A pathway involving HDAC5, cFLIP and caspases regulates expression of the splicing regulator polypyrimidine tract binding protein in the heart

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Summary

Polypyrimidine tract binding protein (PTB) regulates pre-mRNA splicing, having special relevance for determining gene expression in the differentiating muscle. We have previously shown that PTB protein abundance is progressively reduced during heart development without reduction of its own transcript. Simultaneous reduction of histone deacetylase (HDAC) expression prompted us to investigate the potential link between these events. HDAC5-deficient mice have reduced cardiac PTB protein abundance, and HDAC inhibition in myocytes causes a reduction in endogenous expression of cellular FLICE-like inhibitory protein (cFLIP) and caspase-dependent cleavage of PTB. In agreement with this, cardiac PTB expression is abnormally high in mice with cardiac-specific executioner caspase deficiency, and cFLIP overexpression prevents PTB cleavage *in vitro*. Caspase-dependent cleavage triggers further fragmentation of PTB, and these fragments accumulate in the presence of proteasome inhibitors. Experimental modification of the above processes *in vivo* and *in vitro* results in coherent changes in the alternative splicing of genes encoding tropomyosin-1 (TPM1), tropomyosin-2 (TPM2) and myocyte enhancer factor-2 (MEF2). Thus, we report a pathway connecting HDAC, cFLIP and caspases regulating the progressive disappearance of PTB, which enables the expression of the adult variants of proteins involved in the regulation of contraction and transcription during cardiac muscle development.

Key words: Cardiomyocyte, Differentiation, Polypyrimidine tract binding protein, Myocyte Enhancer Factor-2, Gene expression

Introduction

Polypyrimidine tract binding protein (PTB) is an RNA-binding protein well known for its role in the regulation of alternative splicing of many transcripts (Keppetipola et al., 2012), including those coding for sarcomeric proteins β-tropomyosin, α-tropomyosin, α-actinin and troponin-T (Charlet-B et al., 2002; Mulligan et al., 1992; Pérez et al., 1997; Southby et al., 1999). Global profiling of PTB targets in HeLa cells confirmed that PTB represses many neuronal and striated muscle specific exons in genes encoding cytoskeletal and signaling proteins (Llorian et al., 2010; Spellman et al., 2007; Xue et al., 2009). PTB is also involved in cap-independent protein translation (Mitchell et al., 2001; Sawicka et al., 2008) and our previous results showed that PTB supports apoptotic protein translation in differentiating cardiomyocytes (Zhang et al., 2009). All these results suggest an important contribution of PTB in the post-transcriptional control of gene expression during muscle differentiation. However, regulation of PTB expression has been only partially elucidated. PTB limits its own expression by inhibiting inclusion of exon 11 in its own mRNA inducing its degradation by nonsense-mediated decay in HeLa cells (Wollerton et al., 2004). Downregulation of PTB expression is important for orchestrating regulated splicing programs during neurogenesis (Boutz et al., 2007b). The neuron-specific microRNA miR-124 targets the PTB mRNA, reducing PTB levels in differentiating neurons (Makeyev et al., 2007). Likewise, in differentiating C2C12 myoblasts nPTB expression is downregulated by miR-133 (Boutz et al., 2007a). Thus, it seems that targeting PTB mRNA is important for the control of PTB activity in cell lines and during neuronal development. However, there has been little information regarding the regulation of PTB levels in cardiac muscle. Here, we show that PTB levels in the developing heart are reduced post-translationally by a pathway involving histone deacetylases (HDAC) and the direct cleavage of PTB by caspases.

Class IIa histone deacetylases (HDAC) are involved in the control of gene expression in striated muscle (Haberland et al., 2009). In particular, HDAC4 and 5 bind MEF2 transcription factor, inhibiting its transcriptional activity (Lu et al., 2000). Double deletion of HDAC5 and 9 is embryonic lethal in mice accompanied by defects in the cardiac structure (Chang et al., 2004). It was suggested that this phenotype could ensue from precocious differentiation due to premature derepression of

MEF2 activity (Chang et al., 2004), but the hypothesis remains unexplored.

The caspase-dependent signaling machinery is expressed in the embryonic myocardium but is repressed during heart differentiation (Bahi et al., 2006; Zhang et al., 2009; Zhang et al., 2007). Deficiency in caspase-8 (Varfolomeev et al., 1998), Fas-associated death domain protein (FADD) (Yeh et al., 1998) or cellular FLICE-like inhibitory protein (cFLIP) (Yeh et al., 2000), which regulates the death receptor-dependent pathway, as well as the double deletion of executioner caspases 3 and 7 (Lakhani et al., 2006) induced embryonic lethality with alteration of the cardiac ventricular structure. Cardiac defects of caspase-8-deficient mice including abnormal myocyte death were rescued by *ex vivo* embryo culture and pointed to a role of caspase-8 in the control of cell death during development (Sakamaki et al., 2002). Strikingly, the rate of apoptosis in the FADD and cFLIP mutant hearts was low and similar to wild type tissue but the differentiation process of the ventricles was altered suggesting that these factors have functions unrelated to cell death during heart development (Yeh et al., 2000; Yeh et al., 1998). Despite the relevant contribution of the caspase-dependent signaling to cardiac morphogenesis, its precise function in the heart remains unknown. Caspases have been shown to directly target PTB in cell lines treated with toxic drugs (Back et al., 2002), but there has been no previous indication of a role for such processing in a normal physiological setting.

The MEF2 family of transcription factors, composed of four members termed MEF2A–D, is involved in the transcription of genes required for skeletal and heart muscle development as well as for muscle adaptation to stress (Potthoff and Olson, 2007). MEF2A is the most abundant MEF2 variant in the adult heart (Yu et al., 1992) and its deletion in mice induces death during the first week of life with alterations in cardiomyocyte ultrastructure (Naya et al., 2002). Deletion of MEF2C induces profound morphological defects in the heart (Lin et al., 1997). Finally, deletion of MEF2D hampers cardiac hypertrophy in the adult (Kim et al., 2008). Therefore, MEF2 activity is essential for heart development and cardiac adaptation to stress. An alternative splicing event in *Mef2* transcripts involving the inclusion of a short exon (exon β) occurs in striated muscle and brain, two tissues with MEF2-regulated gene transcription (Yu et al., 1992). Inclusion of exon β increases during C2C12 myoblast differentiation *in vitro* (Yu et al., 1992; Zhu et al., 2005), resulting in translation of a MEF2 variant with stronger transcriptional activity (Yu et al., 1992; Zhu et al., 2005). *In vitro* experiments in HeLa cells showed that PTB induces exon β skipping in *Mef2d* (Llorian et al., 2010) and in cultured C2C12 myoblasts exon β inclusion in *Mef2c* seems to require PTB (Lin and Tarn, 2011). In addition, exon β inclusion was detected in RNA extracts of adult heart yet the timing and mechanisms regulating the splicing of exon β in the heart have not yet been characterized (Yu et al., 1992; Zhu et al., 2005).

Finally, although it is known that PTB is involved in the alternative splicing of several genes encoding structural proteins and, in particular for the actin-binding tropomyosin proteins (Llorian et al., 2010; Mulligan et al., 1992) there was no previous information about the regulation of the alternative splicing of the tropomyosin transcripts during cardiomyocyte differentiation.

In this study, we present *in vivo* and *in vitro* data showing that expression of the splicing repressor PTB in the developing myocardium is reduced through its caspase-dependent cleavage. Upstream control of the caspase activity is influenced by HDAC activity, which in turn regulates the expression of the caspase inhibitor cFLIP, while the downstream consequence of reduced PTB levels is the inclusion of PTB-repressed exons in the transcripts of the structural proteins α- and β-tropomyosin and the MEF2 transcription factors to express the adult variants. These findings reveal a new pathway of regulation of the splicing repressor PTB during heart development.

Results

PTB expression is silenced perinatally during heart development through post-transcriptional mechanisms blocked by HDAC5

We examined the expression pattern of PTB in the rat heart at different development stages. PTB protein levels were reduced rapidly after birth (Fig. 1A), and by day 90, PTB was undetectable. Interestingly, we did not detect significant changes in the PTB mRNA level during the perinatal period of development. In the adult, PTB mRNA levels had decreased by only 40% while PTB protein had already disappeared (Fig. 1B), suggesting post-transcriptional regulation.

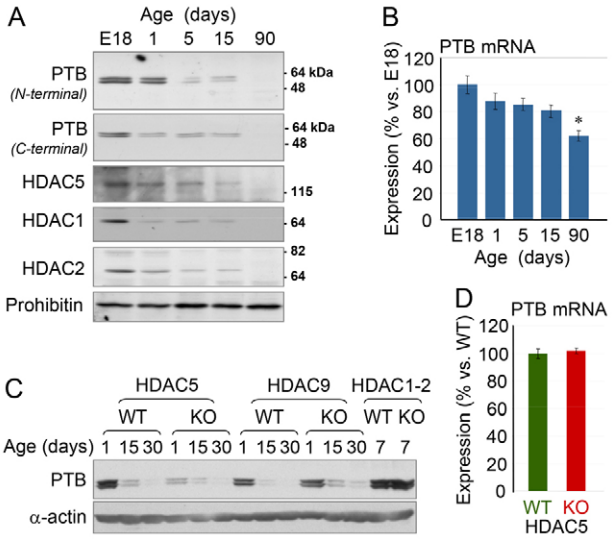

Fig. 1. PTB expression during cardiomyocyte differentiation is regulated post-transcriptionally by HDAC5. (**A**) Expression of PTB and HDAC proteins in heart protein extracts from rats of different ages ranging from embryos (E18) to 3-month-old adults. PTB was analyzed with antibodies against the N-terminal region and C-terminal region. Two alternatively spliced variants of PTB were detected (lower band, PTB1, and upper band, PTB4, translated from a transcript including exon 9). (**B**) PTB transcript abundance was quantified in quadruplicates by real time qRT-PCR in total RNA extracts of the same hearts as in A and referred to the value of the housekeeping gene *Gapdh* amplified in the same reaction. Error bars show s.e.m. from three independent experiments. *$P<0.05$ versus embryo by two-tailed Student's *t* test. (**C**) PTB abundance in protein extracts from hearts of wild type (WT) newborns, young and young adults and HDAC5-deficient (KO) and HDAC9-deficient littermates as well as wild type and HDAC1 and HDAC2 double deficient neonates. Similar results were obtained with two independent sets of samples. (**D**) PTB total transcript abundance was quantified by real time PCR from the hearts of 1-day-old neonatal wild type and HDAC5-deficient mice. Error bars show s.e.m. from three independent experiments.

HDAC proteins are regulators of gene expression in many tissues and, in particular, they have an essential function during heart development (Chang et al., 2004; Montgomery et al., 2007). Mice deficient for Class II HDAC 5 and 9 or Class I HDAC 1 and 2 show propensity to lethal cardiac defects (Chang et al., 2004; Montgomery et al., 2007). Therefore, we measured the expression of HDAC proteins in the heart and observed that HDAC 1, 2 and 5 were abundant in the embryonic myocardium and there was a positive correlation in their temporal expression pattern to that of PTB (Fig. 1A). Consistent with a causal relationship between the decrease in expression of HDACs and PTB, we also observed lower amounts of PTB in the hearts of neonatal mice deficient for HDAC5 expression (Fig. 1C), although PTB transcript levels were similar to wild type age-matched littermates (Fig. 1D). This effect was specific for HDAC5 because neither lack of HDAC9 nor the double deletion of Class I HDAC1 and 2 modified PTB expression significantly *in vivo* (Fig. 1C). These results suggest that HDAC5 influences the post-transcriptional regulation of PTB in the heart *in vivo*. However, given its molecular function as a histone deacetylase it is unlikely that it acts directly as a positive effector of PTB expression.

HDAC sustains PTB expression in cardiomyocytes through inhibition of caspase-dependent PTB cleavage

To further explore the relationship between HDAC activity and PTB expression, we investigated the effects of treating postnatal rat cardiomyocytes with the HDAC inhibitors, sodium butyrate (NaB) or trichostatin-A (TSA) (Fig. 2A,B). Both treatments led to a reduction of full length PTB protein abundance, with no effects upon PTB mRNA levels at 72 hours (Control: 1.0±0.0; NaB:1.2±0.2; TSA:1.1±0.1; $n=3$). However, the reduction in full length PTB was accompanied by the appearance of faster migrating doublet (Fig. 2A, left panel), as detected with an antibody obtained against the C-terminal region of PTB. The size of the faster migrating PTB doublet (~40 kDa) observed in cardiomyocytes treated with HDAC inhibitors is consistent with cleavage by caspases between the RRM1 and RRM2 domains of PTB (Keppetipola et al., 2012). This effect was not observed in human embryonic kidney 293 (HEK293) cells (Fig. 2A, right-hand panel), which express high levels of Class I HDAC but lack detectable expression of HDAC5. Consistent with PTB cleavage by caspases in cardiomyocytes treated with HDAC inhibitors, the pan-caspase inhibitor z-VAD-fmk abolished appearance of the smaller PTB fragments, preserving the abundance of the full length form (Fig. 2A). Caspase-dependent PTB cleavage induced by HDAC inhibition was not associated with a significant increase in cell death (Fig. 2B). If caspases play a physiological role in PTB cleavage *in vivo*, caspase deficiency should lead to increased abundance of PTB in the developing heart. Consistent with this prediction, PTB was more abundant in the heart of cardiac-specific caspase-3 and -7-deficient mice than in their wild type age-matched controls (Fig. 2C), while the abundance of the PTB transcript was unchanged (Fig. 2D).

The preceding data indicate that caspases are responsible for the reduction in PTB levels during cardiac development. However, it is not clear how the activity of caspases upon PTB is regulated. Given that disruption of cFLIP, a natural inhibitor of caspase activity, interferes with heart development (Yeh et al., 2000), we next aimed to assess the contribution of cFLIP to HDAC inhibitor-induced PTB cleavage *in vitro*. We found that cFLIP expression is downregulated during cardiomyocyte differentiation (Fig. 3A, left panel) and that the heart of HDAC5-deficient mice express lower levels of cFLIP than age-matched wild type mice (Fig. 3A, right panel). These results suggested that HDAC could be required for cFLIP expression in the developing heart. cFLIP mRNA (Fig. 3B) and protein (Fig. 3D) expression was reduced in cardiomyocytes treated with HDAC inhibitors and cFLIP overexpression or treatment with the caspase-8 inhibitor z-IETD-fmk prevented HDAC inhibitor-induced PTB cleavage (Fig. 3C). cFLIP overexpression also prevented fragmentation of PTB into a smaller fragment of ~25 kDa (Fig. 3D). Taken together, our results demonstrated a role of the extrinsic pathway of apoptosis, unrelated to cell death and regulated by HDAC, in the control of PTB expression in the myocardium.

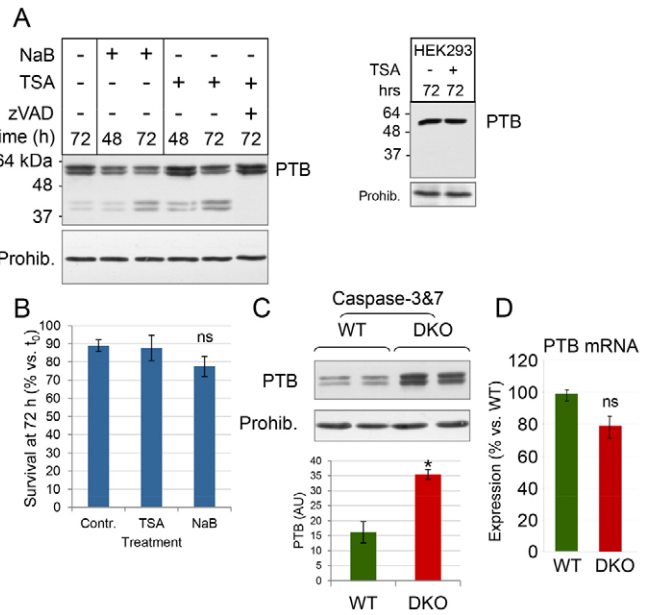

Fig. 2. HDAC inhibition induces the caspase-dependent cleavage of PTB. (**A**) Rat neonatal ventricular cardiomyocytes were treated with HDAC inhibitors NaB (5 mM) or TSA (100 nM) for 48 or 72 h in the presence or absence of the pan-caspase inhibitor z-VAD-fmk (50 μM). PTB abundance was detected with an antibody against the C-terminal region. Right-hand panel: HEK293 cells were treated for 3 days with TSA, and PTB was detected in total protein amounts with the same antibody. The experiments were repeated four times with comparable results. Prohib., prohibitin (used as a loading control). (**B**) Cell survival was counted by the Trypan Blue exclusion assay in cardiomyocyte cultures treated for 3 days with either TSA or NaB at the doses reported above or cultured without drugs (Contr.). Values are expressed as percentage of cell survival versus control plates processed before addition of the drugs. Each experiment was performed in triplicate. $n=3$, error bars show s.e.m. ns, not significant changes versus controls (paired Student's t test). (**C**) PTB abundance was analyzed in total protein extracts of hearts from young wild type (WT) and cardiac-specific caspase-3 and -7-deficient mice (DKO). Lower panel: western blot densitometric analysis (AU, arbitrary units). *$P<0.05$ versus WT by two-tailed Student's t test. (**D**) Quantitative real time PCR of PTB transcript in the same hearts as in C. $n=3$, Error bars show s.e.m. ns, not significant changes versus WT by two-tailed Student's t test.

Caspase-dependent cleavage of PTB triggers its proteasome-dependent degradation

Upon performing a time course of PTB degradation in cardiomyocytes treated with HDAC inhibitors, a small fragment of

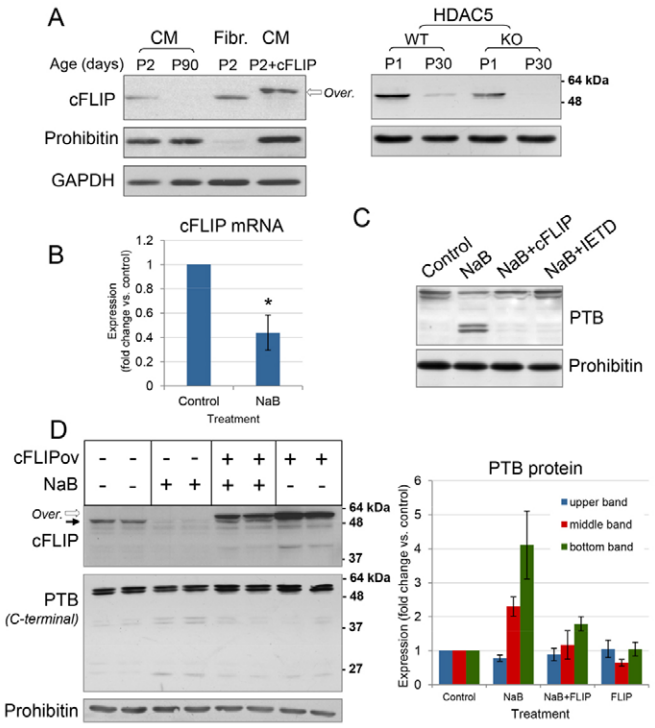

Fig. 3. HDACs block caspase-dependent cleavage of PTB in cardiomyocytes by supporting expression of the endogenous caspase inhibitor cFLIP. (**A**) Left-hand panel: cFLIP abundance was detected in isolated rat neonatal (P2) and adult (P90) cardiomyocytes (CM), neonatal fibroblasts (Fibr.) and neonatal cardiomyocytes overexpressing FLAG-cFLIP (Over., overexpressed FLAG-cFLIP band). Because fibroblasts express very low levels of prohibitin, GAPDH expression is also shown as a loading control. Right-hand panel: cFLIP abundance was detected in total cardiac protein extracts of neonatal (P1) and young (P30) wild type (WT) and HDAC5-deficient (KO) mice. (**B**) Rat neonatal ventricular cardiomyocytes were treated with the HDAC inhibitor NaB (5 mM) for 24 h and cFLIP transcript abundance was analyzed by quantitative real time PCR. $n=3$, error bars show s.e.m. $^*P<0.05$ versus control by two-tailed Student's t test. (**C**) PTB protein abundance was assessed with an antibody against the C-terminal region of PTB in protein extracts from cardiomyocytes transduced with empty viruses or viruses inducing cFLIP overexpression (cFLIP) treated or not with the HDAC inhibitor NaB (5 mM) for 24 h in the presence or absence of the caspase-8 inhibitor z-IETD-fmk (IETD) (20 μM). (**D**) Cardiomyocytes were transduced with empty viruses or viruses inducing cFLIP overexpression (cFLIPov) and were treated or not with the HDAC inhibitor NaB (5 mM) for the last 24 h. Expression of cFLIP (the white arrow shows overexpressed FLAG-cFLIP and the black arrow shows endogenous cFLIP) and PTB was analyzed in total protein extracts. The bar graph shows the densitometric analysis of PTB protein abundance from experiments shown in D. Error bars show s.e.m of $n=4$ experiments (upper band, full length PTB; middle band, ~40 kDa fragment; bottom band, ~25 kDa fragment).

~25 kDa was observable at later time points (Fig. 4A). This suggests that the caspase-dependent fragments of PTB previously observed (Fig. 3) are being further processed. It has been reported that caspase-dependent fragmentation could trigger the proteasome-dependent degradation of the targeted protein (Demontis et al., 2006; Plesca et al., 2008). Therefore, we assessed if PTB was degraded by the proteasome. In the presence of the proteasome inhibitor lactacystin we observed an accumulation of the smallest PTB fragments produced during NaB treatment (Fig. 4B), suggesting that PTB fragments generated by caspase activity were further degraded by the proteasome.

Abundance of PTB determines the use of alternatively regulated exons in the mRNA of the transcription factor MEF2 and the structural protein tropomyosin during cardiomyocyte differentiation

The reduction in PTB levels during cardiac development is likely to be responsible for numerous changes in alternative splicing (Llorian et al., 2010). We first assessed whether alternative splicing of known PTB-regulated alternative splicing events were coherently regulated in our different developmental, knockout and overexpression experimental models. Mutually exclusive alternative splicing of exons 6 and 7 in the *Tpm2* gene, which encodes β-tropomyosin, has long been known to be regulated by PTB (Llorian and Smith, 2011; Mulligan et al., 1992), and is mis-regulated upon knockdown of PTB in HeLa cells (Llorian et al., 2010; Mulligan et al., 1992). Splicing of mutually exclusive exons 8 and 9 in the *Tpm1* gene, which codes for α-tropomyosin, is also affected by PTB knockdown (Llorian et al., 2010; Mulligan et al., 1992), and PTB CLIP tags are located between the two exons of both genes indicating that PTB directly regulates these events (Llorian et al., 2010; Spellman et al., 2007; Xue et al., 2009). We therefore analyzed *Tpm1* and *Tpm2* splicing in postnatal cardiomyocytes overexpressing PTB, in heart extracts from different developmental stages and in hearts of mice deficient for HDAC5 and caspases 3 and 7 (Fig. 5). Expression of *Tpm1* exon 9 and *Tpm2* exon 7 was reduced in cultured neonatal cardiomyocytes overexpressing PTB as expected (Fig. 5B,G), while the expression of the alternatively spliced exon was accordingly upregulated. During early postnatal development of the heart, the expression of the PTB-repressed exons of *Tpm1* and *Tpm2* increased in the myocardium (Fig. 5C,H) in agreement with the reduction of PTB abundance (Fig. 1A). *Tpm2* exon 7 was later downregulated, coinciding with the reduction of TPM2 expression in the myocardium (Marston and Redwood, 2003). Consistent with the low expression of PTB in the HDAC5-deficient heart, *Tpm1* exon 9 and *Tpm2* exon 7 were more abundantly expressed than in the hearts of age-matched wild type mice (Fig. 5D,I). Conversely, these exons were less abundant in the hearts of caspase-3- and -7-deficient mice, which express high levels of PTB, than in the hearts of age-matched wild type mice (Fig. 5E,J). Taken together, our results showed that changes in the signaling pathway regulating PTB expression influenced the alternative splicing of genes encoding the cardiac structural proteins TPM1 and TPM2.

Finally, we were interested in exploring the possibility that the MEF2 transcription factors may constitute important targets of regulation by altered PTB levels. MEF2 proteins are expressed abundantly in the developing heart, with MEF2A being most highly expressed at later stages of development (Fig. 6A). In muscle, brain and in differentiated C2C12 cells, *Mef2* transcripts may contain an alternatively spliced exon (β) encoding a short glutamic acid-rich sequence (e.g. SEEEELEL in MEF2A) that gives rise to MEF2 proteins with stronger transcriptional activity (Yu et al., 1992; Zhu et al., 2005) (Fig. 6B; supplementary material Fig. S1). Previous reports have shown that PTB can regulate different members of the *Mef2* family in HeLa, C2C12 and N2A cells (Boutz et al., 2007b; Lin and Tarn, 2011; Llorian et al., 2010). Our results showed that during heart development, exon β was progressively included in *Mef2a* and *Mef2d* transcripts at the same time as PTB protein levels decreased in the myocardium to form the transcript variants most abundant in the adult heart (the rat sequence has been deposited in GenBank

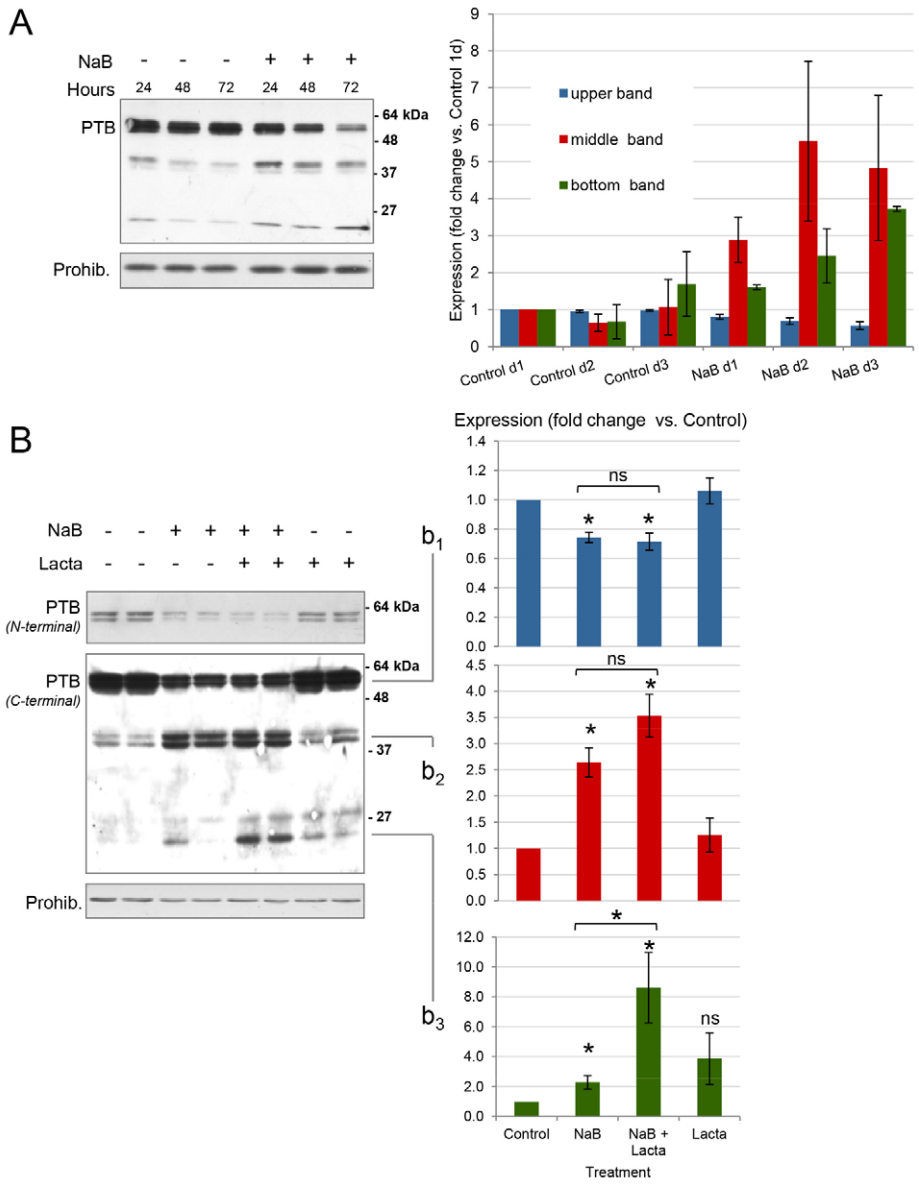

Fig. 4. Caspase-dependent cleavage of PTB triggers its proteasome-dependent degradation. (**A**) Time course of PTB processing in rat neonatal cardiomyocytes treated with the HDAC inhibitor NaB (5 mM). The bar graph shows the densitometric analysis of PTB protein abundance. Error bars show s.e.m of $n=3$ experiments (upper band, full length PTB; middle band, ~40 kDa fragment; bottom band, ~25 kDa fragment). (**B**) Effect of the addition of the proteasome inhibitor lactacystin (Lacta) (2 μM) on PTB processing during treatment with the HDAC inhibitor NaB (5 mM) for 72 h. Right-hand panels: Densitometric analysis of PTB protein abundance. b_1, full length PTB; b_2, ~40 kDa band; b_3, ~25 kDa band. Error bars show s.e.m of $n=4$ experiments performed in duplicate. *$P<0.05$ versus control by two-tailed Student's t test. Prohib., prohibitin; ns, not significant.

under accession no. GU646868), while the most abundant *Mef2c* variant lacked this exon during the timescale of the experiment (Fig. 6B). Overexpression of either PTB1 or PTB4 in neonatal cardiomyocytes, which express low levels of these proteins, induced exon β skipping in *Mef2a* and *Mef2d*, but not in *Mef2c* (Fig. 6C). Moreover, deficiency of HDAC5, which induced premature reduction in PTB abundance, caused increased exon β inclusion in *Mef2* (Fig. 6D), whereas cardiomyocyte-specific deficiency of executioner caspase-3 and -7, which induced abnormal abundance of PTB (Fig. 2C), caused reduced exon β inclusion in *Mef2* (Fig. 6E). There is therefore a good inverse correlation between PTB abundance and inclusion of *Mef2a* and *Mef2d* exon β. UV-crosslinking experiments in HeLa nuclear extracts, where PTB is abundantly expressed and influenced exon β splicing (supplementary material Fig. S2A), showed that PTB crosslinked only weakly to human *Mef2a* and *Mef2d* RNAs fragments containing exon β and their respective intronic flanking regions (supplementary material Fig. S2B). This suggests that PTB might act indirectly to influence *Mef2* alternative splicing, or alternatively that PTB binding to *Mef2* transcripts might be promoted by other factors in developing mouse cardiomyocytes. However, taken together, these results showed that PTB expression, controlled by HDAC and caspases, determined the inclusion of exon β in *Mef2a* and *Mef2d* transcripts in differentiating myocytes.

Discussion

This study reveals a novel signaling network that regulates the progressive disappearance of the splicing repressor PTB during cardiac muscle differentiation involving the action of caspases, which influences alternative splicing of muscle-enriched transcription factors MEF2A and MEF2D. The results presented here show that HDAC regulate the caspase-dependent cleavage of PTB inducing its degradation by the

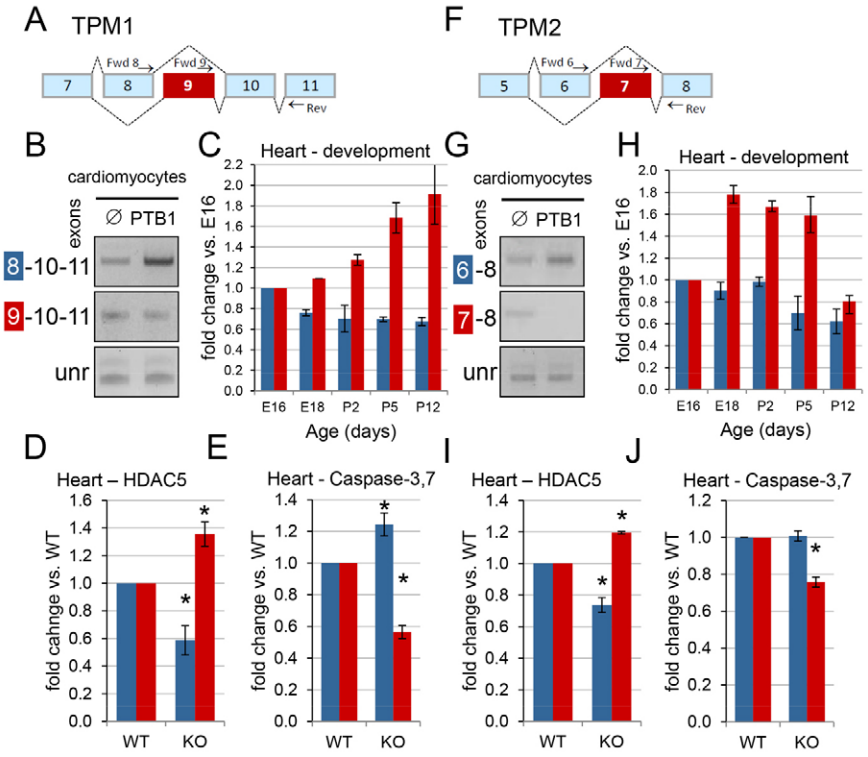

Fig. 5. PTB regulates alternative splicing of tropomyosin-1 and tropomyosin-2 in the developing heart. (**A**) Diagram showing possible transcripts generated by splicing of mutually exclusive exons in *Tpm1*, indicating the PTB-repressed exon in red and the position of the primers used for RT-PCR. (**B**) Inclusion of exons 8 and 9 in the *Tpm1* transcripts in empty virus-transduced () and PTB1-overexpressing neonatal rat cardiomyocytes. unr, upstream of n-ras (used as a loading control). (**C**) Abundance of the *Tpm1* variants including the PTB-repressed exon 9 (red) or the alternatively spliced exon 8 (blue) in reverse-transcribed total cardiac RNA extracts of rats at different ages (from embryonic day 16 to postnatal day 12). Bars show relative values to embryonic day 16 (E16). (**D**) Inclusion of *Tpm1*'s exons 8 and 9 in the hearts of embryonic E18 wild type (WT) and HDAC5-deficient (KO) mice, expressed as fold change versus WT. (**E**) Inclusion of *Tpm1*'s exons 8 and 9 in the hearts of neonatal wild type (WT) and caspase-3,7-deficient (KO) mice, expressed as fold change versus WT. (**F**–**J**) Same experiments as in (A–E) for *Tpm2*'s exons 6 and 7. Each bar indicates the mean and error bars show s.e.m of results obtained from three independent hearts. *$P<0.05$ versus WT.

proteasome. The reduced PTB levels then lead to increased inclusion of PTB-repressed exons (Fig. 7).

Though the importance of PTB in the regulation of relevant splicing events influencing the expression and function of many genes, including some coding for structural proteins relevant for muscle cells, is well established (Charlet-B et al., 2002; Mulligan et al., 1992; Pérez et al., 1997; Southby et al., 1999), knowledge about the signaling pathways that control PTB expression in muscle cells is more limited, and is mainly derived from studies in the C2C12 myoblast cell line (Boutz et al., 2007a; Lin and Tarn, 2011). In differentiating neurons a switch between expression of PTB and the neuronal paralog nPTB is driven by the neuronal micro-RNA miR-124, which downregulates PTB expression leading to a series of neuronal specific alternative splicing events (Boutz et al., 2007b; Makeyev et al., 2007). In C2C12 cells, the protein RBM4 promotes skipping of PTB exon 11 and nPTB exon 10, leading to nonsense-mediated mRNA decay (NMD) and reduced PTB and nPTB levels (Lin and Tarn, 2011). In addition, miR-133 reduces nPTB expression (Boutz et al., 2007a).

Here, we demonstrate a novel mechanism for the control of PTB expression in primary cardiomyocytes and in the rodent heart *in vivo*. Caspase-dependent cleavage of PTB is demonstrated *in vitro* in neonatal cardiomyocytes (Fig. 2A) and is suggested to regulate PTB abundance in the heart *in vivo*, because cardiac-specific caspase-3 and -7 null mice express more PTB than wild type mice (Fig. 2B). Caspases were previously shown to cleave PTB in cell lines treated with toxic drugs (Back et al., 2002), but here we show that this cleavage occurs as part of a normal developmental program. By reducing PTB abundance, caspase activity could contribute to the control of many PTB-dependent splicing events in the perinatal period of heart development. This could be involved in the deleterious effects caused by *in vivo* deletion of key regulators of the extrinsic apoptotic signaling, which obstruct heart development without affecting the rate of cardiac cell death (Yeh et al., 2000; Yeh et al., 1998). The expression of the caspase-8 inhibitor cFLIP was downregulated during cardiac perinatal development and also in the heart of HDAC5-deficient mice compared with wild type mice, suggesting that cFLIP expression could contribute to the control of caspase-dependent PTB cleavage *in vivo*. Consistent with this hypothesis, PTB cleavage induced by HDAC inhibitors in cardiomyocytes was blocked by overexpressing cFLIP-L or by adding a caspase-8 inhibitor, in agreement with the contribution of the extrinsic pathway to PTB degradation. These results together with previous reports showing that caspase-3 and caspase-9 regulate differentiation of C2C12 myoblasts *in vitro* (Fernando et al., 2002; Murray et al., 2008) support a role of the caspase-dependent cell signaling during muscle differentiation. Although PTB cleavage induced by HDAC inhibition produced C-terminal fragments of ~40 kDa, we did not detect the presence of these fragments *in vivo* in correlation to the reduction of full length PTB in the developing heart. However, the increased abundance of PTB proteins in the heart of cardiac-specific caspase-3 and -7 double knockout strongly supports a role of caspases in the control of PTB expression *in vivo*. The results presented here also suggest that the initial cleavage of PTB by caspases triggers further PTB degradation by the proteasome. Caspase-dependent processing inducing further degradation of the target has been demonstrated for other proteins (Demontis et al., 2006; Plesca et al., 2008).

The regulated programs of alternative splicing in developing heart involve numerous factors in addition to PTB; bioinformatic analysis of exons that are co-regulated during cardiac development indicated enrichment of binding sites for CELF, MBNL and FOX proteins in addition to PTB (Kalsotra et al.,

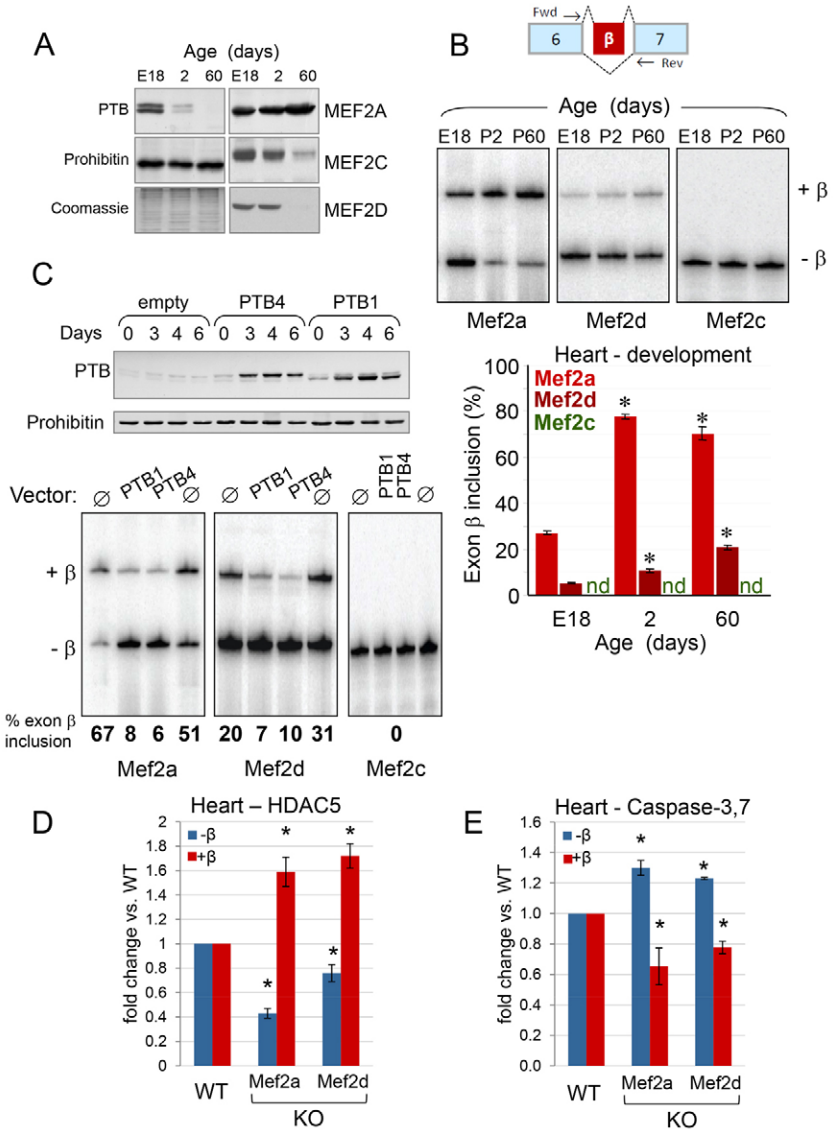

Fig. 6. PTB expression determines skipping of exon β in *Mef2a* and *Mef2d* transcripts in cardiomyocytes. (**A**) Expression of PTB and MEF2 (A,**C**,**D**) in cardiac protein extracts of rat embryos (E18), 2-day-old neonates and 2-month-old adults, with an antibody raised against the N-terminal region of PTB. Similar results were obtained in two analyses using independent samples. Prohibitin was used as a loading control. (**B**) A *Mef2* alternative splicing event occurring during heart development (see a more detailed explanation in supplementary material Fig. S1). Exon β inclusion in *Mef2a*, *Mef2c* and *Mef2d* transcripts was analyzed by radioactive qRT-PCR with primers flanking the alternatively spliced exon in total mRNA samples from hearts of rat embryos (E18), 2-day-old neonates and 2-month-old adults. + β and − β indicate inclusion or exclusion of exon β, respectively. Bar graph: quantification of radioactive products obtained by qRT-PCR. nd: not detected. Error bars show s.e.m. from three independent samples. *$P \leq 0.05$ versus E18 by two-tailed Student's *t* test. (C) Effects of PTB overexpression in cardiomyocytes on *Mef2* exon β splicing. Upper panels: time course of PTB1 and PTB4 overexpression in P4 cardiomyocytes. Neonatal cardiomyocytes were transduced with empty lentiviruses (Ø) or lentiviruses inducing overexpression of PTB, and PTB abundance was monitored by western blot analysis. Lower panels: *Mef2* exon β splicing. Total RNA was extracted at day 4 post-transduction, and exon β inclusion in *Mef2a*, *Mef2c* and *Mef2d* transcripts was analyzed by radioactive qRT-PCR. Two independent experiments were performed with similar results. (D) Analysis of exon β inclusion (−β, not included; +β, included) in *Mef2* transcripts was assessed in total RNA extracts from hearts of 2-day-old wild type (WT) and HDAC5-deficient mice (HDAC5 KO) and is expressed as fold change compared with WT. (**E**) Analysis of exon β inclusion (−β, not included; +β, included) in *Mef2* transcripts was assessed in total RNA extracts from hearts of 1-month-old wild type (WT) and cardiac-specific caspase-3 and -7-deficient mice (KO) and is expressed as fold change compared with WT. Error bars in D and E show s.e.m. of data from three hearts per genotype. *$P < 0.05$ versus WT by two-tailed Student's *t* test.

2008). Indeed, all of these proteins were also implicated in the computationally assembled muscle 'splicing code' (Barash et al., 2010; Llorian and Smith, 2011). Curiously, while the preceding report demonstrated changes in the levels of CELF, MBNL and FOX proteins, no changes in levels of PTB expression were observed during post-natal heart development in mouse (Kalsotra et al., 2008). The reasons for this discrepancy between our observations and those of Kalsotra et al. (Kalsotra et al., 2008) are unclear. Nevertheless, we reproducibly observed a rapid decrease in PTB protein abundance in mouse and rat hearts after birth, using two independent PTB-specific antibodies against two different regions of the protein, whose specificity has been previously confirmed [Fig. 2A and (Zhang et al. (2009)].

In addition, our findings expand the current knowledge about the relevance of PTB in the biology of striated muscle by showing its role in *Mef2* alternative splicing in the developing heart. Thus, although it is known that PTB regulates splicing of many genes involved in muscle contraction, our data reveal that PTB is also involved in the splicing of *Mef2a* and *Mef2d*, which encode transcription factors that are important for the control of gene expression during cardiac muscle differentiation and for the adaptation of the cardiac muscle to stress (Kim et al., 2008).

MEF2 and HDAC are mutually regulated factors involved in the control of gene expression during heart development (McKinsey and Olson, 2005; Potthoff and Olson, 2007). Deficiency in either MEF2A or MEF2C induces profound alterations in heart morphology, and MEF2D deficiency hampers the normal response of the heart to stress in the adult (Kim et al., 2008; Lin et al., 1997; Naya et al., 2002). Inclusion of exon β in *Mef2* transcripts generates mRNAs coding for MEF2 variants bearing an acidic peptide in the transcription activation domain (Yu et al., 1992) (supplementary material Fig. S1) that enhances transcriptional activity of MEF2 (Zhu et al., 2005). On the other hand, mice deficient for Class II HDAC 5 and 9 or Class I HDAC 1 and 2 show propensity to lethal cardiac defects (Chang et al., 2004; Montgomery et al., 2007). Class II HDACs directly bind to MEF2 and inhibit MEF2-dependent gene transcription contributing to the regulation of muscle-specific gene expression (Lu et al., 2000). Given the essential role of MEF2 in heart development, it seems that adjusting MEF2 activity through

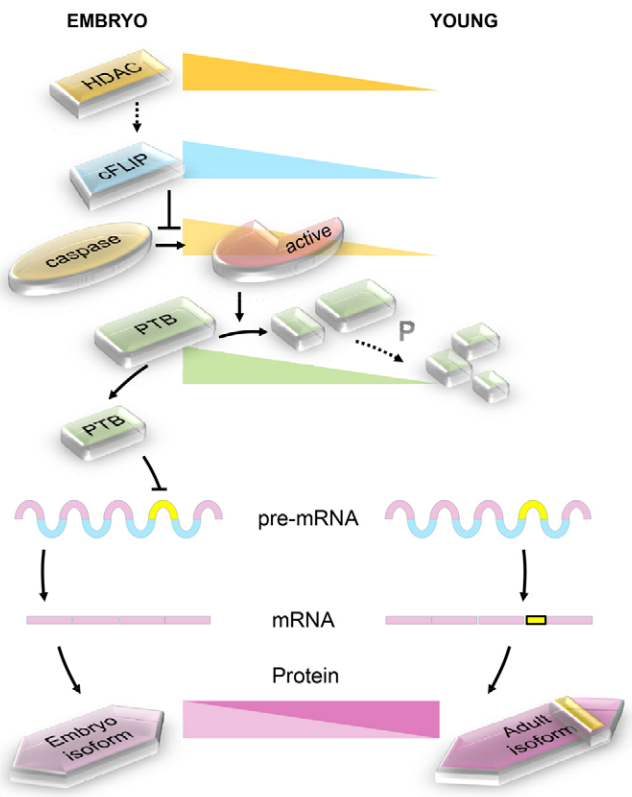

Fig. 7. Model of the regulation of alternative splicing by HDAC and caspases through the control of PTB abundance during heart development. The expression of HDAC decreases during perinatal cardiomyocyte differentiation. Developmental downregulation of HDAC induces the reduction of cFLIP expression, allowing caspases to cleave the splicing repressor PTB. Caspase expression is reduced at the transcriptional level during heart development (Zhang et al., 2007). Caspase-dependent PTB cleavage triggers its degradation by the proteasome. Low levels of PTB in the postnatal heart permits inclusion of PTB-repressed exons in pre-mRNAs, switching to the adult variants of genes such as those encoding the transcription factor MEF2 and the structural protein α-tropomyosin.

regulation of exon β splicing can be relevant for the correct contribution of MEF2-dependent transcription, regulated by Class II HDAC, during heart organogenesis. Our results showed that exon β inclusion in *Mef2a* and *Mef2d* occurs progressively during heart development. We also observed an inverse relationship between exon β inclusion and PTB expression and show reduced PTB expression perinatally in the hearts of rats and mice. Furthermore, we show that PTB expression is sustained by HDAC5, thus linking HDAC5 with the regulation of MEF2A and MEF2D activity through the control of mRNA splicing. These results unveil an additional new pathway by which HDAC can influence MEF2 activity without direct interaction.

The reason for exon β inclusion in the transcript of *Mef2a* and *Mef2d* but not in *Mef2c* in the heart is unknown but our results show that exon β inclusion in *Mef2a* and *Mef2d* is regulated by PTB. MEF2C is essential for the heart only during early development (Lin et al., 1997), and our results show that PTB is abundant during that period. Intrigued by the lack of exon β inclusion in *Mef2c* we verified the presence of this exon in the *Mef2c* gene (*Mef2c* mRNA NCBI entries including exon β are: human, NM_002397; mouse, NM_025282; and rat, XM_001056692). Our in silico analysis of the sequences flanking exon β in the *Mef2* sequences showed putative PTB binding motifs in all three genes; however, we found also a potential Fox-1 binding motif GCAUG (Jin et al., 2003) near both ends of exon β only in the *Mef2c* sequence. Fox-1 is abundant in differentiated muscle and can induce exon skipping (Fukumura et al., 2007). This could contribute to exon β skipping in *Mef2c* in the absence of PTB. However the role of Fox-1 in *Mef2c* exon β skipping is speculative and would require further investigation. On the contrary, MEF2A plays a key role during the late phase of myocardial differentiation (Naya et al., 2002), precisely coinciding with the reduction of PTB expression and exon β inclusion, as we show here. Exon β inclusion in MEF2A seems correlated to its trans-activating activity (Yu et al., 1992) suggesting that it could be essential for the correct function of MEF2A *in vivo*. Furthermore, we showed that a significant fraction of the transcript for MEF2D, which plays a relevant role in stress-induced gene expression in the adult (Kim et al., 2008), includes exon β in a PTB-inhibitable manner progressively after birth. Interestingly, we have recently found increased expression of MEF2A and MEF2D in myocytes treated with hypertrophic agonists and the hypertrophied adult heart (Ye et al., 2012). It can be hypothesized that, in the heart, exon β inclusion is important to define the activity of MEF2A and MEF2D when they are essential, but not in MEF2C, and that this splicing event is regulated by different mechanisms depending on the gene, with PTB being a relevant regulator of exon β splicing in *Mef2a* and *Mef2d* transcripts.

In this work we also show that two splicing events affecting the tropomyosin genes 1 and 2, which were known to be regulated by PTB abundance in other cell types (Llorian and Smith, 2011; Mulligan et al., 1992), are also influenced by PTB in the developing myocardium *in vivo* and neonatal cardiomyocytes *in vitro*. Furthermore, our results demonstrate that experimental alteration of the novel signaling pathway controlling PTB abundance in the heart consistently influences PTB-dependent alternative splicing, adding experimental evidences for the link between HDAC, caspases and PTB in the control of alternative splicing in the heart.

In conclusion, our data show that abundance of PTB in cardiomyocytes is dependent on HDAC5 through the regulation of its FLIP-inhibitable, caspase-dependent cleavage and that progressive reduction of PTB expression permits the inclusion of PTB-repressed exons to change gene isoform expression in the heart during cardiac muscle differentiation. These findings contribute to the understanding of the role of caspases in myocyte differentiation, revealing the involvement of HDAC5 in its regulation and suggesting a previously unknown pathway for the control of gene expression by HDAC, involving the splicing repressor PTB.

Materials and Methods

Animal, tissues and cell cultures

The investigation with experimental animals conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by our Experimental Animal Ethic Committee. For analysis of protein expression we used hearts from Sprague–Dawley rats (Charles-River) housed in our Experimental Animal Facility at the University of Lleida. Full caspase-7-deficient, cardiac-specific caspase-3-deficient mouse strain was generated by sequential crossing of caspase-7 null mice (Lakhani et al., 2006) with caspase-3 floxed mice (a gift from Richard Flavell, Yale University School of Medicine, New Haven, CT) and then with the Nkx2.5-Cre mouse strain (a gift from Eric Olson, University of Texas Southwestern

Medical Center, Dallas, TX). Genotypes were analyzed by PCR. HDAC5 and HDAC9 strains (Chang et al., 2004) and a HDAC1 and HDAC2 double mutant strain (Montgomery et al., 2007) were housed in their laboratories of origin. Hearts were dissected minced into small cubes, rinsed with cold phosphate buffered saline and snap-frozen into liquid nitrogen. Rat neonatal cardiomyocytes were obtained from the ventricles of 2 to 4-day-old pups as described elsewhere (Bahi et al., 2006). HEK293 cells were cultured and used both for experiments and for virus production as reported previously (Bahi et al., 2006).

Pharmacological treatments, lentivirus production and cell transduction

The HDAC inhibitors NaB (cat. no. B5887) and TSA (cat. no. T8552) were purchased from Sigma. The caspase inhibitors z-VAD-fmk (cat. no. 550377) and z-IETD-fmk (cat. no. 550380) were from BD Pharmingen. The proteasome inhibitor lactacystin (L6785) was from Sigma. Lentiviral particles empty or for inducing overexpression of human PTB1 and PTB4 and mouse c-FLIP-L were prepared as previously reported (Zhang et al., 2009) and cardiomyocytes were treated after 3 days of transduction as described elsewhere (Bahi et al., 2006).

RNA extraction, and real time and quantitative RT-PCR

For heart and cardiomyocytes, total RNA was obtained from frozen tissues or cell pellets with the RNeasy Mini Kit (Qiagen). RNA concentration measurements and reverse transcription were done as described (Bahi et al., 2006; Zhang et al., 2009). Quantitative real time PCR was performed in a iCycler iQ PCR detection system and iQ v.3 and iQ v.5 software (BioRad), using the TaqMan Gene Expression Master Mix (cat. no. 4369016) and the Gene Expression Assays Mm00943334_m1 and Rn00821112_m1 to amplify genes encoding mouse and rat PTB, respectively and Rn00589205_m1 to amplify rat cFlip, with simultaneous amplification of *Gapdh* as a loading control (Applied Biosystems). For radioactive quantitative PCR, reverse transcriptase reactions were carried out using 1 µg of total RNA, oligo dT and Superscript II (Invitrogen) following manufacturer's instructions. PCR reactions were carried out with 10 pmol of forward primer and 4 pmol of ^{32}P-labeled reverse primer (supplementary material Table S1) and 1 U/µl of Taq polymerase, under the following conditions: 94℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s, 25 or 30 cycles. 1/20 of the PCR was separated on 8% polyacrylamide urea gel. Gels were dried and exposed in phosphorimager cassettes. PCR band ratios were determined using a Molecular Dynamics PhosphorImager and shown as a fraction of the total. Data shown are means±SD, $n=3$.

Protein extraction, SDS-PAGE and western blot

Protein expression was analyzed in protein extracts diluted in Tris-buffered 2% SDS solution at pH 6.8 and SDS-PAGE was performed as described (Bahi et al., 2006). Antibody specifications are described in supplementary material Table S2. Western blots were performed as reported previously (Bahi et al., 2006).

Author contributions

J.Y., M.L., M.C., A.R. and R.M. performed the experiments; J.X.C., R.B.-D. and C.W.S. facilitated logistics and contributed helpful ideas; R.A.F., E.N.O. and C.W.S. contributed reagents and experimental models; C.W.S. assisted in writing the article; and D.S. developed the hypothesis, designed the experiments and wrote the article.

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