

Two Isoforms of the *Drosophila* RNA Binding Protein, How, Act in Opposing Directions to Regulate Tendon Cell Differentiation

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Summary

Differential RNA metabolism regulates a wide array of developmental processes. Here, we describe a mechanism that controls the transition from premature *Drosophila* tendon precursors into mature muscle-bound tendon cells. This mechanism is based on the opposing activities of two isoforms of the RNA binding protein How. While the isoform How(L) is a negative regulator of Stripe, the key modulator of tendon cell differentiation, How(S) isoform elevates Stripe levels, thereby releasing the differentiation arrest induced by How(L). The opposing activities of the How isoforms are manifested by differential rates of mRNA degradation of the target *stripe* mRNA. This mechanism is conserved, as the mammalian RNA binding Quaking proteins may similarly affect the levels of Krox20, a regulator of Schwann cell maturation.

Introduction

Posttranscriptional regulation based on differential RNA metabolism is an important mechanism for temporal and spatial regulation of gene expression (Wilusz et al., 2001). A wide array of developmental and physiological processes is regulated by activation or repression of translational activity. These include the formation of the anterior-posterior axis in the *Drosophila* embryo (van Eeden and St Johnston, 1999; Parisi and Lin, 2000; Curtis et al., 1995), sex determination in *C. elegans* (Goodwin and Evans, 1997; Puoti et al., 1997), and erythrocyte differentiation (van Leyen et al., 1998; Ostareck et al., 2001) and iron homeostasis in mammals (Hentze and Kuhn, 1996). Posttranscriptional mechanisms are regulated by RNA binding proteins, which bind to the nascent transcripts, often at their 3' untranslated region (UTR), and control various aspects of RNA metabolism, including splicing, stability, nuclear export, localization, and/or association with the translation machinery (Jan et al., 1997; Gavis et al., 1996; Wharton et al., 1998).

Drosophila Held out wing (How) is a KH domain RNA binding protein that controls muscle function as well as tendon cell differentiation (Baehrecke, 1997; Zaffran et al., 1997; Lo and Frasch, 1997). The How protein is expressed at high levels in *Drosophila* embryonic tendon cells as well as in mesodermal derivatives. In *how*

mutant embryos, the heart muscles do not operate properly, and the somatic muscle pattern is abnormal, leading to embryonic lethality (Zaffran et al., 1997; Baehrecke, 1997). How contains a single maxi-KH domain, characteristic of the Signal Transduction and Activation of RNA (STAR) family of RNA binding proteins (Vernet and Artzt, 1997).

The KH domain, initially defined in the heterogeneous nuclear RNA-ribonucleoprotein complex (hnRNP K) (Siomi et al., 1993), is a conserved region that is often repeated within the sequence of RNA binding proteins and which has been implicated in RNA binding activity (Musco et al., 1996). Prototypic members of the STAR family include *quaking* (*qki*), a gene whose product is required for maturation of Schwann cells and oligodendrocytes in mammals (Ebersole et al., 1996), *gld1*, encoding a protein that is essential for oocyte differentiation and meiotic prophase progression in *C. elegans* (Jones and Schedl, 1995; Jan et al., 1999), and Sam68, a protein involved in cell cycle progression in mammals (Lock et al., 1996). The molecular basis for the activity of the STAR family members has yet to be elucidated. The activity of Quaking, at least in part, is based on its effect on RNA stability of target RNAs, such as Myelin Basic Protein (MBP) mRNA in Schwann cells (Li et al., 2000).

We previously showed that How mediates tendon cell differentiation by negatively regulating *stripe* mRNA levels (Nabel-Rosen et al., 1999). Stripe, an EGR (early growth response) family transcription factor, is a key regulator of tendon cell differentiation (Volk, 1999). Initially, the expression of Stripe in tendon precursors is positively induced by the Hedgehog and EGF receptor pathways and negatively regulated by the Wingless pathway (Piepenburg et al., 2000; Hatini and DiNardo, 2001). Subsequently, during later developmental stages (i.e., stages 14–16), Stripe expression is maintained at high levels only in muscle-associated tendon cells, leading to their maturation (Becker et al., 1997). Prior to muscle binding, Stripe levels are kept low in tendon precursors, presumably due to the negative regulation by How (Nabel-Rosen et al., 1999, and see below). Following muscle binding, Stripe levels are elevated in response to Vein, a *Drosophila* neuregulin-like ligand, produced by the approaching myotube. Vein activates the EGF receptor pathway in the muscle-bound tendon cell, leading to tendon cell differentiation (Yarnitzky et al., 1997).

The *how* gene encodes two protein isoforms produced by alternative splicing, How(L) and How(S). The How proteins are identical along most of their coding sequence, including the conserved maxi-KH domain, but differ at the C-terminal region (Lo and Frasch, 1997). How(L) contains a unique tail of 36 amino acids, which carries an evolutionarily conserved nuclear retention signal (Nabel-Rosen et al., 1999; Wu et al., 1999), whereas How(S) contains two unique stretches, one of 14 amino acids, starting at amino acid position 269, (G.V. and T.V., unpublished data) and a second of a six amino acid peptide at the C-terminal domain; neither of these

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stretches are evolutionarily conserved (Lo and Frasch, 1997; T.V., unpublished data). In contrast to the nuclear localization of How(L), How(S) is distributed in both the cytoplasm and nuclei of cells, and its expression is elevated during late stages of embryonic development (Nabel-Rosen et al., 1999). Our previous experiments showed that How proteins associate with the 3'UTR of *stripe* mRNA. Moreover, overexpression of How(L) in the embryonic ectoderm of wild-type embryos leads to downregulation of Stripe levels, consistent with the idea that How(L) acts as a repressor (Nabel-Rosen et al., 1999). We suspected that How(S) might not exhibit repressive activity similar to How(L), since it is expressed in mature tendon cells, and its expression is upregulated following overexpression of Vein in the embryonic ectoderm.

Previously, we showed that *how* is required for the arrest of tendon cell maturation through the activity of How(L). Here, we address the function of How(S), and the molecular basis of its activity. We show that, in contrast to How(L), How(S) elevates the levels of Stripe, and that this activity is mediated through the 3'UTR of *stripe* mRNA. Moreover, How(S) counteracts the repression by How(L) when these isoforms are coexpressed. The mechanism of repression/facilitation by How proteins is based on their effect on *stripe* mRNA decay. We suggest that tendon cell differentiation is arrested when the abundance of How(L) isoform is high compared to that of How(S) isoform and is facilitated following the upregulation of How(S). Our results further suggest that this two-way switch mechanism is not unique to How proteins; a similar mechanism may be implemented by QKI proteins while controlling Schwann cell maturation.

Results

How Activity Is Mediated by the 3'UTR of *stripe* mRNA

Our previous analysis, using pulldown assays, showed that both How isoforms associate specifically with the 3'UTR of *stripe* mRNA. Overexpression of Stripe with its endogenous 3'UTR in the ectoderm, using the *en-gal4* driver, results in a delay of about 2–3 hr in Stripe expression. This delay is due to the repression of Stripe by How(L), which is present at low levels in all ectoderm cells. In *how* mutant embryos, the delay in Stripe expression is not observed (Nabel-Rosen et al., 1999).

To test whether the How-dependent delay in Stripe expression is mediated by the 3'UTR of *stripe* mRNA, we generated transgenic flies carrying the *stripe* coding sequence without its 3'UTR. The delay in Stripe expression is eliminated in embryos expressing this construct, under regulation of the *en-gal4* driver (Figure 1). When taken together with our previous results (see above), this experiment indicates that How inhibits Stripe expression through its association with the *stripe* 3'UTR. Notably, the elevated levels of Stripe lacking its 3'UTR in the transgenic embryos may be detected already at the mRNA level, as revealed by in situ hybridization. This suggests that Stripe regulation is implemented at the mRNA level.

How(L) and How(S) Exhibit Antagonistic Effects on the Expression of *gfp-sr3'UTR* Reporter RNA

Stripe stimulates its own expression and, in addition, induces How(L) expression. Therefore, the alteration in Stripe expression described above may not directly reflect the effect of How proteins. In order to analyze the net effects of both How isoforms on their target RNAs, we produced transgenic flies carrying a reporter gene consisting of *gfp* fused to the 3'UTR of *stripe* (*gfp-sr3'UTR*). These flies were recombined to the *en-gal4* driver and crossed to flies carrying either *UAS-How(L)* or *UAS-How(S)*.

The effects brought about by the How proteins on the *gfp-sr3'UTR* were tested by measuring the levels of GFP protein in embryos carrying the *gfp-sr3'UTR* reporter combined with either How(L) or How(S). The GFP levels were compared to control embryos carrying a nonrelevant *UAS-flp* construct in addition to the *gfp-sr3'UTR* reporter. Examination of these embryos shows that the levels of the GFP protein are significantly reduced in embryos expressing How(L) relative to the levels in the control embryos (Figure 2). In contrast, the GFP levels are significantly elevated in embryos expressing How(S) relative to the levels in the control embryos. Quantitation of GFP expression levels was performed by measuring the mean intensity fluorescence of each of the stripes in the engrailed domain in several embryos. These measurements show that the addition of How(S) roughly doubled the GFP intensity, while the addition of How(L) lowered the GFP intensity to about half the intensity of the control.

To assess whether the How proteins act at the mRNA level, we performed in situ hybridization with a *gfp* probe to visualize the *gfp* mRNA in the embryos carrying the various constructs. The alterations in the levels of the GFP protein are reflected by corresponding differences at the mRNA level (Figure 2), suggesting that the regulation of How proteins is implemented by controlling the mRNA levels. To confirm that the differences detected in GFP levels are mediated by the 3'UTR of *stripe* mRNA, we analyzed the effect of How(L) or How(S) on the levels of GFP protein and *gfp* mRNA in embryos carrying a *gfp* reporter that utilizes the 3'UTR of the pUAST vector taken from SV-40(T) (Brand and Perrimon, 1993). In these embryos, we could not detect any significant regulation by How of the levels of either the GFP protein or *gfp* mRNA (Figure 2).

These results verify that How(L) represses and How(S) facilitates the expression of their target RNA and that the 3'UTR of *stripe* mRNA mediates these activities. The fact that both the protein and the RNA levels of the target gene are altered makes it likely that the activity of How proteins is implemented at the mRNA level.

How(S) and How(L) Exhibit Opposing Effects on the Endogenous Stripe Levels

We next sought to determine the effects of both How proteins on endogenous Stripe levels by driving their expression in tendon cells of *how* mutant embryos lacking functional How, utilizing the *sr-gal4* driver. The resulting Stripe levels are significantly reduced in embryos expressing How(L) and elevated in embryos expressing How(S) (Figure 3). These results indicate that How(L)

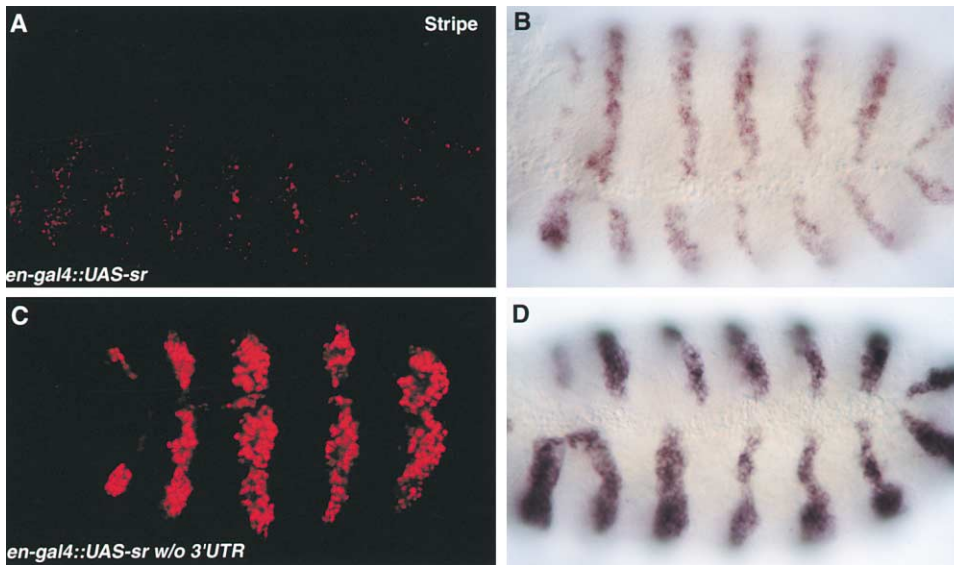


Figure 1. Deletion of *stripe* 3'UTR Leads to Elevated Levels of Ectopic Stripe Protein and mRNA

Embryos at late stage 11 carrying either an intact *UAS-stripe* cDNA construct (A and B) or *UAS-stripe* lacking the 3'UTR (C and D) were driven to express *stripe* by the *en-gal4* driver. The embryos were stained with anti-Stripe antibody (A and C) to visualize the Stripe protein or hybridized with a *stripe* Dig-labeled DNA probe to detect *stripe* mRNA (B and D). Note that the levels of *stripe* protein and mRNA are lower in embryos carrying the *stripe* with its 3'UTR than in embryos carrying *stripe* without its 3'UTR.

and How(S) exhibit opposing activity not only on the *gfp-sr3'UTR* reporter but also in vivo, altering endogenous Stripe levels.

We wished to examine whether How(S) can overcome the effect of the endogenous How(L) that is present in wild-type premature tendon cells at a relatively early developmental stage (stage 12). For that purpose, we used the *tub-gal4* driver. Premature tendon precursors of wild-type embryo overexpressing How(S) exhibit significantly higher levels of Stripe compared to those of their wild-type counterparts. This suggests that How(S) can overcome the repression of endogenous How(L) in premature tendon cells, leading to elevated levels of its target *stripe* mRNA. This facilitation may be due to its ability to antagonize How(L) repression and/or by How(L)-independent activity of How(S). Conversely, overexpression of How(L) in wild-type embryos (utilizing the *stripe-gal4* driver) at stage 14–15, when Stripe levels in wild-type embryos are high, led to a significant reduction of Stripe levels.

Maturation of Tendon Cells Is Inversely Correlated with the Relative Expression Levels of How Isoforms

Our results predict that maturation of tendon cells depends on the elevation of How(S) and the lowering of How(L). To evaluate the relative levels of How(L) and How(S) in tendon cells during the transition from premature to mature state of differentiation, we needed to isolate the tendon cells from embryos at early and late stages. This selection is essential, since the expression of How in other tissues may mask the tendon-specific How expression. To this end, we used FACS to separate GFP-positive tendon cells taken from embryos expressing *UAS-gfp* driven by the tendon-specific *sr-gal4* driver.

Embryos at the age of 7–11 hr after egg laying (AEL) and at 16–18 hr AEL were collected and dissociated into a single cell suspension (see Experimental Procedures). These cells were separated into GFP-positive and -negative subpopulations by FACS. The enrichment of GFP-positive cells is demonstrated by ~10-fold enhancement in the GFP levels detected by Western analysis using anti-GFP antibody reacted with cell extract from unsorted and sorted cell populations (Figure 4). The GFP-positive tendon cells were analyzed by Western analysis with anti-How antibody (Figure 4). This analysis shows that at 7–11 hr AEL, when most of the tendon cells are at the precursor state of differentiation, the levels of How(L) are higher than those of How(S). Conversely, at 16–18 hr AEL, when most of the tendon cells are at the mature state of differentiation, How(S) isoform becomes abundant. The true levels of How(S) may be higher than is apparent from our assay, due to a weaker reactivity of our anti-How antibody with How(S) (G.V. and T.V., unpublished data). Thus, maturation of tendon cells is correlated with the reduction of the repressor, How(L), and elevation of the facilitator, How(S).

The Activity of How Proteins Is Based on Their Opposing Effects on mRNA Decay of Their Target RNA

To gain further insight into the mechanism of How protein activity, we followed the alterations in protein and mRNA levels of the *gfp-sr3'UTR* reporter in Schneider cells expressing How(L), How(S), or both. To this end, Schneider cells were cotransfected with the *gfp-sr3'UTR* reporter together with either or both How constructs. The levels of GFP protein detected by Western analysis are significantly reduced in the presence of

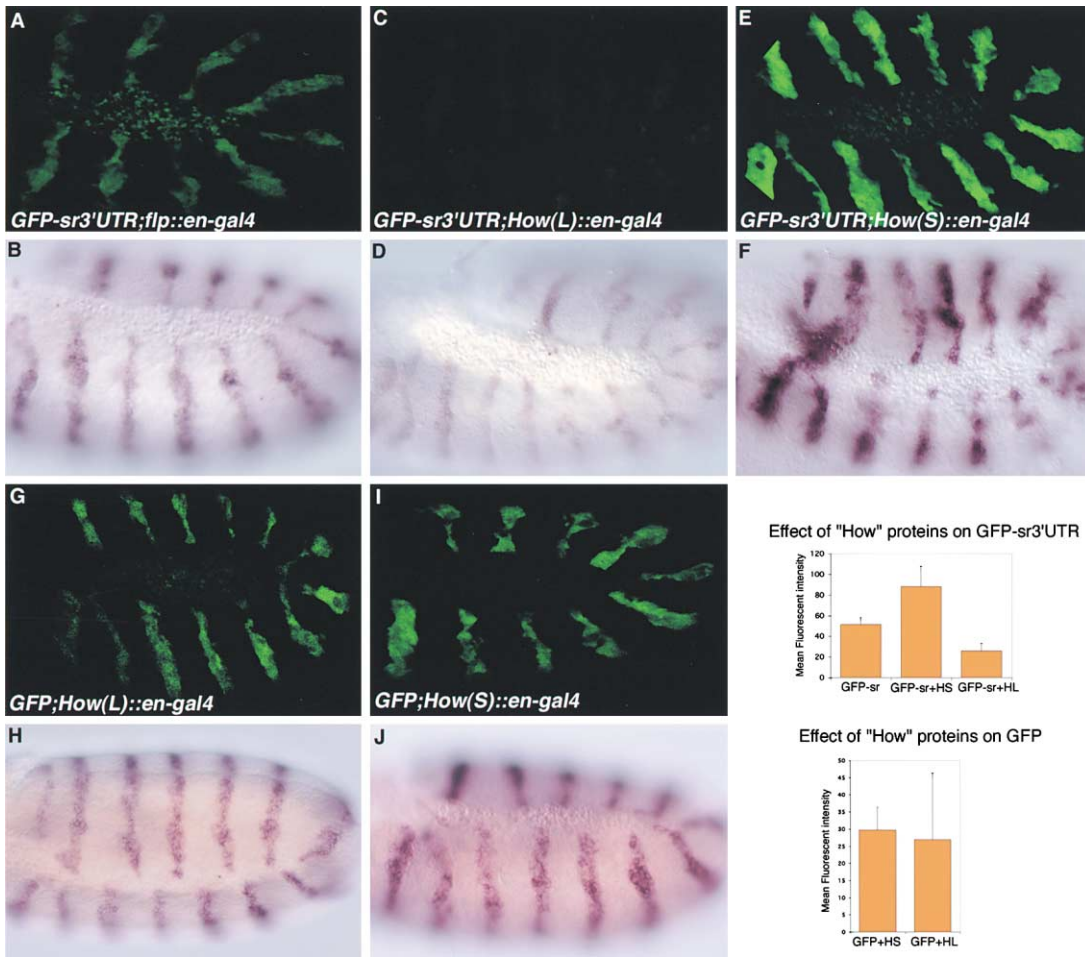


Figure 2. The Effect of How Proteins on *gfp-sr3' UTR* Reporter

Embryos at late stage 11 carrying *UAS-gfp-sr3' UTR* (A–F) or *UAS-gfp* (G–J), together with the *en-gal4* driver (A–J), are shown. In addition, the embryos carry either a control *UAS-flp* construct (A and B), *UAS-How(L)* construct (C, D, G, and H), or *UAS-How(S)* construct (E, F, I, and J). The GFP protein levels in all embryos were visualized in live, dechorionated embryos by confocal microscopy using the same settings (laser power, iris, and gain). The *gfp* mRNA is detected by in situ hybridization with Dig-labeled *gfp* DNA probe. All in situ hybridization experiments were carried with the same *gfp* probe, and incubation times with the alkaline-phosphatase substrate were equal. The charts shown in the right lower panel summarize measurements of the average mean fluorescence intensity of 8–9 engrailed bands from three different embryos with or without *stripe 3' UTR*, utilizing LaserSharp MRC-1024 software. Note that the GFP fluorescence intensity and the mRNA levels are elevated in embryos carrying How(S) (E and F) and reduced in embryos carrying How(L) (C and D) compared to those in the control (A and B). The GFP levels in embryos carrying a control *gfp-SV403' UTR* reporter were not altered in the presence of either How(L) or How(S).

How(L) and are elevated in the presence of How(S) (Figure 5A). Densitometric measurements show that, on average, the activity of How(L) reduced the GFP levels by ~50% ($n = 3$) relative to those of the control, whereas How(S) elevated the GFP levels by ~50% ($n = 3$) (Figure 5B). These results are comparable with the measurements of the GFP intensity in the embryos shown in Figure 2. No significant variations in the GFP levels were detected when a *gfp* construct lacking the *stripe 3' UTR* was coexpressed with either of the How proteins. Interestingly, when both How proteins were cotransfected together with the *gfp-sr3' UTR* reporter, the GFP levels were restored to the control levels, suggesting that How(S) is capable of counteracting the repression mediated by How(L). Thus, the opposite effects of the two isoforms are balanced.

Northern analysis with the cells described above provided similar results. Following 2 days of cotransfection, the mRNA of *gfp-sr3' UTR* was lower in the presence of How(L) and higher in the presence of How(S) (Figure 5C shows a typical result of three repetitions of the experiment). To further analyze whether How proteins affect mRNA turnover or stability, we examined the rate of mRNA decay of the reporter *gfp-sr3' UTR* mRNA in cells expressing How(L) or How(S) following a short pulse of transcription and compared it to that of a control *gfp* lacking *sr3' UTR*. Cells stably transfected with each of the How proteins (controlled by metallothionein promoter) were transiently transfected with *gfp-sr3' UTR* or *gfp* reporters under heat shock promoter regulation. The levels of the *gfp* mRNA were analyzed at various time points following 24 hr induction of How proteins (by the

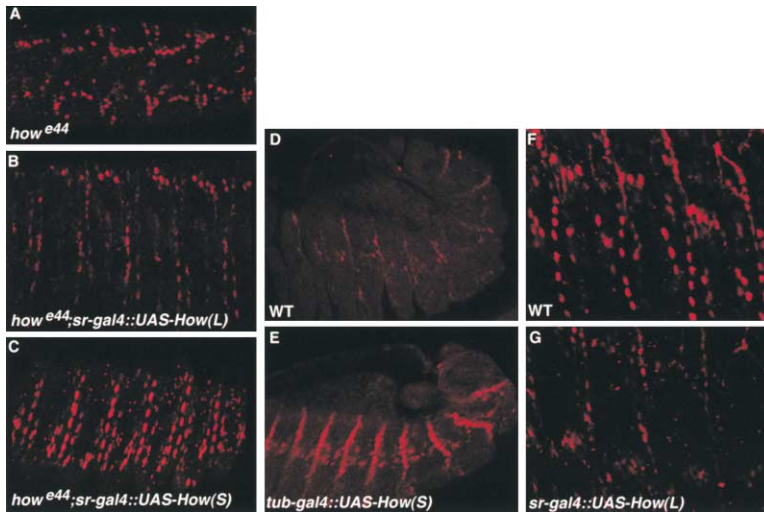


Figure 3. How Proteins Alter the Levels of Endogenous Stripe

Stripe levels are reduced in *how^{e44}* mutant embryo expressing How(L) in tendon cells (compare [B] to [A]) and elevated in *how^{e44}* mutant embryo expressing How(S) in tendon cells (compare [C] to [A]). *stripe-gal4* driver was used in both cases to drive the expression of each of the How proteins. Overexpression of How(S) in wild-type embryo (stage 12) by the *tub-gal4* driver leads to significant elevation of endogenous Stripe levels (compare [E] to [D]). Conversely, overexpression of How(L) in tendon cells of wild-type, stage 16 embryo (utilizing the *stripe-gal4* driver) leads to a significant reduction of Stripe levels (compare [G] to [F]).

addition of CuSO₄) succeeded by a short (15 min) heat shock pulse. To eliminate possible variations due to the transfection efficiency, an equal number of the transiently transfected cells was plated for each time point. We find that How(L) affects the rate of decay of the *gfp-sr3'UTR* mRNA levels. Forty-five minutes following the heat shock treatment, the *gfp* mRNA is hardly detected, and, after 4 hr, no signal is observed, in contrast to a control *gfp* reporter. In the presence of How(S), on the other hand, the decay of the *gfp-sr3'UTR* mRNA levels is attenuated following the heat shock pulse [Figures 5D and 5E show a typical pattern of the mRNA decay of three repetitions of the pulse/chase experiment done either in the presence of How(L) or How(S)]. These results suggest that, while How(L) activity leads to higher rate of degradation of the target mRNA, How(S) activity stabilizes the target mRNA, leading to its accumulation.

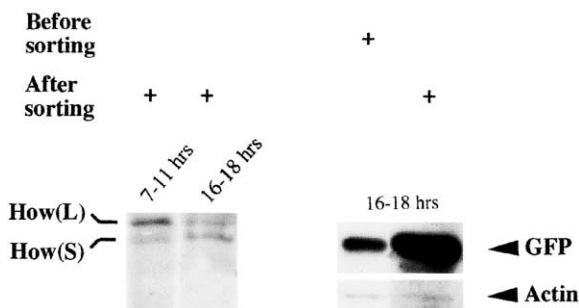


Figure 4. The Relative Levels of How(L) and How(S) Shift during Tendon Cell Maturation

The two left lanes show Western analysis with anti-How antibody with an equal number of FACS-sorted tendon cells dissociated from embryos at the age of 7–11 hr AEL or 16–18 hr AEL. These tendon cells were sorted from embryos induced to express tendon-specific GFP, using the *sr-gal4* driver together with *UAS-gfp*. Note that at 7–11 hr, the longer form of How is the prominent band and that, at 16–18 hr, the longer form is significantly reduced and the shorter form is more pronounced. The two right lanes show Western analysis with anti-GFP and anti-actin antibodies applied on the same lanes, reacting with protein extract from the embryos before and after FACS sorting. Note the enrichment in the GFP band after sorting.

The Nuclear Retention Signal Is Essential for the Repression Manifested by How(L)

As we and others have previously shown, a conserved stretch of seven amino acids present in both How(L) and QKI-5 (REHPYQR) carries a nuclear retention signal (Nabel-Rosen et al., 1999; Wu et al., 1999). To assess the contribution of this sequence to the repression potential of How(L), we constructed mutant forms of How(L) that either lack the entire nuclear retention sequence or carry various missense mutations in that sequence. The mutated constructs were cotransfected together with the *gfp-sr3'UTR* reporter into Schneider cells, and the GFP intensity as well as the nuclear retention of the mutated How(L) were examined. This analysis indicates that the retention of How(L) in the nucleus is correlated with its ability to repress the GFP levels. These results are summarized in Figure 6B, and a representative result is shown in Figure 6A. A single missense mutation (tyrosine to glycine) that alters the nuclear retention of How(L) also abrogates its ability to repress the GFP levels. In contrast, when the same tyrosine was mutated to phenylalanine, both the nuclear retention and the repression by How(L) were preserved. Thus, nuclear retention is essential for the repression potential of How(L).

Quaking Isoforms Exhibit Contrasting Activities in the Regulation of a *gfp-krox20* 3'UTR Reporter

Mammalian Schwann cell differentiation and *Drosophila* tendon cell differentiation are regulated by a partially homologous set of genes (Volk, 1999). *krox20* (also named *egr2*), a key gene in the terminal differentiation of Schwann cells, may function in a manner similar to Stripe (Warner et al., 1998; Topilko et al., 1994). Both Stripe and Krox20 share a conserved, triple zinc finger, DNA binding domain typical of EGR family members. In view of the similarity between Quaking (QKI) and How proteins, we next investigated whether QKI isoforms could bind and regulate the expression of Krox20 in an antagonistic manner similar to that of How(S) and How(L). Thus, the nuclear QKI-5, which is most similar to How(L) and carries a nuclear retention signal, may

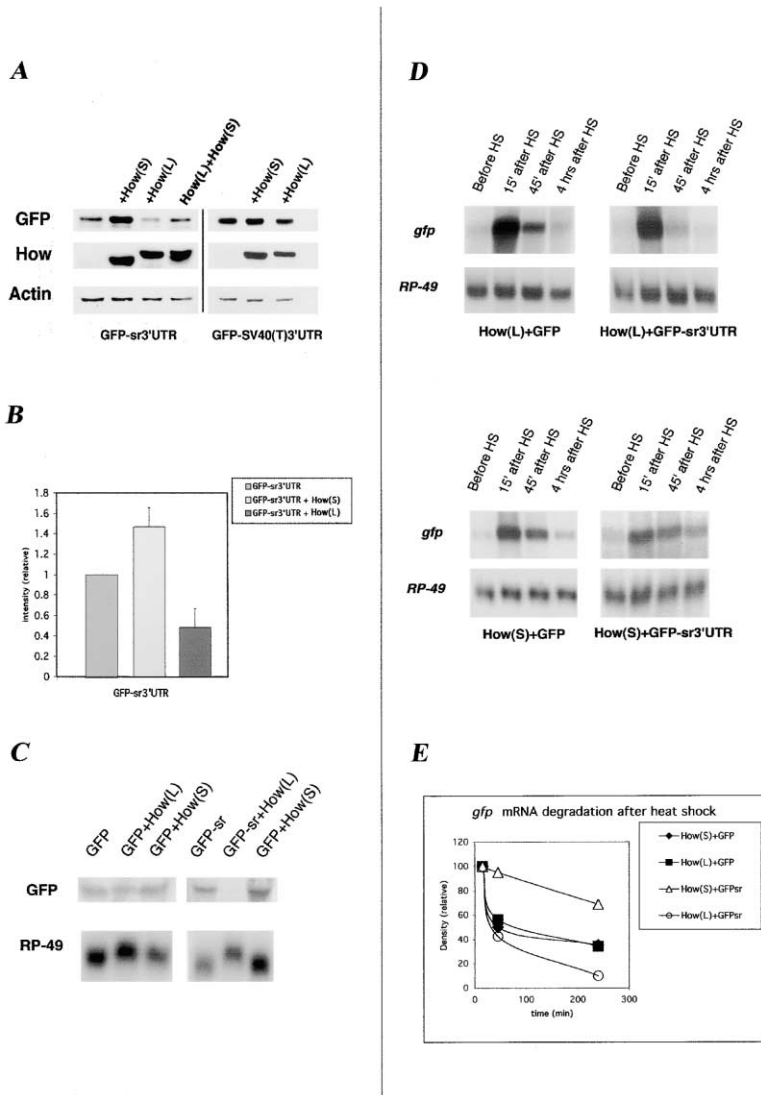


Figure 5. How Proteins Affect the Levels of GFP Protein through an Effect on *gfp* mRNA Stability

(A) Western analysis with anti-GFP antibody of protein extracts taken from Schneider cells that were cotransfected with either *gfp-sr3'UTR* (four left lanes) or *gfp-SV40(T)3'UTR* (three right lanes). In addition, the cells were cotransfected with *how(S)*, *how(L)*, or with both constructs. The bands of How and actin in each sample are detected by anti-How or anti-actin antibodies. Note that the levels of GFP (fused to *sr3'UTR*) are elevated in the presence of How(S), reduced in the presence of How(L), and restored to control levels in the presence of both. The levels of GFP lacking *sr3'UTR* are not affected in the various treatments.

(B) The average intensity calculated from densitometric measurements of the GFP bands normalized to the actin bands in three different transfection experiments.

(C) Northern analysis with *gfp* probe showing the *gfp* mRNA steady state levels of cells transfected with *gfp-sr3'UTR* (three right lanes) or *gfp-SV40(T)3'UTR* (three left lanes), either alone (first lane) or together with How(L) (second lane) or How(S) (third lane). Control mRNA levels of the ribosomal protein *RP-49* are shown respectively. Note that the *gfp-sr3'UTR* mRNA is high in cells coexpressing How(S) and reduced in cells coexpressing How(L). The control *gfp-SV40(T)3'UTR* mRNA levels are not altered in the presence of How proteins.

(D) The rate of decay of *gfp-sr3'UTR* mRNA differs in the presence of How proteins. Cells stably transfected with each of the How proteins and transiently transfected with *gfp-sr3'UTR* or *gfp* reporters, under heat shock control, were induced to express the How proteins. The RNA extracted from whole cell lysate of each sample was collected at different time points following a short pulse of transcription of the *gfp* reporters performed by 15 min of heat shock. The rate of *gfp* mRNA decay is shown for *gfp* + How(L), *gfp-sr3'UTR* + How(L), *gfp* + How(S), and *gfp-sr3'UTR* + How(S), respectively. Control mRNA levels of ribosomal protein *RP-49* are shown for each sample.

sr3'UTR + How(L), *gfp* + How(S), and *gfp-sr3'UTR* + How(S), respectively. Control mRNA levels of ribosomal protein *RP-49* are shown for each sample.

(E) Quantitative analysis of the Northern shown in (D). The intensity of each of the GFP bands is normalized according to the amount of the *RP-49* in the lane and to the *gfp* mRNA level 15 min following the heat shock treatment. Note that the *gfp-sr3'UTR* is degraded faster than the control *gfp* in the presence of How(L) and is more stable in the presence of How(S).

repress while QKI-6 and QKI-7 may facilitate the expression of their target RNAs. Indeed, Figure 7 shows that in Schneider cells, QKI-5 repressed both *gfp-krox20* and *gfp-sr3'UTR*. In contrast, only a slight reduction of the GFP levels was observed when cells were transfected with *gfp* fused to a nonrelevant 3'UTR. In contrast, QKI-6 and QKI-7 facilitated the expression of both *gfp-krox20* and *gfp-sr3'UTR* reporters and did not affect the control GFP levels. We did not detect any apoptotic effect of QKI-7 (as described in Pilotte et al. [2001]) in the Schneider cells. However, the QKI-7 isoform used in this study differs slightly from QKI-7 used by Pilotte's group and is also not recognized by the anti-QKI-7 antibody described in Hardy et al. (1996). These results suggest that QKI proteins may regulate *Krox20* expression in

Schwann cells in a manner similar to that of How proteins.

Discussion

The transition from a premature to mature state of cell differentiation is a significant step toward organogenesis, yet the molecular mechanisms that mediate this transition are not fully understood. *Drosophila* tendon precursors undergo maturation following their binding to muscle cells. Previously, we showed that How(L) activity arrests tendon precursor differentiation (Nabel-Rosen et al., 1999). In this paper, we address the function of How(S), showing that it exhibits an opposite effect on tendon cell differentiation, both by counteracting How(L)

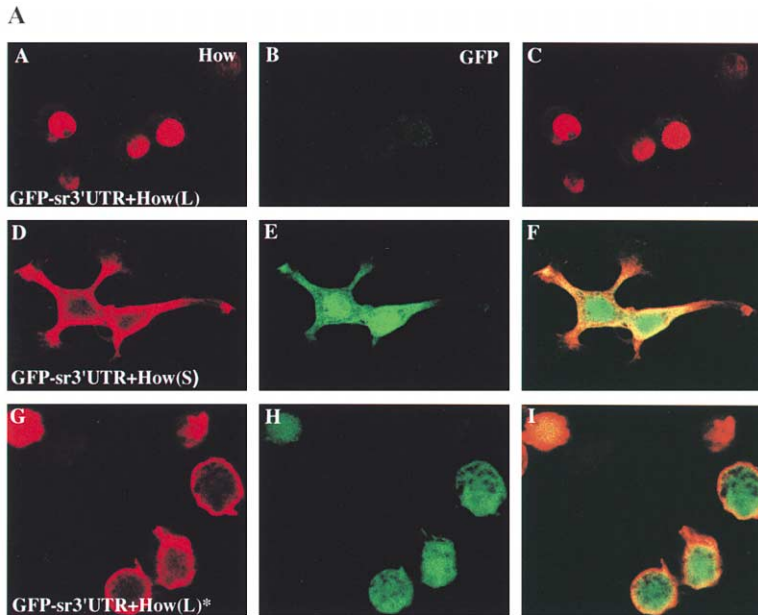
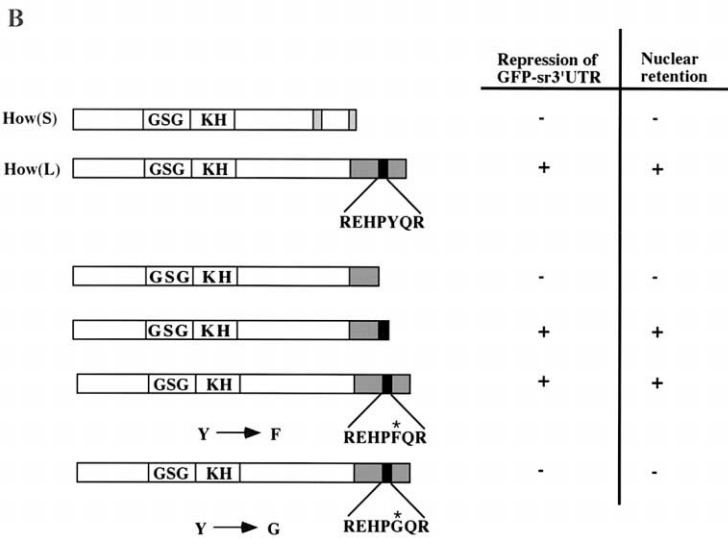


Figure 6. The Nuclear Retention of How(L) Is Essential for Its Repression Activity

(A) The GFP levels (green in [B], [E], and [H]) are shown in Schneider cells cotransfected with *gfp-sr3'UTR* and How(L) (A–C), How(S) (D–F), or with a mutated form of How(L) (G–I), in which the tyrosine in the nuclear retention signal was mutated into glycine. Immunofluorescent labeling with anti-How antibody (A, D, and G) shows the nuclear retention of How(L). In addition, the cytoplasmic and nuclear distribution of both How(S) and mutated How(L) is demonstrated. Note that the GFP levels are not reduced in cells transfected with mutated How(L) that is not retained in the nucleus.

(B) A summary of cotransfection experiments with the *gfp-sr3'UTR* reporter and various mutated forms of How(L). The schemes show the details of the truncation of How(L). The GFP intensity in each transfection experiment is compared to control cells transfected with the *gfp-sr3'UTR* construct alone.



repression and through positive upregulation of Stripe levels. Thus, the products of the *how* gene in *Drosophila* are part of a two-way switch, which on the one hand prevents tendon precursors from undergoing premature differentiation prior to their encounter with muscles and on the other hand facilitates differentiation of the muscle-bound tendon cells.

Since How proteins are expressed in other tissues, including muscles, heart, glial cells, and chordotonal organs, we suggest that in these tissues How proteins may similarly regulate the transition between premature and mature differentiation states. Moreover, this two-way switch mechanism may be conserved in vertebrates and operate during Schwann cell maturation.

Possible Mechanism of How Activity

RNA binding proteins of the STAR family may regulate gene expression at various levels, e.g., at the level of

nuclear export of the target mRNA, at the level of mRNA stability, and at the translation level (Vernet and Artzt, 1997; Lewis et al., 2000; Ostareck et al., 2001). The How proteins appear to exert their activity through their effect on mRNA stability. How(L) appears to induce rapid degradation of the target RNA, an activity that is tightly coupled to its nuclear retention and depends on the presence of the nuclear retention signal that is conserved in QKI-5. In our previous analysis, we suggested that How(L) may prevent nuclear export of its target mRNA. Indeed, in embryos overexpressing How(L), we occasionally detect the mRNA of *gfp-sr3'UTR* in the nucleus (data not shown). Presently, we are unable to determine whether the primary effect of How(L) is retention of the target mRNA in the nucleus followed by degradation of the target mRNA or vice versa. As shown above, How(S) increases the stability of the same target RNA. The fact that How(S) is present both in the nucleus

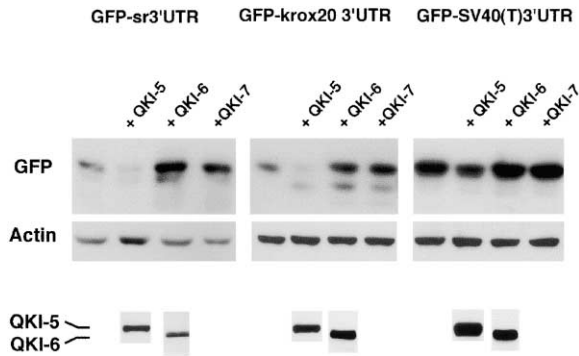


Figure 7. Quaking Proteins Affect the Protein Levels of Either *gfp-sr3'UTR* or *gfp-krox20 3'UTR*

Western analysis with anti-GFP antibody of Schneider cells transfected with either *gfp-sr3'UTR* (four left lanes), *gfp-krox20 3'UTR* (four middle lanes), or *gfp-SV40(T)3'UTR* (four right lanes). The total amount of cell extract in each sample is normalized by reaction with anti-actin (bands at the bottom). Note that the levels of GFP are low in cells coexpressing QKI-5. GFP is elevated in cells coexpressing QKI-6 or QKI-7 relative to the control in cells coexpressing either *gfp-sr3'UTR* or *gfp-krox20 3'UTR* but not in cells coexpressing *gfp-SV40(T)3'UTR*.

and in the cytoplasm raises the possibility that the association of How(S) with its target RNA during and following its nuclear export leads to mRNA stabilization. A number of RNA binding proteins possess both nuclear and cytoplasmic functions (Wilkinson and Shyu, 2001), e.g., proteins that affect both mRNA export and mRNA stability. Similarly, How proteins may affect both nuclear-cytoplasm shuttling and mRNA stability. The differential association of each of the How proteins with distinct protein partners presumably leads to their opposing effects on mRNA stability. A possible mechanism for the counteraction effect of How(S) may arise from its association with How(L), eliminating the repression by How(L). Indeed, How(S) and How(L) are coprecipitated from Schneider cells following their cotransfection together with the *gfp-sr3'UTR* (G.V., unpublished data).

A recent report suggests that a sequence (TGE) in the 3'UTR of *tra-2*, essential for Gld-1 binding, mediates deadenylation and poly(A)-dependent translation repression (Thompson et al., 2000). Poly(A) deadenylation may also lead to mRNA degradation. Since a sequence motif that is partially related to TGE is also present in the *stripe* and *krox20 3'UTR*, degradation of the target mRNA by How(L) may be based on a similar mechanism. Recently, the two cytoplasmic hnRNPs, K and E1, have been shown to inhibit translation of *lipoxigenase* mRNA by preventing its attachment to the 60S ribosomal unit (Ostareck et al., 2001). We do not exclude the possibility that How(S), in addition to its positive effect on mRNA stability, may also facilitate translation efficacy.

The Relative Proportion of How(L) and How(S) Levels Are Critical for the Differentiation Switch

Our results suggest that the relative amount of How(L) and How(S) during different stages of embryonic or adult development regulate the switch between the premature and mature state of differentiation of tendon cells.

In early stages of embryonic development, How(L) prevails, *Stripe* expression is downregulated, and differentiation is arrested. In later stages of embryonic development, How(S) is upregulated, overriding How(L) repression and facilitating *Stripe* expression. The difference in *stripe* mRNA levels may be further enhanced by *Stripe* transcriptional autoregulation. What could be the mechanism that regulates the relative levels of How proteins during tendon cell maturation? Northern analysis performed by Lo (Lo and Frasch, 1997) suggests that the total levels of How(L) mRNA are low throughout embryonic development, relative to those of How(S) mRNA. At the protein level, the proportion of the two proteins is inverted; How(S) protein levels are low and increase only during late stages of embryonic development. This suggests that How(S) is posttranscriptionally regulated. Indeed, we find that transgenic flies carrying How(S) with its unique 3'UTR exhibit almost undetectable levels of How(S) protein following induction by the *gal4* driver. When this 3'UTR is deleted, the expression levels of How(S) are significantly higher. Previous analysis suggested that the expression of How(S) is elevated by Vein-mediated activation of the EGF receptor pathway in tendon cells following muscle-tendon association (Nabel-Rosen et al., 1999). The molecular link between EGF receptor activation and the upregulation of How(S) has yet to be elucidated. A recent report suggesting that ERK phosphorylation of hnRNP-K drives cytoplasmic accumulation of hnRNP-K (Habelhah et al., 2001) may be of relevance if, similarly to hnRNP-K, How(S) undergoes ERK-dependent phosphorylation.

Similarity between the Activity of How and Quaking Proteins

The mechanism described here for the activity of How proteins appears to be conserved in evolution. Our analysis suggests that QKI proteins regulate the expression of target genes in opposing directions. QKI-5 represses while QKI-6 and QKI-7 facilitate the expression of target RNAs. Moreover, based on our results, it appears that *krox20* mRNA is an endogenous target for QKI activity. We suggest that QKI proteins regulate the transition from premature to mature Schwann cells by repressing or elevating the levels of *krox20* as well as those of additional target mRNAs. Thus, it is likely that the relative proportions of the inhibitor (QKI-5) and facilitators (QKI-6 and QKI-7) determine the state of Schwann cell differentiation. It is already known that QKI-5 is highly expressed in premature Schwann cells throughout embryonic development and that QKI-6 and QKI-7 are upregulated during Schwann cell maturation (Hardy et al., 1996), consistent with the possibility that the later are positive regulators of Schwann cell maturation. *Krox20* has been recently shown to induce a wide array of genes, many of which are essential for myelination (Nagarajan et al., 2001). Loss of *krox20* is associated with human myelinopathies (Warner et al., 1998). If QKI proteins modulate the level of *Krox20* during Schwann cells maturation, then reduction in QKI levels may result in abnormal *Krox20* levels during Schwann cell myelination. Indeed, reduced levels of QKI (presumably all the isoforms) observed in *qki^Δ* mice lead to severe myelination defects (Ebersole et al., 1996).

A recent study in mice has identified the mRNA of myelin basic protein (MBP) as a target for Quaking activity (Li et al., 2000). In adult *qki^v* mice, the levels of MBP are reduced, as a result of destabilization of MBP mRNA. The fact that, at the adult stage, QKI-6 and QKI-7 [equivalent to How(S)] are the predominant isoforms suggests that their activity, like that of How(S), is required for stabilization of MBP mRNA. Apparently, the levels of QKI-5 in this mutant are high enough to carry on embryonic development. It would be essential in future studies to analyze the effect of each of the QKI isoforms on target mRNAs in Schwann cells.

Are there other QKI homologs in *Drosophila*? Out of ten proteins related to *quaking* recently described in the *Drosophila* genome (Di Fruscio et al., 1998; Fyrberg et al., 1998; Lasko, 2000), *how* shows the highest similarity to Quaking and therefore may represent its true ortholog.

In summary, we describe a mechanism by which different isoforms of the same RNA binding protein act in an antagonistic manner to regulate a switch in cell differentiation, i.e., transition from a premature to a mature state of differentiation of *Drosophila* tendon cells. This mechanism appears to be conserved and to operate during maturation and myelination of Schwann cells.

Experimental Procedures

Fly Strains

Fly stocks used were as follows: *howe44* (Baehrecke, 1997); *gal4* lines included *stripe-gal4* (G. Morata, Madrid) and *engrailed-gal4* (A. Brand, Wellcome/CRC Institute, Cambridge); *tubulin-gal4*, *UAS-gfp*, *UAS-gfp-mCD8*, and *UAS-flp* (Bloomington *Drosophila* Stock Center). The following lines were constructed in our lab: *UAS-StripeB*, *UAS-How*, *UAS-Stripe* without 3' UTR, *UAS-How* without 3' UTR and *UAS-gfp-stripe* 3' UTR. How(S) and How(L) cDNAs were obtained from M. Frasch (Mount Sinai Medical Center, NY). All cDNAs were introduced into the fly germ line by a standard P element transformation method. The following lines were created and used to obtain the different genetic backgrounds: *UAS-gfp-sr3' UTR/TM3Sb* flies were crossed to *en-gal4*. The progeny of this cross were further crossed to either *UAS-How(L)* or *UAS-How(S)* without 3' UTR, or to a control *UAS-flp* homozygous fly strain. All the progeny of the second cross contained *UAS-How* or *UAS-flp*, while only embryos that contained both *en-gal4* and *UAS-gfp-sr3' UTR* expressed GFP. Additional fly lines produced by standard crosses were as follows: *UAS-How(L)/UAS-How(L);how⁶⁴⁴/TM3-ftz-lacZ* and *UAS-How(S)/UAS-How(S);how⁶⁴⁴/TM3-ftz-lacZ*.

Immunochemical Reagents

Primary antibodies included the following: anti-Stripe (Becker et al., 1997) and anti-How (Nabel-Rosen et al., 1999) antibodies prepared in our lab; anti-GFP antibodies purchased from Boehringer-Mannheim; anti-actin antibodies purchased from Sigma; and anti-QKI-specific antibodies obtained from K. Artzt (Austin, Texas).

Secondary antibodies included Cy3, Fluorescein, and HRP-conjugated anti-guinea pig or anti-rat (Jackson).

Whole-Mount Embryonic Staining

Antibody staining was performed essentially as described previously (Ashburner, 1989) except that the embryos were fixed with 3% paraformaldehyde.

In situ hybridization was performed by the method of Tautz and Pfeifle (1989) using a Digoxigenin-labeled DNA probe. Fluorescent-labeled preparations were visualized using a Bio-Rad MRC 1024 confocal microscope coupled to a Zeiss Axiovert 135 M microscope. Bright field and fluorescent digital images were processed using Photoshop 5.5 (Adobe Systems, California).

Western and Northern Analysis

Western analysis was performed according to standard procedures as described in Volk (1992). Super Signal chemiluminescent substrate (Pierce) was used for signal detection. Embryos used for Western analysis were picked by hand and crushed in RIPA buffer. The RIPA-soluble fraction was isolated, boiled in sample buffer, and further processed for Western analysis.

For Northern blots, the total RNA was extracted from transfected Schneider cells 2–3 days after transfection using TriReagent. Equal amounts of total RNA (20 μ g) were loaded in each lane. ³²P-labeled *gfp* and *Drosophila rp-49* probes were used to detect the mRNA transcripts.

Sorting of Tendon Cells by FACS

Embryos expressing GFP in tendon cells were staged, collected, and dechorionated. The embryos were dissociated using a glass rod in Schneider medium, washed, and centrifuged at 1500 rpm for 10 min. Cells were then resuspended in Trypsin-EDTA (Sigma, UK) and incubated for ~30 min. The cells were then recentrifuged and the Trypsin was replaced by Schneider medium containing 10% FCS. In order to obtain a single-cell suspension, the cells were first filtered through a 40 μ m net and subsequently pipetted with a fine pulled Pasteur pipette. Propidium iodide was added to the cell suspension prior to sorting, and dead cells were excluded together with the rest of the non-GFP-expressing cells by the FACS. After sorting, GFP-positive tendon cells were centrifuged for 30 min at 3000 rpm, resuspended in sample buffer, boiled, and further processed to Western analysis.

DNA Constructs

UAS-stripe lacking the 3' UTR was produced by PCR using *stripeB* as template DNA; this construct included the entire open reading frame of *stripeB*.

UAS-gfp-stripe 3' UTR was produced by ligation of *stripe* 3' UTR (nt 3316–4465) into an Egfp containing pUAST vector.

The two truncated How(L) mutants were produced by PCR using a forward primer starting 30 nt before the first ATG and reverse primers starting either at position 1469 (just before the nuclear retention signal) or at position 1499 (just after the nuclear retention signal). The nucleotide numbers refer to the cDNA sequence representing GenBank accession number AF003106.

The mutated How(L) carrying a point mutation in the tyrosine of the nuclear retention signal was produced by two-step PCR. First, a How(L) fragment was produced by PCR using the 5' forward primer (see above) and a reverse primer starting at position 1469 (CTTAGTCGCCAAACGAC). Two shorter fragments containing a Y to F or Y to G mutation were produced using two distinct forward primers (GTCGTTTGGCGACTAACCGCGAGCATCCCTTCAGCGA GCAACGGTC and GTCGTTTGGCGACTAACCGCGAGCATCCCGG TCAGCGAACGGTC) and a reverse primer (GCTCTAGAGCTTCGC CGATATGGGTG). Then, the How(L) fragment was combined with each of the shorter fragments containing the point mutations by PCR using the forward 5' and reverse 3' common primers.

The *gfp-krox20* construct was produced by PCR. A fragment of the entire GFP open reading frame was produced by PCR, and a partially overlapping fragment of the entire *krox20* 3' UTR (accession number X06746) was synthesized. These fragments were then fused by PCR using the 5' forward and 3' reverse primers at the 5' end of GFP and 3' end of *krox20*. (The template DNA for the *krox20* 3' UTR was obtained from P. Gilardi-Hebenstreit [INSERM, ECOLE Normale Supérieure, Paris]).

The sequence of all the DNA constructs described was verified by DNA sequencing.

S2 Schneider Cell Transfections

For the transfection with mutated How(L), S2 Schneider cells were transiently transfected by the calcium phosphate precipitate method, incubated overnight, washed twice with Schneider medium, seeded on coverslips, and allowed to adhere for 2 hr. The cells were heat-shocked for 30 min at 37°C, allowed to recover for an additional 4 hr, fixed with 4% paraformaldehyde, and visualized by confocal microscopy.

For Western and Northern blot analysis of the effect of How(L)

and How(S) on *gfp-sr3'UTR* expression, cells were transfected by the calcium phosphate precipitate method with the various UAS constructs together with a Bluescript vector containing GAL-4 under the actin promoter. In each experiment, the amount of pUAST vector in the different transfections was equalized using an empty pUAST vector. Stable lines of How(L) or How(S) containing Schneider cells were obtained by cotransfection of hygromycin-resistant and metallothionein How(S)- or How(L)-containing plasmids. Selection of positive cells was performed in the presence of hygromycin. Induction of How(L)/How(S) proteins was by addition of 200 μ M CuSO₄ to the medium.

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