



# A novel thyroid hormone receptor isoform, TR $\beta$ 2-46, promotes SKP2 expression and retinoblastoma cell proliferation

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Retinoblastoma is a childhood retinal tumor that develops from cone photoreceptor precursors in response to inactivating *RB1* mutations and loss of functional RB protein. The cone precursor's response to RB loss involves cell type-specific signaling circuitry that helps to drive tumorigenesis. One component of the cone precursor circuitry, the thyroid hormone receptor  $\beta$ 2 (TR $\beta$ 2), enables the aberrant proliferation of diverse RB-deficient cells in part by opposing the down-regulation of S-phase kinase-associated protein 2 (SKP2) by the more widely expressed and tumor-suppressive TR $\beta$ 1. However, it is unclear how TR $\beta$ 2 opposes TR $\beta$ 1 to enable SKP2 expression and cell proliferation. Here, we show that in human retinoblastoma cells TR $\beta$ 2 mRNA encodes two TR $\beta$ 2 protein isoforms: a predominantly cytoplasmic 54-kDa protein (TR $\beta$ 2-54) corresponding to the well-characterized full-length murine Tr $\beta$ 2 and an N-terminally truncated and exclusively cytoplasmic 46-kDa protein (TR $\beta$ 2-46) that starts at Met-79. Whereas TR $\beta$ 2 knockdown decreased SKP2 expression and impaired retinoblastoma cell cycle progression, re-expression of TR $\beta$ 2-46 but not TR $\beta$ 2-54 stabilized SKP2 and restored proliferation to an extent similar to that of ectopic SKP2 restoration. We conclude that TR $\beta$ 2-46 is an oncogenic thyroid hormone receptor isoform that promotes SKP2 expression and SKP2-dependent retinoblastoma cell proliferation.

Cancers are caused by abnormalities in oncogenes or tumor-suppressor genes that initiate and advance tumorigenesis. At the initiation step, cell type-specific circuitry may sensitize cells to the initial oncogenic insult. Understanding how cell type-specific circuitry sensitizes to oncogenic changes may enable rational cancer prevention and treatment approaches.

Retinoblastoma is a childhood retinal tumor that has provided insights into the role of cell type-specific circuitry in tumor initiation (1). Most retinoblastomas are thought to arise from cone photoreceptor precursors in response to biallelic inactivation of the *RB1* gene and loss of functional RB protein (2, 3). Human cone precursor circuitry may sensitize to *RB1* mutation via intrinsic high expression of oncoproteins, such as MYCN and MDM2, and cone lineage transcription factors, such as retinoid X receptor- $\gamma$  (RXR $\gamma$ )<sup>3</sup> and thyroid hormone receptor  $\beta$ 2 (TR $\beta$ 2) (2, 4). RXR $\gamma$  and TR $\beta$ 2 normally mediate cone photoreceptor differentiation (5, 6) but promote cone precursor proliferation and retinoblastoma genesis after RB loss (2, 4). RXR $\gamma$  enables retinoblastoma cell survival in part by inducing MDM2 expression via a human-specific *MDM2* promoter element (4). However, the oncogenic role of TR $\beta$ 2 is enigmatic.

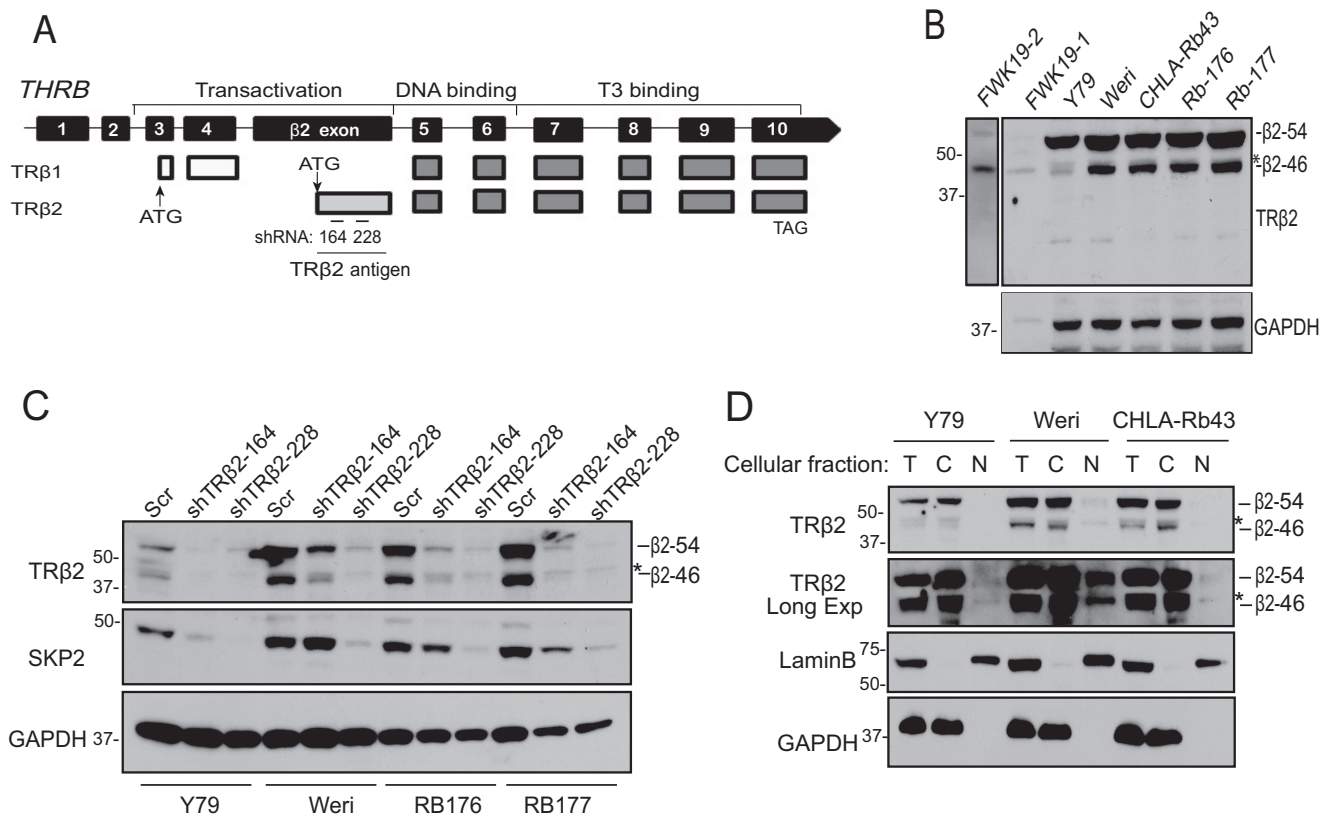
TR $\beta$ 2 is highly expressed in a limited number of cell types, including cone photoreceptor precursors, pituitary cells, and cochlear hair cells (7–9), each of which aberrantly proliferates in response to RB loss (2, 10, 11). Indeed, TR $\beta$ 2 is required for proliferation of retinoblastoma cells and enhances growth of *Rb1*-null mouse pituitary tumors, whereas ectopic TR $\beta$ 2 enabled proliferation of RB-depleted neuroblastoma cells (12). TR $\beta$ 2 appears to promote RB-deficient human retinoblastoma as well as Rb-deficient mouse pituitary tumors by antagonizing the highly related, more widely expressed, and tumor-suppressive thyroid hormone receptor TR $\beta$ 1 (12, 13). TR $\beta$ 2 and TR $\beta$ 1 are produced from the same *THRB* gene but use alternative transcriptional promoters and N-terminal coding exons (8, 14). They both have canonical nuclear hormone receptor structure with an N-terminal “A/B” corepressor and coactivator–

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<sup>3</sup> The abbreviations used are: RXR $\gamma$ , retinoid X receptor- $\gamma$ ; TR $\beta$ , thyroid hormone receptor  $\beta$ ; SKP2, S-phase kinase-associated protein 2; T<sub>3</sub>, triiodothyronine; PI, phosphatidylinositol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; uORF, upstream open reading frame; BN, bidirectional EFl $\alpha$ -Neo.



**Figure 1. Two predominantly cytoplasmic TRβ2 isoforms in human retinoblastoma and fetal retina cells.** *A*, *THRB* structure (top) and distinct N-terminal TRβ2 and TRβ1 coding sequences (bottom). “ATG” indicates previously identified translation start sites. Positions of TRβ2 shRNAs and antibody immunogen are indicated. *B*, Western blot of TRβ2 and GAPDH loading control in retinoblastoma cells and week 19 retinae. The positions of molecular weight markers (left) and TRβ2-46, the nonspecifically recognized \*, and TRβ2-54 are indicated. *C*, Western blot of TRβ2, SKP2, and GAPDH loading control in the indicated cell lines after transduction with TRβ2 shRNAs (*shTRβ2-164* and *shTRβ2-228*) or a nontargeting control (*Scr*) at day 5 postinfection. *D*, Western blot of TRβ2, nuclear marker Lamin B, and cytoplasmic marker GAPDH in total cell lysate (T) or cytoplasmic (C) or nuclear (N) fractions of Y79, Weri-1, and CHLA-RB43.

binding domain, a central DNA-binding domain, and a C-terminal T<sub>3</sub>-binding domain (Fig. 1A). However, their distinct A/B domains mediate distinct interactions and effects (15–17), including enhanced TRβ2-mediated transactivation (18). Additionally, TRβ1 can inhibit PI 3-kinase signaling (19, 20) and was found to suppress liver and mammary tumors via induction of the NCoR transcriptional corepressor (21). Meanwhile, TRβ2 was found to oppose a TRβ1-dependent down-regulation of the F-box protein SKP2 (12), which is required for production of RB-deficient tumors (22, 23). However, it is unclear how TRβ2 opposes TRβ1 to enhance SKP2 expression and cell proliferation. Here, we report that these functions are mediated by a novel N-terminally truncated and cytoplasm-localized TRβ2 isoform.

## Results

### A novel TRβ2 isoform in retinoblastoma and fetal retina

We initially examined TRβ2 expression in retinoblastoma cell lines Y79, WERI-1, RB176, RB177, and CHLAVC-RB43. Western blotting with a TRβ2 antibody (Fig. 1A) revealed two major species, here referred to as TRβ2-54 and TRβ2-46, with apparent molecular masses of 65 and 56 kDa (Fig. 1B). TRβ2-54 predominated in Y79, whereas both TRβ2-54 and TRβ2-46 were prominent in the other lines. The TRβ2 antibody also detected several minor species, including one referred to as “\*,”

migrating slightly behind TRβ2-46 (Fig. 1B). TRβ2-54 and TRβ2-46 were confirmed to be TRβ2 isoforms based on their down-regulation in four retinoblastoma cell lines after transduction with TRβ2 shRNAs (Fig. 1C). In contrast, \* was unaffected by TRβ2 shRNAs and deemed to represent a cross-reacting protein or a TRβ2 species that resists knockdown.

Because retinoblastomas are derived from cone precursors (2, 3) and TRβ2 is solely detected in cone precursors in the developing human retina (4, 24), we examined whether TRβ2-54 and TRβ2-46 are expressed in the cone precursor cell of origin. In Western blots, the main TRβ2 species in developing retina comigrated with retinoblastoma cell TRβ2-46, whereas a less abundant species comigrated with TRβ2-54 (Fig. 1B). Because of unavoidable sample limitations, a lower amount of fetal retina protein was loaded, and all bands, including the GAPDH loading control, migrated more slowly than their counterparts in retinoblastoma samples. A similar ratio of TRβ2-46 and TRβ2-54 comigrating species was detected in three retinae (Fig. 1B and data not shown). The high TRβ2-46 and low TRβ2-54 in human retina differ from what was seen in mouse retina where only one specifically recognized species was reported (25). As cones comprise ~2–3% of human retinal cells, TRβ2-46 and TRβ2-54 are more highly expressed in cone precursors than appears from analyses of whole-retina lysates.

**Exclusive cytoplasmic localization of TR $\beta$ 2-46**

TR $\beta$ 1 is mainly detected in the nucleus but can shuttle between cytoplasmic and nuclear compartments (26, 27) and undergo T<sub>3</sub>-induced cytoplasm-to-nucleus translocation (20). TR $\beta$ 2 is also thought to be mainly nuclear (26); however, by immunostaining, TR $\beta$ 2 was perinuclear or cytoplasmic in later stages of mouse cone differentiation (25) and was mainly cytoplasmic in human cone precursors and retinoblastoma cells (2, 4, 24). To define the subcellular localization of the different TR $\beta$ 2 isoforms, retinoblastoma cells were subjected to cytoplasmic and nuclear fractionation and TR $\beta$ 2 immunoblotting. Separation of nuclear and cytoplasmic components was confirmed by detection of GAPDH solely in cytoplasmic fractions and Lamin B in nuclear fractions (Fig. 1D). As in past immunostaining analyses, the vast majority of TR $\beta$ 2-46 and TR $\beta$ 2-54 were in the cytosol in three retinoblastoma cell lines (Fig. 1D). However, after long exposures, TR $\beta$ 2-54 and the nonspecific \* species were also detected in nuclear fractions, whereas the more rapidly migrating TR $\beta$ 2-46 was detected solely in cytoplasmic fractions (Fig. 1D).

**TR $\beta$ 2-46 translation initiates at methionine 79**

The full-length human TR $\beta$ 2 transcript corresponding to the well-characterized murine *Tr $\beta$ 2* is represented by GENCODE transcript ENST00000280696.9. This RNA is predicted to encode a polypeptide of 54.4 kDa (UniProt P10828, isoform  $\beta$ 2). We previously found that transduction of RB177 cells with TR $\beta$ 2 cDNA containing the same open reading frame mainly increased expression of TR $\beta$ 2-54, based on its comigration with the major endogenous TR $\beta$ 2 species (12). Thus, we sought to define the origin of the smaller TR $\beta$ 2-46.

We first assessed whether TR $\beta$ 2-46 resulted from differential splicing. To do so, we amplified cDNA from two retinoblastoma cell lines with forward primer F1 positioned at the 5' end of the predicted TR $\beta$ 2 coding sequence and reverse primers R1–R6 in each downstream exon (Fig. 2A). In both lines, each primer pair amplified a single PCR product of the predicted sizes (Fig. 2B), suggesting that there were no novel splice sites between the known TR $\beta$ 2 exons. We next evaluated whether alternative 5' exons are spliced to the TR $\beta$ 2 exon by amplifying RB176 cDNA using reverse primer R1 and forward primers F2–F6 (Fig. 2A). This generated PCR products of the predicted sizes using the F2 and F4 primers with 5' ends at nucleotides –102 and –301, respectively (Fig. 2, B and C). The 825-nucleotide PCR product made with the F4–R1 primers indicated that *THRB* RNA that encodes TR $\beta$ 2 had a 5'-UTR of  $\geq$ 301 nucleotides. This is 68 nucleotides longer than the murine ortholog (RefSeq NM\_009380.3) (Fig. 2C) but within a previously deduced 377-nucleotide 5'-UTR in the mouse pituitary *Tr $\beta$ 2* transcript (14). The 825-nucleotide PCR product obtained using the F4–R1 primer pair was sequenced and confirmed to contain the predicted 301-nucleotide 5'-UTR (Fig. 2C). Thus, we confirmed that human retina expresses a *THRB* RNA encoding TR $\beta$ 2 but did not detect novel splicing events that might produce TR $\beta$ 2-46.

We then examined whether TR $\beta$ 2-46 used an alternative translation initiation codon in the TR $\beta$ 2 exon. We focused on

the in-frame ATG<sup>Met</sup> codons 69 and 79 (Fig. 2C), which were predicted to encode proteins of 47 and 46 kDa, respectively. We tested whether such proteins were produced by cotransducing Y79 cells with TR $\beta$ 2 shRNA, to deplete endogenous TR $\beta$ 2, and with cDNAs encoding either TR $\beta$ 2 wildtype (WT) or mutants lacking codons 1–69 (TR $\beta$ 2- $\Delta$ 1–69) or with Met-69 and/or Met-79 ATG<sup>Met</sup> codons changed to GCG<sup>Ala</sup> (TR $\beta$ 2-M69A, TR $\beta$ 2-M79A, and TR $\beta$ 2-M69A/M79A; Fig. 2D). We then compared the migration of the ectopic TR $\beta$ 2s with that of endogenous TR $\beta$ 2-46 and TR $\beta$ 2-54. Transduction of TR $\beta$ 2-WT and TR $\beta$ 2-M69A produced proteins comigrating with TR $\beta$ 2-54 and TR $\beta$ 2-46 (Fig. 2D), indicating that both species can be produced in the absence of ATG<sup>Met</sup> codon 69. In contrast, TR $\beta$ 2-M79A and TR $\beta$ 2-M69A/M79A produced a protein comigrating with TR $\beta$ 2-54, and TR $\beta$ 2- $\Delta$ 1–69 produced a protein comigrating with TR $\beta$ 2-46 (Fig. 2D). These data indicate that TR $\beta$ 2-54 initiates translation at methionine 1 and TR $\beta$ 2-46 initiates at methionine 79.

**TR $\beta$ 2-46 but not TR $\beta$ 2-54 promotes SKP2-mediated cell cycle progression**

Having identified the two TR $\beta$ 2 isoforms, we examined their roles in retinoblastoma cell proliferation. In past analyses, TR $\beta$ 2 knockdown with each of six shRNAs impaired proliferation and survival of four retinoblastoma cell lines (Refs. 4 and 12 and data not shown). Impaired proliferation was associated with diminished SKP2 expression and impaired S-phase entry and was partially rescued by ectopic SKP2, indicating that SKP2 is an important TR $\beta$ 2 target (12).

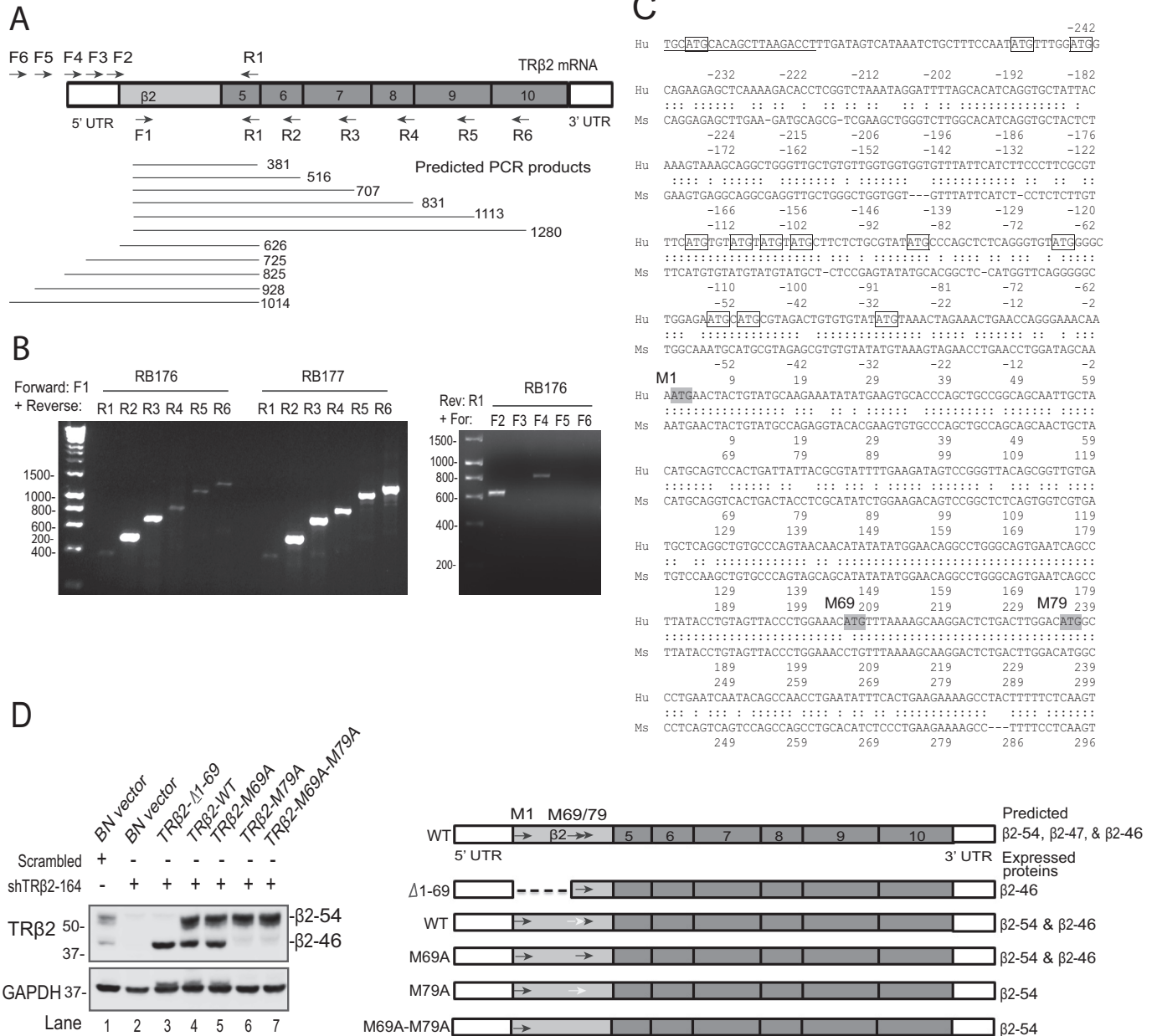
Here, we examined the abilities of the different TR $\beta$ 2 isoforms to complement endogenous TR $\beta$ 2 loss. We first confirmed the prior observations in the context of a TR $\beta$ 2 knockdown and complementation assay. TR $\beta$ 2 knockdown and cotransduction of the BN vector caused an  $\sim$ 80% decrease in SKP2 protein but no change in SKP2 RNA (Fig. 3, A–C), confirming that TR $\beta$ 2 sustains SKP2 expression at the post-transcriptional level (12). TR $\beta$ 2 knockdown followed by nocodazole treatment at days 4.0–4.5 decreased the proportion of S/G<sub>2</sub>/M-phase cells from 50 to 16% (Fig. 3D), confirming that TR $\beta$ 2 is needed for G<sub>1</sub>-to-S progression. Concordantly, TR $\beta$ 2 knockdown impaired Y79 cell proliferation and survival (Fig. 3E).

In cells with endogenous TR $\beta$ 2 knockdown, ectopic TR $\beta$ 2-WT and TR $\beta$ 2-46 partially restored SKP2 levels, G<sub>1</sub>-S progression, S-phase entry, and proliferation, whereas TR $\beta$ 2-54 failed to do so (Fig. 3, A, B, D, and E). TR $\beta$ 2-WT and TR $\beta$ 2-46 did not fully restore SKP2 to endogenous levels, possibly due to the inability to precisely replicate the endogenous TR $\beta$ 2 levels or cell cycle-dependent expression (28). Ectopic SKP2 more fully restored SKP2 protein (Fig. 3, A and B, lane 6) but did not further restore cell cycle progression or proliferation, implying that TR $\beta$ 2-46 restored sufficient SKP2 to elicit SKP2-mediated cell cycle changes. Thus, TR $\beta$ 2-46 but not TR $\beta$ 2-54 promoted SKP2 expression and cell cycle progression.

**TR $\beta$ 2-46 increases cytoplasmic SKP2 stability**

Having determined that TR $\beta$ 2-46 enhances expression of SKP2 protein but not SKP2 RNA, we investigated whether it does so by regulating SKP2 stability. We also examined whether





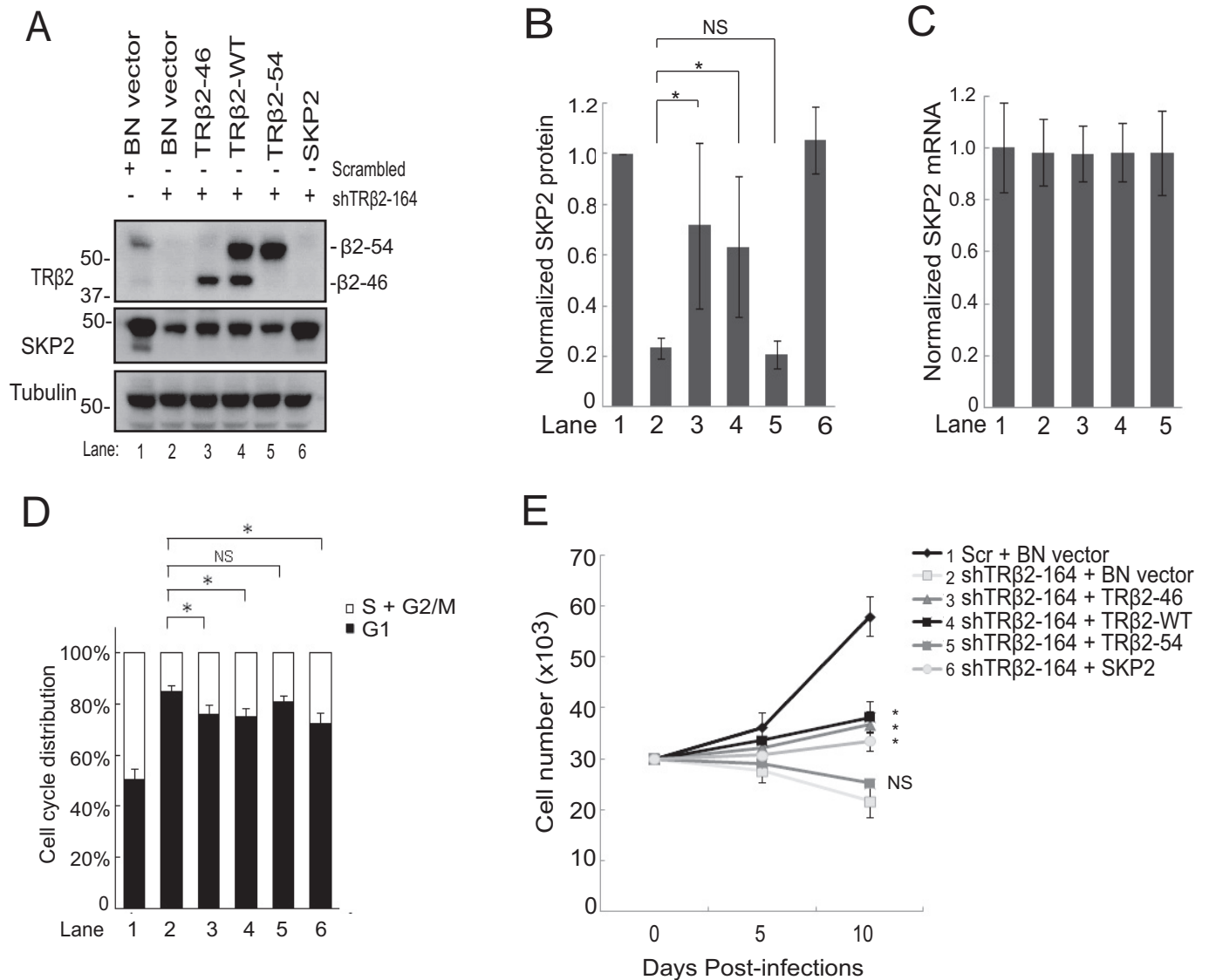
**Figure 2. TRβ2-46 is translated from methionine 79.** *A*, TRβ2 exon structure, PCR primer locations, and predicted PCR products. *B*, PCR products from RB176 and RB177 cDNAs using the primers shown in *A*. *C*, alignment of human (*Hu*) RB176 cDNA amplified using primers F4 (*underlined*) and R1 with mouse (*Ms*) TRβ2 cDNA ENST00000280696.9. Start codons Met-1, Met-69, and Met-79 are shaded, and ATGs in the human 5'-UTR are boxed. *D*, production of TRβ2-46 or TRβ2-54 from WT and mutant TRβ2 cDNAs. *Left*, Western blot analysis of TRβ2 at 4 days after cotransduction of Y79 cells with scrambled or TRβ2-164 shRNA and with WT or mutant TRβ2 cDNAs or empty BN vector. *Right*, structures of TRβ2-WT, TRβ2-Δ1–69, TRβ2-M69A, TRβ2-M79A, and TRβ2-M69A/M79A. Arrows, potential translation initiation codons that were present in each construct and required (*black*) or not required (*white*) for TRβ2-46 and TRβ2-54 expression.

TRβ2-46 regulates SKP2 in the nucleus or in the cytoplasm as both compartments have been implicated in SKP2 function (29–33). Through cell fractionation we found that ~80–90% of SKP2 was located in the cytoplasm of vector-transduced Y79 retinoblastoma cells (Fig. 4A). After TRβ2 depletion, SKP2 declined and was seen solely in the cytoplasm. Ectopic TRβ2-46 partially restored cytoplasmic but not nuclear SKP2 (Fig. 4A) despite that, in this experiment, ectopic TRβ2-46 partially localized to the nucleus, likely due to its higher-level expression.

To assess whether TRβ2 enhanced SKP2 stability, Y79 cells were cotransduced with TRβ2 shRNAs and either the BN vec-

tor or BN-TRβ2-46. On day 4, cells were treated with cycloheximide to suppress protein synthesis, and the rate of SKP2 decay was examined. In this setting, TRβ2 knockdown and SKP2 down-regulation were intentionally modest as needed to retain sufficient SKP2 to observe its half-life. As such, we observed little effect of TRβ2 knockdown on SKP2 stability, whereas ectopic TRβ2-46 stabilized and increased SKP2 expression (Fig. 4B).

The high TRβ2-46 expression and SKP2 regulation in retinoblastoma cells raised the possibility that TRβ2-46 might promote SKP2 expression in the developing retina. Indeed, immunostaining revealed high-level SKP2 and TRβ2 in the cytoplasm



**Figure 3. TRβ2-46 but not TRβ2-54 promotes SKP2 expression, cell cycle progression, and proliferation.** Y79 cells were coinfecting with lentivirus expressing scrambled (*Scr*) or *TRβ2-164* shRNA and with vectors expressing TRβ2-46, TRβ2-54, or both (with TRβ2-WT) or expressing SKP2. A and B, cells were harvested at day 4 for Western blotting of TRβ2, SKP2, and tubulin loading control (A) and quantification of SKP2 normalized to tubulin (B). The immunoblot is representative of five experiments. Bar heights are means, and error bars are standard deviations of five experiments. C, SKP2 mRNA expression assessed by RT-PCR in triplicate and normalized to *ACTB*. Error bars are standard deviation of three technical replicates. D, cell cycle distribution after infection as in A and treatment with 100 μg/ml nocodazole from day 4 to 4.5. \*,  $p < 0.05$ , unpaired two-tailed *t* test. E, cell numbers after plating 30,000 cells/well of each culture 0, 5, or 10 days post-transfection. Error bars, standard deviation of three technical replicates (\*, comparing with curve 2,  $p < 0.05$ ; NS, not significant).

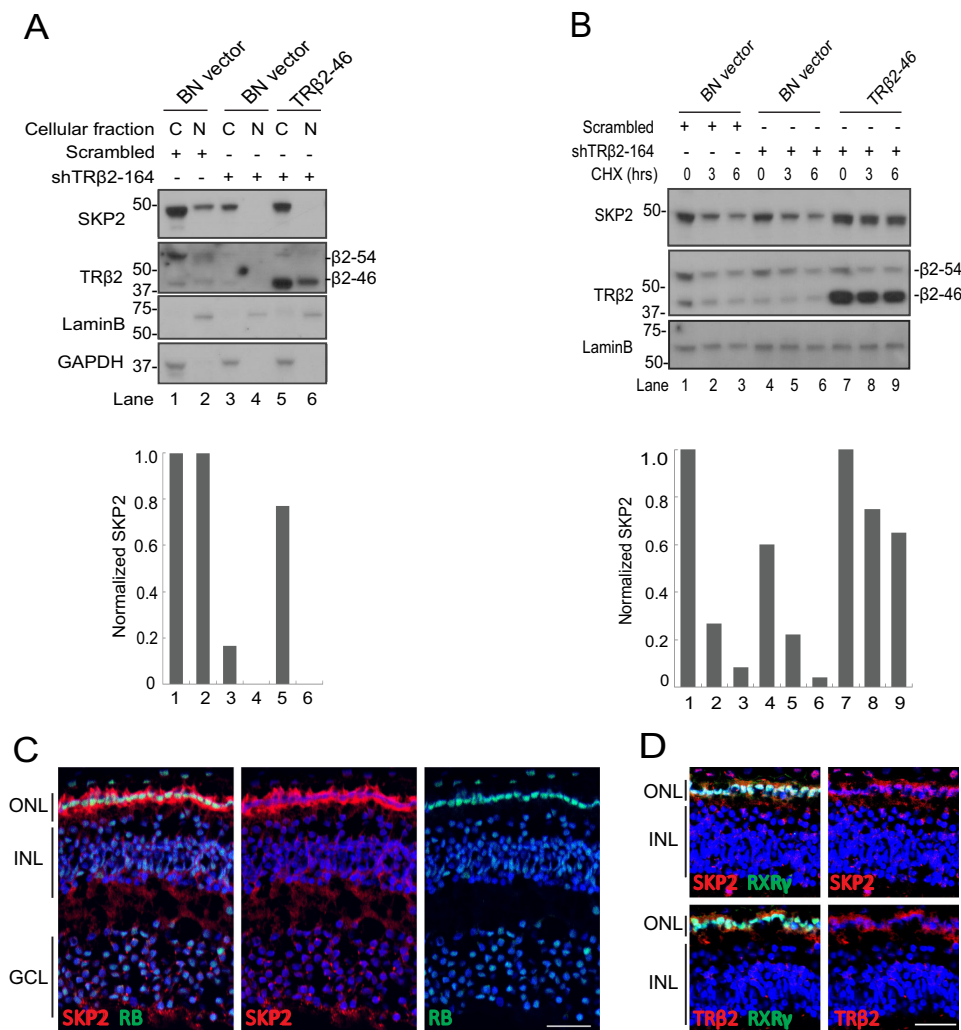
of maturing cone precursors relative to all other cell types (Fig. 4, C and D), consistent with SKP2 regulation by the cone-specific TRβ2-46 isoform.

## Discussion

We report that retinoblastoma cells express two functionally distinct TRβ2 isoforms, here designated TRβ2-46 and TRβ2-54 according to their predicted molecular masses. Both isoforms were encoded by a *THRB* transcript orthologous to the well-characterized mouse *THRB* RefSeq isoform 2 via alternative translation initiation, with the canonical TRβ2 initiation codon used to produce TRβ2-54 and methionine 79 used to produce TRβ2-46. Methionine 79 is conserved in mice, and a TRβ2 protein of similar electrophoretic mobility appeared to be present in pituitary extracts from WT but not *Trβ2*<sup>-/-</sup> mice

(25), suggesting that TRβ2-46 might be expressed in mouse pituitary. However, no faster-migrating TRβ2 was evident in mouse retinae (25, 34), suggesting that species-specific mechanisms might promote TRβ2-46 expression in human cone precursors and cone precursor-derived retinoblastoma cells.

The mechanism that regulates the translation initiation of TRβ2-54 and TRβ2-46 is currently unclear. As one possibility, 5'-UTR sequences and upstream open reading frames (uORFs) can influence translation initiation (35–37). Retinoblastoma cell *THRB* cDNA had a 5'-UTR of at least 301 nucleotides, including multiple uORFs that are conserved in mice (Fig. 2C), suggestive of a conserved regulatory role. If the 5'-UTR and uORFs do indeed regulate alternative initiation then species differences in *cis*-acting sequences or *trans*-acting factors must underlie the predominant TRβ2-46 in human and TRβ2-54 in mouse retinae.



**Figure 4. TR $\beta$ 2-46 increases cytoplasmic levels of SKP2.** Y79 cells were subjected to TR $\beta$ 2 knockdown and rescue with *BN-TR $\beta$ 2- $\Delta$ 1–69* encoding TR $\beta$ 2-46. **A**, effect of TR $\beta$ 2-46 on SKP2 subcellular localization. Cell lysates were harvested on day 5 and fractionated for Western blot analysis of TR $\beta$ 2, SKP2, Lamin B, and GAPDH. **Bottom**, quantification of nuclear and cytoplasmic SKP2 normalized to vector-transduced controls. **B**, effect of TR $\beta$ 2-46 on SKP2 stability. At 4 day after infection, cells were treated with 20  $\mu$ g/ml cycloheximide (CHX) for 0, 3, or 6 h to inhibit protein synthesis, and lysates were harvested for SKP2 and TR $\beta$ 2 Western blot analysis. **Bottom**, quantification of SKP2 normalized to Lamin B. **C** and **D**, presumptive fovea of 18-week developing human retina, showing high cytoplasmic SKP2 (red) costained with nuclear RB (green) (**C**) and in the same region as high cytoplasmic TR $\beta$ 2 in serial sections (**D**) in maturing cone precursors (marked by RXR $\gamma$ ; green). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bars, 40  $\mu$ m.

Translation of TR $\beta$ 2-46 from methionine 79 eliminates N-terminal structures that are implicated in the enhanced transcriptional activity of TR $\beta$ 2 relative to TR $\beta$ 1 (16, 18, 38). As this enhanced transcriptional activity is implicated in regulation of the hypothalamic–pituitary–thyroid axis (39) and in long and medium wavelength cone gene expression and differentiation (6, 40), TR $\beta$ 2-46 seems unlikely to drive these transcriptional programs. Furthermore, the lack of TR $\beta$ 2-46 in developing mouse retina (25, 34) suggests that TR $\beta$ 2-46 is not needed for cone development processes that are shared between mice and humans. Instead, TR $\beta$ 2-46 may participate in human-specific processes such as foveagenesis or expression of a cone precursor proliferation–related program (2, 3).

Our cell fractionation analyses revealed that the vast majority of TR $\beta$ 2-54 and virtually all TR $\beta$ 2-46 are cytoplasmically located in human retinoblastoma cell lines. This is in accord with the prior immunodetection of cytoplasmic TR $\beta$ 2 in retinoblastomas and human cone precursors (2, 4, 24) and suggests

that the cytoplasmic TR $\beta$ 2 immunostaining was authentic. The high levels of cytoplasmic TR $\beta$ 2-46 also suggest that TR $\beta$ 2-46 has a cytoplasmic role. In retinoblastoma cells, TR $\beta$ 2-46 but not TR $\beta$ 2-54 partially sustained SKP2 expression, S-phase entry, and proliferation (Figs. 3 and 4). Although the underlying mechanism is not yet defined, it may be relevant that cytoplasmic SKP2 can be induced by AKT signaling (32, 41–43) and that cytoplasmic TR $\beta$ 1 can suppress AKT signaling via inhibition of PI 3-kinase (19, 20). Because TR $\beta$ 2 increased SKP2 expression by antagonizing TR $\beta$ 1 (12), we speculate that TR $\beta$ 2-46 sustains SKP2 by opposing TR $\beta$ 1-mediated inhibition of PI 3-kinase activity. The high SKP2 expression in human cone precursor cytoplasm (Fig. 4C) is consistent with the possibility that TR $\beta$ 2-46 also promotes cytoplasmic SKP2 expression in the developing retina.

The TR $\beta$ 2-46–mediated up-regulation of SKP2 seems likely to contribute to retinoblastoma initiation and propagation. Indeed, SKP2 is required for the cone precursor proliferative

response to RB loss (2) as well as for retinoblastoma cell proliferation (23). Moreover, down-regulation of SKP2 in RB-depleted cells may provide an important barrier to development of RB-deficient tumors, whereas intrinsic TRβ2 expression enables SKP2 expression and RB-deficient malignancies (12). In retinoblastoma cells, TRβ2-46 increased cytoplasmic SKP2 expression along with cell proliferation. This was unexpected because SKP2 is thought to promote proliferation in part by mediating p27 degradation in the nucleus. However, SKP2 also has roles in the cytoplasm where it can ubiquitylate and activate AKT (31) and can promote degradation of the proapoptotic FOXO1/3 and the tumor-suppressive E-cadherin (32, 33).

In summary, we identified a novel TRβ2-46 isoform that is highly expressed in human but not mouse cone precursors. We demonstrate that TRβ2-46 is highly expressed in cone precursor-derived retinoblastoma cells and is critical to retinoblastoma cell SKP2 expression and proliferation. Thus, TRβ2-46 is a cell type-specific factor that is intrinsically expressed in the retinoblastoma cell of origin and collaborates with the cancer-initiating RB loss to enable tumorigenesis.

## Experimental procedures

### Cell lines and retinal tissues

Y79 and Weri-RB1 cells were from the ATCC. RB176, RB177 (4), and CHLAVC-RB43 (44) were as described. Following informed consent, fetal eyes were obtained from authorized sources with approval of the Children's Hospital Los Angeles Institutional Review Board.

### Cell culture

Retinoblastoma cells were cultured in RB culture medium as described (4). Cells were synchronized at metaphase by addition of nocodazole (Sigma-Aldrich, M1404) to 100 ng/ml. Protein synthesis was blocked by addition of cycloheximide (US Biological, C8500) to 20 μg/ml.

### Subcellular fractionation and Western blotting

Subcellular fractionation was as described (45). For Western blotting, RB cells or minced retina was incubated with lysis buffer (10 mM Tris, pH 8.0, 140 mM NaCl, 1% Nonidet P-40, 0.1 mM EDTA with protease and phosphatase inhibitors (Roche Applied Science)) on ice for 10 min and centrifuged at 20,000 × g for 10 min at 4 °C, and supernatant was collected. 30 μg of retinoblastoma cell protein, 10 μg of retina lysate, and 5 μg of molecular weight markers (Bio-Rad, 161-0317) were separated by SDS-PAGE. Antibodies to human TRβ2 amino acids 1–110 (sc-67123), SKP2 (sc-7164), GAPDH (sc-32233), and Lamin B (sc-6216) were from Santa Cruz Biotechnology, and α-tubulin antibody was from Sigma-Aldrich (T5168). Secondary antibodies with chemiluminescence or fluorescence signals were quantified by Imagine Studio Lite and normalized to loading controls.

### TRβ2 cloning and mutagenesis

*BE-Neo-TRβ2-WT* was produced from RB176 cDNA by PCR amplification of a 825-bp *THRB* cDNA fragment with primers F4 and R1 (Fig. 2A) and replacement of the corresponding cod-

ing sequence and the 14-nucleotide 5'-UTR of *BE-Neo-TRβ2* (12) using In-Fusion (Clontech). *BE-Neo-TRβ2-Δ1–69* was made with In-Fusion using primers TRβ2-Δ1–69 F (5'-CAA-AATGTTTAAAAGCAAGGACTCTGACTTGG) and TRβ2-Δ1–69 R (5'-CTAGAAACTGAACCAGGGAAACAAAATGTTTAAAAG). *BE-Neo-TRβ2-M69* was made with In-Fusion using primers TRβ2-M69A F (5'-GAAACGCGTTTAAAAGCAAGGACTCTGACTTGG; mutated bases underlined) and TRβ2-M69A R (5'-TTTAAACGCGTTTCCAGGGTAACTACAGGTA). *BE-Neo-TRβ2-M69A/M79A* was made by PCR amplification of *BE-Neo-TRβ2-M69* using primers TRβ2-M79A F (5'-CTTGACGCGGCCCTGAATCAATACAGC; mutated bases underlined) and TRβ2-M79A R (5'-AGGGCCGCGTCCAAGTCAGAGTCCTTGCT). *pLKO-shTRβ2–228* and *pLKO-shTRβ2–164* were described previously (12) and designated according to the first target nucleotide after the canonical initiation ATG (4).

### TRβ2 RNA analysis

RB176 RNA was isolated, and cDNA was produced and PCR-amplified as described (44) using the following primers: F6, 5'-TTTCATGCTTAAGCTGTCAACC; F5, 5'-AAATGCATCTTAGCAGCTTACG; F4, 5'-TGCATGCACAGCTTAAGACCT; F3, 5'-AGCACATCAGGTGCTATTACA; F2, 5'-GCTTCTCTGCGTATATGCCCA; F1, 5'-TATGCAAGAATATATGAAGTGCA; R1, 5'-GTGGCTTTGTCACCACACAC; R2, 5'-TGATTTTCGCGTGAAGTTGTC; R3, 5'-TTT-TGATGAGCTCCCATTC; R4, 5'-TCAACCTTCCACCTTCTGG; R5, 5'-GACATGCCAGGTCAAAGAT; R6, 5'-TCACGTGGTGTTCGGTAA. PCR products were separated on agarose gels and visualized by ethidium bromide staining.

### Lentivirus production and infection

Lentiviruses were produced by transfection of  $2 \times 10^7$  293T cells similar to that described previously (4). Virus was harvested at 60 h, concentrated 50-fold by centrifugation at 25,000 rpm for 90 min, and suspended in RB medium. 500 μl of concentrated virus was used to infect  $5 \times 10^5$  Y79, WERI-1, or RB177 cells in 500 μl of filtered conditioned RB medium with 4 μg/ml Polybrene (Sigma-Aldrich) followed by gentle pipetting 20 times. At 18 h after infection, cells were diluted in an equal volume of conditioned RB medium. Infected cells were selected starting 48 h after infection with 2 μg/ml puromycin for 48–72 h or with 200 μg/ml G418 for 4–7 days and fed every 2–3 days by replacing two-thirds of the media.

### Cell cycle and cell proliferation analyses

Cells were fixed in 70% ice-cold ethanol for 1–16 h at 4 °C, pelleted by centrifugation at 10,000 × g for 10 s, resuspended in propidium iodide (10 μg/ml in PBS and 100 μg/ml RNase (Invitrogen)), incubated for 30 min at 37 °C, and analyzed using a BD Canto flow cytometer with >20,000 gated events per sample. Cell cycle distributions were defined using FACSDiva version 6.1.3. Proliferation was evaluated by cell counting using a hemocytometer.

### Statistical analysis

All data were from at least three independent biological repeats. One-way analysis of variance was used to determine



whether there were differences among the means of three or more groups, and then an unpaired two-tailed *t* test was performed to identify where the differences occurred between groups (Fig. 3, B, D, and E). A *p* value <0.05 was considered significant.

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