-RBP/Translin binds to consensus chromosomal DNA breakpoint junctions as an octameric ring and recognizes DNA breaks at genomic hotspots (2, 7, 8). TB-RBP also binds to consensus RNA sequences present in many brain and testis mRNAs (6, 9–11) and links specific mRNAs to microtubules (10, 12, 13). In the testis TB-RBP forms a complex with the Tet ATPase to transport specific mRNAs from nuclei to cytoplasm and through intercellular bridges (4). This ribonucleoprotein complex contains the kinesin KIF 17b, a testicular motor protein controlling transcription of CREM-regulated male germ cell mRNAs (14). The level of Translin has been demonstrated to closely parallel the proliferative state of somatic cells with its induced overexpression in HEK 293 cells accelerating cell proliferation (15). TRAX, first characterized as a Translin-like protein (16), forms DNA and RNA binding complexes with Translin (17, 18). TRAX has been proposed to function in DNA repair in conjunction with the nuclear matrix protein C1D (19).

Recently, we generated mice with a functional deletion of the TB-RBP gene (20). The mice are growth retarded and show defects in behavior and fertility. Many of the germ cells in the testis cannot proceed beyond first meiotic metaphase suggesting a defect in chromosome segregation and cytokinesis. Using 14.5-day-old embryos from heterozygous crosses, we have developed lines of MEFs from TB-RBP null animals and from their littermates. As in TB-RBP-deficient mice, these cells lack both TB-RBP and TRAX, although TRAX mRNA levels are not reduced. We find that early passage null MEFs have slower rates of proliferation with a block in the G2 stage of the cell cycle. Reintroduction of TB-RBP increases the rate of cell proliferation and stabilizes the TRAX protein. Transgenic and transfection experiments indicate that TRAX protein is dependent upon TB-RBP for stabilization and both TB-RBP and TRAX are needed for normal cell proliferation.

EXPERIMENTAL PROCEDURES

Generation of MEFs—TB-RBP-deficient MEFs were derived from TB-RBP null mice generated by gene trapping (20). MEFs were produced using established protocols (21). Briefly, embryos were removed from pregnant heterozygous mice at 14.5 days and rinsed separately in sterile PBS. Fetal membranes, placenta, head, and soft tissues were discarded, and the remaining tissues were rinsed in fresh PBS. Each embryo was minced and incubated at 37 °C for 5 min in 2 ml of 0.25% trypsin/EDTA. Debris was removed by passing the cell suspension through a 100-μm cell strainer (BD Biosciences Labware, Franklin Lakes, NJ). Supernatants were plated in culture dishes containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 1× non-essential amino acids (Invitrogen, Rockville, MD). The cells were split before reaching confluency and genotyped by PCR and Western blotting. For long-term cultures, passage three MEFs were plated at 5 × 10⁴ in 100-mm dishes and were split every 4–5 days.

Plasmid Constructs—Wild type TB-RBP was subcloned into pEGFP-C2 (Clontech, Palo Alto, CA) (17). Mouse TRAX cDNA was ob-
tained by RT-PCR from mouse testis RNA using a pair of primers containing EcoRI and BamHI sites and subcloned into pDsRed2-N1 and pEGFP-N1 (Clontech, Palo Alto, CA). To clone truncated forms of TB-RBP, pEGFP/TB-RBP was used as a template. PCR products were amplified with the 5′/H11032 primer, gcc gtc gac atg tct gtg agc gag, and 3′/H11032 primer, gcc gga tcc ctt cac atc gta ctt (for TB-RBP-204), or 3′/H11032 primer, gcc gga tcc att gat gaa agt aga (for TB-RBP-175), and cloned into pEGFP-C1 producing pEGFP/TB-RBP-204. The pLenti6/V5-D-TOPO constructs were cloned using the following primers: 5′/H11032 primer, cac cat ggt gag caa ggg cga g, and 3′/H11032 primer, gcc cag tta tct aga tcc ggt gga (for EGFP); 5′/H11032 primer, cac cat gtc tgt gag cga g, and 3′/H11032 primer, gcc gtc gac atg tct gtg agc gag or 3′/H11032 primer, gcc gga tcc att gat gaa agt aga (for TB-RBP and TB-RBP-204); 5′/H11032 primer, cac cat ggt gag caa gga and 3′/H11032 primer, gcc cag tta tct aga tcc ggt gga (for TRAX), respectively.

Cell Culture and Transfections—The day before transfection, TB-RBP-deficient MEFs were seeded at 1×10⁵ cells per multiwell six-well culture plate. For some transfections, spontaneously immortalized null MEFs were used to verify the potential of TB-RBP to stabilize TRAX. LipofectAMINE 2000 (Invitrogen, Rockville, MD) (2 l) and plasmid (1 µg) were preincubated with 50 l of Optimum I (Invitrogen) for 5 min and then gently vortexed and incubated at RT for 20 min. The total 100-µl mixture was added to each well of a 6-well plate, dropwise.

Whole cell lysates were harvested for Western blotting. For cell lines that stably express GFP/TB-RBP, GFP/TRAX, or GFP, transfections were performed with plasmid (3 µg) and pTK-Hyg (1 µg) (Clontech, Palo Alto, CA) combined with 8 µl of LipofectAMINE 2000. Transfected TB-RBP-deficient MEFs were split 72 h before selection with hygromycin B (Roche Applied Science, Indianapolis, IN). After 2 weeks, positive colonies were identified by fluorescence microscopy and picked using clone cylinders (Sigma, St. Louis, MO). To rescue slow growing early passage MEFs, passage 4–5 MEFs were plated onto 96-well plates at 1.5×10³ per well. The next day, the MEFs were infected with a ViraPower™ Lentiviral Expression System with constructs containing TB-RBP and TB-RBP-204, respectively (Invitrogen).

Western and Northern Blotting—Whole cell lysates from passage 4 MEFs were lysed in radioimmune precipitation assay buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and freshly added 1/10 volume of protease inhibitor mixture; Sigma, St. Louis, MO) in PBS, incubated on ice for 20 min, and centrifuged at 12,000 g for 10 min at 4 °C. Protein concentrations were quantitated with a BCA Protein Assay Kit (Pierce, Rockford, IL). Aliquots (20 µg) were mixed with 2× SDS loading buffer containing 0.35 µl 2-mercaptoethanol, subjected to 10% SDS-PAGE, and then transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The primary antibodies, mouse anti-α-tubulin and anti-β-actin (Sigma, St. Louis, MO), affinity purified anti-TB-RBP (6) and affinity purified anti-TRAX (20) were used. Horseradish peroxidase-conjugated anti-mouse IgG antibody, protein A, and

![FIG. 1](https://example.com/fig1.png)

**A.** TB-RBP-deficient MEFs grow more slowly than MEFs from wild type and heterozygous littermates. A, growth kinetics of primary MEFs. Passage 3 MEFs (5×10⁵) were cultured in 100-mm dishes and counted at successive times as indicated. Open diamonds, +/+; open squares, +/−; open triangles, −/−. B, transfection with TB-RBP corrects the slow growth rate. Early passage MEFs were infected with lentivirus containing TB-RBP or TB-RBP-204. The proliferation of the MEFs was measured 48 and 72 h after infection using the One Solution Proliferation Assay. The value 100% in the ordinate represents the proliferation rate of the control null MEFs. The asterisk indicates a significant difference for TB-RBP infection (p < 0.01) compared with the mock and TB-RBP-204 infection.
the ECL Detection Kit (Amersham Biosciences, Piscataway, NJ) were used to visualize each protein.

Total RNA from MEFs was purified, and aliquots (15 μg) were loaded in a 1.0% formaldehyde denaturing agarose gel, and Northern blotting was performed using the NorthernMax kit (Ambion, Austin, TX). DNA probes encoding the open reading frames (0.6 kb for TB-RBP and 0.8 kb for TRAX) were PCR-amplified using the following primers: TB-RBP, 5′-atg cggt tct ctt gcc ggc cag and 3′-gcc gtc cgg atg aag gaa gaa and 5′-gcc gaa gag and 3′-gcc gaa gag and 5′-gcc gag cgt ctc. The probes were labeled with Ready-To-Go DNA Labeling Beads and purified through ProQuant G-50 Micro Columns (Amersham Biosciences). Ribosomal RNA, stained with ethidium bromide, served as a loading control.

Ubiquitination Analyses—The day before transfection, TB-RBP-deficient MEFs were plated in 6-well plates and pEGFP/TRAX was delivered by LipofectAMINE 2000. Three hours after transfection, MG-132 (Calbiochem, La Jolla, CA) was added. Forty-eight hours later, whole cell lysates were prepared in radioimmune precipitation assay buffer containing freshly added protein inhibitor inhibitor mixture. In the studies examining the recovery of endogenous TRAX, MG-132 was added 24 h before the cells were harvested. Immunoprecipitations were performed as previously described (22). Briefly, the TB-RBP-deficient MEFs were lysed in immunoprecipitation buffer and incubated at 4°C for at least 30 min. The lysates were passed through a 20-gauge needle multiple times to shear genomic DNA, and the samples were clarified by centrifugation. The supernatants were pre-cleared with protein A-agarose beads (Invitrogen) at 4°C for 1 h. TRAX antibody (3 μg/ml) was added to the pre-cleared supernatant and incubated at 4°C overnight with shaking. Protein A-agarose beads were added for an additional 2 h, and the extracts were centrifuged at 2000 × g for 5 min. The agarose bead pellets were washed three times with PBS containing 0.1% Tween, 2× SDS loading buffer was added, and the samples were boiled for 10 min. Following electrophoresis in a 10% SDS gel, the proteins were transferred onto polyvinylidene difluoride membranes, and primary (rabbit anti-TRAX) and secondary (horseradish peroxidase-conjugated protein A) antibodies were added. The proteins were visualized with the ECL Detection System. To detect ubiquitinated TRAX, mouse anti-ubiquitin antibody (Zymed Laboratories Inc., San Francisco, CA) was used as the primary antibody.

Immunofluorescence—TB-RBP-deficient MEFs were grown on twowell chamber slides (Lab-Tek, Campbell, CA). Fluoresce micrographs of live cells or cells fixed with 4% freshly prepared paraformaldehyde were taken 24 h after transfection and counter stained with 4′,6-diamidino-2-phenylindole.

Double Thymidine Synchronization of HeLa Cells and Fluorescence-activated Cell Sorting Analysis—HeLa cells (ATCC, Manassas, VA) were grown in DME with 10% fetal bovine serum. When HeLa cells reached a sub-confluent density, 2 mM thymidine was added and the cells were grown for 19 h. The cells were then washed with pre-warmed PBS three times and grown 10 h in complete medium before adding 2 mM thymidine again. Seventeen hours later, the cells were washed three times, complete medium was added, and the cells were harvested. For the cell cycle analysis, the MEFs were trypsinized, resuspended in complete DMEM, centrifuged at 1500 rpm for 5 min, and washed twice with ice-cold PBS. The cells were resuspended in 1 ml of 20°C methanol and incubated for 30 min on ice. The cells were then centrifuged at 1500 rpm for 4°C for 5 min, supernatants were aspirated, and 400–500 μl of propidium iodide staining solution (0.15 mM propidium iodide, 0.1% Triton X-100, and 1 mg/ml RNase (Sigma, St. Louis, MO)) was added. Samples were sorted using an EPICS XL (Beckman-Coulter, Inc., Miami, FL).

Transgenic Expression of TRAX—A FLAG-tagged cDNA encoding the complete open reading frame of human TRAX (hTRAX) (~0.9 kb) was inserted between 1.4 kb of the mouse PGK2 promoter and a SV40 poly(A) tail sequence (~0.5 kb). The expression plasmid construct was amplified in E. coli and purified by CsCl gradient ultracentrifugation (23). The purified hTRAX DNA construct was injected into pronuclei of fertilized mouse eggs by the Transgenic and Chimeric Mouse Facility at the University of Pennsylvania. Mouse pups were genotyped by PCR using genomic DNA isolated from their tails. Six founder lines containing the transgene (lines 1, 6, 20, 34, 36, and 37) were selected and analyzed further for expression of the TRAX transgene in testes. Homozygous male mice were generated by breeding hemizygous males and females of line 36. Males and females were selected from different litters of founder line 36. Among male progeny that harbor the transgene, potential homozygous males were identified by the production of all hemizygous offspring in the next generation when mated with wild type female mice. Total RNA and cytoplasmic protein extracts were prepared from the testes of transgenic animals and used for Northern and Western blotting as previously described (6). Mouse testes were fixed in Bouin’s solution (Sigma, St. Louis, MO), and paraffin-mounted slides stained with hematoxylin and eosin were prepared by the Pathology Department of the Children’s Hospital at the University of Pennsylvania.

RNA Interference Treatment—siRNAs, designed to target mouse TB-RBP and TRAX mRNAs, were synthesized (Dharmacon, Lafayette, CO). The criteria for designing and methodology to introduce the siRNAs into cells was as described previously (24, 25). Briefly, for each 96-well assay, the day before transfection 4–5 × 10^5 cells in 100 μl were plated in each well; LipofectAMINE 2000 (1 μl) and 2 μl of a 40 μM stock solution of siRNA were preincubated with 100 μl of Optimun I separately for 5 min, then mixed and vortexed gently and incubated at 37°C for an additional 20 min. The mixture was then evenly added to 96-well plates (30 μl per well in triplicate). Forty-eight hours later, cell proliferation was analyzed using the One Solution Proliferation Assay (Promega, Madison, WI). The siRNAs used in this report are as follows: TB-RBP1, aatgctgagacaggaatgct; TRAX1, aatcatttcagcaggagctgg; TRAX2, aagagctgaggcttgct; and GFP, aatgctgagacaggaatgct.

**RESULTS**

**TB-RBP-deficient MEFs Show a Reduced Growth Rate**—TB-RBP-deficient MEFs were derived from 14-day embryos produced by mating mice heterozygous for the TB-RBP gene. Within 4 days of culture, MEFs from TB-RBP null embryos exhibited a reduced growth rate compared with their wild type and heterozygous littermates (Fig. 1A).

The slower growth rate of TB-RBP-deficient MEFs resulted from the loss of TB-RBP as it could be reversed by the reintroduction of TB-RBP at 48 h post-transfection (left three columns, Fig. 1B) and 72 h post-transfection (right three columns, Fig. 1B) post-infection. This rescue required the full-length TB-RBP, because a truncated form of TB-RBP, TB-RBP-204, which lacks the C terminus and cannot form multimers (32), was unable to correct the cell proliferation deficiency of the null MEFs (Fig. 1B).

**TB-RBP-deficient MEFs Show a Coordinate Loss of TRAX**—Among the TB-RBP-interacting proteins, Translin-associated protein X (TRAX) is its most closely related protein (16). As
seen in tissues isolated from TB-RBP null mice (20). TB-RBP and TRAX protein levels were reduced to about 50% or to non-detectable levels in multiple cultures of MEFs from heterozygous or null littermates, respectively (Fig. 2A). Similar reductions in TB-RBP mRNA levels were seen in the MEFs from heterozygous and null littermates (Fig. 2B). However, no decreases in TRAX mRNA levels were seen in the same total RNA preparations from wild type, heterozygous or null MEFs, suggesting that the reduction of TRAX protein occurred post-transcriptionally (Fig. 2B).

TRAX Is Degraded in MEFs by the Ubiquitination Pathway—MG-132 is a cell-permeable proteasome inhibitor that reduces the degradation of ubiquitin-conjugated proteins in mammalian cells (26, 27). When TB-RBP null MEFs were cultured in the presence of MG-132, TRAX protein became detectable (Fig. 3). After forty-eight hours in culture, substantial levels of both transfected TRAX-GFP and endogenous TRAX were detected compared with untreated control cells (Fig. 3A). At MG-132 concentrations of 500 nM, massive cell loss was seen. In the control cells and cells incubated with MG-132, a putative breakdown product of TRAX was seen (see arrowhead in Fig. 3A). In addition to endogenous TRAX, null MEFs incubated with high concentrations of MG-132 contain slower migrating forms of TRAX (Fig. 3B). The two slower migrating forms of TRAX have estimated molecular masses of about 8 and 16 kDa greater than the 33-kDa molecular mass of TRAX. An additional high molecular weight aggregate of TRAX is often seen in Western blots (left panel, Fig. 3C). The incremental increases of about 8 kDa and the aggregate of TRAX suggest that we could be detecting ubiquitinated forms of TRAX.

To confirm that the slower migrating forms of TRAX are ubiquitinated, an aliquot of the extract analyzed in Fig. 3C was immunoprecipitated with an affinity-purified TRAX antibody and immunoblotted with an ubiquitin antibody. Two ubiquitinated forms of TRAX and a polyubiquitinated high molecular form of TRAX bands were detected (right panel, Fig. 3C).

Reintroduction of Full-length TB-RBP Stabilizes TRAX—Immunoprecipitation and yeast two-hybrid assays have demonstrated protein-protein interactions between full-length TB-RBP and full-length TRAX but not between truncated forms of TB-RBP or TRAX (28). An intact leucine zipper in TB-RBP is essential for homo-oligomer and hetero-oligomer formation. To determine whether loss of TRAX protein was directly caused by the absence of TB-RBP protein in the MEFs from TB-RBP null mice, a construct encoding a fusion protein of the full-length TB-RBP sequence and GFP was transfected into TB-RBP-deficient MEFs (Fig. 4A). We reasoned that the expression of exogenous TB-RBP would stabilize endogenous TRAX in the TB-RBP-deficient cells. Concomitant with the appearance of TB-RBP 24 h after transfection, TRAX was detected and continued to increase 72 h after transfection (Fig. 4B). No stabilization was seen with an equally abundant GFP transfection control.

To confirm that the reappearance of TRAX required interaction between TB-RBP and TRAX, additional transfections into
cytomegalovirus promoter, also were unable to stabilize endogenous TRAX (data not shown).

In addition to being able to rescue TRAX in TB-RBP-deficient cells by transient transfection, TRAX was also detectable in a stably transfected TB-RBP null MEF cell line expressing a GFP fusion form of wild type TB-RBP (Fig. 4D). This rescue was specific for TB-RBP, because no TRAX was detected in a control stably transfected cell line expressing GFP (Fig. 4D). The reappearance of TRAX protein in these TB-RBP null MEFs appears to be regulated post-transcriptionally, because, although a high level of TB-RBP mRNA was induced, the endogenous TRAX mRNA level did not increase in the stably transfected cells (Fig. 4E). These data indicate that TRAX stabilization requires direct interaction between TRAX and TB-RBP.

**TB-RBP and TRAX Colocalize**—Immunofluorescence of transfected cells confirms that full-length, but not truncated forms of TB-RBP, interact with endogenous TRAX (Fig. 5). Full-length TB-RBP expressed in null MEFs is primarily concentrated in the perinuclear region often forming punctate structures whereas TB-RBP-204 is widely dispersed in cells without visible punctate structures (Fig. 5A). Our previous studies indicated TRAX colocalized with Golgi-associated proteins in the perinuclear regions of male germ cells (29). Full-length TRAX (red) and full-length TB-RBP (green) colocalized in both live (upper panels) and fixed null MEFs following transfection (lower panels) (Fig. 5B).

The Level of TRAX Protein Closely Parallels TB-RBP throughout the Cell Cycle—In HeLa cells, TB-RBP mRNA levels have been demonstrated to be cell cycle-dependent while TRAX mRNA levels are not (30). Our findings that TRAX protein stability is dependent upon TB-RBP levels suggest that they will be coordinately regulated during the cell cycle. To test this, we have used Western blotting to analyze a panel of proteins in synchronized HeLa cells (Fig. 6). HeLa cells were released from a double thymidine block, and aliquots of cells with changing populations of G1/G0, S phase, and G2/M cells

---

**Fig. 4.** TRAX returns in TB-RBP-deficient MEFs upon reintroduction of TB-RBP. A, schematic representation of TB-RBP protein. TB-RBP-175 and TB-RBP-204 represent truncated forms of TB-RBP with a partial deletion of the leucine zipper domain, respectively. TB-RBP and TRAX interact with endogenous TRAX (Fig. 5). Similar results were obtained following immunoprecipitation of TRAX from extracts containing full-length and truncated forms of TB-RBP (data not shown). Short-term transfections into null MEFs expressing TRAX alone from pEGFP/TRAX or pCI-TRAX, a mammalian expression vector with a cDNA for GFP, were transfected into TB-RBP-deficient MEFs expressing TRAX alone from pEGFP/TRAX. After 24 h of transfection, the photographs were taken under fluorescence microscopy. B, colocalization of TB-RBP and TRAX in TB-RBP-deficient MEFs. pDsRed/TRAX (red) and pEGFP/TB-RBP (green) were cotransfected into TB-RBP-deficient MEFs. Forty-eight hours after transfection, cells were fixed and stained with 4',6-diamidino-2-phenylindole. Upper panels, live cells; lower panels, cells fixed with 4% paraformaldehyde. Scale bar, 20 μm.

---

**Post-transcriptional Regulation of TRAX**

12609

---

**Fig. 5.** Colocalization of TB-RBP and TRAX. A, expression patterns of full-length and truncated TB-RBP. Left panel, cells transfected with pEGFP; middle panel, cells transfected with pEGFP/TB-RBP; right panel, cells transfected with pEGFP/TB-RBP-204. After 24 h of transfection, the photographs were taken under fluorescence microscopy. B, colocalization of TB-RBP and TRAX in TB-RBP-deficient MEFs. pDsRed/TRAX (red) and pEGFP/TB-RBP (green) were cotransfected into TB-RBP-deficient MEFs. Forty-eight hours after transfection, cells were fixed and stained with 4',6-diamidino-2-phenylindole. Upper panels, live cells; lower panels, cells fixed with 4% paraformaldehyde. Scale bar, 20 μm.

---

**Fig. 6.** Western blotting to analyze a panel of proteins in synchronized HeLa cells. HeLa cells were released from a double thymidine block, and aliquots of cells with changing populations of G1/G0, S phase, and G2/M cells...
were collected (Fig. 6A). Western blotting revealed TRAX protein levels mirrored TB-RBP throughout the cell cycle with both showing reduced levels at G2/M (Fig. 6B). Assaying the blots for control marker proteins revealed that the level of PCNA increases following thymidine release, maximal expression of cyclin B was seen in G2/M, and tubulin and actin levels are constant (Fig. 6B). Quantitation of the TB-RBP and TRAX protein bands in Fig. 6B is shown in Fig. 6C. These data demonstrate that, despite major differences in TB-RBP and TRAX mRNA levels throughout the cell cycle, the protein levels are closely coordinated.

**TRAX Cannot Be Overexpressed in the Testes of Transgenic Mice**—To examine the relationship between TRAX and TB-RBP in an in vivo model, TRAX-expressing transgenic mice were created using the testis-specific PGK-2 promoter (31). PGK-2 is first expressed in late stage meiotic germ cells and is most abundant in the post-meiotic round spermatids. Six different founder lines (lines 1, 6, 20, 34, 36, and 37) carrying a FLAG-tagged human TRAX transgene were identified by PCR genotyping.

Exogenous hTRAX transcripts of 1.4 kb and endogenous TRAX mRNAs of 2.4 kb were detected from the total testis RNA of three hemizygous founder lines (lines 6, 20, and 36) and a homozygous male derived from line 36 (Fig. 7A). Low levels of exogenous TRAX mRNA were detected in testes from lines 1, 34, and 37, and males from founder lines 6 and 36 expressed exogenous mRNA levels comparable to endogenous TRAX mRNA levels. Immunoblot analysis showed that the slower migrating FLAG-tagged exogenous hTRAX proteins were also expressed in the testes of some of the founders (Fig. 7B). In general, the levels of exogenous TRAX in the hemizygous transgenic males were low and often not representative of the mRNA levels (see founder lines 6 and 36). Quantitation of the TRAX immunoblots revealed that the combined amounts of endogenous and exogenous TRAX proteins were similar to the TRAX protein level in testes of wild type mice (Fig. 7C). The constancy of the total TRAX protein is especially evident in the homozygous male where the endogenous TRAX protein decreased in proportion to exogenous TRAX (Fig. 7B). No change was seen in

---

**Fig. 6.** TRAX follows TB-RBP levels throughout the cell cycle. A, HeLa cells were released from a double thymidine block, and cells were collected for 14 h at 2-h intervals. During this period of time the cells advanced from G1 through S and G2/M phases. B, aliquots of cells from each time point indicated in A were analyzed by Western blotting for a panel of proteins. C, quantitation of each protein band from B was performed by densitometry using ImageQuaNT software.
the level of TB-RBP in the homozygous male, suggesting that "extra" TRAX that could not be protected by TB-RBP was degraded in the germ cells of the transgenic mice. Consistent with the normal ratio of the TB-RBP-TRAX proteins in the testes, all of the transgenic male mice were fertile and histological sections of their testes revealed normal spermatogenesis (data not shown). Thus, we see in an *in vivo* model the amount of TB-RBP controls the amount of TRAX protein as we have seen in transfected TB-RBP-deficient MEFs (Fig. 4).

**TB-RBP and TRAX Are Both Essential for Normal Cell Proliferation**—We have demonstrated that the absence of TB-RBP in MEFs leads to a reduced rate of cell proliferation and to the loss of TRAX. The reintroduction of TB-RBP leads to normal cell proliferation while also stabilizing TRAX. To determine whether TB-RBP or TRAX or both proteins influence cell proliferation, we have selectively reduced TB-RBP or TRAX with specific siRNAs introduced into NIH 3T3 cells. Consistent with our findings comparing null and heterozygous MEFs, a reduction of TB-RBP leads to a reduced level of TRAX (Fig. 8A, lane 5). Although reduction of TRAX protein with two different siRNA sequences does not lead to a reduction of TB-RBP (Fig. 8A, lanes 3 and 4), the transient reduction of either TB-RBP or TRAX with specific siRNAs reduces the cellular proliferation rates of the NIH 3T3 cells compared with control and GFP siRNA-transfected cells. This

---

**Fig. 7.** TRAX levels are maintained at a constant level in transgenic mice. A, Northern blot of TRAX expression in transgenic and wild type mice. Total testis RNAs (20 μg) were hybridized with a PCR-amplified mouse TRAX coding region probe to detect exogenous (~1.4 kb) and endogenous (~2.4 kb) TRAX mRNAs. B, immunoblot analysis of TRAX and TB-RBP in transgenic and wild type mice. TB-RBP and TRAX were detected in testis cytoplasmic extracts (100 μg) by Western blotting. C, quantitation of total endogenous and exogenous TRAX from three independent Western blots was performed by densitometry using ImageQuanNT software.
reduction of growth rate was statistically significant in three independent experiments (Fig. 8B). These data suggest that TB-RBP is essential in regulating the amount of TRAX in cells and both proteins can influence cell proliferation.

DISCUSSION

TB-RBP/Translin is ubiquitously expressed in the mouse and has been proposed to function as both a DNA- and RNA-binding protein suggesting multiple functions. Its associated partner TRAX, first characterized as a Translin-like protein (16), forms DNA- and RNA-binding complexes with Translin (17, 18) and has been proposed to function in DNA repair in conjunction with the nuclear matrix protein C1D (19).

The level of TRAX in both cultured cells and in vivo appears to be exquisitely dependent upon the level of TB-RBP. As seen in TB-RBP null mice (20), TRAX is absent in MEFs from TB-RBP null mice and is reduced to 50% in heterozygous MEFs, despite normal TRAX mRNA levels. Except for the coordinated loss of TRAX, other TB-RBP-interacting partners such as the kinesin KIF17b (data not shown), the TER ATPase, and a cytoskeletal γ-actin are present at normal levels in null MEFs (28). Using MG-132, a reversible proteasome inhibitor (27, 32), we have been able to stabilize endogenous and transfected TRAX in the absence of TB-RBP for short periods of time. GFP fusion forms of TRAX are more stable than TRAX expressed alone. Our detection of slower migrating forms of ubiquitinated TRAX suggests that the loss of TRAX in the absence of TB-RBP occurs by degradation in proteasomes. We don’t know how TRAX binding to TB-RBP prevents this process, but ubiquitinated bands of TRAX are detected when we incubate wild type MEFs with MG-132, suggesting that endogenous TRAX levels are also regulated by ubiquitination in wild type mouse cells (data not shown).

Conversely, chloroquine, a lysosome inhibitor, did not stabilize TRAX in TB-RBP-deficient MEFs under similar experimental conditions (data not shown).

Reintroduction of TB-RBP into null MEFs stabilizes TRAX protein with no increase in TRAX mRNA level. A truncated form of TB-RBP, TB-RBP-204, which cannot form homo-oli-
Post-transcriptional Regulation of TRAX

...does not stabilize TRAX. Consistent with a need for protein-protein interaction, yeast two-hybrid and glutathione S-transferase fusion protein assays (28) and the colocalization of TB-RBP and TRAX in TB-RBP null MEFs (Fig. 5) provide compelling evidence for direct interaction between these proteins. The coordinate expression of TB-RBP and TRAX is also seen throughout the cell cycle in synchronized HeLa cells (Fig. 6). Although microarray studies with HeLa cells reveal that TB-RBP mRNA level fluctuates through the cell cycle while TRAX mRNA stays constant (30), we find that the protein level of TRAX mirrors closely the protein pattern of TB-RBP (Fig. 6), perhaps with “extra” TRAX being removed by ubiquitination. Our inability to alter TRAX levels in the testis by transgenesis further suggests a close stoichiometric interaction between TB-RBP and TRAX. Although some transgenic mice express similar levels of exogenous and endogenous TRAX mRNAs, no increase in TOTAL TRAX protein was seen. In fact, there was a compensatory decrease in endogenous TRAX protein for the additional transgenic TB-RBP and TRAX. Our RNA interference experiments suggest that a given amount of TB-RBP can stabilize only a given amount of TRAX. Quantitation of TB-RBP and TRAX in extracts of male germ cells has revealed equimolar amounts of the two proteins (data not shown), consistent with the loss of “extra” TRAX.

Imbalances between TB-RBP and TRAX appear detrimental and affect cell proliferation. Mice lacking TB-RBP are born 10–30% smaller than their wild type or heterozygous littermates and exhibit a wide range of deficiencies, including reduced fertility and behavioral abnormalities (20). Consistent with the smaller size of the TB-RBP-deficient mice, MEFs derived from the TB-RBP null embryos show decreased proliferation. Fluorescence-activated cell sorting analysis indicates this reduction in proliferation is caused by a build-up of cells in the G2 phase of the cell cycle (data not shown). In addition to the reduced cell proliferation seen in the MEFs lacking TB-RBP (and presumably other somatic cells from TB-RBP null mice), this proliferation deficiency may be the cause of the large loss of germ cells in male TB-RBP null mice (20).

Translin, the human orthologue of TB-RBP, has been proposed to be involved in chromosomal translocation breaks and binding to genomic hotspots (1). As part of this study, we have challenged the TB-RBP null MEFs with mitomycin C (to 800 ng/ml), bleomycin (to 19 μM), UV (to 400 J/m2), and x-irradiation (to 4 Gy), but were unable to detect any differences between wild type and TB-RBP null MEFs in terms of cell survival or number of DNA breaks and gaps (data not shown). Similarly, TB-RBP-deficient mice show normal B and T cell development suggesting an alternative mechanism exists for the proposed involvement of Translin in Ig/TCR rearrangements (20). We propose that, in mouse somatic cells such as the MEFs, TB-RBP and TRAX primarily play essential roles in cell proliferation and cytokinesis.

In addition to the reduced cell proliferation of MEFs, the loss of TB-RBP leads to deficiencies in fertility, behavior, skin, and growth in mice (20) At the cellular level, reductions of TB-RBP protein lead to equivalent reductions of TRAX, raising the question whether the cellular and animal phenotypes we see result from loss of TB-RBP, loss of TRAX, or loss of both TB-RBP and TRAX. Our RNA interference experiments suggest both proteins influence cell growth. As expected, when TB-RBP is decreased in NIH 3T3 cells, we see a compensatory decrease in TRAX (Fig. 8). This relationship is not reciprocal, because no decrease in TB-RBP protein level is seen when TRAX protein is reduced. Reduced cell proliferation is also seen in long-term cultures of wild type MEFs, which contain lower amounts of TB-RBP (data not shown). Interestingly, the reduction of either TB-RBP (and TRAX) alone causes a reduction in cell proliferation. The effect on cell proliferation following either TB-RBP or TRAX reduction is suggestive of the importance for a complex of the two proteins. Transient transfections with TRAX into TB-RBP null MEFs cause the cells to lose viability suggesting TRAX alone may be toxic and TB-RBP is needed to both stabilize and neutralize TRAX toxicity. The dependence of TRAX on TB-RBP protein appears to be evolutionarily conserved from Schizosaccharomyces pombe to humans, because both proteins are widely expressed. Because increased levels of TRAX may be deleterious to normal cell survival, mutations or translocations that stabilize TRAX protein without TB-RBP interaction may lead to genetic disorders. For instance, intergenic splicing in the human TRAX locus (chromosome 1q42), resulting in fusion transcripts of TRAX and DISC1 (disrupted in schizophrenia 1), has been implicated in inherited schizophrenia (34). Therefore, to prevent the formation of unregulated “stable” forms of TRAX that do not interact with TB-RBP, perhaps a failsafe mechanism for degradation of TRAX in the absence of TB-RBP has evolved. In addition to precisely regulating TRAX levels, TB-RBP and TRAX interactions appear to be required for the intracellular distribution of the proteins, because TRAX contains a functional nuclear localization signal and TB-RBP a functional nuclear export signal. We also cannot exclude the possibility that TB-RBP functions as a chaperone for the proper folding of TRAX (35, 36). We conclude that TRAX levels are stringently imbalanced between wild type and TB-RBP null MEFs in terms of cell survival. Mutations or translocations that stabilize TRAX protein may be deleterious to normal cell survival, raising the importance of TB-RBP to both stabilize and neutralize TRAX toxicity. The dependence of TRAX on TB-RBP protein appears to be evolutionarily conserved from Schizosaccharomyces pombe to human, because both proteins are widely expressed. Because increased levels of TRAX may be deleterious to normal cell survival, mutations or translocations that stabilize TRAX protein without TB-RBP interaction may lead to genetic disorders. For instance, intergenic splicing in the human TRAX locus (chromosome 1q42), resulting in fusion transcripts of TRAX and DISC1 (disrupted in schizophrenia 1), has been implicated in inherited schizophrenia (34). Therefore, to prevent the formation of unregulated “stable” forms of TRAX that do not interact with TB-RBP, perhaps a failsafe mechanism for degradation of TRAX in the absence of TB-RBP has evolved. In addition to precisely regulating TRAX levels, TB-RBP and TRAX interactions appear to be required for the intracellular distribution of the proteins, because TRAX contains a functional nuclear localization signal and TB-RBP a functional nuclear export signal. We also cannot exclude the possibility that TB-RBP functions as a chaperone for the proper folding of TRAX (35, 36). We conclude that TRAX levels are stringently regulated by TB-RBP at a post-transcriptional level and TB-RBP and TRAX are closely interacting partners that both contribute to cell proliferation.

Acknowledgments—We thank Dr. J. McCarrey (University of Texas, San Antonio) for providing the PGK-2 promoter and Dr. Jean Richa (Transgenic Facility of the University of Pennsylvania) for producing the TRAX transgenic mice.

REFERENCES

1. Aoki, K., Suzuki, K., Sagano, T., Tatsuka, T., Nakahara, K., Kuge, O., Omori, A., and Kasai, M. (1995) Nat. Genet. 10, 167–174

2. Kasai, M., Matsuzaki, T., Katayanagi, K., Omori, A., Mazzarri, R. T., Stremlinger, J. L., Aoki, K., and Suzuki, K. (1997) J. Biol. Chem. 272, 11402–11407

3. Kwon, Y. K., and Hecht, N. B. (1993) Mol. Cell. Biol. 13, 6547–6557

4. Morales, C. R., Lefrancois de, C., Chennathukuzhi, V., El-Alfy, M., Wu, X., Yang, J., Gerton, G. L., and Hecht, N. B. (2002) Dev. Biol. 246, 480–494

5. Aoki, K., Inazawa, J., Takahashi, T., Nakahara, K., and Kasai, M. (1997) Genomics 43, 237–241

6. Wu, X. Q., Gu, W., Meng, X., and Hecht, N. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5640–5645

7. VanLoock, M. S., Yu, X., Kasai, M., and Engelman, E. H. (2001) J. Struct. Biol. 1358, 55–66

8. Pascal, J. M., Hart, P. J., Hecht, N. B., and Robertus, J. D. (2002) J. Mol. Biol. 319, 1049–1057

9. Gu, W., Wu, X. Q., Meng, X. H., Morales, C., El-Alfy, M., and Hecht, N. B. (1998) Mol. Reprod. Dev. 49, 219–228

10. Han, J. R., Wu, G., and Hecht, N. B. (1995) Biol. Reprod. 53, 707–717

11. Yang, J., Chennathukuzhi, V., Miki, R., O’Brien, D. A., and Hecht, N. B. (2003) Biol. Reprod. 68, 853–859

12. Han, J. R., Yu, G. K., and Hecht, N. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9550–9554

13. Wu, X. Q., Meng, X. H., Morales, C., El-Alfy, M., and Hecht, N. B. (2000) Mol. Reprod. Dev. 62, 720–725

14. Chennathukuzhi, V., Morales, C. E. M., and Hecht, N. B. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 15566–15571

15. Ishida, R., Okado, H., Sato, H., Shimori, C., Aoki, K., and Kasai, M. (2002) FEBS Lett. 525, 105–110

16. Aoki, K., Ishida, R., and Kasai, M. (1997) FEBS Lett. 401, 109–112

17. Chennathukuzhi, V., Kurbahra, Y., Bray, J. D., and Hecht, N. B. (2001) J. Biol. Chem. 276, 13256–13263

18. Finkenstadt, P. M., Kang, W. S., Jeon, M., Taira, E., Tang, W., and Baraban, M. (2000) J. Neurochem. 74, 1754–1762

19. Erdemir, T., Bilican, B., Oncel, D., Goding, C. R., and Yavuzer, U. (2002) J. Cell Sci. 115, 207–216

20. Chennathukuzhi, V., Steen, J. M., Abel, T., Donlon, S., Yang, S., Miller, J. P., Allman, D. M., Simmons, R. A., and Hecht, N. B. (2003) Mol. Cell. Biol. 23, 6419–6434

21. Hooper, M. C. (1987) in Teratocarcinomas and Embryonic Stem Cells: A
Post-transcriptional Regulation of TRAX

22. Yang, S., Sun, Y., and Zhang, H. (2001) *J. Biol. Chem.* 276, 4889–4893
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor
24. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) *Nature* 411, 494–498
25. Yang, S., Tutton, S., Pierce, E., and Yoon, K. (2001) *Mol. Cell Biol.* 21, 7807–7816
26. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994) *Cell* 78, 761–771
27. Jensen, T. J., Loo, M. A., Pind, S., Williams, D. B., Goldberg, A. L., and Ricordan, J. R. (1995) *Cell* 83, 129–135
28. Wu, X. Q., Lefrancois, S., Morales, C. R., and Hecht, N. B. (1999) *Biochemistry* 38, 11261–11270
29. Bray, J. D., Chennathukuzhi, V. M., and Hecht, N. B. (2002) *Genomics* 79, 799–808
30. Whitfield, M. L., Sherlock, G., Saldanha, A. J., Murray, J. I., Ball, C. A., Alexander, K. E., Matese, J. C., Perou, C. M., Hurt, M. M., Brown, P. O., and Botstein, D. (2002) *Mol. Biol. Cell* 13, 1977–2000
31. McCarrey, J. R. (1987) *Gene (Amst.)* 61, 291–298
32. Wiertz, E. J., Jones, T. R., Sun, L., Bogyn, M., Geuze, H. J., and Ploegh, H. L. (1996) *Cell* 84, 769–779
33. Wu, X. Q., Xu, L., and Hecht, N. B. (1998) *Nucleic Acids Res.* 26, 1675–1680
34. Millar, J. K., Christie, S., Semple, C. A., and Porteous, D. J. (2000) *Genomics* 67, 69–77
35. Hartl, F. U., and Hayer-Hartl, M. (2002) *Science* 295, 1852–1858
36. Nicola, A. V., Chen, W., and Helenius, A. (1999) *Nat. Cell Biol.* 1, 341–345