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Tra2β as a novel mediator of vascular smooth muscle diversification

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Abstract

Transformer splicing regulatory proteins determine the sexually dimorphic traits of Drosophila. The role of the vertebrate homologues of Tra-2 in phenotypic specification is undefined. We are using the alternative splicing of the MYPT1 E23 exon as a model for the study of smooth muscle diversification into fast and slow contractile phenotypes. Tra2β mRNA and protein is expressed at up to 10-fold higher levels in fast smooth muscle tissues such as the rat portal vein (PV) and small mesenteric artery (MA), in which E23 is spliced, as compared to the slow smooth muscle tissues of the large arteries and veins, in which E23 is skipped. Tra2β is up-regulated up to 10-fold concordant with the initiation of E23 splicing as the rat PV and avian gizzard implement the fast program of gene expression in the peri-natal period. In disease models such as portal hypertension and MA high/low flow, the PV and MA1 dynamically down-regulate Tra2β concordant with a shift to E23 skipping and the slow program of gene expression. Tra2β binds to a highly conserved sequence within E23 and *trans*-activates its splicing *in vitro* and *in vivo*; this is abolished with mutation or deletion of this sequence. RNAi mediated knock-down of Tra2β markedly reduces E23 splicing. We propose that Tra2β has been conserved through evolution and re-deployed for the specification of the fast smooth muscle phenotype, and may serve as a novel nodal point for the investigation of this process in developmental and disease models.

Keywords

Transformer; smooth muscle; phenotype; exon splicing; myosin phosphatase

Introduction

Smooth muscle exhibits considerable phenotypic diversity that, as is true for striated muscle, may be dichotomized into fast or slow sub-types based on the rates of contraction and relaxation. Vascular smooth muscle tends to be of the slow phenotype while visceral smooth muscle tends to be of the fast phenotype, though muscles of fast, slow and intermediate phenotypes are present in both vascular and visceral systems. These phenotypes are specified during development, responsive to neural and humoral inputs and mechanical load, and critical to the function of these systems in normal and disease states[1;2].

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Disclosures None.

Much diversity in smooth muscle is generated by the alternative splicing of exons. Myosin heavy and light chains, myosin phosphatase, tropomyosin, caldesmon and calponin isoforms are generated by alternative splicing of exons and are thought to contribute to the functional differences between fast (also described as phasic) versus slow (also referred to as tonic) smooth muscle[3]. We have selected the myosin phosphatase targeting subunit 1 (MYPT1) as a model gene for the study of smooth muscle phenotypic diversity. The cassette-type splicing of a 31 nt 3'alternative exon (MYPT1 E23) (Fig. 1A) is 1) highly tissue-specific. The alternative exon is skipped in the slow smooth muscle of the large arteries and veins, and included in the fast smooth muscle of the Portal Vein (PV) and the small mesenteric arteries, which have an intermediate phenotype. 2) Evolutionarily conserved in mammals and birds. 3) Tightly regulated during development. The PV and avian gizzard, prototypical fast tissues, acquire phasic properties in the peri-natal period[4;5]. At this time these tissues undergo a complete transition from E23 skipping to E23 inclusion as part of the re-programming of gene expression to the fast pattern[6;7]. In contrast the smooth muscle of the large arteries and veins is of the slow phenotype with E23 skipping throughout development, as are smooth muscle cells (SMCs) in culture[6;8] suggesting that this is a default phenotype. 4) Modulates in disease models. In models of portal hypertension (PHT) and altered flow, the PV and mesenteric resistance artery switch to E23 skipping[9;10] as part of a generalized reversion towards the slow phenotype.

The goal of the current study was to identify the control mechanisms for the tissue-specific splicing of E23 in relation to smooth muscle phenotypic diversity. Depending on the alternative exon and cell type, some have proposed combinatorial control by widely expressed splicing regulatory factors to result in tissue-specific splicing. In a few other instances, a highly tissue-restricted factor has a dominant effect on the splicing of alternative exons in those tissues[11]. The most striking example of the latter is sex-determination in the fly. This developmental choice is under the control of the Transformer proteins, which through the regulated splicing of Dsx and Fru alternative exons specify the unique physical and behavioral characteristics of female flies[12]. The vertebrate homologues of the fly Tra-2, Tra-2α and Tra2β, were subsequently identified, and in the case of Tra2α, shown to be functionally equivalent to the fly Tra2[13-16]. Tra2α and β are atypical members of the SR family of RNA binding proteins, and have been suggested to regulate the splicing of a number of vertebrate alternative exons in conjunction with the classical SR proteins[16]. However, in contrast to its clear and potent role in phenotypic specification in flies, a role for Tra in phenotypic specification in higher organisms has not been defined.

Materials and Methods

Oligonucleotides used for RT-PCR are listed in supplementary materials.

Sequence analysis of MYPT1 E23 and flanking introns

We carried out phylogenetic analysis of the MYPT1 E23 and flanking intronic sequence (rat MYPT1: ENSRNOG00000004925) using UCSC genome browser (http://genome.ucsc.edu/). The candidate *cis*-elements of E23 splicing were identified either by manually screening the sequence for known binding motifs or by using web-based data bases including Splicing Rainbow (http://www.ebi.ac.uk/asd-srv/wb.cgi?method=8), RESCUE-ESE (http://genes.mit.edu/burgelab/rescue-ese/) and ESEfinder (http://rulai.cshl.edu/tools/ESE2/). Only those motifs above the threshold values were considered as putative *cis*-elements.

Tissue samples, cell lines and cell culture

Tissue samples were obtained from: 1) normal adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) and the offspring of paired matings. 2) rats where the PV was surgically stenosed to induce PHT[9] 3) rats where alternating second order MAs were ligated

to induce high flow and low flow states in alternating upstream first order MAs[10] 4) White leghorn chickens and embryos prior to hatching (Squire Valley Farm, Cleveland, OH). Animal care and use procedures were approved by the institutional Animal Care and Use Committees at Case Western Reserve University. The rat samples were derived from previously published studies. SMCs were isolated from chicken gizzard and rat Aorta (Ao) and maintained in culture as described[17]. The Human Embryonic kidney (HEK) 293 (Quantum Biotechnology Inc, Quebec, Canada) and A7r5 (embryonic rat aortic SM) cell lines were grown in monolayer in DMEM supplemented with 10% HFBS.

Analysis of Tra2β mRNA levels and splice isoforms

Real time PCR was performed to measure the total abundance of Tra2β. Values were normalized to SRp20 mRNA, which was invariant between samples. Splice variants of Tra2β in rat tissues were analyzed by standard RT-PCR.

Analysis of Tra2β abundance by Western blot

Nuclear extracts (NEs) were prepared from chicken and rat tissues and cultured cells as described[17]. NEs were analyzed by western blot. Antibodies used: polyclonal antibody to the N-terminus of Tra2β (S-18) (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), mAb10 against SR proteins, DH7 (gift from Dr Helfman) against PTB and a GAPDH antibody (Abcam, Cambridge, MA).

RNA-protein binding assay

The RNA-protein binding assay was carried out as previously described[17] using biotin labeled RNA oligos and NEs collected from rat tissues.

Transfection of plasmid DNA and RNA interference assay

The MYPT1 mini-gene was constructed by amplifying the 600 nt mouse genomic fragment containing the E23 and flanking introns by PCR and cloned into the second intron of the rabbit β-globin present in pβG plasmid[18]. Mutations and deletion in E23 within the MYPT1 mini-gene construct were generated using the QuickChange® II Site-Directed Mutagenesis kit (Stratagene) and confirmed by DNA sequencing. A full length Tra2β1 cDNA was amplified from mouse liver RNA using the oligonucleotides described previously[19] and cloned into the pcDNA 3.1expression vector (Invitrogen). Primary SMCs or HEK293 cells were transfected with pβG plasmid containing a wild type or mutant MYPT1 mini-gene and in some experiments a Tra2β expression vector using Lipofectamine2000 (Invitrogen) as described[17]. For *in-vivo* plasmid delivery 6-day old chickens were anesthetized and after laparotomy, the gizzards were injected with a wild type or mutant MYPT1 mini-gene plasmid with or without Tra2β expression vector as described[20].

For RNA interference HEK293 cells were transfected with small interfering RNA (siRNA) synthetic duplex designed for Tra2β (*Silencer*^{R} siRNA, Ambion, Austin, Tx) as described[17]. *Silencer*^{R} GAPDH siRNA and a non-specific siRNA (Ambion) were used as controls for the RNAi assay.

RT-PCR and quantification

The transfected and injected samples were analyzed by RT-PCR for the presence of exonincluded and exon-skipped MYPT1 mini-gene transcripts using the vector specific primer pair as described[17]. The 5'-end of the 3' PCR oligonucleotide was labeled with a Cy3 fluorescent label (IDT) for the visualization of the RT-PCR product. Data are expressed as mean ± SD ($n \geq 3$). Data groups were compared by one-way ANOVA and student's *t-test* using Prism version 4.0. $P < 0.05$ was considered statistically significant.

Results

Sequence analysis of MYPT1 alternative exon E23 and flanking introns

The sequence of the MYPT1 E23 is highly conserved amongst birds and mammals (Fig. 1A) and not identified in the frog (Fig. 1B), zebra fish or worm (not shown) homologues. Characteristics of E23 indicative of a weakly spliced exon include its small size, a 5' splice site which deviates from the consensus GTRAGT, and the absence of a polypyrimidine tract upstream of the 3' splice site (Fig. 1A and accession # AF110176.1). Phylogenetic analysis identified ~160 nt of intronic sequence upstream and ~200 nt of intronic sequence downstream of E23 that were also highly conserved (Fig. 1B). E23 contains consensus *cis*-elements for the classic SR proteins SRp-40, 55 and SC35 (Fig. 1A) that were not affected by the phylogenetic sequence variation at the 2 underlined nt. A manual search identified a putative and conserved Tra2β binding site in E23 similar to a proposed Tra2β consensus sequence of GHVVGANR[21].

Expression of Tra2β correlates with splicing of MYPT1 E23

During the development of the fast smooth muscle phenotype of the rat PV and avian gizzard, Tra2β mRNA is up-regulated 4-8 fold (Fig. 2A), co-incident with the complete switch from E23 skipping to E23 inclusion[6;8]. The developmental up-regulation of Tra2β in the fast smooth muscle is specific as 1) there is no change in the mRNA levels of SRp20, used here for internal normalization and 2) there is no developmental change in Tra2β mRNA abundance in the slow smooth muscle of the large artery like Ao (Supplemental Fig. 1A), in which E23 is skipped[7]. Tra2β mRNA abundance is 5-10 fold higher in the mature fast smooth muscle tissues, the rat PV and chicken gizzard, as compared to the mature slow smooth muscle tissues, the Ao and other large arteries and veins (Fig. 2B). SMCs in culture express lower levels of Tra2β and skip E23 regardless of whether they are derived from the slow Ao (upper panel) or the fast gizzard (lower panel), indicative of a reversion to the slow phenotype. The MA1 is a small resistance artery with a mixed contractile phenotype[9]. It expresses relatively high level of Tra2β mRNA concordant with 80% E23 is inclusion (Fig. 2B).

In two different disease models 1) PV ligature to induce PHT and 2) MA2 ligature to induce high and low flow in the MA1s, we have previously observed shifts from the fast towards the slow programs of gene expression[9;10]. In these models Tra2β mRNA is dynamically downregulated in the PV and MA1 which temporally correlates with changes in E23 splicing (Fig. 3A, B). Thus these models demonstrate excellent correlations in the dynamic shifts in Tra2β mRNA abundance and E23 splicing.

Tra2β isoform expression differs between fast and slow smooth muscle

The alternative usage of exons 2-3 generates three major isoforms of Tra2β (Tra2β1,3,4; Fig. 4A) Tra2β1 codes for the full-length Tra2β protein, while Tra2β and β4 are thought to give rise to a truncated protein lacking the first RS domain of Tra2β[15] We assayed the expression of the splice variants of Tra2β In the PV in the neonatal period there is a transition from a 1:1 ratio of the transcripts that would code for the β1 and β4 isoforms at D3 and D6 to a 10:1 predominance of the transcript that would code for the β1 isoform at D12 and into adulthood (Fig. 4B). The ratio of transcripts that would code for the β1 and β4 isoforms in the Ao is 5:1 (Fig. 4B) The transcript that would code for the β3 isoform is abundant only in PV D6. In the PHT PV model the splicing of the Tra2β alternative exon #2 is re-induced, with a ratio of transcripts for β1to β4 of 2;1 at D3 and 3:1 at D7, and returned to 10:1 by D14 (Fig. 4C). Splice variant isoforms of Tra2β are not detected in the chicken[22].

Tra2β protein abundance during vascular smooth muscle phenotypic specification

Tra2β protein is detected as a single band at ~37 kDa in PV and Ao NEs (Fig. 5A). Tra2β protein abundance in this PV neonatal series relative to the adult PV is: D3 10%, D6 20% and D12 70%. The abundance of Tra2β in the adult Ao is 8% of that in the adult PV, and there is no change in Tra2β protein abundance in the Ao in the neonatal period (Supplemental Data, Fig. 1B). In the PHT model, the abundance of Tra2β protein in the PV is reduced to 10% of control values within 3 days after the PV ligature (Fig. 5B) and returned to the control values at D14. These differences in Tra2β abundance are specific as indicated by the invariance of the classic SR proteins (Figs. 5A, B).

Tra2β binds to the 31 nt MYPT1 E23 exonic sequence

The close correlation between MYPT1 E23 splicing and Tra2β expression in these different models suggested that it may be a tissue-specific enhancer of E23 splicing. In the next set of experiments we tested the ability of Tra2β to bind to and *trans*-activate E23 splicing. Tra2β bound to a 31 nt biotin-labeled RNA oligonucleotide containing the wild type E23 sequence. This binding is detected with NE from adult PV but not adult Ao (Fig. 6A,B). On a longer exposure weak binding of Tra2β is observed in reactions that used PHT PV D3 NE. Tra2β binding is not observed in control reaction that used PV NE and an unrelated RNA sequence (Fig. 6B). SRp55 and SRp40 bound to this 31 nt RNA oligonucleotide in NEs from PV, Ao and PHT PV. A weak singlet at ~30 kDa is observed only in reactions that used PV NE and likely represents the predicted binding of SC35.

A deletion of 6/8 nt of the putative Tra2β binding site (Fig. 6A), leaving the putative SR binding sites intact (ΔTRA) abolished Tra2β binding without affecting SRp55/40/30 binding (Fig. 6C). Mutation of two key central nt of the putative Tra2β motif, from GA to UU[21], also abolished Tra2β binding without affecting SRp 55/40 binding. When a portion of the Tra2β binding site and overlapping SR p55/40/30 binding site were deleted (ΔTra-SR), Tra2β and SRp30 binding is not detected and SRp40 binding is significantly reduced while SRp55 binding is only modestly reduced. A mutation of the SR binding site immediately 5' to the Tra2β binding site (SRmut1) significantly reduced SRp 55/40/30 binding but did not affect Tra2β binding. Mutation of both the 5' and 3' SR binding sites, while leaving the Tra2β binding site intact (SRmut2), nearly completely abolished the binding of the SR proteins and Tra2β (a faint band for SRp55 was detected at long exposures). In summary, Tra2β binds to the predicted 8 nt *cis*-element in E23, and this binding is lost when the adjacent SR *cis*-elements are mutated.

Tra2β trans-activates MYPT1 E23 splicing

To test the functional role of Tra2β in MYPT1 E23 splicing, we inserted a MYPT1 mini-gene that contains the mouse E23 exon and conserved flanking intronic sequence (~300 nt each of upstream and downstream sequence) between two constitutive β-globin exons in the pβG vector (Fig. 7A). Transfection of this construct into cultured chicken gizzard or rat aortic SMCs resulted in a very low level of E23 splicing (Fig. 7B, upper panel), consistent with nearly exclusive skipping of the endogenous E23. In contrast injection of this construct into the gizzard smooth muscle of 1 week old chickens resulted in a significant level of splicing of E23 (Fig. 7B), consistent with splicing of the endogenous E23 in this fast smooth muscle. In either context, cotransfection of a Tra2β expression plasmid significantly increased mini-gene E23 splicing (lower panel), with a linear relationship between the amount of Tra2β expression vector and mini-gene E23 splicing (Fig. 7C).

To test the role of the Tra2β and SR binding *cis*-elements in E23 splicing, the mutations and deletions that reduced Tra2β and SR binding (Fig. 6) were introduced into the MYPT1 mini-gene construct. Deletion or mutation of the Tra2β binding site (ΔTRA, TRAmut) completely or nearly completely abolished E23 splicing in the gizzard smooth muscle (Fig. 7D). These

constructs are unresponsive to Tra2β co-transfection (Fig. 7D), demonstrating the necessity of the *cis*-element for Tra2β activation of E23 splicing. A deletion of the 5' portion of the Tra2β*cis*-element and adjacent SR *cis*-element (ΔTRA-SR) completely abolished E23 splicing and again the co-transfection of the Tra2β expression plasmid had no effect. A 2 nt mutation to the 5' SR binding site (SRmut1) that severely diminished SR binding but had no effect on Tra2β binding also markedly reduced E23 splicing in the gizzard smooth muscle. This construct is also unresponsive to co-transfection with the Tra2β expression plasmid. In summary, Tra2β *trans*-activates splicing of the MYPT1 E23 dependent upon both the Tra2β *cis*-element and the adjacent SR *cis*-element.

Endogenous Tra2β is required for MYPT1 E23 splicing

In screening Tra2β expression in various cell types we observed that Tra2β is 4-6 fold more abundant in the HEK293 cell line as compared to the cultured SMCs. In these cells, and in contrast to the cultured SMCs, there is also increased splicing of E23 in the context of the transfected MYPT1 mini-gene (Fig. 8B). Co-transfection of the Tra2β expression plasmid further increased exon-inclusion. To test the function of endogenous Tra2β in the regulation of E23 splicing, we used RNA interference (RNAi) to knock-down Tra2β. This resulted in a 50% to 75% reduction in Tra2β protein at 48-96 hours after transfection (Fig. 8A) that is specific as indicated by the invariance of PTB and the lack of effect of a scrambled (NS) siRNA or siRNA against GAPDH (not shown). Knock-down of Tra2β by RNAi reduced E23 splicing to a level similar to that of the cultured RASMCs (Fig. 8B). The control siRNAs had no significant effect. These results indicate that endogenous Tra2β is necessary for the splicing of MYPT1 E23 in the context of the mini-gene construct.

Discussion

In the current study we propose Tra2β as a novel mediator of the fast smooth muscle phenotype based on 1) its concordant expression with the splicing of MYPT1 E23, a marker of the fast smooth muscle phenotype, in multiple developmental and disease models. This relationship is conserved between two classes of animals, birds and mammals that diverged several hundred million years ago, supporting its functional significance. 2) The ability of Tra2β to bind to E23 and *trans*-activate E23 splicing in a sequence-dependent manner. To the best of our knowledge this is the first factor to be implicated in the specification of the fast muscle phenotype, in smooth or any other muscle lineage.

Prior studies of Tra2β in vertebrates have suggested that it is ubiquitously expressed and may regulate the splicing of a number of exons. A major limitation of these studies is the absence of an analysis of Tra2β in relation to tissue-specific or developmentally regulated splicing of exons. There has also been some suggestion that Tra2β may be enriched in certain tissues, e.g. in the nervous system[23] and testes[24]. This proposed more tissue-specific expression and function of Tra in vertebrates is consistent with its role in the fly. Tra and Tra-2 are required in female *Drosophila* for the suppression of the development of the male-specific abdominal muscle (also known as the muscle of Lawrence, MOL)[25], while the other muscles develop normally, and the development of female-specific behaviors and reproductive organs[26]. Interestingly, the MOL is more highly innervated than the other muscles, and it uniquely fails to develop in the absence of innervation[27], perhaps presaging the role of innervation in the development of the fast smooth muscle phenotype[4].

A model for Tra2β's function in tissue-specific splicing of exons in vertebrate smooth muscle specification is suggested by the expression, binding and mini-gene splicing data. In cells where Tra2β expression is low, such as Ao and cultured SMCs, Tra2β binding to E23 was not detected, and mutation of the Tra2β binding site did not affect the low level of E23 inclusion. In tissues where Tra2β expression is induced up to 10-fold, such as rat PV and avian gizzard, Tra2β

binding to E23 was evident, and mutation of this site reduced E23 splicing to the level in the low-Tra2β expressing cells. In contrast the ubiquitous classic SR proteins bound to E23 in both fast and slow phenotypes but apparently were not sufficient to activate E23 splicing. These results are consistent with a model in which Tra2β and SR proteins synergistically bind and activate alternative exon splicing[28], with Tra2β having a lower binding affinity but more potent activation of splicing of a weak exon[29]. In this model Tra2β plays a dominant role as a tissue-specific splicing factor, but combinatorial control in conjunction with ubiquitously expressed factors is also operative. While we did not specifically measure the affinity of the *cis*-element for Tra2β binding, the absence of Tra2β binding in NEs where its abundance is low, e.g. the Ao and cultured SMCs, is consistent with a low affinity of Tra2β for this *cis*-element, though we cannot exclude the possibility of an inhibitor of Tra2β binding in these extracts. The Tra2β *cis*-element identified in E23 is based on the consensus sequence of GHVVGANR[21], a modification of the previously described Tra2 binding sequence consisting of GAA repeats[16]. The rather degenerate or non-specific nature of these binding sequences would seem to be at odds with the proposed highly specific role of Tra2β in regulating exon splicing, an issue that may be resolved with further definition of Tra2β target exons and binding sequences.

A limitation of this study is that the tests of MYPT1 E23 splicing were performed in transient transfection assays *in vitro* and *in vivo* with an ~600 nt MYPT1 mini-gene construct. The level of splicing of the MYPT1 mini-gene E23 in the gizzard was less than that of the endogenous gene. This is most likely due to the out-titration of splicing factors in this transient transfection system, as also observed in our prior experiments with a different construct[20]. Consistent with this interpretation, co-transfection of the Tra2β expression plasmid further increased MYPT1 mini-gene E23 splicing in the gizzard tissue, from 30% to 50%, suggesting that under the conditions of this transient transfection experiments Tra2β levels were limiting. Given that Tra2β does not drive E23 splicing to 100%, as well as the multitude of splicing decisions that are made in phenotypically diverse SMCs, it seems quite likely that additional factors will be involved. A related question is the full repertoire of exon splicing that Tra2β may regulate in vivo. Our initial sequence analysis has identified potential Tra2β binding-sites in other smooth muscle alternative exons including Caldesmon, Calponin and m-Vinculin. Identifying the full repertoire of exons under the control of Tra2β in vivo can be addressed by loss-of-function studies. The correlation of MYPT1 E23 splicing and Tra2β expression reported here in the rat and chicken is also observed in the mouse (data not shown), indicating that it will be a useful model to address this question.

In contrast to striated muscle, scarce attention has been paid to the question of the specification of muscle phenotypes in the development of the vascular system and their modulation in disease, despite its likely high significance with respect to vascular function. Tra2β may serve as a novel nodal point to address these questions. A good candidate for upstream control is a 559 nt sequence within the Tra2β intron 1 that is an ultra-conserved sequence (UCS), with 95-100% sequence identity between birds and mammals[30]. A high percentage of UCS function in the regulation of tissue-specific gene transcription and developmental phenotypic specification[30;31] and it will be of great interest to test the Tra2β UCS in this regard. Of note, the tissue-restricted expression (splicing) of *Drosophila* Tra is under the control of Sxl, a pathway that is not evolutionarily conserved, even within Dipterans[32], while more distantly related organisms lack homologues of sxl. This is consistent with the theory of “bottom up” evolution of gene regulatory networks[33], in which gene regulatory factors maintain their functional properties, in this case tissue-specific splicing of exons, and are co-opted by different upstream inputs to regulate new gene networks as lineages diversify through evolution (also see[34]). Downstream of Tra only a few bona fide targets have been identified, including *dsx* and *fruity* in *Drosophila*, consistent with its specialized role in this organism. These downstream factors have been conserved and expanded through evolution and constitute the DMRT and BTB-ZF transcription factor gene families, respectively, in higher vertebrates. The current

study provides a foundation and rationale for investigation of Tra2β gain-and-loss of function studies in the mouse in order to further define the program of exon splicing that is regulated by Tra2β *in vivo*. Such studies may provide a link between exon splicing and transcription in the control of the smooth muscle phenotype in development and disease.

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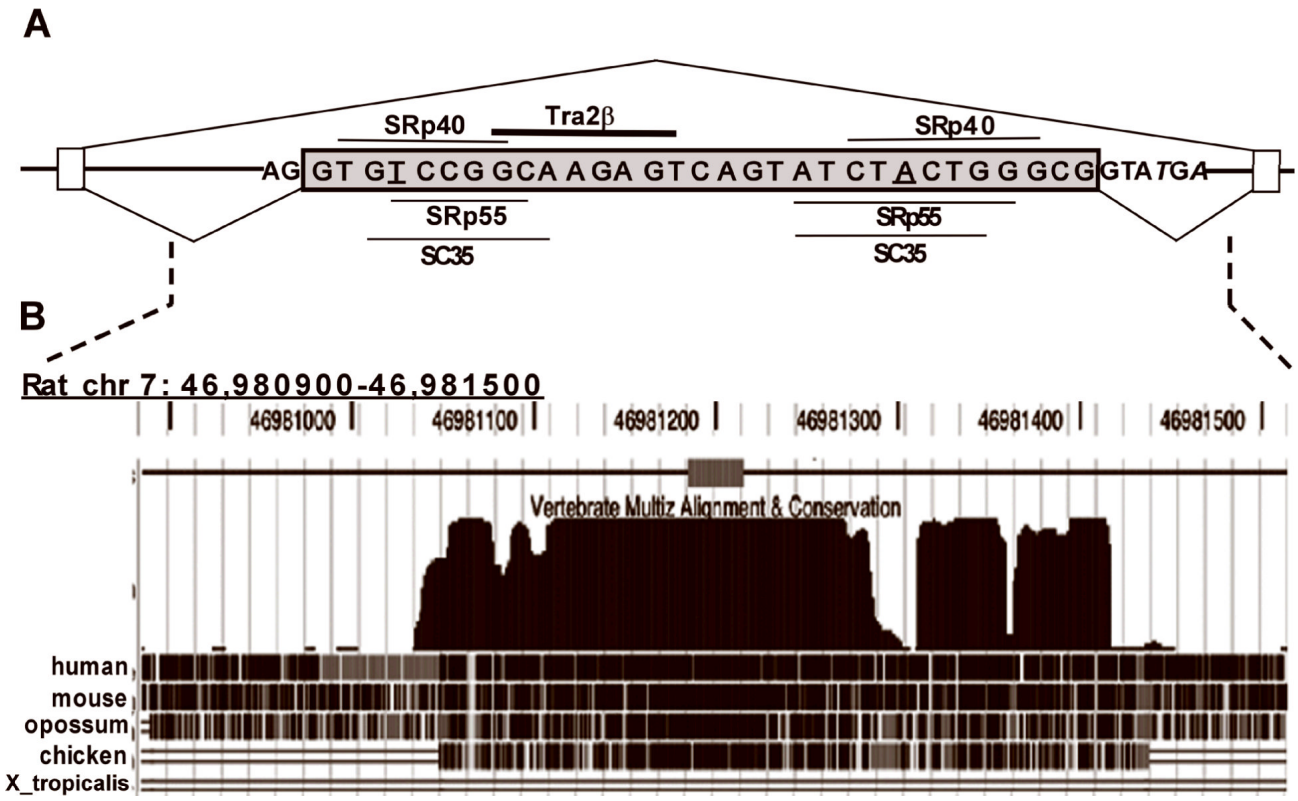

Fig.1.

MYPT1 3' E23 and flanking intronic sequence. **A)** Diagram of rat MYPT1 E23 sequence (gray box) and exon splicing. Phylogenetic sequence variation is underlined. Putative binding sites for splicing regulatory proteins are indicated above and below the exon. The splice site sequences are shown; nucleotides that deviate from the consensus are italicized. **B)** Phylogenetic analysis of rat MYPT1 genomic sequence consisting of E23 and flanking introns. Gray solid box,E23; line,flanking introns. The black histogram represents the evolutionary conservation among vertebrates. Horizontal lines, gaps in the alignment.

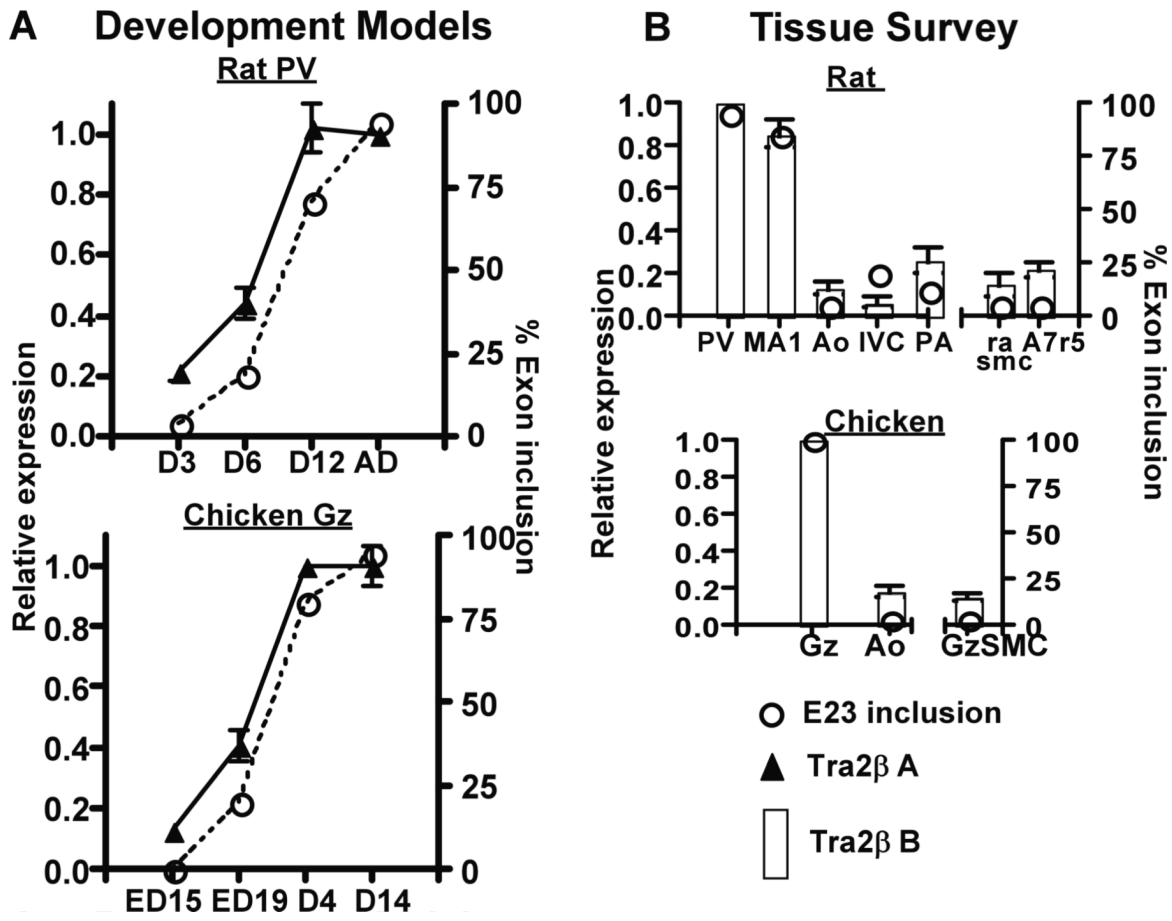

Fig.2.

Concordance in Tra2β mRNA levels and MYPT1 E23 inclusion in smooth muscle. Tra2β mRNA abundance was measured by real-time PCR. The %E23 inclusion as measured previously is shown for reference purposes. **(A)** Tra2β increases 4-8 fold in the peri-natal period in the PV (upper panel) and Gz (lower panel) concordant with a complete switch to E23 inclusion **(B)** Tra2β is 5-10 fold more abundant in the mature PV, MA1 and Gz compared to large arteries and veins (Ao, IVC, PA), where E23 is nearly exclusively skipped. Cultured SMCs show low levels of Tra2β mRNA and skipping of E23. Values on the y-axis represent expression of Tra2β relative to **A)** and **B)** adult PV (upper panel) or Gz (lower panel), normalized to SRp20. Data is displayed as the mean ± S.D. ($n \geq 3$). PV, Portal vein; Gz, gizzard; MA1, first order mesenteric artery; Aorta, Ao; IVC, inferior vena cava; PA, pulmonary artery; rasmc, rat aortic SMCs; GzSMC, gizzard SMCs; D, days post birth; ED, embryonic day, AD, adult.

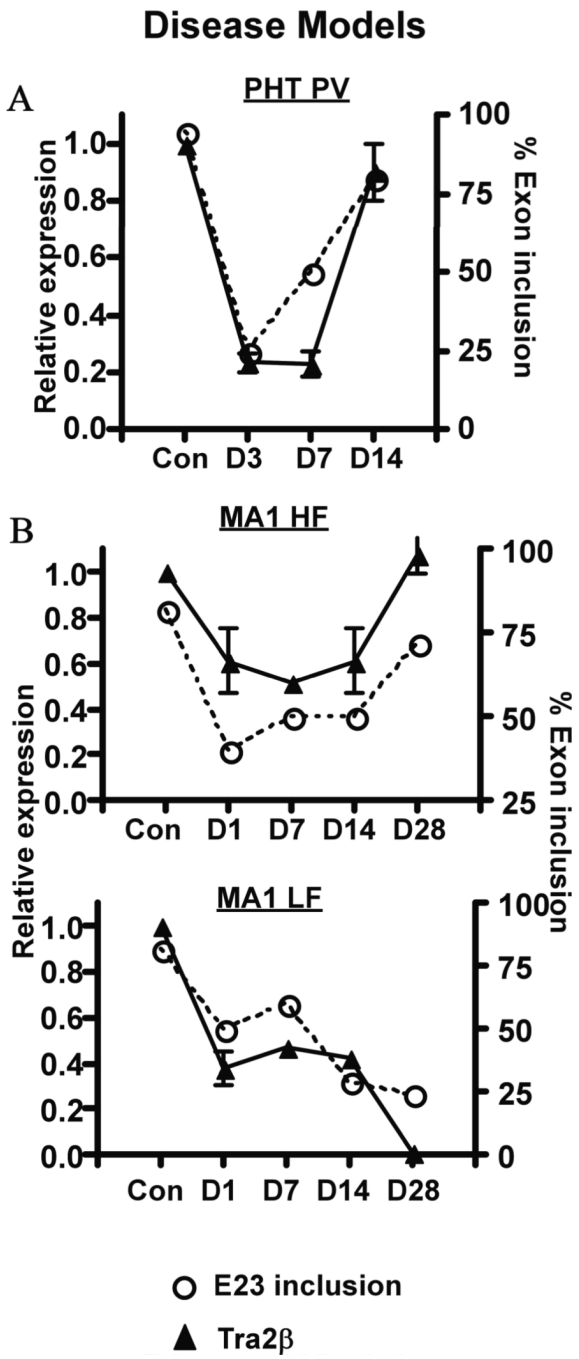

Fig.3.

Concordance in Tra2β mRNA levels and MYPT1 E23 inclusion in disease models. In **A)** PHT PV and **B)** HF and LF MA1 there is dynamic down-regulation in Tra2β mRNA concordant with a switch to skipping. Values on the y-axis represent expression of Tra2β relative to **A)** control PV and **B)** control MA1, normalized to SRp20. Data is displayed as the mean±S.D. (n ≥ 3). PHT, portal hypertension; HF, high-flow; LF, low-flow. D, Days after ligature of PV (PHT) or second order MA (MA1 HF/LF).

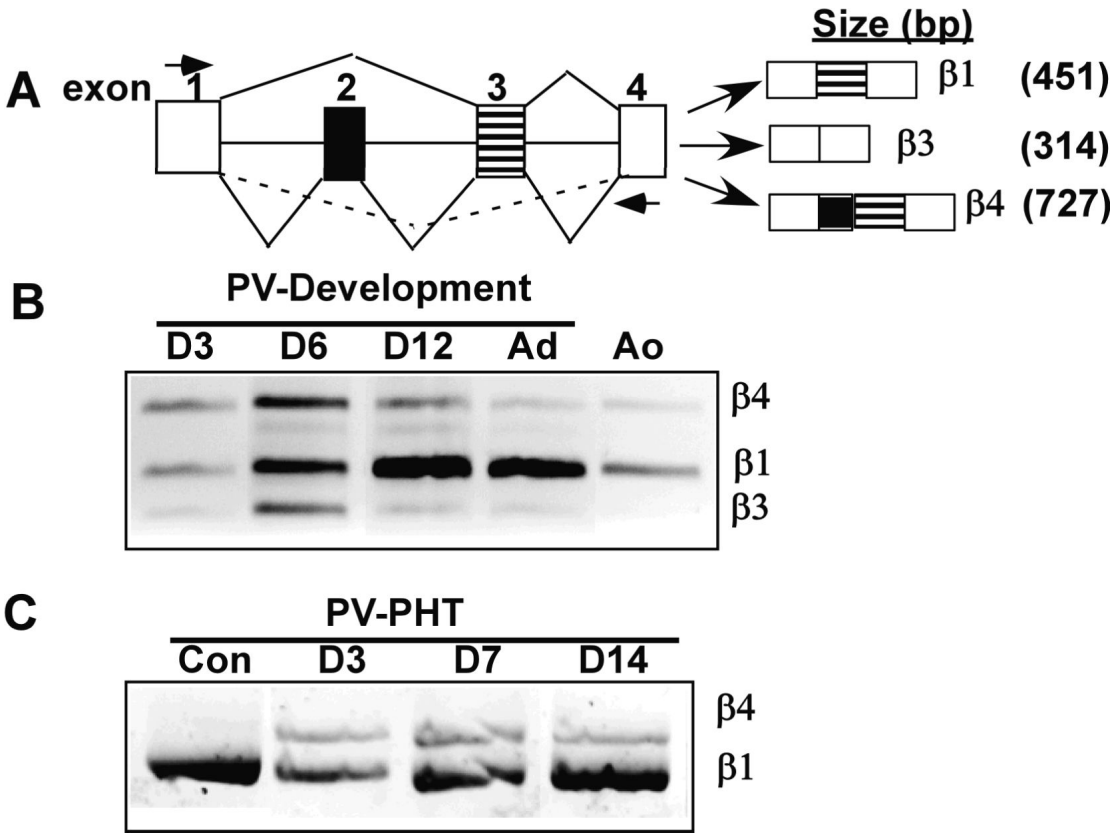

Fig.4.

Tra2β isoforms in PV development and a disease model. **A)** Alternative splicing of exons 2 (black box) and 3 (striped box) gives rise to three major transcripts. The arrows indicate the positions of primers. The expected size of the PCR product (in bp) is indicated. Tra2β isoforms expression was analyzed in **B)** PV development and **C)** PHT PV. Representative gels are shown (n=3). bp, base pair.

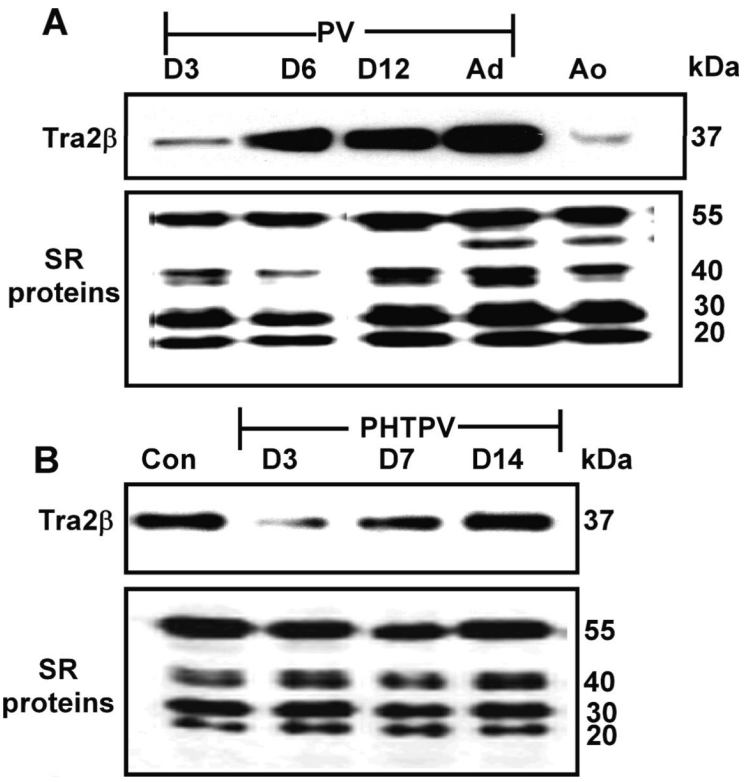

Fig.5.

Concordant up-regulation of Tra2β protein and MYPT1 E23 inclusion. Nuclear extracts from **A)** rat PV post-natal development and mature aorta and **B)** PV PHT were analyzed by Western blotting. Membranes were probed with antibodies to detect Tra2β and SR proteins. Approximate size of each band in kDa is indicated. A representative blot is shown (n=4).

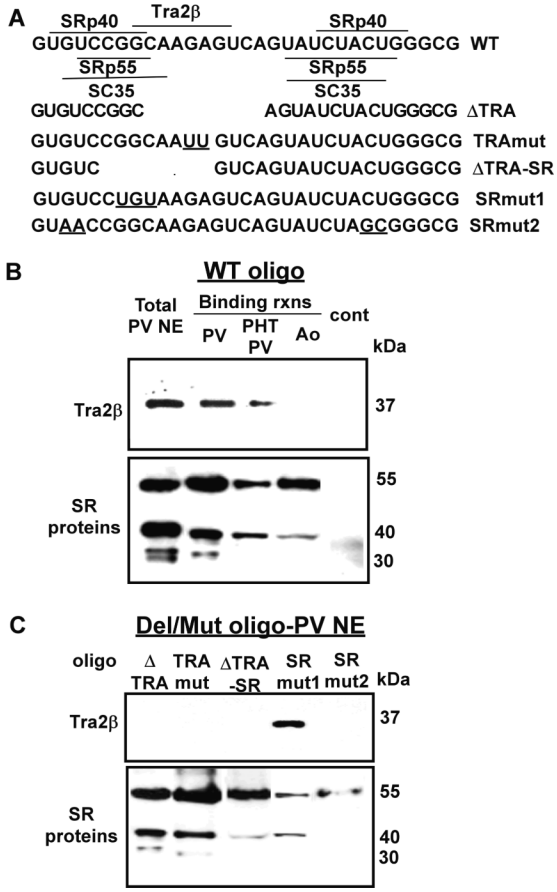

Fig.6.

Tra2β binds to the MYPT1 E23 in RNA pull-down assays. **A)** Sequence of wild type E23 and mutations and deletions of putative Tra2β and SR binding sites. **B)** The WT biotin-labeled RNA oligonucleotide was incubated with NE from adult rat PV, Ao, or D3 PHT PV. RNA-bound proteins were eluted and detected by western blotting. **C)** Oligonucleotides with deletion or mutation of Tra2β and/or SR binding sites were mixed with PV NE and analyzed as in B. The approximate size of the bound protein is indicated in kDa. A representative blot is shown (n=3). WT, wild type; Δ, deletions; mut, mutation; cont, control (unrelated RNA sequence).

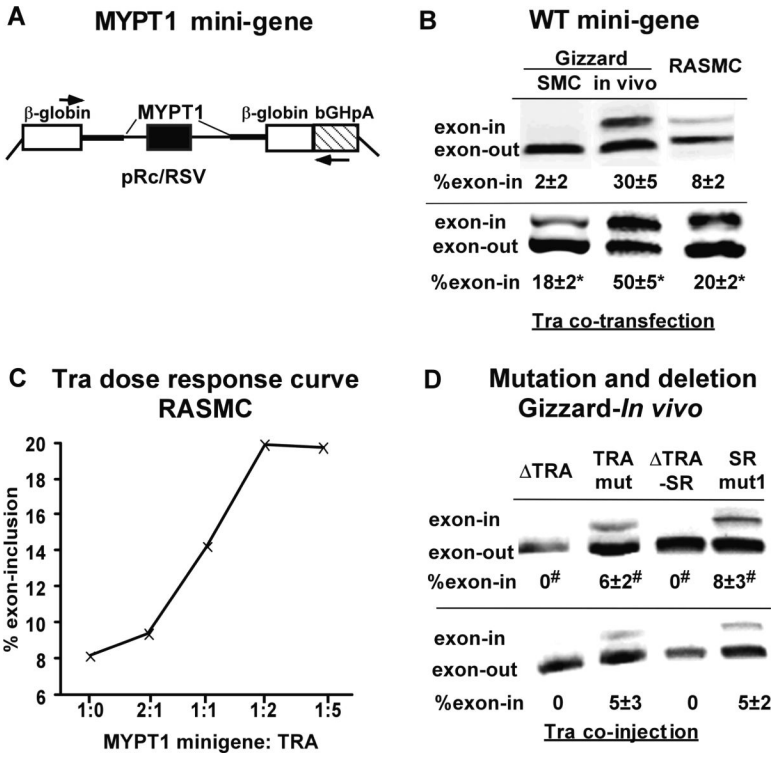

Fig.7.

Tra2β trans-activates MYPT1 E23 splicing dependent upon the exonic Tra2β *cis*-element. **A)** Diagram of MYPT1 mini-gene construct. Open box,βglobin exons; solid box, E23 exon; connecting lines,introns (thicker line, β globin, thinner line,MYPT1). Arrows indicate the locations of the Primers. **B)** The wild-type MYPT1 mini-gene plasmid either alone (upper panel) or with a Tra2β expression plasmid (lower panel), was transfected into cultured chicken Gz or rat aortic SMCs, or injected into the gizzard *in vivo*. **C)** Dose response studies of Tra2β expression plasmid co-transfected with the wild type mini-gene into the RASMCs. A stoichiometry of MYPT1 minigene:Tra2β of 1:2 was optimum and was used in the other experiments. **D)** MYPT1 mini-gene mutation and deletion analysis. MYPT1 mini-gene constructs alone (upper panel) or with Tra2β expression plasmid (lower panel) were injected into the gizzard *in vivo*. RNA harvested 24 h post-transfection, and ratios of MYPT1 exon-in to exon-out mini-transcripts determined by RT-PCR. A picture of a representative gel is shown with quantification (n=3-6). **P*<0.01 (WT+TRA vs. WT only), #*P*<0.01 (Δ/mut vs. WT)

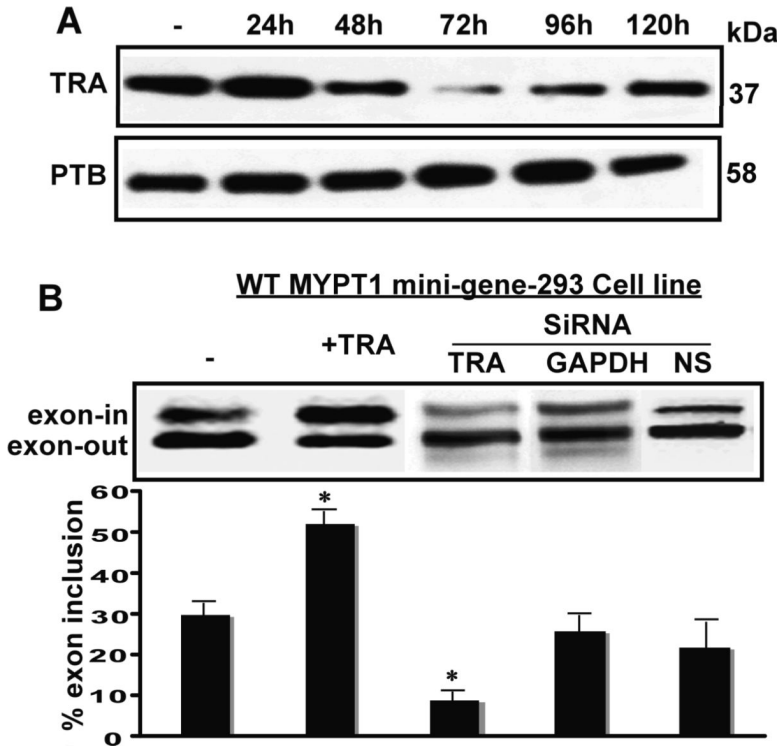

Fig.8.

Knock-down of Tra2β reduces MYPT1 E23 splicing. **A)** Tra2$^{\beta}$. was knocked down in cultured HEK293 cells by siRNA. NEs were collected at 24h to 120h post transfection and Tra2$^{\beta}$. level assessed by western blotting. Constant PTB levels are shown as internal controls. **B)** The wild-type MYPT1 mini-gene plasmid alone or with the Tra2β expression plasmid was transfected into 293 cells. The MYPT1 mini-gene plasmid was also transfected into 293 cells 48 h after treatment with siRNA against Tra2β, or GAPDH or ns siRNA as negative controls. RNA was isolated and ratios of MYPT1 exon-in to exon-out mini-gene transcripts were determined. Representative gels are shown with quantification as the mean ± S.D. (n≥3). *P<0.01 (vs. control), NS, non-specific.