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Muscle-dependent maturation of tendon cells is induced by post-transcriptional regulation of *stripeA*

Gloria Volohonsky[1], Gundula Edenfeld[2], Christian Klämbt[2] and Talila Volk[1,*]

Terminal differentiation of single cells selected from a group of equivalent precursors may be random, or may be regulated by external signals. In the *Drosophila* embryo, maturation of a single tendon cell from a field of competent precursors is triggered by muscle-dependent signaling. The transcription factor Stripe was reported to induce both the precursor cell phenotype, as well as the terminal differentiation of muscle-bound tendons. The mechanism by which Stripe activates these distinct differentiation programs remained unclear. Here, we demonstrate that each differentiation state is associated with a distinct Stripe isoform and that the Stripe isoforms direct different transcriptional outputs. Importantly, the transition to the mature differentiation state is triggered post-transcriptionally by enhanced production of the *stripeA* splice variant, which is typical of the tendon mature state. This elevation is mediated by the RNA-binding protein How(S), with levels sensitive to muscle-dependent signals. In *how* mutant embryos the expression of StripeA is significantly reduced, while overexpression of How(S) enhances StripeA protein as well as mRNA levels in embryos. Analysis of the expression of a *stripeA* minigene in S-2 cells suggests that this elevation may be due to enhanced splicing of *stripeA*. Consistently, *stripeA* mRNA is specifically reduced in embryos mutant for the splicing factor Crn, which physically interacts with How(S). Thus, we demonstrate a mechanism by which tendon cell terminal differentiation is maintained and reinforced by the approaching muscle.

KEY WORDS: Splicing, Tendon cells, *stripe*, *how*, *Drosophila*

INTRODUCTION

The development of the contractile system in the *Drosophila* embryo is based on the tight coordination between the differentiation of somatic muscles and their epidermal tendon attachment cells (Volk, 1999). Somatic muscles are specified in the mesoderm, while tendon precursor cells are defined as a group of ectodermal cells expressing the Early growth response (EGR)-like transcription factor Stripe (Frommer et al., 1996; Hatini and DiNardo, 2001; Piepenburg et al., 2000). Once each of these cell types has been specified, it undergoes tissue-specific differentiation, which is tightly coordinated and occurs following the encounter between the two cell types. Specifically, the newly formed myotube migrates toward the tendon precursor cell in response to guidance cues provided by the tendon cell (Schnorrer and Dickson, 2004). Once the approaching myotube reaches its destination, it provides a differentiation signal (the EGFR ligand, Vein), which triggers the maturation of the tendon cell (Yarnitzky et al., 1997). This maturation is essential for both the formation of adherens junctions between tendon and muscle cells to tightly hold the two cell types together and for developing tendon resistance and elasticity to accommodate muscle contractions during larval movements (Subramanian et al., 2003).

stripe, the earliest tissue-specific gene detected in the tendon precursor cells is required for the appropriate differentiation of tendon cells, and in its absence, tendon cells do not form. Moreover, in *stripe* homozygous mutant embryos, muscle migration and adhesion is abrogated, resulting in the disruption of the entire somatic muscle pattern and embryonic lethality (Frommer et al., 1996). Reciprocally, ectopic Stripe expression in the ectoderm drives the expression of an array of tendon-specific genes outside their normal domain of expression (Becker et al., 1997; Vorbruggen and Jackle, 1997). Thus, *stripe* is a key factor in tendon cell specification and differentiation.

At early embryonic stage 11, *stripe* transcription is regulated by signaling pathways involved in ectodermal segment polarity, such as the Hh, Wg and EGFR signaling pathways (Hatini and DiNardo, 2001; Piepenburg et al., 2000). However, Stripe protein expression is detected only at later stages (11-12), and is significantly elevated at stages 14-16 (Becker et al., 1997; Frommer et al., 1996). At these stages, high protein levels of Stripe are maintained only in muscle-bound tendon cells. In addition, the *stripe* gene has been shown to produce two spliced variants, *stripeA* and *stripeB* (Frommer et al., 1996). StripeB coding sequence is included within StripeA, and both splice variants share a similar 3′ UTR. However, StripeA contains a unique N-terminal domain as well as unique 5′ UTR. In situ hybridization showed that *stripeB* is detected already at stage 11, while *stripeA* appears at later developmental stages in a subset of tendon precursor cells. An enhancer sequence located upstream to the *stripeA* 5′ UTR was shown to drive expression of a reporter gene at a pattern similar to StripeB starting at embryonic stage 10 (Piepenburg et al., 2000). This suggests that the *stripe* locus is transcriptionally active already at embryonic stage 10, and post-transcriptional mechanisms may control its mRNA accumulation and couple them to the state of differentiation of the tendon cell. Distinct promoters for each of the *stripe* variants have not been identified yet but could also affect their expression profile.

A post-transcriptional mechanism controlling *stripe* mRNA levels is based on the activity of the RNA-binding protein Held out wing (How) (Nabel-Rosen et al., 1999). How is a member of the Star (Signal transduction and RNA control) family, which includes the *Caenorhabditis elegans* protein Gld-1 and the mammalian protein Quaking (Vernet and Artzt, 1997). These proteins are essential for the control of transition between differentiation states, including the transition from mitosis to meiosis and sex determination mediated by Gld-1 in *C. elegans* (Crittenden et al., 2002; Crittenden et al., 2003; Hansen et al., 2004), and the switch to maturation of Schwann cells in

[1]Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel. [2]Institut für Neurobiologie, Universität Münster, Badestrasse 9, D-48149 Münster, Germany.

*Author for correspondence (e-mail: lgvolk@weizmann.ac.il)

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the peripheral nervous system and oligodendrocytes in the central nervous system mediated by Quaking in mammalian species (Ebersole et al., 1996; Hardy, 1998; Larocque et al., 2005). In *Drosophila*, How regulates mesoderm invagination, muscle-dependent tendon cell differentiation and heart rate (Baehrecke, 1997; Lo and Frasch, 1997; Nabel-Rosen et al., 1999; Zaffran et al., 1997).

The *how* gene is spliced into two isoforms: How(L) and How(S) (Lo and Frasch, 1997) share the same RNA-binding domain but differ at their C-terminal region. The How(L) isoform is maternally contributed and is expressed during earlier stages of embryonic development. The How(S) isoform is detected at high levels in tendon cells following muscle binding (Nabel-Rosen et al., 1999) and in cardiac tissue. While both How(L) and How(S) bind the same target mRNA at the 3′ UTR, their activity is in opposing directions; How(L) binding to mRNA leads to mRNA degradation, while How(S) binding to the same target leads to its stabilization (Nabel-Rosen et al., 2002).

In homozygous *how* mutant embryos, Stripe protein (analyzed by an antibody that recognizes both Stripe proteins) is detected in a higher number of tendon cells at late embryonic stages, suggesting that the selection of a single tendon precursor to undergo terminal differentiation does not occur (Nabel-Rosen et al., 1999). We have previously shown that How proteins bind to the 3′ UTR of *stripe*, which is shared by both *stripe* variants, and thus affect the stability of *stripe* mRNA (Nabel-Rosen et al., 2002). We have also shown that overexpression of StripeB in the ectoderm leads to elevation of How(L) (Nabel-Rosen et al., 1999), and that StripeB enhances the β-gal expression of *stripe* enhancer trap where the *P*-element is located close to the *stripeB* unique 5′ UTR (Becker et al., 1997).

Here we address the specific function of each Stripe protein isoform in tendon cell differentiation, and the contribution of How proteins to the post-transcriptional regulation of each Stripe isoform. We show that the expression of the two Stripe isoforms is tightly regulated and is linked to maturation of tendon cells. StripeB is expressed continuously from the precursor stage, while StripeA protein is detected only in the muscle-bound mature state. Consistently, each Stripe protein exhibits distinct biological activities and transcription output. Importantly, we demonstrate that the elevation of StripeA depends on the activity of How(S); embryos homozygous for *how* show specific reduction of the *stripeA* mRNA and protein levels, a phenotype that is shared with embryos lacking the splicing factor Crooked neck (Crn). Consistent with a role for How(S) in *stripeA* splicing, we demonstrate that How(S) promotes the splicing of *stripeA*-specific exons in S-2 cells. In tendon cells, How(S) is elevated only upon muscle binding, and its expression is further reinforced by StripeA activity, thus driving a single muscle-bound tendon to express high StripeA levels, leading to its irreversible maturation.

MATERIALS AND METHODS

Staining of embryos

Primary antibodies used: Anti-Myosin heavy chain (MHC) (P. Fisher, Stony Brook, NY), anti-Stripe, anti-Shot, anti-How, anti-StripeA (all produced in our lab), and anti-Slit (Hybridoma Center). The anti-StripeA was raised against GST fusion protein with the unique 218 amino acids at the N-terminus of StripeA. Secondary antibodies included Cy3, Fluoresceine or HRP-conjugated anti-guinea pig, anti-rat or anti-mouse (Jackson, USA). In situ hybridization was performed as previously described (Nabel-Rosen et al., 1999) using RNA probes complementary to the unique 3′ UTRs of each *how* splice variant.

RT-PCR analysis of total RNA extracts

Total RNA was extracted from transfected cells 16 hours after transfection or from embryos at stage 15-16. About 8×10^6 cells or 100 embryos were used for each extraction. RNA was extracted with the Nucleospin RNA II kit (Machery Nagel). For RT-PCR, cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche) with 2 ugr template RNA. RT-PCR was performed using the LightCycler FastStart DNA Master SYBR Green I (Roche) kit according to the manufacturer's instructions. As a control, primers specific for the ubiquitous transcription factor eIF4A or Tubulin, were used, with a similar amount of total RNA. The following primers were used in the RT-PCR reaction: for stripeA levels SrA504F-TGGACTACAGATGAAAATCC and SrA726R-GCGGT-TAGTTTGATTGATTC. For StripeB, SrB328F-CGCAGACCGACTAC-TAGGA and SrB566R-CTTGAACAGACAGGTGTCG. For the in vivo splicing assay, SrAint509F-CCTAGCCAGACCAGCTTTC and SrAint745R-GGTAAACGATCACTTTTTGGT were used to detect the spliced form. To detect unspliced mRNA, a different reverse primer, SrAint785R-GTAATAAATTCGGCCCCGC was used. All mRNA levels were normalized to the level of a housekeeping gene in the same sample.

In vitro binding assay

The protein-RNA binding assay was performed as described previously (Nabel-Rosen et al., 1999). An intron sequence unique to SrA, with and without the How response element, was used as a template, mixed with in-vitro-translated How(S) or How(S)^{e44} HA-tagged proteins (TNT Coupled Reticulocyte Lysate System, Promega). Binding was performed by adding ~1 μg biotin-labeled RNA to 5 μl translated How proteins. The magnetic beads were then isolated, washed, boiled in sample buffer and analyzed by western analysis with anti-HA antibodies (monoclonal, 1:2000 dilution, Covance). As a nonspecific RNA control, we used RNA transcribed from GFP cDNA and *stripe* intronic sequence without the putative How-binding site.

Fly strains

yw (wild type), *how^{stru}*, *how^{e44}* (Baehrecke, 1997), *mys^{XG43}*, *UAS-How*(*S*) (Nabel-Rosen et al., 1999), *UAS-Hid*, *ptc-gal4*, *en-gal4* and *69B-gal4, mef-gal4*. *UAS-StripeA*, and *UAS-StripeB* (created in our lab). *UAS-Crn* (Klaembt Lab, Muenster, Germany).

Constructs

All HA-tagged proteins were created by PCR with a reverse primer containing the HA sequence (5′-TCATGCGTAATCTGGAACATCG-TATGGGTAN$_{18}$-3′) and 18 bp overlapping the 3′ end of the specific gene. How^{e44} mutant constructs were created by PCR. The nuclear localization signal (NLS) of SV40 large T was added to HA-tagged How constructs by using a 3′ primer that contains the sequence encoding the NLS (5′-TCAATCTACCTTTCTCTTCTTTTTTGGATCTGCGTAATCTGGAAC-ATC-3′) or the mutated NLS (5′-TCAATCTACTGCTCTTGCTGCT-GCTGGATCTGCGTAATCTGGAACATC-3′).

RESULTS

StripeA is detected only in muscle-bound tendon cells

We performed quantitative RT-PCR on embryos at different developmental stages and found that while *stripeB* was expressed constitutively, *stripeA* mRNA was elevated only at late developmental stages (not shown). To further examine whether StripeA protein is elevated only in muscle-bound tendon cells, we raised antibodies to the N-terminal unique domain of StripeA (we verified the unique reactivity of this antibody by staining embryos overexpressing each Stripe isoform using the *69B-gal4* driver, see below). Although this antibody recognizes only StripeA, previous antibody (Becker et al., 1997) raised against the coding region of StripeB, recognizes both Stripe isoforms. We compared the pattern of expression of each of the Stripe isoform in embryos before, and following, muscle binding (Fig. 1). At stage 14, in regions where muscle binding to tendon cells did not take place, StripeB was detected in all the tendon precursor cells, while StripeA levels were extremely low (Fig. 1A-D). At stage 15, StripeA expression gradually increased and was observed in muscle-bound tendon cells (Fig. 1E-H). At stage 17, following the establishment of the entire

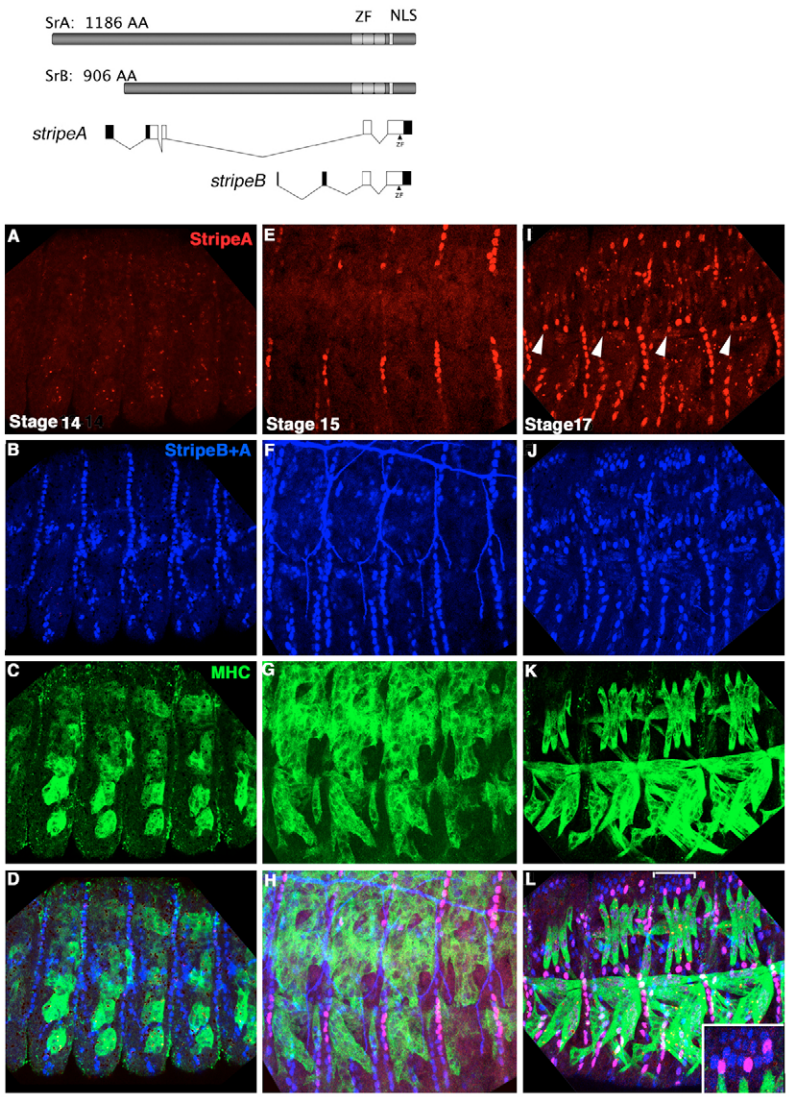

Fig. 1. *stripeA* and *stripeB* mRNAs show distinct expression profiles during embryonic development. Upper panel: schematic representation of the coding sequences of StripeA and StripeB isoforms, and the genomic region of the *stripe* gene (non-coding exons are shown in black and coding exons in white). Lower panel: whole embryo staining of stage 14 embryo, before muscle-tendon interaction (**A-D**), stage 15 (**E-H**), where part of the muscles are attached, or stage 17 embryos, where muscle-tendon interaction has been established (**I-L**). Staining was with anti-StripeA (red, A,E,I); anti-Stripe (blue, B,F,J; an antibody recognizing both Stripe isoforms); and anti-Myosin heavy chain (MHC, green, C,G,K). D,H and L are the corresponding merged images. Arrowheads in I point to the three muscle-bound tendon cells expressing high StripeA levels. The inset in L represents high magnification of the bracket showing about 14 blue-labeled tendon precursor cells, from which only three cells that are in close proximity with the three lateral transverse muscles express StripeA. NLS, nuclear localization signal; ZF, zinc finger.

somatic muscle patterning, StripeA was significantly elevated in all the muscle-bound tendon cells. StripeB was still detected in the neighboring tendon precursor cells (Fig. 1I-L). This result supports a model in which StripeA is significantly elevated only in muscle-bound tendon cells.

Analysis of the *myospheroid* mutant embryos, in which muscles detached from tendon cells shortly following their initial association due to the lack of functional integrin receptors, revealed that StripeA levels were significantly reduced, whereas StripeB levels were not changed (Fig. 2). In addition, ablation of the muscles by driving the cell apoptotic inducer Hid into all muscles by the muscle-specific driver *mef2-gal4*, led to a complete ablation of StripeA in regions lacking muscles (see arrows in Fig. 2I,J,L), or to its significant reduction in regions where the somatic muscles are still detected. Although it is not clear whether the muscle died before its attachment to the tendon, this experiment shows that StripeA expression is maintained only in muscle-bound tendon cells.

We thus conclude that the two Stripe isoforms exhibit differential expression patterns; StripeB is expressed following the initial specification of the tendon precursors at stage 12 of embryonic development, whereas StripeA expression is uniquely expressed only following muscle binding to the tendon cells at later embryonic stages.

Ectopic expression of Stripe isoforms leads to distinct effects on somatic muscle patterning

To assess the biological activity of the distinct Stripe proteins, we tested the effect of each Stripe isoform on the overall pattern of the somatic musculature, following overexpression in the entire ectoderm. Previous studies showed that overexpression of StripeB leads to a subtle effect on muscle migration toward their attachment sites (Becker et al., 1997; Vorbruggen and Jackle, 1997). We produced transgenic flies expressing *UAS-stripeA*, and repeated this experiment using the pan-ectodermal *69B-gal4* driver, in combination with either *UAS-stripeA* or *UAS-stripeB*, comparing their effect on muscle patterning. Staining of embryos overexpressing each of the Stripe isoforms with anti-Stripe antibody (recognizing both Stripe isoforms) revealed that the levels of the ectopic Stripe directed by both UAS constructs were comparable (Fig. 3). Overexpression of StripeA led to a severe disruption of the somatic muscle pattern (Fig. 3C-F). Moreover, 52% (n=89) of the embryos exhibited an additional phenotype in which germ band retraction was arrested. We also noted that in these embryos, overexpression of StripeA altered the shape of the cells in the ectoderm, and they looked narrower. Notably, embryos that did undergo germ band retraction also showed severe muscle pattern defects, suggesting that this phenotype did not stem from germ band

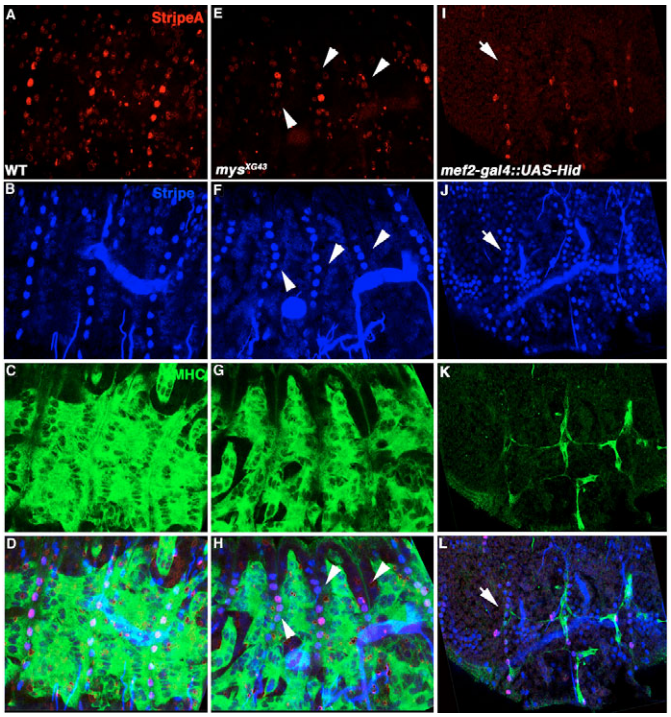

Fig. 2. StripeA is hardly detected when muscle-tendon interaction is abrogated. Dorsal view of stage 16 wild-type (**A-D**) or *myospheroid* (*mys*) mutant (**E-H**) embryos or embryos overexpressing Hid in muscle cells (**I-L**), stained with anti-StripeA (red, A,E,I) anti-Stripe (Blue, B,F,J) anti-Myosin heavy chain (MHC; green, C,G,K). The corresponding merged panels are shown in D,H and L. Arrowheads (E,F,H) or arrows (I,J,L) show that in *mys* mutants, or in regions where muscle are lacking, all tendon precursors express StripeB, but StripeA is significantly reduced or completely absent.

retraction defects per se. By contrast, overexpression of StripeB did not affect germ band retraction and cell shape, and the effect on muscle path-finding was less severe (Fig. 3G,H). Similarly, a more severe effect of StripeA overexpression relative to StripeB, was also detected using the *en-gal4* driver (not shown).

This experiment suggests that each of the Stripe isoforms regulates distinct sets of downstream genes. Importantly, StripeA premature expression affects not only muscle patterning but also normal morphogenetic movements such as germ band retraction, at early developmental stages.

Stripe proteins differ in their transcriptional output

To address whether Stripe proteins activate/repress a differential set of downstream genes, we further examined the induction of a set of tendon-specific genes following overexpression of each Stripe isoform. To this end, the expression of *short stop*, *slit* and *how* was analyzed. We used *ptc-gal4*, *69B-gal4* or *en-gal4* to drive either *UAS-stripeB* or *UAS-stripeA* in the ectoderm of embryos at different stages.

A differential effect of each of the Stripe isoforms is detected on the expression levels of Short stop (Shot), a cytoskeletal protein required for late stages of tendon cell maturation (Strumpf and Volk, 1998; Subramanian et al., 2003). Using the *ptc-gal4* driver, we detected a significant elevation of Shot protein as induced by StripeA, and no elevation by StripeB (Fig. 4). Notably, although the embryos were at the same developmental stage (stage 14-15), the overall shape of the ectodermal cells in embryos overexpressing StripeA was more elongated relative to control embryos or embryos overexpressing StripeB (see the outlines of the cells, as marked by staining with the membrane-associated marker Shot, in high magnification in Fig. 4D′,E′,F′). Using the *en-gal4* driver we consistently detected high induction of Shot by StripeA, and a very low Shot induction by StripeB (not shown). The levels of the ectopic Stripe in the embryos overexpressing either StripeA or StripeB were comparable (as shown in Fig. 3), supporting a significant positive effect of stripeA, and almost no effect of StripeB, on Shot levels.

Slit, a secreted protein essential for guiding neuronal and muscle migration toward target tissues is expressed by midline glial cells as well as by tendon cells (Kramer et al., 2001). Importantly, Slit activity in the segmental border tendon cells takes place before muscle attachment to the tendon cell, and thus may be induced by the early StripeB activity. To address the effect of Stripe proteins on Slit expression, we tested Slit expression following ectopic expression of Stripe proteins driven by *69B-gal4*. In a wild-type embryo, Slit protein expression is somewhat diffuse around the Stripe-expressing cells. Following ectopic expression of either Stripe B or StripeA, the expression of Slit was strongly induced (verified by microarray analysis, not shown) and appeared in a spike-like structure in the entire ectoderm (Fig. 5). As StripeB is expressed earlier in tendon precursors, it appears that the relatively early expression of Slit surrounding the tendon cells is probably due to StripeB activity, while StripeA may be essential for the maintenance of Slit in the muscle-tendon junction site.

Next, we tested the effect of Stripe proteins on the levels of the RNA-binding protein, How (Fig. 5). We performed in situ hybridization with probes specific to each of the How variants in embryos overexpressing Stripe using the *en-gal4* driver. The results show that whereas both Stripe isoforms induced elevation of How(L), only embryos overexpressing StripeA showed elevation in How(S) mRNA (Fig. 5H,I,K,L), indicating that StripeA, but not StripeB, elevates How(S) levels.

Tissue-specific transcription factors often exhibit autoregulatory activities that ensure their constitutive expression following an initial external input. StripeB was shown previously to autoregulate its own expression as inferred from its ability to induce β-gal expression through the *stripe* enhancer trap (Becker et al., 1997), thus creating a positive transcriptional autoregulatory control of its expression. To test whether StripeB controls StripeA, or vice versa, we performed an RT-PCR analysis on embryos overexpressing Stripe proteins under the *ptc-gal4* driver. To monitor endogenous *stripe* levels, we used primers specific for their unique 5′ UTR, which are not included within the pUAST *stripe* cDNA constructs, used to produce the transgenic flies. While this analysis demonstrated that StripeB positively regulates its own expression, and elevated endogenous *stripeB* levels to about fourfold (confirming previous data), it induced a 50% reduction of *stripeA* levels (Fig. 5M). Conversely, overexpression of *stripeA* induced no significant changes in *stripeA* levels, or in *stripeB* levels.

These results are consistent with the notion that the Stripe proteins may share a set of genes that are commonly activated, e.g. *slit* and *thrombospondin* (A. Subramanian and T.V., unpublished), as well as an additional set of genes that differ in their sensitivity to each of the Stripe isoforms. Moreover, it appears that distinct mechanisms control the expression of each of the Stripe proteins. While StripeB is autoregulatory, StripeA does not exhibit such activity.

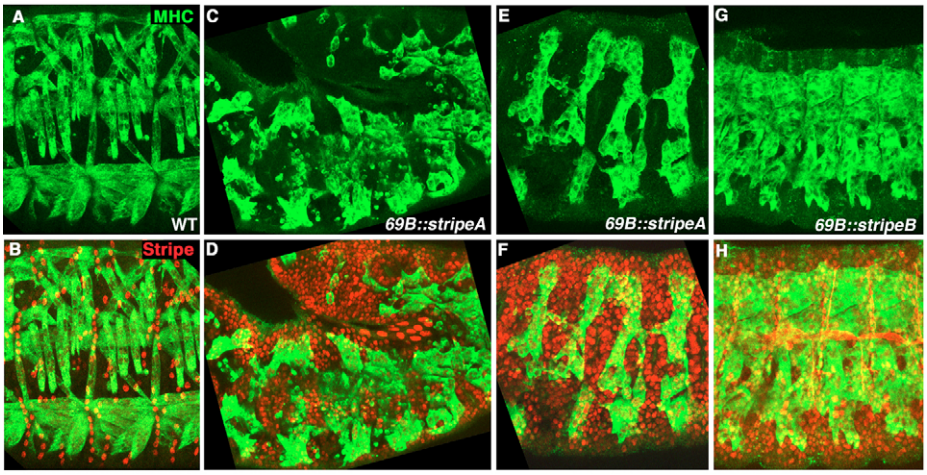

Fig. 3. Overexpression of StripeA leads to severe disruption of the somatic muscle pattern, and arrest of germ band retraction. Wild-type embryos (**A**,**B**) or embryos overexpressing StripeA (**C-F**) or StripeB (**G**,**H**), using the pan ectodermal driver *69B-gal4,* were double labeled with Myosin heavy chain (MHC; green), or with Stripe (red). Panels A,C,E,G show the somatic muscle pattern, whereas panels B,D,F,H show the corresponding merge of MHC and Stripe labeling. Note the disruption of muscle pattern (E,F) and the inhibition of germ band retraction (C,D) in embryos overexpressing StripeA.

Taken together, these experiments demonstrate that in addition to commonly induced genes, StripeA induces the expression of genes characteristic of the mature tendon state, such as *shot* and *how*(*S*). The strong muscle phenotype observed following the expression of StripeA might result from the combination of overexpressing both the early and late genes.

How is required for the control of StripeA and StripeB isoforms

Previously we showed that in *how* mutant embryos the levels of StripeB were elevated, suggesting that How acts to repress *stripeB* mRNA levels in a wild-type situation. We wished to address whether How represses or facilitates StripeA-specific levels. Our results showed that in *how* mutant embryos at stage 16 of embryonic development StripeA protein levels were specifically reduced (Fig. 6B). Staining with the antibody recognizing both Stripe isoforms did not show significant changes in the intensity of staining in the *how* mutants. Consistent with the reduced levels of StripeA in how^{stru} mutant embryos, the levels of Shot (shown to be a target for StripeA) were significantly reduced (Fig. 6F). We performed quantitative RT-PCR on embryos at stage 16 [14-16 hours after egg laying (AEL)], and found that *stripeA* mRNA levels were reduced about fourfold relative to wild-type embryos (Fig. 6L). Conversely, *stripeB* mRNA levels showed about a fourfold elevation in the *how* mutant embryos (Fig. 6L), consistent with our previous results (Nabel-Rosen et al., 1999). These results suggest that in wild-type embryos How is required for elevation of *stripeA* mRNA levels.

Previously, we showed that when expressed ectopically in the entire ectoderm, How(L) represses *stripeB* levels. To further address whether How proteins exhibit differential effects on StripeA levels, we overexpressed each How isoform in the ectoderm and followed StripeA expression in the segmental border tendon cells. We found that whereas How(L) repressed StripeA levels (similar to the case for StripeB), How(S) induced elevation of StripeA levels (Fig. 6I-K). This effect was also demonstrated at the mRNA level by performing quantitative RT-PCR on mRNA extracted from embryos overexpressing How(S) with *stripeA*-specific primers Fig. 6M). By contrast to an expected elevation in *stripeB* mRNA levels following overexpression of How(S), we detected a slight decrease in the mRNA levels (Fig. 6M). This may be explained by an indirect positive effect of How(S) on a negative regulator of *stripeB*.

We conclude that How is required for the elevation of StripeA levels following muscle-dependent maturation of tendon cells, and that How(S) appears to be the isoform required for this elevation.

The splicing of *stripeA* might be mediated by How(S)

We next wished to elucidate the level of mRNA metabolism at which How(S) controls the elevation of *stripeA* mRNA. Quaking, the mammalian homolog of How, had been shown to affect myelin basic protein mRNA nuclear export and stability (Larocque et al., 2002; Li et al., 2000), as well as the splicing of myelin-associated glycoprotein in the nervous system (Wu et al., 2002).

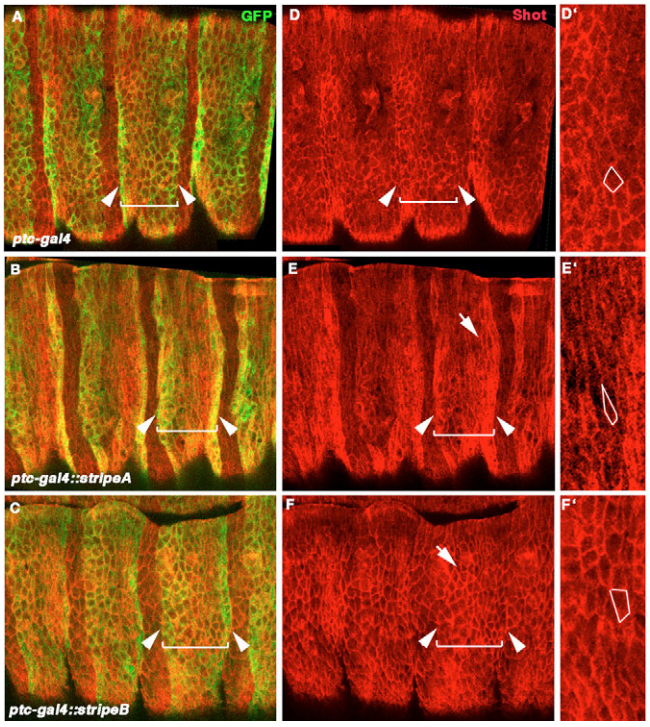

Fig. 4. Differential effect of StripeA and StripeB on Short stop levels. Stage 14-15 wild-type embryos (**A**,**D**,**D′**), or embryos overexpressing StripeA (**B**,**E**,**E′**) or StripeB (**C**,**F**,**F′**) together with GFP driven by *ptc-gal4* were labeled for GFP (green, A,B,C) and Shot (red, D-F′). Only StripeA induced the ectopic expression of Shot. Arrowheads and brackets mark the *ptc-gal4* domain. D′,E′ and F′ represent higher magnification of D,E,F. Note the change in cell shape induced by StripeA (arrows in E,F and white outlines marking a single cell in each panel).

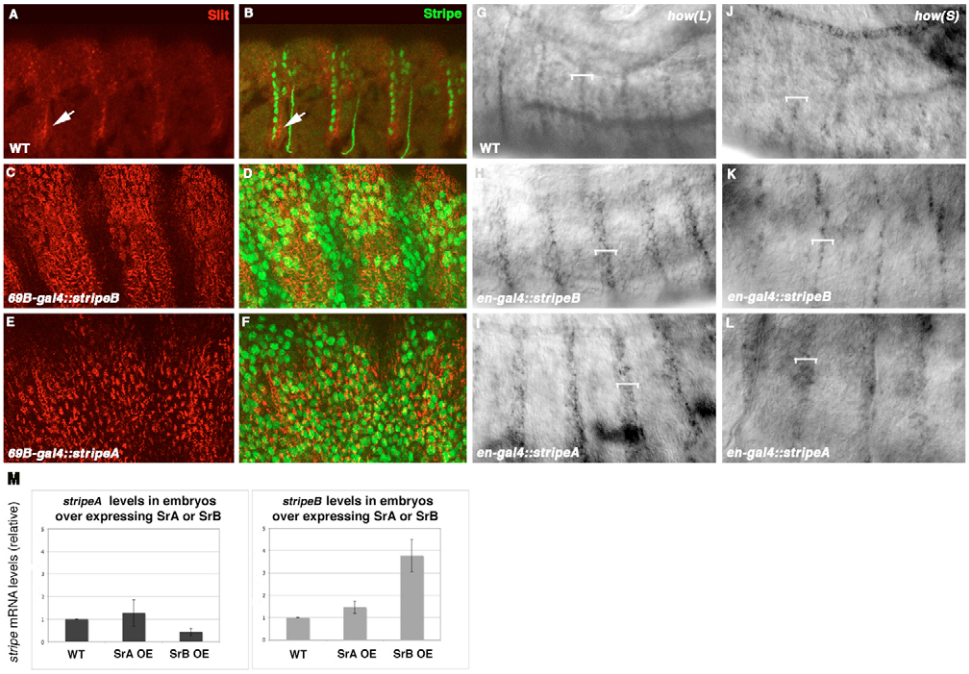

Fig. 5. StripeA and StripeB exhibit differential transcriptional output. (**A-F**) Stage 15 wild-type embryos (WT; A,B) or embryos overexpressing StripeB (C,D) or StripeA (E,F) driven by the pan-ectodermal driver *69B-gal4* were double-labeled for Slit (red, A,C,E) and for Stripe (green) (merged view is shown in B,D,F). Arrows in A and B show Slit expression pattern close to the Stripe-expressing cells. A significant elevation of Slit is detected in both overexpressing embryos. (**G-L**) In situ analysis of wild-type embryos (G,J) or embryos overexpressing StripeB (H,K) or StripeA (I,L) using *en-gal4* driver with probes specific to *how*(*L*) (G-I) or *how*(*S*) (J-L) mRNAs. While both StripeA and StripeB induce the expression of *how*(*L*) *mRNA,* only StripeA induces the expression of *how*(*S*) mRNA. (Brackets indicate the segmental border expression domain). (**M**) The effect of StripeA or StripeB overexpression driven by *ptc-gal4* driver on the endogenous levels of *stripeA* or *stripeB* mRNA levels in 13-16-hour-old embryos, as measured by RT-PCR performed on RNA samples using primers specific for the endogenous *stripeA* or *stripeB* mRNA.

To address the possible involvement of How in the splicing of *stripeA*, a 5 kb minigene composed of a genomic fragment that includes part of the 3′ end of the *stripeA* first exon and part of the 5′ end of the second exon, together with the entire intronic sequence in between these exons, was sub-cloned into a pUAST expression vector (Fig. 7A). Importantly, the *stripeA* minigene did not contain the 3′ UTR that is common to *stripeA* and *stripeB* and was shown previously to be responsive to How activity, thus eliminating any possible effect of How on the stability of the spliced RNA of the minigene through How binding to the 3′ UTR. The efficiency of the splicing reaction of the two exons was monitored in Schneider cells (S-2 cells) transfected with this construct, using a set of primers representing the expected spliced (599F, 5020R) RNA species (see the scheme in Fig. 7A). The levels of the spliced and unspliced RNAs were measured by RT-PCR in the presence of various How isoforms that were co-transfected into S-2 cells. The results showed that How(S) induced a threefold elevation of the *stripeA*-specific fragment, whereas in the presence of How(L), the levels of this fragment were slightly reduced. This is consistent with the active involvement of How(S) in the splicing of *stripeA*-specific exons.

How(S) is normally distributed both in the nucleus and the cytoplasm. To address whether this sub-cellular distribution is essential for its effect on *stripeA* splicing, we tested the ability of an artificial How(S) protein forced to be in the nucleus by the addition of a classical NLS sequence at its C-terminus to facilitate the splicing of *stripeA*-specific exons. We confirmed, by staining of S-2 cells (not shown), that the How(S)-NLS is mostly detected in the nucleus. Importantly, How(S)-NLS did not facilitate *stripeA*-specific splicing, and in its presence, splicing levels were slightly reduced, similar to the levels in the presence of How(L) (Fig. 7B). Thus, the cytoplasmic localization of How(S) is essential for its function in regulating *stripeA*.

Previously, we showed that a mutated form of How(L), How(L)^{YtoG}, in which the nuclear retention of How(L) is abrogated, does not exhibit repressive activity. When tested in the splicing assay, we found that this cytoplasmic How(L)^{YtoG} slightly elevated the levels of the spliced fragment (Fig. 7B). Western analysis showed that the levels of all the various transfected How proteins in each transfection experiment were comparable (Fig. 7C).

The *stripeA* intronic sequence contains a potential binding site for How, as characterized recently in our lab (Israeli and T.V., unpublished). We therefore tested for the possible physical association of How(S) with *stripeA* intronic sequences. A fragment of 200 bp from the first intron of *stripeA*, containing the putative How-binding site, was transcribed, labeled with biotin and added to a mixture containing HA-tagged How(S). As a negative control we used a mutated How(S)^{e44} form, which imitates the ethane methyl sulfonate-induced severe *how*^{e44} allele. The molecular defect in this allele is a missense mutation (R to C) in the RNA-binding domain. In our protein-RNA binding assay the mutated How^{e44} protein does not bind RNA (Nabel-Rosen et al., 1999). The RNA-protein complexes were precipitated on avidin beads, and the presence of How was detected by western analysis with anti-HA antibodies. Specific binding of How(S) but not of the mutant How(S)^{e44} to the *stripeA*-specific intron was detected (Fig. 7D). Thus, How(S) may mediate its positive effect on the RNA levels of the *stripeA* genomic fragment following a physical association between these two elements.

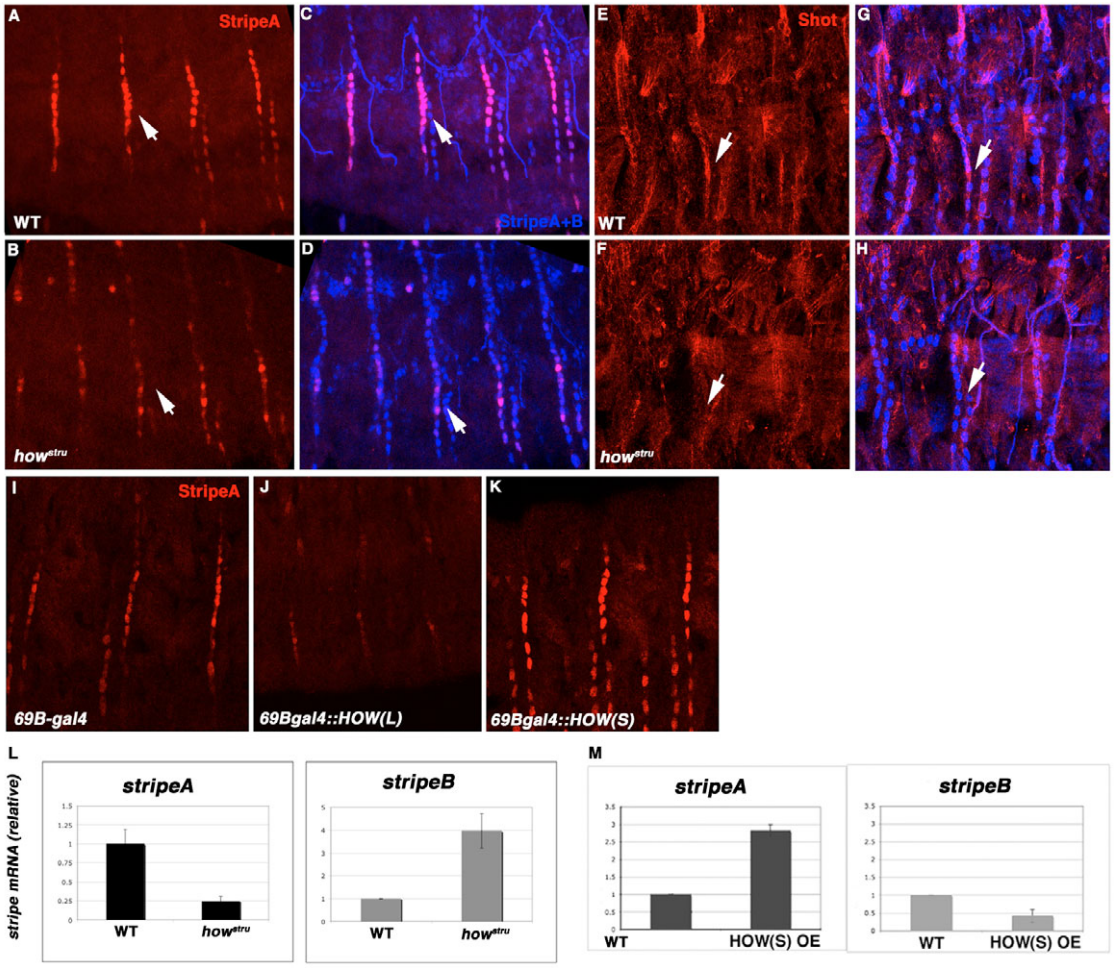

Fig. 6. How(S) is required for *stripeA* elevation. (**A-H**) Wild-type embryo (A,C,E,G) or *how*^{stru} mutant embryo (B,D,F,H) at early stage 16 stained for anti-StripeA (red, A,B) and anti-Stripe (blue, C,D). C and D are the corresponding merged images of A and B. Embryos stained for anti-Shot (red, E,F) and anti-Stripe (blue, G,H). G and H are the corresponding merged images of E and F. Tendon cells of the ventral longitudinal muscles are shown. A significant reduction of StripeA and Shot is detected in the *how* mutant embryos. (**I-K**) Embryos carrying either the *69B-gal4* driver alone (I), together with How(L) (J) or together with How(S) (K) stained with anti-StripeA antibody. Tendons of the ventral longitudinal muscles are shown. (**L**) RT-PCR performed on RNA extracted from *how*^{stru} mutant embryos (selected by their negative GFP staining) or wild-type embryos at the age of 14-16 hours AEL, using primers specific for either *stripeA* (left panel) or *stripeB* (right panel). The total RNA of each sample was normalized against *tubulin* levels. (**M**) RT-PCR with *stripeA*- (left panel) or *stripeB*- (right panel) specific primers, performed on RNA extracted from embryos overexpressing How(S) using the *stripe-gal4* driver. The embryos were at 14-16 hours AEL.

The results of these experiments support the involvement of How(S) in facilitating the splicing of *stripeA*, and suggest that the correct subcellular distribution of How is crucial for its positive effect on *stripeA*. We cannot, however, exclude a possible additional effect of How(S) on *stripeA* stability.

The splicing factor Crn may be involved in the How(S)-dependent elevation of *stripeA* levels

In a parallel study we have identified a functional and biochemical interaction between How(S) and the splicing factor Crn in the process of peripheral glial cell maturation (Edenfeld et al., 2006). Moreover, our results suggest that the interaction between How(S) and Crn occurs in the cytoplasm, consistent with our analysis (Fig. 7) that only cytoplasmic How(S) exhibits a positive enhancement of the spliced *stripeA* minigene. Based on these observations, we went on to test the contribution of Crn to the specific elevation of *stripeA* during tendon cell maturation. To this end, we tested the relative levels of *stripeA* and *stripeB* by quantitative RT-PCR 14-16 hours AEL on *crn* homozygous mutant embryos relative to wild-type embryos at the same age. We compared the level of each of the *stripe* mRNA variants to *tubulin* (*αTub84B*) mRNA levels in each sample. This eliminates a non-specific reduction in the total mRNA levels caused by the lack of Crn. We detected about 40% reduction of *stripeA* levels in the *crn* homozygous mutant embryos. No reduction was detected in *stripeB* levels (Fig. 8A). The reduction of *stripeA* in *crn* mutant embryos is consistent with the idea that the production of *stripeA* during tendon cell maturation depends on *stripeA* splicing. Further support for the requirement of Crn to tendon cell maturation is a consistent reduction in the levels of Shot in tendon cells of stage 16 *crn* mutant embryos (Fig. 8D). We showed above that StripeA is sufficient to induce Shot expression, and its expression is characteristic of the mature state of tendon cells (Fig. 4). Here we show that Shot levels are also reduced in *crn* mutants, consistent with the reduction of *stripeA* in these embryos. Staining of *crn* mutant embryos with antibodies to Stripe (recognizing both

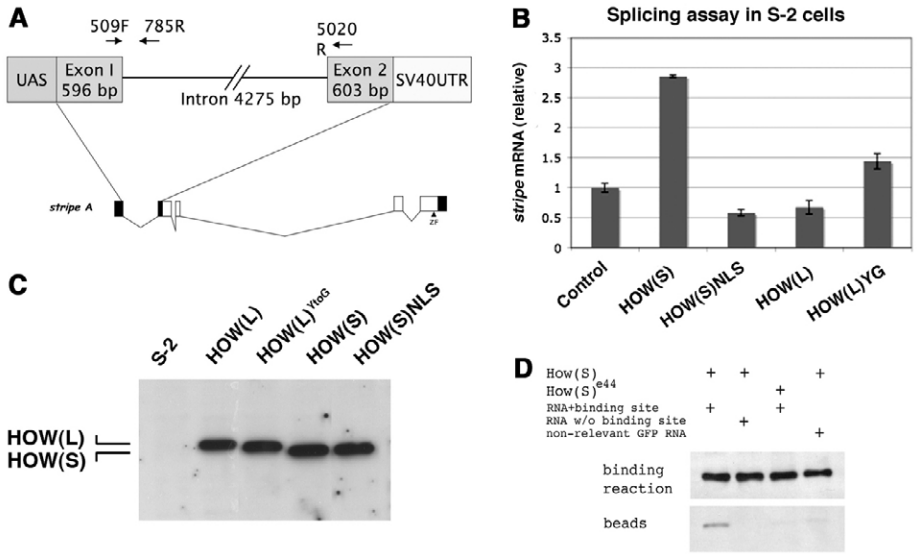

Fig. 7. How(S) facilitates the splicing of *stripeA* minigene. (**A**) S-2 (Schneider) cells transfected with *stripeA* minigene containing the intronic sequences of *stripeA* flanked by partial sequences of its 3′ an 5′ exons, were co-transfected with different forms of How proteins. (**B**) The splicing of the two exons was monitored by RT-PCR using primers specific for the two flanking exons (509F and 5020R, see scheme). All RNA samples were normalized using *tubulin*-specific primers. In the presence of wild-type How(S) the splicing reaction was facilitated by threefold. By contrast, a mutant form of How(S) containing an NLS did not induce such elevation, nor did the nuclear-specific How(L) form. A mutant How(L), which is also cytoplasmic, did induce a mild elevation (1.5-fold). (**C**) Western analysis of the transfected S-2 crude extracts using anti-HA antibody to detect transfected How constructs. (**D**) A protein-RNA binding assay was performed on in vitro transcribed *stripeA* intronic RNA sequences labeled with biotin and precipitated with HA-tagged in vitro translated How(S) or mutated form of How(S), How(S)^{e44}, which does not bind RNA. Western analysis shows the presence of HA-tagged How(S) in the beads only in the presence of *stripeA* intronic sequences, while its levels are equal in all the binding reactions.

isoforms) showed no reduction in total Stripe levels (not shown), supporting a specific effect of Crn on Shot levels. Importantly, the reduction of Shot in *crn* mutants is comparable with the reduction of Shot in *how* mutants (see Fig. 6F).

The levels of How in tendon cells of *crn* mutant embryos appeared the same (Fig. 8F,H). Similarly, there was no change in the subcellular localization of How in *crn* mutants, indicating that the levels of both How(L) and How(S) are unaffected in these mutant embryos.

These results support the idea that a Crn-How-dependent splicing event is essential for tendon cell maturation.

DISCUSSION

This study demonstrates the involvement of post-transcriptional control in a cell-differentiation process that must be coupled to muscle-tendon interaction. Terminal differentiation of tendons involves a major reorganization of the microtubule and actin networks (Subramanian et al., 2003). Such processes are presumably not compatible with embryonic morphogenetic movements such as germ band retraction. Thus, it is essential to spatially and temporally restrict differentiation to single muscle-bound tendon cells. Indeed, our results show that premature overexpression of StripeA in the entire ectoderm leads to severe defects in germ band retraction.

Stripe was shown previously to mediate both the determination of precursor cells as well as their maturation and ability to undergo specific temporal and spatial regulation (Becker et al., 1997; Frommer et al., 1996). Our findings suggest both negative and positive feedback loops, based on post-transcriptional regulation of *stripe* splice variants that on one hand maintain non-bound tendon cells at the precursor state, and on the other hand enable irreversible differentiation of muscle-bound tendons.

Whereas some tissue differentiation processes (e.g. tracheal development) initiate upon the expression of a key transcription factor, which autoregulates its own expression, thus leading to a unidirectional differentiation route, other cells (e.g. cells in the proneural region) go through an intermediate stage of a field of competent precursors, in which only additional local interactions lead to irreversible differentiation. Maturation of tendon cells follows the latter path, although the selection mechanism is based on regulation at the post-transcription level.

We suggest the following model to explain the transition between the two phases of tendon cell development: the initial expression of *stripeB* is induced by segment polarity-dependent signals. StripeB defines a set of tendon precursor cells. StripeB then reinforces its own expression and in addition induces How(L) expression, which in turn suppresses *stripeB* mRNA levels, thus keeping StripeB levels constant throughout embryonic development. This is supported by our experiments, which show that StripeB overexpression leads to elevation in How(L) and in *StripeB* itself. Following myotube extension and adhesion to a tendon precursor cell, How(S) levels are elevated in the muscle-adherent tendon cells, presumably due to EGFR activation (Nabel-Rosen et al., 1999). How(S) associates with the splicing factor Crn and the complex shuttles into the nucleus, where it binds to *stripeA* intronic sequences and elevates its mRNA levels, by enhancing its splicing and maintaining the stability of the spliced mRNA. The resulting muscle-bound tendon cell expresses high StripeA levels, which further drive the expression of genes required for terminal tendon differentiation (e.g. *shot*, *how*), as inferred from StripeA overexpression experiments. This regulatory mechanism couples muscle binding and tendon cell maturation, while preventing differentiation of additional, non-bound, precursors.

The activity of How proteins in regulating Stripe levels

RNA-binding proteins can function as adaptor units promoting the assembly of large protein complexes that control the various aspects of RNA metabolism. How, together with Quaking and GLD-1, belongs to the Star family of RNA-binding proteins, the members of which often regulate more than one facet of RNA metabolism. For

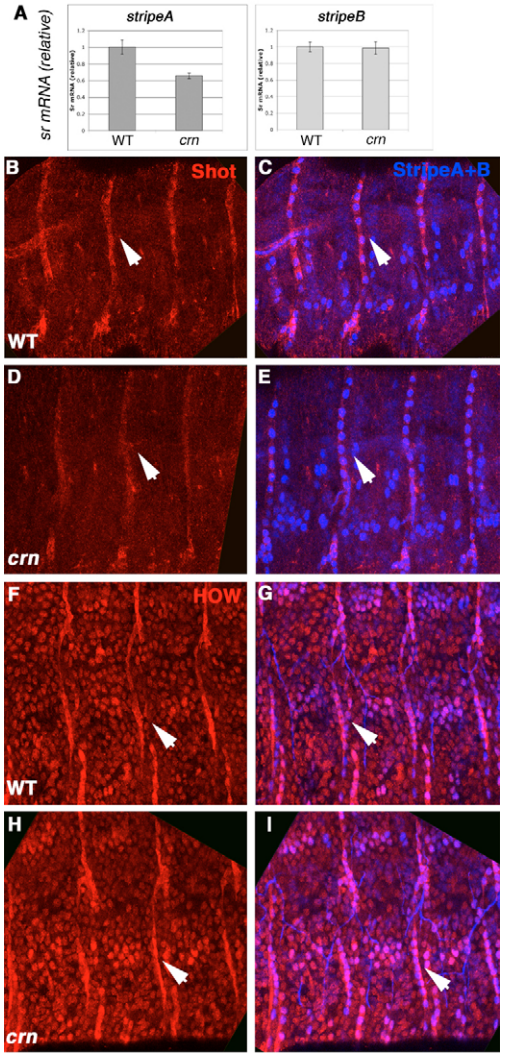

Fig. 8. *stripeA* mRNA levels are specifically reduced in *crn* mutant embryos. (**A**) RT-PCR with *stripeA*- and *stripeB*-specific primers, performed on RNA extracted from *crn* homozygous mutant or from wild-type embryos at 14-16 hours AEL. The mRNA levels were normalized against *tubulin* mRNA levels in each sample. A specific reduction of *stripeA* is detected. (**B-E**) Stage 16 wild-type (B) or *crn* mutant (D) embryos stained for Shot (red) and Stripe (Blue, C,D); C and D are the corresponding merged images. A significant reduction of Shot is detected in *crn* mutant embryos. (**F-I**) Stage 16 wild-type (F) or *crn* mutant (H) embryos stained for How (red) and Stripe (blue, G,I); G and I are the corresponding merged images. Note that there is no change in How levels in *crn* mutants. Arrowheads mark the tendon cells of the ventral longitudinal muscles.

example, GLD-1 has been suggested to regulate mRNA stability as well as translation of some of its targets (Jan et al., 1999). Similarly Quaking controls mRNA stability (Li et al., 2000) as well as RNA splicing (Wu et al., 1999), and possibly also mRNA nuclear export and localization (Larocque et al., 2002). It appears that How proteins also exhibit a wide range of activities on RNA metabolism. While the effect of How(L) and How(S) on *stripe* mRNA stability has been demonstrated previously (Nabel-Rosen et al., 2002), this study suggests that How(S) has an additional activity in regulating the splicing of *stripeA*. Consistent with our study, How has been identified in a dsRNA-based screen for alternative splicing regulators, as a protein required for specific splicing of exons within two out of five tested genes, *paralytic* (exons A/I), and *Dscam* (exon 4), in S-2 cells (Park et al., 2004). Our previous studies suggested that the ability of How proteins to stabilize stripe mRNA is mediated by the 3′ UTR of *stripe* (Nabel-Rosen et al., 2002). However, the splicing of *stripeA* appears to be regulated by its specific intronic sequences.

By contrast to How(L), which is localized specifically in the nucleus, How(S) is distributed both in the nucleus and the cytoplasm. However, when How(S) is retained in the nucleus by the addition of an NLS sequence, it loses its effect on the mRNA levels of its target. What could be the molecular explanation for the involvement of How(S) in splicing? We suggest that How(S) binds to a cytoplasmic splicing factor and recruits it to the nucleus, where it is targeted to bind *stripeA*-specific intronic sequences. This may enhance the splicing of *stripeA*-specific exons. A candidate splicing factor is Crn. Crn is a general, well-conserved splicing factor that is expressed by a wide range of cell types and is distributed both in the nucleus and the cytoplasm (Chung et al., 1999). In a parallel study, we have demonstrated that *crn* and *how* mutants exhibit closely related phenotypes, affecting glial cell maturation. Importantly, we show that both Crn and How(S) proteins [but not How(S)-NLS] co-precipitated from S-2 cell extracts, indicating that both proteins are associated in a common protein complex in the cytoplasm (Edenfeld et al., 2006). In addition, when Crn is myristoylated and transfected into S-2 cells together with How(S), How(S) is relocated to the membrane (Edenfeld et al., 2006). Furthermore, in *crn* mutants StripeA, but not StripeB, levels are reduced, and this is reflected in the reduction of Shot levels.

These results support a model in which How(S) interacts with Crn in the cell cytoplasm, shuttles into the nucleus and facilitates *stripeA* splicing, and possibly mRNA stability, leading to StripeA protein elevation. A similar mechanism may operate in the Quaking-dependent facilitation of myelin-associated glycoprotein splicing.

In summary, we describe a molecular mechanism that is based on post-transcriptional control, by which cell differentiation is induced and maintained by local interactions with neighboring cells.

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