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The cleavage of HuR interferes with its transportin-2-mediated nuclear import and promotes muscle fiber formation

P Beauchamp[1,3], C Nassif[1,3], S Hillock[2], K van der Giessen[1], C von Roretz[1], BJ Jasmin[2] and I-E Gallouzi*[,1]

Although the function of posttranscriptional processes in regulating the expression of genes involved in muscle fiber formation (myogenesis) is well accepted, the mechanisms by which these effects are mediated remain elusive. Here, we uncover such a mechanism and show that during myogenesis, a fraction of the posttranscriptional regulator human antigen R (HuR) is cleaved in a caspase-dependent manner in both cell culture and animal models. Disruption of caspase activity in cultured myoblasts or knocking out the *caspase-3* gene in mice significantly reduced HuR cleavage and the cytoplasmic accumulation of HuR in muscle fibers. The non-cleavable isoform of HuR, HuRD226A, failed to reestablish the myogenic potential of HuR-depleted myoblasts. HuR cleavage generates two fragments: HuR-cleavage product 1 (HuR-CP1) (24 kDa) and HuR-CP2 (8 kDa). Here, we show that one of these fragments (HuR-CP1) binds to the HuR import factor transportin-2 (TRN2) allowing HuR to accumulate in the cytoplasm. As this cytoplasmic accumulation is required for the promyogenic function of HuR, our data support a model, whereby during the transition phase from myoblasts to myotubes, a proportion of HuR is cleaved to generate HuR-CP1. By interfering with the TRN2-mediated import of HuR, this CP helps non-cleaved HuR accumulate in the cytoplasm thus promoting myogenesis.
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One of the main features of muscle tissue is its ability to repair itself upon injury by initiating a process known as myogenesis, which replaces damaged muscle fibers.[1] Myogenesis involves the sequential activation of myogenic regulatory factor (MRF) genes,[2] which trigger the fusion of embryonic muscle cells (myoblasts) to form muscle fibers (myotubes).[1] MRFs such as MyoD, myogenin, myogenic factor 5, and MRF4 belong to a family of transcription factors that are responsible for stimulating the expression of promyogenic genes.[2] Upon activation of myogenesis, MyoD is the first MRF to be upregulated and continues to be expressed in myotubes. MyoD then collaborates with myogenin to promote the fusion of myobasts, leading to muscle fiber formation.[1,3]

Although the signal transduction pathways that activate the expression of MRFs at the transcriptional level are well described, transcription alone is not sufficient to maintain the high levels of their respective proteins required during the myogenic process.[1,3,4] Others and we have suggested that posttranscriptional events are important for muscle development and maintenance.[5,6] Messages encoding MyoD and myogenin proteins contain *cis*-elements, known as AU-rich elements (AREs), in their 3′untranslated regions (3′UTRs). AREs modulate the turnover of several mRNAs in muscle and other cell types by mediating their association with RNA-binding proteins such as the human antigen R (HuR) protein.[7,8] In fact, upon the induction of myogenesis, HuR associates with the 3′UTR of *acetylcholinesterase*, *MyoD*, and *myogenin* mRNAs, leading to their stabilization.[5,6,9] Therefore, posttranscriptional regulatory events involving interactions between AREs in myogenic mRNAs and HuR protein have crucial functions during myogenesis.

Recent observations have indicated that the promyogenic function of HuR involves its import receptor transportin-2 (TRN2). At the initial steps of myogenesis, HuR is localized to the nucleus, whereas during cell fusion and muscle fiber formation, HuR accumulates in the cytoplasm,[5] coinciding with the interruption of the association of HuR with TRN2.[10] Both the dissociation of the HuR/TRN2 complex and the depletion of TRN2 expression stabilize *MyoD* and *myogenin* mRNAs and promote myoblast differentiation.[10]

Recently, we observed that the rapid cytoplasmic accumulation of HuR also occurs in cells undergoing apoptosis in response to several stresses.[11] Indeed, under lethal conditions, cells initiate the caspase-3/7-mediated cleavage of HuR at the Asp (D) 226 residue, generating two cleavage products (CPs): HuR-CP1 (24 kDa) and HuR-CP2 (8 kDa). In HeLa cells, HuR-CP2, but not HuR-CP1, associates with the apoptosome activator PHAPI to promote apoptosis, whereas

[1]Biochemistry Department, McGill University, Rosalind and Moris Goodman Cancer Center, Montreal, Quebec, Canada and [2]Department of Cellular and Molecular Medicine and Centre for Neuromuscular Disease, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada
*Corresponding author: I-E Gallouzi, Department of Biochemistry, McGill University, 3655 Promenade Sire William Osler, McIntyre Building room 906A, Montreal, Quebec, Canada H3G 1Y6. Tel: +1 514 398 4537; Fax: +1 514 398 7384; E-mail: imed.gallouzi@mcgill.ca
[3]These authors contributed equally to this work.
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Abbreviations: HuR, human antigen R; TRN2, transportin-2; MRFs, myogenic regulatory factors; AREs, AU-rich elements; HuR-CP, HuR cleavage product; caspase-CP, caspase cleavage product; AP, antennapedia; GFP, green fluorescence protein; Rec, recombinant; GST, gluthation *S*-transferase; RRM, RNA recognition motif; ActD, actinomycin D
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overexpression of the non-cleavable isoform of HuR, HuR-D226A, delayed this fatal outcome. Interestingly, several studies have shown the implication of important apoptotic players such as caspase-3 and p53 in regulating myogenesis.[12–14] It has been shown that caspase-3 enhances the rate of myofiber formation by specifically cleaving the promyogenic mammalian steril 20-like kinase,[3] leading to its activation.[12] The fact that caspase-3 promotes myogenesis [12,15] raises the possibility that caspase-mediated cleavage could be part of the regulatory mechanisms modulating the promyogenic function of HuR.

In this study, we tested this possibility and present data showing that the caspase-mediated cleavage of HuR is crucial for myogenesis. We showed that this caspase activity generates HuR-CP1, which interferes with the TRN2-mediated import of full-length HuR into the nucleus, thereby promoting muscle fiber formation.

Results

HuR is cleaved in a caspase-dependent manner, both in cultured C2C12 cells and in mice, during myogenesis. To assess whether HuR is cleaved by caspases during myogenesis, we followed HuR expression throughout the differentiation of a well-established murine myogenic cell line, C2C12.[16,17] To ensure that any cleavage of HuR was not the result of contamination with apoptotic muscle cells,[18] the C2C12 cells, differentiated or not, were washed several times to remove the small number of non-adherent dead cells that are normally seen during the myogenic process.[15] The 4′,6-diamidino-2-phenylindole (DAPI) staining of the remaining adherent cells showed that >98% of them had a normal nuclear morphology (Supplementary Figure S1). Western blot analysis of the differentiating C2C12 cells with the anti-HuR monoclonal antibody[19] detected full-length HuR and a second band at 24 kDa, which began appearing at day 1 of myogenesis with a maximum intensity at day 4 (Figure 1a and b). The size of this band corresponds to the size of the HuR cleavage product 1 (HuR-CP1) that was earlier seen in HeLa cells during caspase-induced apoptosis.[11] We also observed that this additional band reached its maximum levels at day 4 of the differentiation process, a time during which muscle cells generated, as expected,[15] the active isoform of caspase-3, caspase-3-CP (Figure 1a and b).

Next, we assessed whether the HuR-CP1 band would also be detected during the regeneration of skeletal muscle in mice. The tibialis anterior (TA) muscle of 4-week-old caspase-3 +/+ or −/− mice was treated with 10 μM cardiotoxin, which induces muscle degeneration followed by regeneration[20,21] (Supplementary Figure S2). Following the expression of HuR during muscle regeneration, we detected the same 24 kDa band seen in differentiating C2C12 cells (Figure 1c, lanes 1–4 and d). This 24 kDa band was absent in differentiating C2C12 cells that were exposed to the caspase inhibitor zVAD[22] (Figure 1e) and was significantly reduced in the TA muscle of caspase-3 −/− mice (Figure 1c, lanes 5–8 and d). The same reduction in the cleavage of HuR was also observed in the extensor digitorum longus (EDL) muscle of caspase-3 −/− mice (data not shown). Although as expected,[5] HuR level was very low in untreated TA muscles (Figure 1c, lanes 1 and 5), the decreased HuR-CP1 levels observed in cardiotoxin-treated TA muscles of caspase-3 −/− mice or in zVAD-treated myotubes (Figure 1c and e) correlated with a drastic reduction in myogenin protein levels. We then determined how much of total HuR is cleaved during myogenesis in our cell culture and animal models. Using the ImageQuant software and by calculating the ratio of band intensities corresponding to HuR-CP1 over total HuR levels, we determined that in differentiated muscle fibers, HuR-CP1 represents ~11% of total HuR (Figure 1b) and that this number is slightly higher (~15%) in mice (Figure 1d). Therefore, these observations indicate that during muscle fiber formation, in both cultured cells and muscle tissue from mice, one or more members of the caspase family are responsible for the cleavage of 10–15% of total HuR.

Our earlier study indicated that although the C-terminal region of the human HuR harbors three potential cleavage sites (D226, D254, and D256), only the D226 residue is targeted by caspases-3 and -7 in HeLa cells undergoing apoptosis.[11] As these three D residues are also conserved in murine HuR (Supplementary Figure S3), we investigated whether any of these aspartates are the sites of cleavage by caspases in muscle cells. Each D residue was replaced by alanine (A) and their expression in differentiating C2C12 cells was determined (Figure 2a and b). Western blot using the anti-green fluorescence protein (anti-GFP) antibody showed that cleavage occurs at the same level as GFP-tagged wild-type HuR for the GFP-HuRD254A and GFP-HuRD256A mutants, but is very week for the mutant GFP-HuRD226A (Figure 2b, compare lane 4 with lanes 1, 2, and 3 and see Figure 1a and b). Using the same calculation method described above (Figure 1), we observed that the cleavage efficiency of GFP-HuRD226A is 10-fold less then the cleavage efficiency of the other HuR isoforms. These observations indicate that in muscle cells, the D226 residue represents the main, but not the unique, cleavage site for HuR that is targeted by caspases during the differentiation process.

Caspase-mediated cleavage of HuR is needed for its promoyogenic function. We next investigated the impact of the D226A mutation on the promyogenic function of HuR. To achieve this, we assessed whether HuR or HuRD226A would reestablish myogenesis in HuR-depleted C2C12 cells.[6] To ensure high uptake efficiency of wtHuR and HuRD226A in muscle cells, these isoforms were conjugated to the antennapedia (AP) cell-permeable peptide[6] (Supplementary Figure S4) and the chimeras were then introduced into HuR-depleted C2C12 myoblasts. Our data show that all the AP-conjugated HuR isoforms were taken up by the cell with the same efficiency and remained stable inside the cell for >8 h (Supplementary Figure S4). The effect of AP-HuR and AP-HuD226A on myogenesis was assessed by phase contrast (Figure 3b) and by immunofluorescence experiments in which we followed the expression of the myoglobin protein, a known marker of muscle differentiation (Figure 3c).[6] Myoglobin expression was also used to determine the fusion index (Figure 3d),

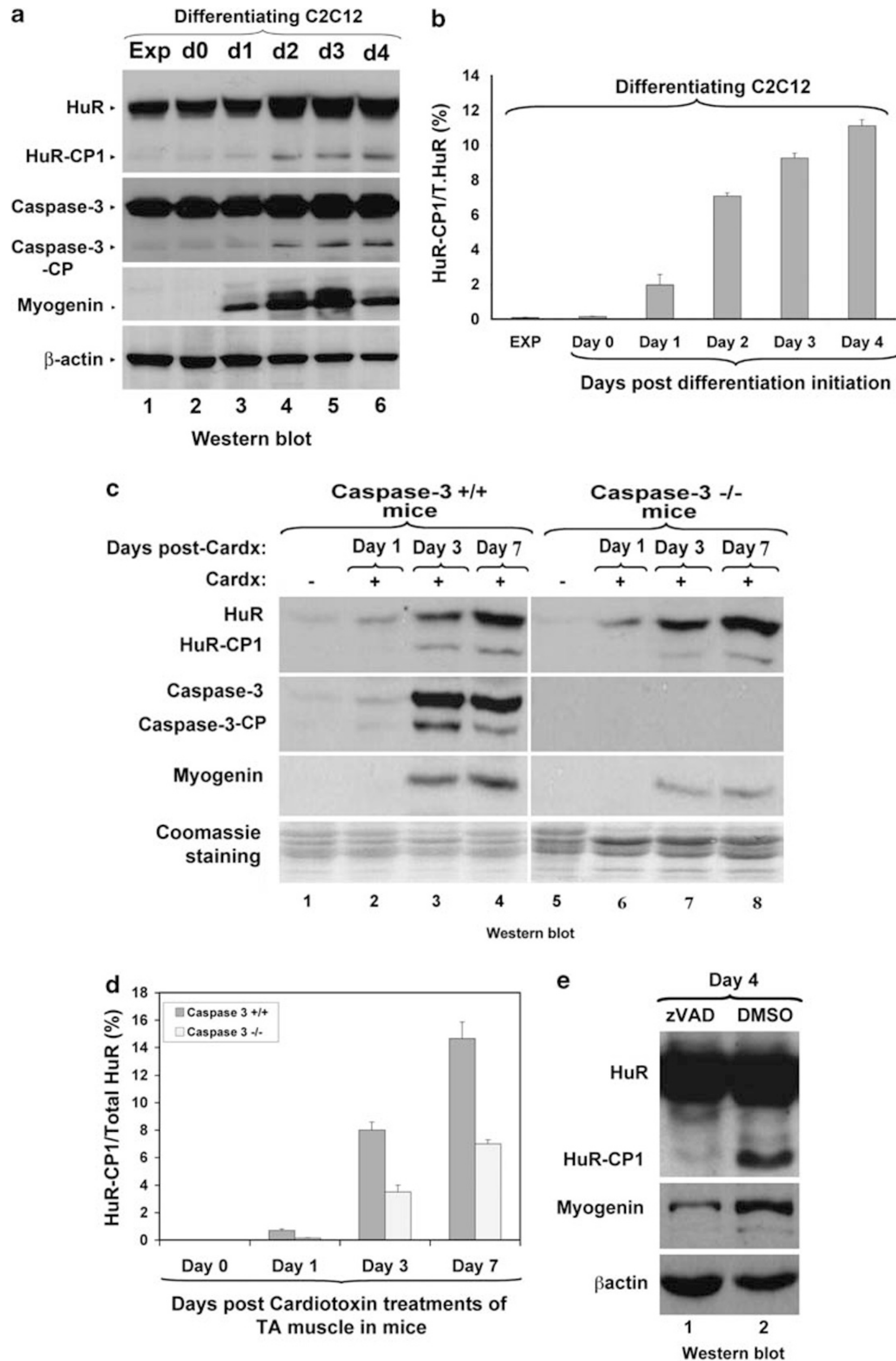


Figure 1 HuR-CP1 is detected at late stages of myogenesis both in differentiating C2C12 cells and in regenerating muscle fibers in mice. (**a**) Western blot analyses were performed using 100 μg of total protein extracts from exponentially growing (Exp) and differentiating C2C12 cells days (d) 0–4. The blot was probed with the monoclonal anti-HuR(3A2), caspase-3, myogenin, and β-actin antibodies. (**b**) The levels of HuR-CP1 and total HuR were quantified using the ImageQuant software. Levels were then standardized against β-actin for each time point and plotted as the percentage $+/-$ the standard error of the mean (S.E.M.) of three independent experiments. (**c**) Western blot analysis was performed using 80 μg of total protein extracts from regenerating TA muscles of two wild-type (WT) and caspase-3 $-/-$ mice *(Casp3tm1Flv*/J mice) for each time point. Regeneration was induced by injecting 10 μM cardiotoxin directly into TA muscles. Blots were probed with antibodies against HuR, caspase-3, and Myogenin. TA muscles were isolated from untreated ($-$) and at days 1, 3, and 7 postinjection. (**d**) The levels of HuR-CP1 in (**c**) were calculated as described in (**b**) except that they were standardized against the band intensities of the coomassie blue staining for each sample. Data are represented as $+/-$ S.E.M. of three independent experiments. (**e**) C2C12 myotubes on day 3 of differentiation were treated with the pan-caspase inhibitor zVAD for 16 h, and total cell extracts were prepared on day 4 of differentiation for western blotting to probe for HuR, myogenin, and β-actin

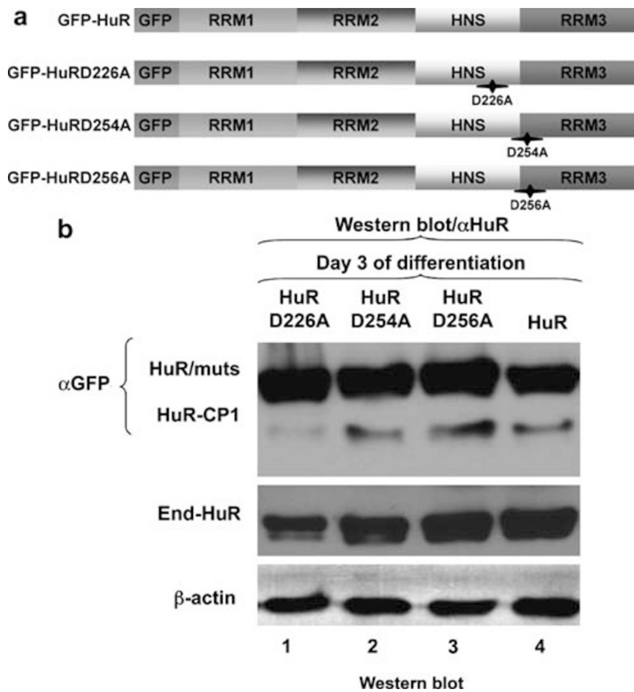


Figure 2 The D22A residue represents the major site that is targeted by caspases to cleave HuR during the differentiation of C2C12 muscle cells. (**a**) Schematic representation of GFP-tagged wild-type HuR (GFP-HuR) and GFP-tagged HuR mutants (GFP-HuRD226A, GFP-HuRD254A, GFP-HuRD256A). (**b**) C2C12 cells were transfected with GFP-HuR, -HuRD254A, -HuRD256A, and -HuRD226A mutants as indicated. The cells were induced for differentiation for 3 days, then 40 μg of total cell extracts were prepared and run on SDS-PAGE for western blot analysis with the anti-GFP (upper panel) and β-actin antibodies (lower panel). The same blot was also probed with the anti-HuR antibody (3A2) to detect endogenous HuR (second panel)

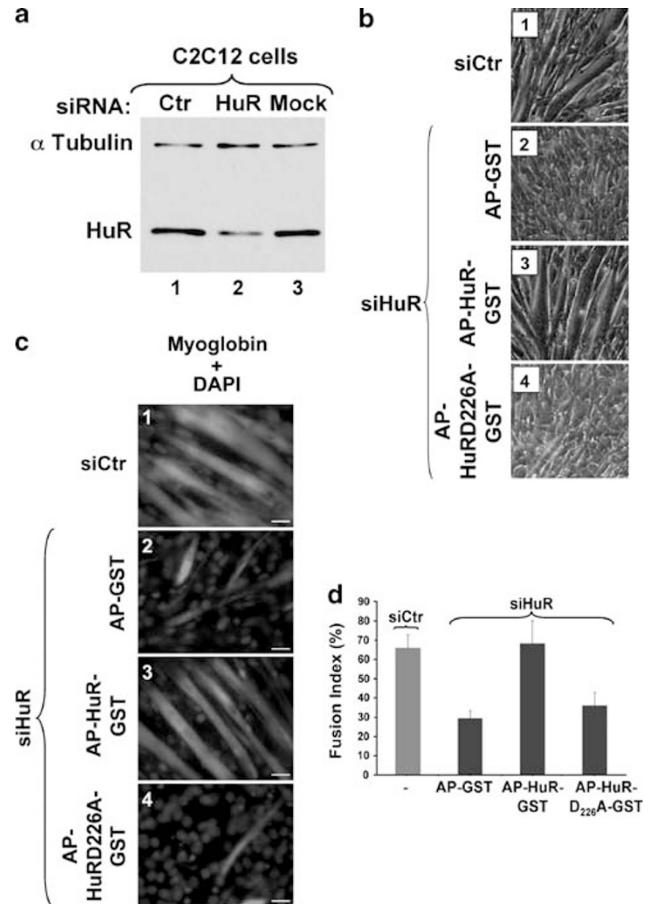


Figure 3 The cleavage of HuR at D226 residue is required for its ability to promote myogenesis. (**a**) C2C12 cells treated with siRNA(si)-HuR or si-control (Ctr) as described [6] were induced to differentiate; 5 μg of total cell extract of cells on day 0 of differentiation was used for western blot analysis with the anti-HuR and anti-α-tubulin (loading control) antibodies. Representative western blots of three independent experiments are shown. (**b**) AP-conjugated-GST, AP-HuR-GST, or AP-HuRD226A-GST were added twice to siHuR- or siCtr-treated C2C12 cells (transfected 24 h before differentiation initiation and at the induction of differentiation). AP-GST and AP-HuR-GST were used, respectively, as negative and positive controls. Phase-contrast pictures of a single representative field of view for each cell treatment on day 3 of differentiation are shown. (**c**) siHuR- or siCtr-treated C2C12 cells in the presence of 50 nM AP-GST, AP-HuR-GST, or AP-HuRD226A-GST were fixed at day 3 of differentiation and immunofluorescence staining was performed against myoglobin and using DAPI. A single representative field for each cell treatment is shown. Bars, 20 μm. (**d**) The fusion index (percent differentiation) was determined by dividing the number of nuclei in myoglobin-positive myotubes by the total number of nuclei in a given microscopic field. For each experiment at least five different fields were counted. Error bars represent the S.E.M. of two independent experiments

which is a commonly used indicator for muscle differentiation efficiency.[23] As earlier shown,[6] depleting HuR expression by ~70% (Figure 3a) significantly reduced the efficiency of muscle fiber formation (panel 2 in Figure 3b and c). The rescue experiment, however, showed that unlike AP-HuR, the AP-HuRD226A mutant failed to rescue myogenesis in siRNA-HuR-treated myoblasts (Figure 3b–d). Therefore, these observations clearly show that an efficient cleavage of HuR by caspases is required for HuR to promote myogenesis.

Association between HuR-CP1 and TRN2 prevents the nuclear import of HuR in muscle cells. Recently, we showed that in HeLa cells undergoing apoptosis, the cleavage of HuR occurs only in the cytoplasm.[11] We tested whether this is also the case during myogenesis, and showed that HuR-CP1 is generated only in the cytoplasmic fraction of differentiating muscle cells (Figure 4a). We also observed that HuR-CP1 levels become maximal in the cytoplasmic fraction at a time when HuR levels in the cytoplasm becomes clearly visible (Figure 4, compare lanes 5, 6, 7 with lane 8). In addition, blocking caspase activity with 10 μM zVAD for 16 h at day 2 of differentiation of C2C12 cells resulted in the nuclear sequestration of HuR (Figure 4b). The implication of caspases in the cytoplasmic accumulation of HuR in differentiated muscle fibers was also confirmed using our animal model. Indeed, we performed nuclear and cytoplasmic fractionation experiments using differentiated EDL muscle fibers that were isolated from caspase-3 +/+ and −/− mice. We observed that in the EDL muscles from caspase-3 −/− mice, the cytoplasmic levels of HuR were significantly lower than those from caspase-3 +/+ mice (Figure 4c–e). Of note, the low levels of total extracts used from each fraction did not allow the detection of HuR-CP1 or

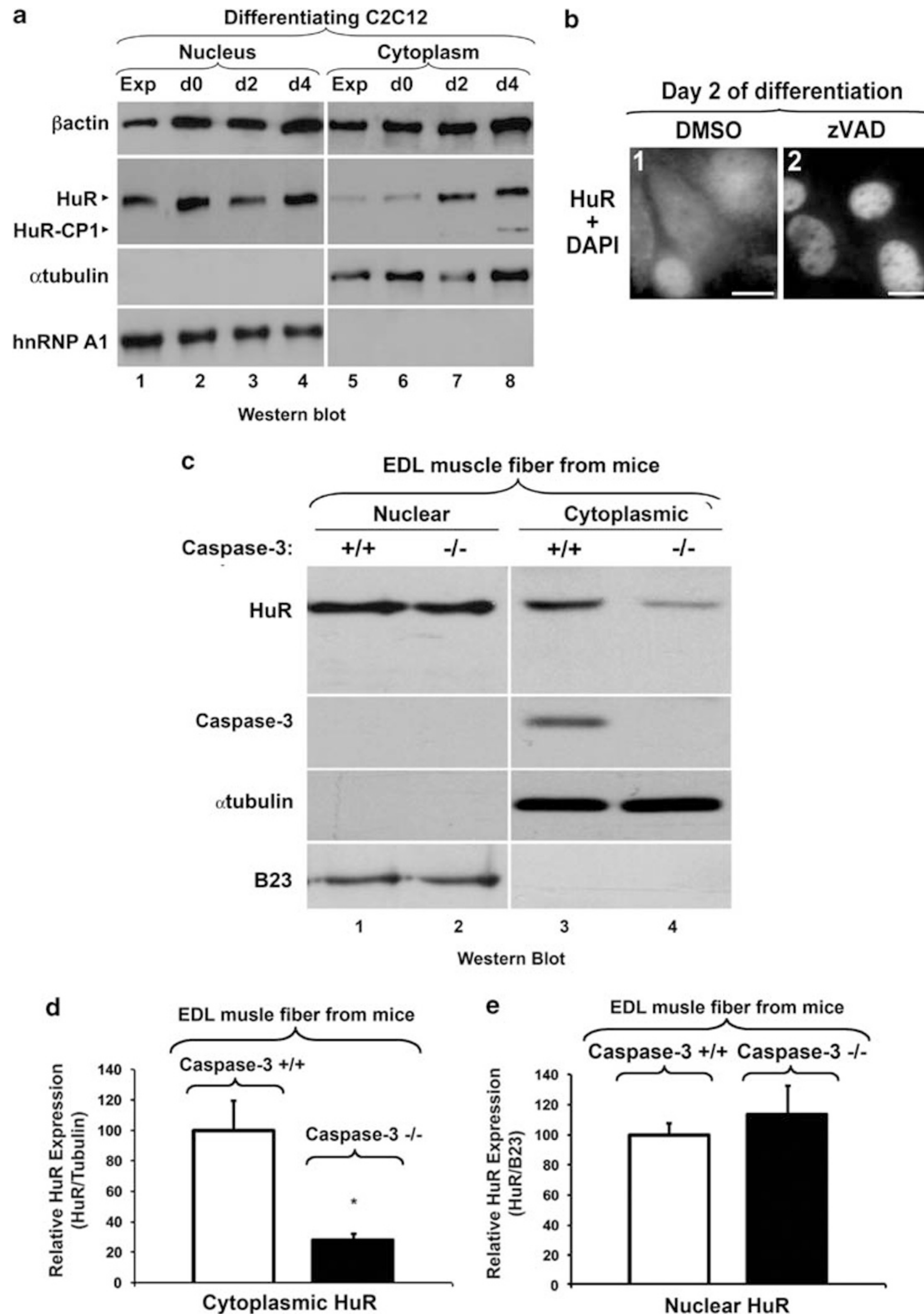


Figure 4 The caspase-3-dependent cleavage of HuR occurs in the cytoplasm of muscle fibers both in differentiated C2C12 cells and in mice. (**a**) Nuclear/cytoplasmic fractionation was performed during C2C12 differentiation, and western blotting analysis was performed on 35 μg of cell extract for each time point. Membranes were probed with antibodies against HuR, β-actin, hnRNP A1 (a nuclear marker), and α-tubulin (a cytoplasmic marker). Representative western blots of two independent experiments are shown. The white lines indicate intervening lanes that have been removed. (**b**) C2C12 cells treated with DMSO or zVAD on induction of differentiation were fixed at day 2, and analyzed by immunofluorescence staining for DAPI and against HuR. Representative images from two independent experiments are shown. Bars, 20 μm. (**c**) Representative western blot of the subcellular distribution of HuR in control (+/+) and caspase-3-deficient (−/−) mice. Nuclear and cytoplasmic extracts were obtained from EDL muscle. Western blot analysis was performed using 30 μg of both nuclear and cytoplasmic extracts. As shown, blots were probed for HuR, caspase-3, the nuclear marker B23 (nucleophosmin), and the cytoplasmic marker α-tubulin. (**d**, **e**) Quantification of the levels of HuR in the cytoplasmic (**d**) and nuclear (**e**) fractions from EDL muscles. Means (+/− S.E.) are shown and expressed as percent of control value. Asterisk denotes a significant difference ($P<0.05$; $n=4$)

caspase-3-CP in the cytoplamic fraction of EDL muscles from caspase-3 +/+ mice. Together, these observations suggest that caspase activity is involved in the cytoplasmic accumulation of HuR during myogenesis.

Earlier experiments have shown that the amount of full-length HuR that associates with TRN2 during myogenesis is around 10% of total HuR.[10] This corresponds to the amount of HuR-CP1 generated in differentiating C2C12 cells (~11%).

Moreover, the two amino-acid residues (Pro 222 and Met 223) of HuR that are required for HuR binding to TRN2[24] are located in the C-terminal region of HuR-CP1 (Figure 2a; Supplementary Figure S4a). These observations, combined with the fact that the cleavage of HuR during myogenesis occurs at the same time as and when the HuR/TRN2 complex is disrupted (between days 3 and 4),[10] raised the possibility that by cleaving a portion of HuR, muscle cells generate HuR-CP1 to block the TRN2-mediated import of HuR. To test this possibility, we performed immunoprecipitation experiments using anti-TRN2 antibody on total extracts of differentiating C2C12 cells that were earlier transfected with the GFP-HuR, -HuR-CP1, or -HuR-CP2 isoforms (Supplementary Figure S5). Western blot analysis using the anti-HuR antibody (3A2) showed that HuR and HuR-CP1 interact with TRN2 (Figure 5a). As the epitope recognized by the 3A2 antibody is located in the first RNA recognition motif (RRM) of HuR,[19] HuR-CP2 could not be detected using 3A2 (Figure 5a, lower panel), and, therefore, the same blots were then probed with the anti-GFP antibody. Although the association between HuR and HuR-CP1 with TRN2 was confirmed (Figure 5b, middle panels), we did not detect any interaction between HuR-CP2 and TRN2 (Figure 5b, compare bottom panel with top panel). The fact that both HuR and HuR-CP 1 associated with TRN2 to the same extent is a good indication that this CP has the ability to disrupt the HuR/TRN2 complex. To test this possibility, we performed an *in vitro* competition assay in which we disrupted the association between gluthation *S*-transferase (GST)-TRN2 and recombinant (Rec)-HuR using Rec-HuR-CP1 or Rec-HuR-CP2. Recombinant proteins had their GST tags removed using the PreScission Protease (GE Healthcare, Quebec, Canada) (Supplementary Figure S6). The association of Rec-HuR to GST-TRN2 beads as well as the disruption of this complex by Rec-HuR-CP1 or -HuR-CP2 was determined *in vitro* using western blot analysis with the anti-HuR monoclonal antibody. As expected, Rec-HuR associated to the GST-TRN2 beads, but not to empty GST beads (Figure 5c, compare lane 2 with lane 4). Our competition experiment, however, has shown that an increasing concentration of Rec-HuR-CP1, but not Rec-HuR-CP2, was able to compete away the association of HuR with GST-TRN2 (Figure 5d, compare lanes 1–5 with lanes 6with 10). These observations clearly show that HuR-CP1 not only associates with TRN2 both *in vitro* and inside muscle cells, but also has the ability to disrupt the HuR/TRN2 complex.

The observations described above lead us to hypothesize that this CP is also able to disrupt the HuR/TRN2 complex in differentiating muscle cells, which would explain how HuR accumulates in the cytoplasm during muscle fiber formation. Several observations from different groups have shown that at early stages of the differentiation process (exponential and day 0), HuR is mainly localized in the nucleus.[5,6,10] Therefore, if our hypothesis is true, the overexpression of HuR-CP1 in muscle cells at day 0 of myogenesis should result in the cytoplasmic accumulation of HuR. To test this hypothesis, we performed fluorescence experiments in which we followed the cellular localization of GFP-HuR in C2C12 cells at day 0 of differentiation in the presence or absence of AP-HuR-CP1-GST or AP-HuR-CP2-GST. As expected, GFP-HuR, in the presence of AP-GST, localized mainly in the nucleus (Figure 5e, panels 1 and 2). However, there was a significant increase in the cytoplasmic level of GFP-HuR in cells treated with AP-HuR-CP1, but not in those treated with AP-HuR-CP2 (Figure 5e, compare panels 3 and 4 to 5 and 6). Together, these results suggest that by associating with TRN2, HuR-CP1 disrupts the HuR/TRN2 complex leading to the cytoplasmic accumulation of HuR, which was earlier shown to be crucial in the promyogenic function of HuR.[5,6,10] The fact that the overexpression of TRN2 in differentiating myoblasts significantly reduced the efficiency of myogenesis (Figure 6) strongly suggests that an increase in the nuclear import of HuR, as a result of an artificial elevation of TRN2 level, negatively affects muscle fiber formation.

It is well established that HuR promotes muscle fiber formation by stabilizing, in the cytoplasm, ARE-containing messages such as *myogenin*.[5,10,25] HuR exercises this stabilization through its ability to specifically recognize the AREs in the 3′UTR of its target messages.[5,10,25] Therefore, to determine whether the cleavage of HuR affects the fate of its mRNA targets, we assessed the association between HuR-CP1 and HuR-CP2 and the HuR-binding site located in the 3′UTR of *myogenin* mRNA (myogenin ARE). Gel shift experiments using the different GST-tagged HuR isoforms showed that only HuR-CP1, but not HuR-CP2, associated with this ARE to the same extent as full-length HuR (Supplementary Figure S7). These data and the fact that HuR-CP1-induced cytoplasmic accumulation of HuR participates in myogenesis (Figures 5 and 6) raise the possibility that overexpressing HuR-CP1 in differentiating muscle cells could correlate with an increase in the stabilization of the *myogenin* mRNA. To test this possibility, we performed an actinomycin D (ActD) pulse-chase experiment[26] on differentiating C2C12 cells treated with AP-HuR or AP-HuR-CP1. We observed that an excess of AP-HuR or AP-HuR-CP1 in C2C12 cells triggers an increase in the half-life of the *myogenin* mRNA, albeit to a different extent (Figure 7a and b). These observations strongly indicate that HuR-CP1, by blocking the nuclear import of endogenous HuR (Figure 5d), indirectly increases the half-life of the *myogenin* mRNA. However, it is also possible that HuR-CP1, through its RRMs 1 and 2, is able to independently promote myogenesis by directly stabilizing the *myogenin* mRNA. To investigate which of these two possibilities is true, we assessed whether HuR-CP1 is able to rescue the expression of *myogenin* mRNA in HuR knockdown C2C12 cells. We depleted HuR expression with high efficiency ($>$85%) using siRNA-HuR[10] in undifferentiated myoblasts (Figure 7c). These cells were then induced to differentiate in the presence of 50 nM AP-GST, AP-HuR, or AP-HuR-CP1. As expected,[6] in the absence of HuR, C2C12 cells failed to express the *myogenin* mRNA (Figure 7d, compare lanes 1 and 2 with lane 3). However, the expression of this mRNA was rescued by only AP-HuR, but not AP-HuR-CP1 (Figure 7d, compare lanes 6 and 9). Likewise, only AP-HuR, but not AP-HuR-CP1, was able to rescue myogenesis in C2C12 cells depleted of HuR (Figure 7e and f). Together, these data strongly suggest that the stabilization effect of HuR-CP1 on the *myogenin* mRNA is an indirect event, which occurs only through its ability to promote the cytoplasmic localization of HuR.

Discussion

Our investigations have uncovered one of the mechanisms by which posttranscriptional regulatory events promote muscle fiber formation. We show that during myogenesis in cultured muscle cells or during the regeneration of skeletal muscle tissues in mice, the RNA-binding protein HuR undergoes a caspase-mediated cleavage and this proteolytic event is required for myotube formation. The cleavage of HuR generates HuR-CP1, a 24 kDa fragment that, by associating with the HuR import factor TRN2, promotes the cytoplasmic accumulation of HuR and triggers an increase in the half-life of the HuR-mRNA target *myogenin*. Therefore, we suggest a model whereby during myotube formation, a percentage of HuR is cleaved, generating HuR-CP1 that retains the ability to associate with TRN2. Through this association, HuR-CP1 sequesters TRN2 leading to the cytoplasmic accumulation of HuR. In the cytoplasm, HuR stabilizes several mRNA targets such as *myogenin*, thereby participating in the events that lead to myotube formation (Figure 8).

Our earlier observations have shown that the cleavage of HuR is required to promote apoptosis in cells exposed to lethal stresses.[11] In this study, we show that cleavage is also an important regulator of the promyogenic function of HuR. It was intriguing to note that a similar regulatory mechanism is used to mediate two opposite functions of HuR: the induction of cell death and the promotion of cell differentiation. Our results, however, are consistent with the earlier reports implicating effector caspases such as caspases-3 and -9 in both stress-induced cell death and the promotion of muscle cell differentiation.[12,15,27] Here, we show that HuR cleavage is triggered only during the fusion step of myoblasts leading to myotube formation (Figure 1a and b). Interestingly, this is also the case for the activation of both caspases-3 and -9, which occurs only during the fusion step of muscle cells[15] (Figure 1a). Whereas the function of caspases in the differentiation process for a number of cell types is shown,[28] the regulatory mechanisms involved and their substrates remain largely unknown. The fact that caspase-3 has an important function in HuR proteolysis during apoptosis,[11] and our data showing that the cleavage of HuR is significantly reduced in both cardiotoxin-treated TA muscle from caspase-3 −/− mice, as well as in myotubes exposed to zVAD (Figure 1), indicates that HuR could be one of the main substrates of caspase-3 during muscle cell differentiation. Although this is likely to be the case, more studies are needed before a definitive conclusion can be made.

Our data show that although only 10–15% of total HuR is cleaved during the myogenic process both in C2C12 muscle cells and in mice (Figure 1), this event is required for the promyogenic activity of HuR (Figure 3). Indeed, here we show that mutating the D226 residue to A generates an HuR isoform that is not only protected from caspase-induced cleavage ($<1\%$ of HuR-CP1 produced) (Figure 2c), but also failed to reestablish myogenesis in siRNA-HuR-treated myoblasts (Figure 3). This indicates that the fraction of cleaved HuR (10–15%) is crucial for the efficient execution of the myogenic process. Moreover, our data indicate that $\sim 90\%$ of total HuR is not cleaved and that a portion of this uncleaved HuR accumulates in the cytoplasm during myogenesis (Figure 4).[10] Therefore, these results, and the fact that an excess of HuR-CP1 correlates with the stabilization of *myogenin* mRNA (Figure 7a and b), argue that both the cleaved and the non-cleaved isoforms of HuR collaborate together to ensure proper myogenesis.

As a function of caspases and other proapoptotic factors in myogenesis has already been suggested,[12,15] we were not surprised to observe that HuR is cleaved in a caspase-dependent manner in this process. It has been shown that caspases-3 and -9, which are important effectors of apoptosis,[27] have a major function in myogenesis.[12,15] Establishing a link between posttranscriptional regulators such as HuR, and important players in cell death mechanisms such as caspases will increase our understanding of how the skeletal muscle tissues can deal with different extracellular assaults to maintain their integrity. Furthermore, by continuing to investigate the functions of the CPs of HuR, and the regulation of their generation, we will gain a greater understanding of how important posttranscriptional regulators such as HuR can be involved in promoting different vital cellular processes.

Materials and Methods

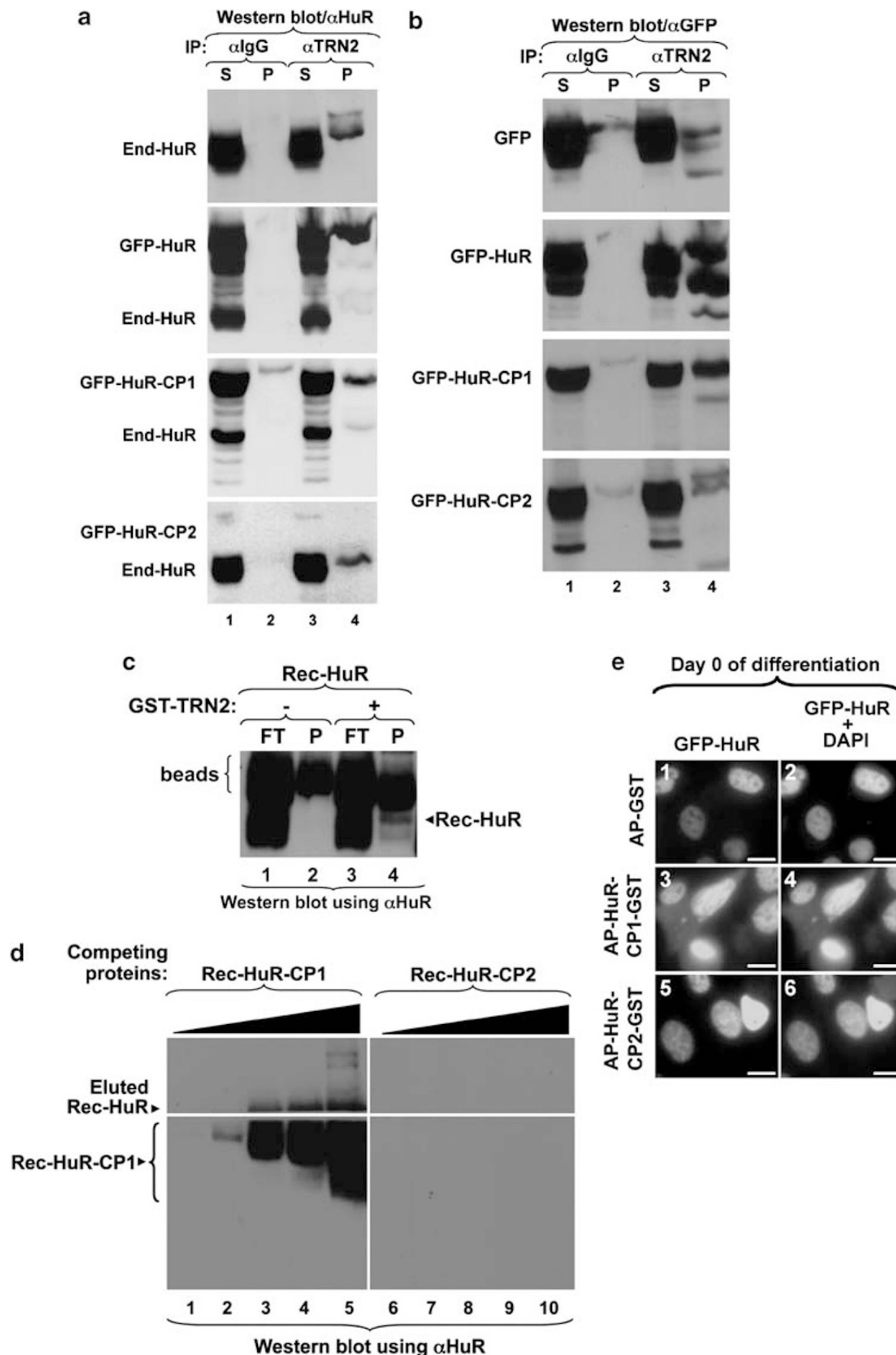
Plasmid construction and protein purification. AP-HuR-CP1-GST and AP-HuR-CP2-GST were generated by cloning HuR-CP1 and HuR-CP2 fragments into the AP/GST vector as described.[6] The PCR amplification of HuR-CP1 and HuR-CP2 was performed using the GST-HuR plasmid as template[29] with the primers HuR-CP1-GST-forward (5′- ggc gcg gcc gca tct aat ggt tat gaa gac cac -3′) and AP-HuR-CP1-reverse (5′- ggc ctc gaggtgatc gac gcc cat ggg -3′), and HuR-CP2-GST-forward (5′- ggc gcg gcc gca cac atg agc ggg ctc tct -3′) and

Figure 5 HuR-CP1 associates with TRN2, disrupts *in vitro* the HuR–TRN2 complex and interferes with the nuclear import of HuR in undifferentiated C2C12 cells. (**a**, **b**) Total extracts from transfected C2C12 cells expressing GFP-HuR, GFP-HuR-CP1, GFP-HuR-CP2, or GFP-HuRD226A were used for immunoprecipitation with anti-TRN2 antibody or IgG control. The immunoprecipitate (P) and the supernatant (S) were analyzed by western blot using antibodies against HuR (**a**) and GFP (**b**). (**a**) End-HuR is the endogenous HuR found in the cells. (**a**, **b**) The position of GFP, GFP-HuR, GFP-HuR-CP1, GFP-HuR-CP2, or GFP-HuRD226A proteins are indicated. (**c**) GST-TRN2 beads were prepared as described in the Material and methods section. A total of 0.225 nmol Rec-HuR was incubated with either beads alone (lanes 1 and 2) or with 0.15 nmol GST-TRN2 beads (lanes 3 and 4). The flow-through (FT) represents the soluble fraction without the beads and the pellet (P) represents the pellet that contains the beads. FT and P fraction were used for western blot with the anti-HuR antibody. (**d**) The P fraction from C was incubated sequentially for 30 min with increasing concentration of Rec-HuR-CP1 or Rec-HuR-CP2 (0.0225, 0.045, 0.225, 0.45, and 1.125 nmol) and the FT of each treatment was collected and used for western blot with the anti-HuR antibody. To avoid the high background that was generated by the increasing amount of Rec-HuR-CP1 when revealing the western blot by ECL, we separated the membrane into two. The upper part was used to reveal the eluted Rec-HuR and the lower part was used to detect the added Rec-HuR-CP1. The membrane revealing the Rec-HuR was exposed for a longer time period than the exposure time used to reveal the Rec-HuR-CP1. Representative western blots of two independent experiments are shown. (**e**) C2C12 myoblasts were transfected with GFP-HuR and 48 h later when the cells were confluent as they were induced for differentiation. Two hours later, these cells were treated with AP-GST, AP-HuR-CP1-GST, or AP-HuR-CP2-GST. Eight hours later, the cells were fixed and stained for DAPI. A single representative field for each cell treatment from three independent experiments is shown. Bars, 20 μm

AP-HuR-CP2-reverse (5′- ggc ctc gag ttt gtg gga ctt gtt ggt ttt -3′), respectively. The Not1/Xho1 fragments of HuR-CP1 and -CP2 PCR products were inserted into the Not1/Xho1 sites of the Gateway AP-GST vector to produce AP-HuR-CP1-GST and AP-HuR-CP2-GST. AP-HuR-GST and AP-HuRD226A-GST were generated and used as earlier described.[11]

The AP-tagged proteins were expressed and produced as described earlier[6] except for the following modifications: the proteins were eluted in a buffer containing 300 mM Tris pH 8.8 and 120 mM NaCl, with 10 mM glutathione for the first elution and 20 mM gluthatione for further elutions.

The GFP-tagged HuR mutants, GFP-HuR, GFP-HuRD226A, GFP-HuRD254A, and GFP-HuRD256A were generated and used as described in Mazroui *et al.*[11] GFP-HuR-CP1 and GFP-HuR-CP2 were generated as follows. DNA coding for the first 226 amino acids of HuR (1–226) (HuR-CP1) or the last 100 amino-acids (227–326) (HuR-CP2) was amplified by PCR, using wild-type HuR cDNA as the template. A BglII site was created at the 3′-end and an EcoRI was generated at the 5′-end of the PCR product for both HuR-CP1 and HuR-CP2. GFP-HuR-CP1 was generated using the following primers forward: 5′-GGC AGA TCT AAT GGT TAT GAA GAC CAC A-3′ and reverse: 5′-GGC GAA TTC TTA ATC GAC GCC CAT GGG-3′. We generated GFP-HuR-CP2



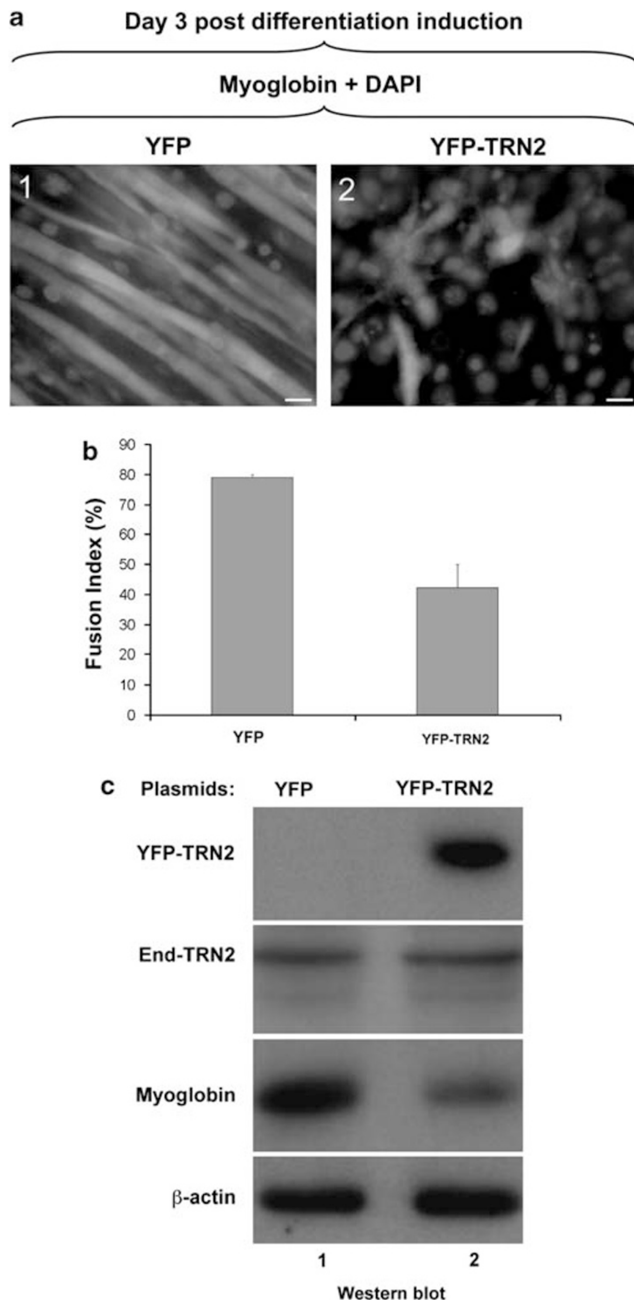


Figure 6 The overexpression of TRN2 in myoblasts significantly reduces their myogenic potential. (**a**) C2C12 myoblasts were transfected twice with YFP or YFP-TRN2, at 50% and again the following day at 75% confluency. Cells were fixed at day 2 of differentiation and DAPI as well as immunofluorescence staining was performed using anti-myoglobin antibody. A single representative field for each cell treatment is shown. Bars, 20 μm. (**b**) The fusion index is shown and was calculated as described above. Error bars represent the S.E.M. of two independent experiments. (**c**) Total cell extracts from differentiated YFP or YFP-transfected C2C12 cells were used for western blot analysis with anti-YFP, -TRN2, myoglobin, and β-actin antibodies. Representative western blots of two independent experiments are shown

using forward: 5′-GGC AGA TCT CAC ATG AGC GGG CTC TCT-3′ and reverse: 5′-GGC GAA TTC TTA GTA AGC TGC GAG AGG AG-3′. The PCR fragments were cloned into the BglII/EcoRI sites of pAcGFP1-C1 vector (BD Bioscience, San Jose, CA, USA) and both fragments inserted in frame with GFP cDNA.

GST-HuR, GST-HuR-CP1, and GST-HuR-CP2 were generated as follows. Full-length HuR or the regions representing its cleavage fragments were amplified by PCR using the GFP-HuR, GFP-HuR-CP1, or GFP-HuR-CP2 plasmids described above. An EcoRI site was created at the 5′-end and a NotI site was generated at the 5′-end of the PCR products. GST-HuR was generated using the following primers – EcoRI-HuR-F: 5′-CCG GAA TTC CGG TCT AAT GGT TAT GAA GAC CAC-3′ and NotI-HuR-R: 5′-GGC GCG GCC GCG CCT TAT TTG TGG GAC TTG TTG GT-3′; GST-HuR-CP1 was generated using EcoRI-HuR-F primer and NotI-HCP1-R: 5′-GGC GCG GCC GCG CCA TCG ACG CCC ATG GGG GA-3′; and GST-HuR-CP2 was generated using EcoRI-HCP2-F: 5′-CCG GAA TTC CGG CAC ATG AGC GGG CTC TCT-3′ and NotI-HuR-R primer. The PCR fragments were cloned into the EcoRI/NotI sites of pGEX-6P-1 vector (GE Healthcare) in frame with GST cDNA.

The GST-conjugated proteins were prepared as described.[29] A prokaryotic vector expressing GST-TRN2 was transfected into competent BL21 bacteria. On IPTG induction, the bacteria were lysed and incubated for 4 h with glutathione sepharose beads (GE Healthcare). The GST-TRN2 beads were then washed at least four times and incubated overnight with 5 μg of Rec-HuR. The GST-tag was removed from GST-HuR, GST-HuR-CP1, and GST-HuR-CP2 using PreScission Protease (GE Healthcare) according to the manufacturer's instructions.

YFP-TRN2 was generated by amplifying TRN2 from GST-TRN2 template by PCR, creating a BamHI and a NotI restriction site at its 3′- and 5′-end, respectively. The PCR fragment was then cloned into the BamHI/NotI sites of YFP-pcDNA3 (generous donation from J Pelletier) in frame at the C-terminal end of the YFP cDNA. Primers used were 5′-CGC GGA TCC GCG GAC TGG CAG CCA GAC GAG-3′ (forward) and 5′-GGC GCG GCC GCG CCC TAG ACC CCA TAG AAA GCC G-3′ (reverse).

Cell culture and transfection, zVAD, and cell-permeable protein treatments. C2C12 cells (ATCC, Manassas, VA, USA) were grown, transfected with siRNAs and differentiation was induced as described earlier.[6,10] To examine caspase-dependent cleavage of HuR by western blotting, zVAD (Sigma-Aldrich, Montreal, QC, Canada) was added directly to C2C12 myotubes on day 3 of differentiation for 16 h, at a final concentration of 10 μM, after which the cells were supplemented with another 10 μM zVAD for 12 h before harvesting on day 4 as described below. To assess the localization of HuR by immunofluorescence, zVAD was added on day 1 of differentiation for 16 h, at a final concentration of 10 μM, and then supplemented with another 10 μM zVAD for 12 h before the cells were fixed on day 2.

The GFP-conjugated constructs were transfected into muscle cell as described.[6,11] For cell-permeable fusion protein experiments, all proteins were generated as described above and added to C2C12 myoblasts at a final concentration of 50 nM both on the day before and the day of differentiation of induction as described.[6]

***In vivo* muscle regeneration.** For regeneration studies, 10 μl of cardiotoxin (Latoxan, Rosans, France) diluted at 10^{-5} M was directly injected into the TA muscle of 4-week-old wild-type or caspase-3 $-/-$ C57BL/10 mice (obtained from Charles River Laboratories, St.-Constant, Canada), as described earlier;[20,30] 1, 3, 7, and 14 days later, cardiotoxin-injected and control-untreated TA muscles were rapidly excised and frozen in melting isopentane precooled with liquid nitrogen (for histological analysis), or directly frozen in liquid nitrogen (for western blots).

Histological analysis was performed on cross-sections of TA muscles that were stained with hematoxylin and eosin, dehydrated in a series of alcohol solutions, cleared with xylene, and mounted using permount (Fisher Scientific, Montreal, QC, Canada). For western blotting, total protein extracts were obtained from whole muscle samples homogenized in 300 ml of RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS Sigma, Montreal, QC, Canada). Protein concentrations were determined using the Bradford Assay (Bio-Rad, Mississauga, ON, Canada). A total of 80 íg of protein extract was resolved by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The blots were first stained with Memcode (Thermo Scientific, Nepean, ON, Canada) to ensure equal loading, subsequently probed with the 3A2 antibody against HuR,[19] or antibodies against caspase-3 (Cell Signaling, Boston, MA, USA) or myogenin (Developmental studies Hybridoma Bank, Iowa, IA, USA) and revealed using ECL (Amersham, Montreal, QC, Canada).

Immunoblotting, immunofluorescence, subcellular fractionation, and preparation of cell extracts. Total cell extracts were prepared as described earlier.[6] The nuclear and cytoplasmic fractions from differentiating C2C12 cells were prepared as described.[6,10] Western blotting was performed as described

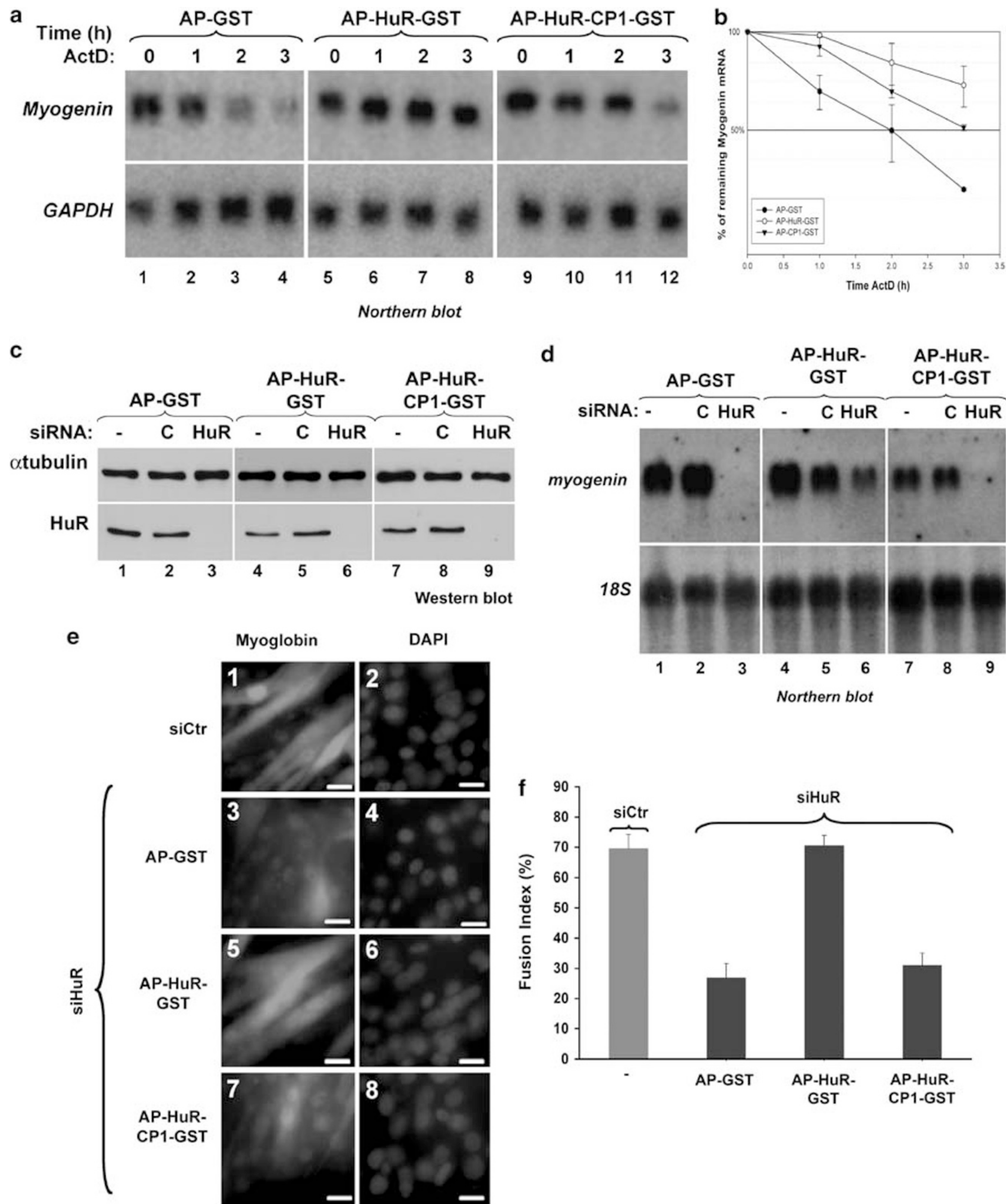


Figure 7 To promote the expression of myogenin mRNA and myogenesis, HuR-CP1 requires endogenous HuR. (**a**) Differentiation was induced in C2C12 cells, which were then treated, 24 h later, with AP-GST, AP-HuR-GST, or AP-HuR-CP1-GST. One hour after treatment with AP conjugates, cells were treated with ActD for 0, 1, 2, or 3 h. The mRNAs of *myogenin* and *GAPDH* (loading control) were detected using northern blot. (**b**) The stability of *myogenin* mRNA was quantified using the ImageQuant software. The percent remaining of *myogenin* mRNA was defined as follow: (1) by dividing the band intensities of *myogenin* over *GAPDH* at each time point, (2) each of these numbers was then normalized against the abundance of *myogenin* mRNA at 0 h of ActD treatment, considered as 100%, and (3) the obtained percentages were plotted on a logarithmic scale. The half-life of the *myogenin* mRNA (50%) was determined by calculating the remaining mRNA relative to the original abundance of message. Error bars represent the S.E.M. of two independent experiments. (**c**, **d**) AP-GST, -HuR-GST, or -HuR-CP1-GST were introduced to control (**c**) siRNA- or HuR-siRNA-treated C2C12 cells that were induced to differentiate for 2 days. Total extracts from these cells were prepared and then used for western blots with anti-HuR and -α-tubulin antibodies (**c**) or northern blot analysis to assess the expression of *myogenin* mRNA and the *18S* (loading control) (**d**). (**e**) Knockdown of HuR expression by siRNAs duplexes, treatment with AP-GST, AP-HuR-GST, and AP-HuR-CP1 and the immunofluorescence experiments were performed as described in Figure 3c. A single representative field for each cell treatment is shown. Bars, 20 μm. (**f**) The fusion index was determined as in Figure 3d. Error bars represent the S.E.M. of two independent experiments

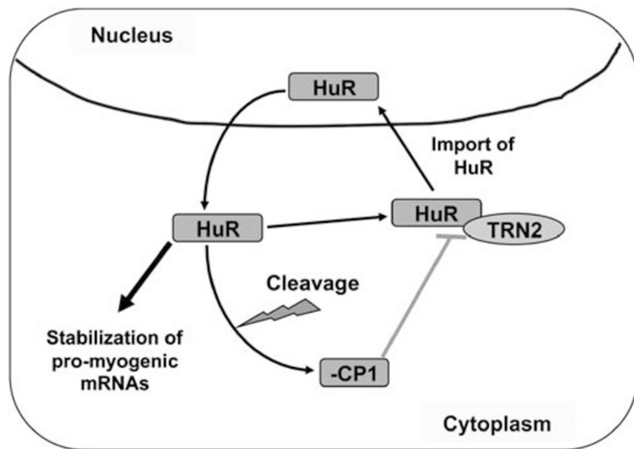


Figure 8 Model depicting how HuR cleavage participates in the promyogenic function of HuR. During the early steps of myogenesis, the nuclear import of HuR is ensured by the import factor TRN2.[10] During fusion, in which myoblasts form myotubes, HuR is needed in the cytoplasm. At this stage, HuR is cleaved by caspases generating HuR-CP1, which in turn blocks the TRN2-mediated import of HuR. HuR-CP1 competes with HuR and forms a stable complex with TRN2. HuR then accumulates in the cytoplasm and stabilizes mRNA such as *myogenin*, thus promoting myogeneis[5,6]

earlier[19] and blots were probed with antibodies against HuR (3A2),[19] caspase-3 (Cell Signaling), myogenin (F5D), MyHC (MF-20) (Developmental studies Hybridoma Bank), α-tubulin (Sigma-Aldrich), GFP (Clonetech, Mountain View, CA, USA), myoglobin (DAKO, Mississauga, ON, Canada), β-actin (Sigma), and hnRNP A1 (Abcam, Cambridge, MA, USA). Immunofluorescence was performed as described earlier[6] using antibodies against HuR, myoglobin, and MyHC and staining with DAPI.

Cytoplasmic and nuclear extracts from whole EDL muscle samples were prepared using the ProteoJET cytoplasmic and nuclear protein extraction kit according to manufactures instructions (Fermentas Life Sciences, Burlington, ON, Canada). Samples were separated by SDS-PAGE and transferred to PVDF membranes. Western blots were performed using antibodies to HuR (3A2[19]), caspase-3, α-tubulin, and B23 (Sigma-Aldrich) and suitable HRP-conjugated secondary antibodies. Blots were exposed using western lightning enhanced luminescence reagent (PerkinElmer, Woodbridge, ON, Canada) and developed with CL-XPosure film (Thermo Scientific) and a Kodak X-OMAT 2000A processor. Blots were subsequently quantified through densitometry analysis (National Institutes of Health ImageJ).

Immunoprecipitation analysis. Total cell lysates for immunoprecipitation were prepared from C2C12 cells that were transfected with GFP, GFP-HuR, GFP-HuR-CP1, and GFP-HuR-CP2 DNA constructs before the initiation of differentiation. C2C12 cells at day 2 of differentiation were scraped in phosphate-buffered saline and centrifuged at 2000 r.p.m. for 5 min at 4 °C. A measure of 2 mg of total cell extracts prepared as described[10] were incubated with protein A beads (GE Healthcare) earlier bound to anti-TRN2 or IgG control (BioCan, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) antibodies as described for 4 h at 4 °C, and then washed with RIPA buffer. The immunoprecipitate was resuspended as described[10] and then analyzed by western blot.

Gel shift assay. Gel shift experiments were performed as described earlier[26] with the following modifications. As a probe, we used a ^{32}P-UTP-labeled cRNA corresponding to the *myogenin* 3′UTR ARE.[6] The labeled cRNA probe was incubated as described earlier[26] with 300 ng of recombinant GST-HuR, GST-HuR-CP1, and GST-HuR-CP2.

Northern (RNA) blot analysis and actinomycin D pulse-chase experiments. Northern blot analysis was performed using 12 μg of total RNA as described earlier[10] with the following modifications. Briefly, after transferring the RNA onto a hybond-N membrane (GE Healthcare), the RNA was UV-cross-linked and the blots were hybridized with *myogenin* and *GAPDH* cDNA probes.[6] The stability of *myogenin* mRNA was assessed by treating C2C12 cells, after 16 h of differentiation, with 50 nM of AP-GST, AP-HuR-GST, or AP-HuR-CP1-GST; 1 h later, cells were treated with the RNA pol II inhibitor ActD (Sigma-Aldrich) at a concentration of 5 μg/ml. Total RNA was isolated from the cells after 0, 1, 2, and 3 h after ActD treatment using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and analyzed by northern blotting.

Conflict of interest

The authors declare no conflict of interest.

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