Development 134, 3473-3481 (2007) doi:10.1242/dev.006080

Post-transcriptional repression of the *Drosophila* midkine and pleiotrophin homolog *miple* by HOW is essential for correct mesoderm spreading

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The even spreading of mesoderm cells in the *Drosophila* embryo is essential for its proper patterning by ectodermally derived signals. In *how* germline clone embryos, defects in mesoderm spreading lead to a partial loss of dorsal mesoderm derivatives. HOW is an RNA-binding protein that is thought to regulate diverse mRNA targets. To identify direct HOW targets, we implemented a series of selection methods on mRNAs whose levels were elevated in *how* germline clone embryos during the stage of mesoderm spreading. Four mRNAs were found to be specifically elevated in the mesoderm of *how* germline clone embryos, and to exhibit specific binding to HOW via their 3′ UTRs. Importantly, overexpression of three of these genes phenocopied the mesoderm-spreading phenotype of *how* germline clone embryos. Further analysis showed that overexpressing one of these genes, *miple* (a *Drosophila* midkine and pleiotrophin heparin-binding growth factor), in the mesoderm led to abnormal scattered MAPK activation, a phenotype that might explain the abnormal mesoderm spreading. In addition, the number of EVE-positive cells, which are responsive to receptor tyrosine kinase (RTK) signaling, was increased following Miple overexpression in the mesoderm and appeared to be dependent on Heartless function. In summary, our analysis suggests that HOW downregulates the levels of a number of mRNA species in the mesoderm in order to enable proper mesoderm spreading during early embryogenesis.

KEY WORDS: Mesoderm, RNA regulation, HOW, Miple, HTL, *Drosophila*

INTRODUCTION

Regulation of RNA metabolism is essential for a variety of developmental processes. The RNA-binding protein (RBP) Held out wing (HOW) is highly expressed in the mesoderm during early embryogenesis. HOW belongs to the STAR (signal transduction and activation of RNA) family (Vernet and Artzt, 1997), which includes the *Caenorhabditis elegans* homolog GLD-1, and the mammalian quaking (QKI, QK) protein. The STAR RBPs are essential for the control of transitional 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 and Schedl, 2006), and the maturation of Schwann cells in the PNS and oligodendrocytes in the CNS mediated by QKI in mammalian species (Ebersole et al., 1996; Hardy, 1998; Larocque and Richard, 2005). In the *Drosophila* embryo, HOW regulates heart-beat rate, mesoderm invagination, muscle-dependent tendon cell differentiation and glial maturation (Baehrecke, 1997; Nabel-Rosen et al., 1999; Zaffran et al., 1997). The *how* gene is differentially spliced into two isoforms, HOW(L) and HOW(S), which share the same signature of the RNA-binding domain but differ at their C-terminal region. Although both HOW(L) and HOW(S) can bind the same mRNA target, they act in opposing directions; binding of HOW(L) to *stripe* mRNA at the 3′ UTR leads to mRNA degradation, whereas the binding of HOW(S) leads to the stabilization of *stripe* mRNA (Nabel-Rosen et al., 2002). In addition to mRNA stabilization/degradation, HOW proteins regulate the splicing of specific targets (Edenfeld et al., 2006; Volohonsky et al., 2007).

Previous analysis showed that *how* mutant germline clone embryos exhibit defects in mesoderm invagination during the beginning of gastrulation (Nabel-Rosen et al., 2005). This defect stems from extra mesodermal cell divisions, due to the elevation of *string* (also known as *cdc25*) mRNA. Despite the defects in mesoderm invagination in *how* mutant germline clone embryos, gastrulation is only delayed and, eventually, all mesoderm cells invaginate beneath the internal surface of the ventral ectoderm. However, in addition to this phenotype, these *how* mutant embryos exhibit abnormalities in mesoderm spreading (Nabel-Rosen et al., 2005). Mesoderm spreading over the ectoderm in the *Drosophila* embryo is essential for the correct specification of the distinct subpopulations of cells of the mesoderm lineage, including somatic, visceral and heart muscles, fat body, gonadal mesoderm, and others (Baylies et al., 1998; Frasch, 1999). Following their invagination from the ectoderm, the mesodermal cells undergo an epithelial-to-mesenchymal transition, adhere to the basal surface of the overlying ectoderm and spread dorsally. Then, differentiation signals from the ectoderm subdivide the mesoderm layer into distinct domains. Proper spreading of the mesoderm depends on FGF signaling, in which the FGF receptor, Heartless (HTL), is expressed by mesodermal cells and is activated by two FGF8-like ligands [Thisbe (THS) and Pyramus (PYR)] produced by the ectoderm layer (Beiman et al., 1996; Gisselbrecht et al., 1996; Gryzik and Muller, 2004; Shishido et al., 1997; Stathopoulos et al., 2004). Despite the ubiquitous expression of HTL and its adaptor protein, Downstream of FGF (DOF, also known as Stumps – FlyBase), in the mesoderm, MAPK activation in the mesoderm layer is spatially restricted. Initially, it is expressed in the cells that adhere to the ectoderm, and later it is elevated in dorsally located cells (Gabay et al., 1997; Wilson et al., 2004; Wilson and Leptin, 2000). The two FGF8-like

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Accepted 17 July 2007

HTL ligands Thisbe and Pyramus exhibit a dynamic expression pattern during mesoderm spreading. *thisbe* mRNA is expressed throughout the neurogenic ectoderm, and *pyramus* mRNA is refined into dorsal and ventral regions of the neurogenic ectoderm (Stathopoulos et al., 2004; Gryzik and Muller, 2004). Both expression patterns do not correlate with the restricted MAPK activation detected in the spreading mesoderm. The factor(s) controlling the spatial regulation of MAPK activation in the mesoderm are yet to be elucidated. It is assumed that additional components, possibly of the extracellular matrix deposited between the ectoderm and the mesoderm, might control the spatial distribution/activation of the secreted FGF ligands, enabling spatially and temporally restricted MAPK activation of the mesodermal cells.

To elucidate the basis for the abnormal mesoderm spreading in *how* mutant embryos, we performed a microarray screen for putative HOW target genes. We expected that the mRNA levels of these genes would be elevated in the mesoderm of the mutant embryos, because only the repressor, HOW(L), is normally expressed at this stage. Four out of 32 potential targets identified in this screen were further analyzed and shown to be specifically elevated in the mesoderm in *how* germline clone embryos, and to bind HOW via their 3′ UTRs. One of these genes, the midkine and pleiotrophin heparin-binding growth factor *miple*, exhibited abnormal mesoderm spreading following its overexpression in the mesoderm, which was correlated with scattered MAPK activation in mesodermal cells. This might explain the aberrant mesoderm spreading observed in *how* mutant embryos.

We therefore suggest that the HOW-dependent negative regulation of mRNA levels of several targets during gastrulation is essential for proper mesoderm spreading.

MATERIALS AND METHODS

Constructs, fly lines and staining procedures

The *UAS-falten*, *-miple* and *-CG31638* constructs were generated by inserting a PCR product composed of the coding regions of these genes fused to a hemagglutinin (HA) tag at their C-terminal end [the templates obtained as ESTs from the *Drosophila* Genomics Resource Center (DGRC)] into the *Eco*RI site of pUAST, and transgenic flies were generated. HOW(L)-HA and HOW(L)$^{R185\ to\ C}$-HA (a mutation changing Arginine 185 into Cysteine) that were used for the in vitro binding assay were as previously described (Nabel-Rosen et al., 2002). The *GFP-miple* 3′ UTR was constructed by insertion of *miple* 3′ UTR into pUAST vector containing *EGFP*. The two point mutations in the HOW response element ACUAA were inserted by standard mutagenesis, creating the sequence ACGGA.

For the production of germline clones (Nabel-Rosen et al., 2005), males carrying *FRT82B,OvoD/TM3Sb* (Bloomington Stock Center) were crossed to females carrying *hs-flp;Dr/TM3,Sb* (Umea Stock Center), and the progeny males carrying *hs-flp;FRT82B,OvoD/TM3,Sb* were crossed to females carrying *FRT82B,how^{e44}/TM3,Sb* (produced in our laboratory by recombination). Larvae of this cross were heat-shocked daily over 3 days for 50 minutes at 37°C, and adult females carrying the *OvoD* construct that had wing blisters were crossed to males carrying *how^{strw}/TM3,2Xtwistgal4,UAS-GFP* (Prout et al., 1997).

Gal4 lines: *Mef2-gal4* (Bloomington Stock Center) and *twist-gal4* (A. Muller, University of Dundee, UK).

For the rescue experiments, flies carrying *htl/TM6*;*UAS-miple/Cyo* were crossed to flies carrying *htl/TM6;mef2-gal4/Cyo.* Both balancers were marked, so the *htl* mutants were identified by negative *β-gal* expression.

Antibodies used include rat anti-HOW (Nabel-Rosen et al.,1999), anti-Myosin heavy chain (-MHC) (P. Fisher, Stony Brook, NY), mouse anti-dpERK (Sigma), rabbit anti-Twist (obtained from S. Roth, Cologne, Germany) mouse anti-HA (Roche), rabbit anti-Even skipped (-EVE) and rabbit anti-Tinman (M. Frasch, Mount Sini, NY). For embryos double stained with anti-dpERK and anti-Twist, fixation was carried out in 8% formaldehyde/PBS and 50 mM EGTA for 25 minutes, anti-dpERK primary antibody incubated for 2 hours at room temperature (RT) in 0.1% Tween/PBS, and secondary antibody (goat anti-mouse biotin; Chemicon) incubated for 60 minutes at RT. It was amplified by streptavidin-HRP for 30 minutes at RT, followed by incubation with tyramide biotin for 20 minutes (both from Perkin Elmer TSA biotin system). Finally, embryos were incubated with streptavidin-Cy3 for 30 minutes at RT (Jackson ImmunoResearch) (Melen et al., 2005). Following this staining, embryos were then stained with anti-Twist antibodies. Secondary antibodies included Cy3, Fluoresceine, or HRP-conjugated anti-rabbit or anti-rat, or-anti mouse (Jackson). For embryos double-labeled with MEF2 and EVE, the embryos were fixed and stained with anti-EVE, and secondary Cy2-conjugated anti-rabbit antibody. Then, the embryos were labeled with anti-MEF2 followed by labeling with Cy3-conjugated anti-rabbit antibody, which also recognized the EVE antibody. This resulted with yellow labeling of the EVE cells and red labeling of the MEF2 cells.

For in situ hybridization, a probe was prepared using T7/T3/SP6 RNA polymerase (according to the relevant ESTs) and the Roche RNA DIG labeling mix. Fixation, hybridization and detection were performed according to http://www.biology.ucsd.edu/~davek/.

Microarray experiments

Sample preparation

Embryo collections were performed every 2 hours from either *y w* or from *how* germline clone mutant females on apple juice/yeast plates at 25°C. Plates were removed and the embryos were aged for an additional 3 hours at 25°C. Because the *how* mutation was established in trans to a balancer chromosome carrying *GFP*, the *how/GFP*-balancer collections contained a mixed population of embryos. Homozygous *how* mutant embryos were separated from their siblings using a fluorescent binocular. Carefully staged embryos that had been aged 3-5 hours in this manner were collected and dechorionated. Total RNA was extracted from sufficient amounts of embryos (~100 embryos) using the Macherey-Nagel Nucleospin RNA II mini kit, following the protocol, and then kept at –70°C. Total RNA was prepared independently five times from embryos of each genetic background in order to better normalize the age of these embryo populations. The RNA samples were then collected and concentrated to give 1 mg of total RNA using the RNA cleanup RNeasy mini kit (Qiagen). The probe preparation, cDNA synthesis, cRNA reactions and hybridization with Affymetrix high-density oligonucleotide arrays for *Drosophila melanogaster* was carried out in the Weizmann Institute microarray unit.

Normalization and statistics

More than 13,500 gene sequences predicted from the annotation of the *Drosophila* genome are represented on the *Drosophila* affymetrix array. Signals were pre-processed using Robust Multichip Average (RMA) algorithms with the default parameters (i.e. RMA model-based background adjustment, quantile normalization, median polish summation of the probe intensities). A total of 690 probe sets on the chip were differentially expressed with $P<0.05$ (*t*-test). Among them, 147 probe sets exhibited a 1.5-fold or greater upregulation in *how* germline clones.

All microarray data were submitted to Gene Expression Omnibus (GEO). The accession number is GSE7772.

Protein-RNA binding assay and western analysis

The protein-RNA binding assay was performed essentially as described (Nabel-Rosen et al., 1999). The entire *miple*, *CG31638*, *falten*, *lap* or *punt* cDNAs (ESTs obtained from DGRC) were used as templates to produce biotin-labeled RNAs (Biotin labeling mix, Roche, and T7/T3 or SP6 polymerase, Promega). The biotin-labeled RNA was purified on a G-50 Sephadex Quick Spin Column (Roche) and then mixed with in vitro-translated HOW(L) or HOW(L)$^{R185\ to\ C}$-HA-tagged proteins (TNT T7 quick coupled transcription/translation system, Promega) and precipitated with magnetic streptavidin beads. Binding was performed by adding approximately 1 μg of biotin-labeled RNA to 5 μl of the translated HOW proteins. Streptavidin magnetic beads were first washed with binding buffer, and 300 μl of the beads was added to each binding reaction for 25 minutes at RT. The magnetic beads were then isolated, washed, and boiled in sample buffer, and the supernatant was loaded on 10% SDS-

polyacrylamide gel and reacted with mouse anti-HA antibodies (1:2000 dilution). Blocking, hybridization and detection were performed using standard protocols.

Transient transfection of SR$^+$ cells

SR$^+$ cells were grown in Schneider's medium supplemented with 10% fetal calf serum (Hyclone) and 1% penicillin-streptomycin (pen-strep) solution. For transfection, cells were seeded at 3.5-5×10^6 cells in 4.5 ml medium per 50 ml flask (Nunc) and allowed to adhere for several hours. Transfection was performed using lipid reagent, according to the manufacturer's protocol (Escort IV, Sigma). A total of 6 μg DNA was used for each transfection. Cells were collected for analysis 48 hours after transfection.

RESULTS

Embryos lacking maternal and zygotic HOW exhibit defects in mesoderm spreading

Previous analysis of *how* germline clone embryos showed that HOW is essential for the arrest of cell cycle progression during mesoderm invagination. We found that, despite this defect, gastrulation does occur, and all mesodermal cells eventually invaginate into the interior of the embryo. However, we noticed that subsequent mesoderm spreading over the ectoderm is abnormal (Nabel-Rosen et al., 2005). To further define the effects of abnormal mesoderm spreading on later stages of mesoderm development, we analyzed the expression pattern of various markers characteristic of the heart, pericardial cells and somatic musculature precursors in *how* germline clone mutant embryos. We first analyzed mesoderm spreading in *how* mutant embryos at stages 7-9, which we labeled with anti-Twist (anti-TWI) antibodies. In contrast to a homogenous distribution of mesodermal cells in wild-type embryos, the distribution of the mesoderm Twist-expressing cells in *how* mutant embryos was uneven. Although in some regions the mesodermal cells migrated dorsally, in other regions, the cells were retarded and did not reach the dorsal ectoderm domain (Fig. 1F). Consistent with abnormal mesoderm spreading, staining for the dorsally located heart cells by anti-Tinman antibodies at later stages (e.g. stages 13-14) revealed 1-2 segments that lacked cardiac and pericardial cells in *how* germline clone mutant embryos (Fig. 1G). In addition, such embryos exhibited 1-2 segments that lacked EVE-positive cells in the dorsal mesoderm (Fig. 1H,I′), and staining for Myosin heavy chain (MHC) revealed 1-2 segments in which some of the more dorsal muscles were missing (Fig. 1J). Analysis of embryos labeled simultaneously for both EVE and MEF2 revealed that, in regions in which cardioblasts (the most dorsal MEF-2-positive cells) were missing, the distribution of EVE-positive pericardial cells was also deranged, but the pattern of more-ventral somatic muscles was normal, supporting a defect in dorsal spreading. However, cells that did migrate dorsally expressed EVE, indicating that the cells were capable of responding to patterning signals (Fig. 1I,I′). The double labeling for EVE and MEF2 was performed sequentially (see Materials and methods for details), because both antibodies were raised in rabbits. Additionally, the cardioblasts of the *how* germline clone embryos were often not ordered as a single line of cells and the EVE-positive cells were not spaced-out evenly, as in wild-type embryos (Fig. 1I, arrow). These phenotypes might stem from later requirements for HOW in this tissue.

Taken together, these results suggest that, in embryos lacking HOW, the mesoderm does not spread correctly and evenly over the ectoderm following gastrulation, resulting in embryos in which 1-2 segments lack dorsal mesoderm structures.

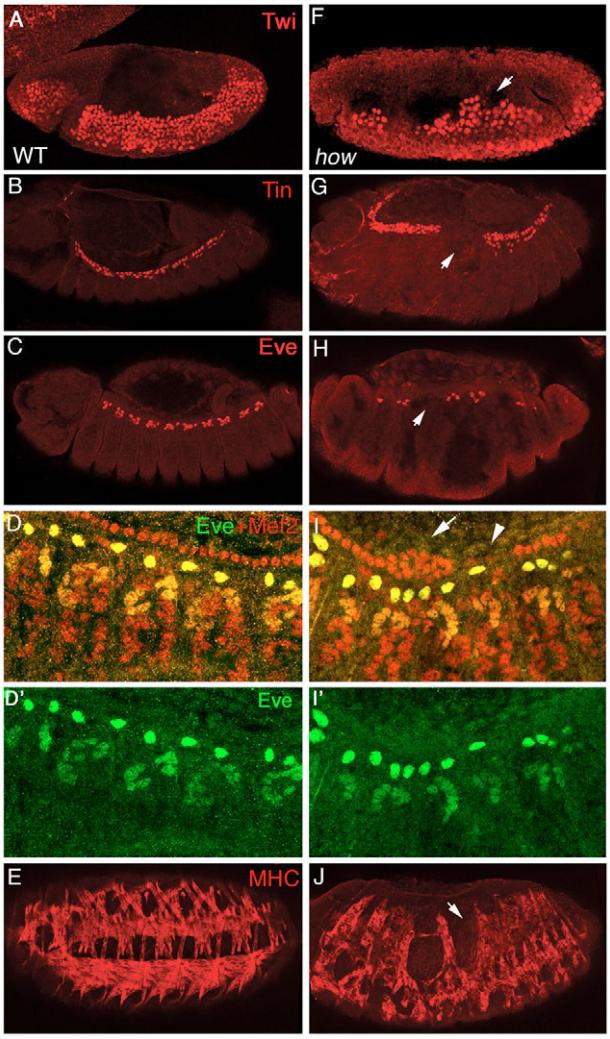

Fig. 1. Defects in mesoderm spreading in *how* germline clone embryos. Whole-mount wild-type (WT; **A-E**) or *how* germline clone (**F-J**) embryos stained for Twist (TWI; A,F), Tinman (TIN; B,G), EVE (C,H), EVE (green) + MEF2 (red) (D,I) (D′ and I′ represent respective single EVE staining of the merged images in D,I), and Myosin heavy chain (MHC; E,J). Arrows indicate uneven mesoderm spreading (F), segments lacking TIN-positive heart cells (G), segments lacking EVE-positive cells (H), uneven distribution of cardioblasts (I) and segments lacking dorsal muscles (H). Arrowhead in I shows a region lacking both cardioblasts and EVE-positive pericardial cells, while more ventral MEF2-positive cells are present. The images in D-I′ were taken in threefold higher magnification to detect the details of the distinct cell types.

The repressor isoform HOW(L) is expressed during mesoderm spreading

To analyze which of the HOW isoforms is responsible for the phenotype of mesoderm spreading, we stained wild-type embryos at different developmental stages with anti-HOW antibodies that recognize both HOW isoforms, and detected positive staining in the mesoderm that persisted during mesoderm spreading (Fig. 2). Western analysis of 3-5-hour-old embryos showed that the larger isoform, HOW(L), was predominantly expressed during the time period of mesoderm spreading (stages 7-9, which occur during the time period of 3-5 hours), and this was confirmed by mRNA in situ hybridization with each of the *how* splice variants (Fig. 2D-F). This

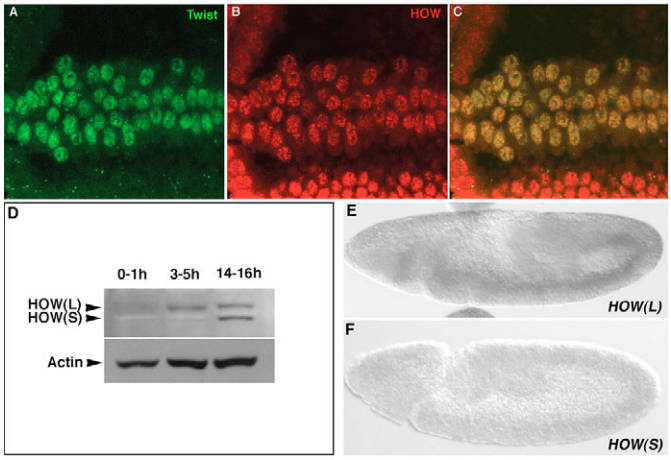

Fig. 2. HOW(L) is the major HOW isoform at stages 6-10. Whole-mount wild-type embryo double labeled for Twist (**A**) and for HOW (**B**). The merged image (**C**) indicates that HOW is expressed in the nuclei of the Twist-positive mesodermal cells. (**D**) Western analysis of embryos at 0-1, 3-5 and 14-16 hours after egg laying with anti-HOW antibodies that recognize both the HOW(L) and HOW(S) isoforms. At early stages, only the HOW(L) isoform is detected. (**E**,**F**) In situ hybridization of wild-type embryos at stage 10 with *how(L)*-specific probe (E) or with *how(S)*-specific probe (F).

suggests that the mesoderm phenotype described above resulted from lack of the HOW(L) isoform, which was previously demonstrated to repress levels of various mRNA species.

Identification of HOW target mRNAs during mesoderm spreading

HOW(L) represses the levels of its target mRNAs (Nabel-Rosen et al., 1999; Nabel-Rosen et al., 2002). Therefore, the mRNA levels of direct HOW targets are expected to be increased in the *how* germline clone mutant embryos. To identify mRNA targets of HOW, we used cDNA microarray hybridization to compare the abundance of mRNA species extracted from 3-5-hour-old *how* germline clone embryos with that of stage-matched wild-type embryos. We used a GFP-marked balancer to identify homozygous (non-fluorescent) *how* mutant embryos (which also lacked maternal HOW). The RNA was extracted from five independent embryo collections, which were sorted separately each time. This was done to reduce fluctuations in RNA levels due to slight changes in the age of the collected embryos. Statistical analysis of the results obtained in the microarray experiment identified 145 mRNAs that showed increased expression equal to or above 1.5-fold. To further select for putative direct HOW target mRNAs, we screened for the presence of a HOW binding sequence at the 3′ UTR of each of the 145 genes (HOW response element, HRE=ACUAA) (Israeli et al., 2007). This analysis reduced the number of putative targets to 49 mRNAs. Further sorting was performed based on the conservation of the HRE sites in *Drosophila pseudoobscura*. A database of the 3′ UTRs present in *D. Pseudoobscura*, and their alignments with the 3′ UTRs of the relevant genes in *Drosophila melanogaster*, was obtained from A. Stark (Stark et al., 2005). Selected genes contained HREs in a conserved sequence along the 3′ UTR of *D. melanogaster* and *D. pseudoobscura* or exhibited the HRE at another location in the 3′ UTR. This reduced the number of potential HOW targets to 32 (see Table 1). We chose to focus on five genes out of these 32; the sequences of these five genes indicated possible roles in the process of mesoderm spreading: *miple*, *falten*, *CG31638*, *lap* and *punt*. *miple* codes for a secreted heparin binding domain protein (see below); *falten* encodes a protein with potential GTPase activity; CG31638 exhibits partial homology to Myosin (according to protein Blast); LAP (like AP-180) contains an ENTH domain that binds phosphatidylinositol phosphates (PtdIns) to modulate Clathrin adaptors in endocytosis; and *punt* codes for a type II TGFβ receptor. In situ hybridization with probes representing these five genes revealed that the levels of the first four mRNAs were significantly elevated in the *how* germline clone mutant embryos, consistent with the microarray results (Fig. 3), whereas *punt* mRNA did not show a significant elevation in these mutants (data not shown). Each of these five candidates was also tested for direct binding to HOW via their 3′ UTR using a protein-RNA binding assay (Nabel-Rosen et al., 1999). The 3′ UTR of each gene was isolated, transcribed, labeled with biotin, purified and further tested for its ability to co-precipitate with in vitro-translated HOW(L). The mRNAs of the four genes that were upregulated in the mutant *how* embryos in the mesoderm – *miple*, *falten*, *CG31638* and *LAP* – exhibited specific binding to HOW via their 3′ UTRs, but did not bind a point-mutated HOW

Table 1. Potential mRNA targets of HOW

Fold of change*	Gene	Conserved	DM only	DP only
8.3	***CG1221* (*miple*)**	0	1	2
5.7	*CG11357*	0	2	0
4.6	*CG5973*	0	1	3
3.0	*CG4193*	0	1	1
3.0	*CG18543*	0	1	1
2.7	*CG11143*	0	1	1
2.6	*CG6207*	0	1	3
2.4	*CG30035*	0	1	1
2.3	*CG13795*	0	1	1
2.2	*CG3158*	1	0	0
2.1	*CG5621*	1	0	0
2.0	*CG14889*	2	1	7
1.9	***CG9670* (*falten*)**	2	1	0
1.9	*CG11064*	0	1	3
1.8	*CG10460*	0	1	2
1.8	*CG2065*	0	1	0
1.8	*CG13920*	1	0	0
1.8	*CG7272*	1	1	1
1.8	***CG31638***	1	1	2
1.8	*CG6448*	0	1	0
1.7	CG5992	0	2	1
1.7	*CG1633*	0	1	1
1.7	*CG18661*	0	1	0
1.7	*CG1667*	0	1	5
1.6	*CG17734*	0	1	2
1.6	*CG9629*	0	1	1
1.6	***CG2520* (*lap*)**	1	4	3
1.6	*CG10120*	0	1	1
1.6	*CG11267*	0	1	0
1.6	***CG7904* (*punt*)**	2	0	0
1.6	*CG32920*	0	2	2
1.5	*CG18319*	0	3	0
1.5	*CG2803*	0	1	1
1.5	*CG7334*	0	1	2
1.5	*CG10691*	1	1	0
1.5	*CG8327*	0	2	0
1.5	*CG13928*	0	2	3

The five genes that were analyzed further because their sequences indicated possible roles in the process of mesoderm spreading are shown in bold. *Indicates the fold of change of expression in *how* germline clone mutant embryos compared with wild type. Conserved, HOW response element location is conserved between *D. melanogaster* and *D. pseudoobscura*; DM only, HOW response element appears only in *D. melanogaster*; DP only, HOW response element appears only in *D. pseudoobscura*.

(HOW$^{R185\ to\ C}$), which molecularly mimics the severe *how^{e44}* allele, which is incapable of RNA binding (Fig. 3I). The *punt* 3′ UTR did not show binding to HOW, nor did its mRNA elevate in the *how* mutant embryos, thus it does not represent a direct target for HOW activity.

We conclude that *miple*, *falten*, *CG31638* and *lap* represent potential direct targets for negative regulation by HOW, because their mRNA levels were elevated in the mesoderm of *how* mutant embryos, and their 3′ UTRs bound to HOW.

If HOW repression of these genes is essential for mesoderm spreading, we would expect the aberrant mesoderm spreading phenotype detected in *how* germline clone mutant embryos to be phenocopied by overexpression of these genes in the mesoderm. To address this possibility, we produced transgenic flies carrying *miple*, *falten* and *CG31638* under the control of *gal4*-binding *UAS* sites. Driving the expression of these proteins (without their 3′ UTRs) with the *twist-gal4* driver led to various degrees of mesoderm spreading defects (Fig. 4A and data not shown). We decided to further focus on the phenotype obtained by overexpressing *miple*, because it exhibited the most prominent and consistent mesoderm spreading phenotype (Fig. 4A, compare to wild-type Twist expression in Fig. 1A).

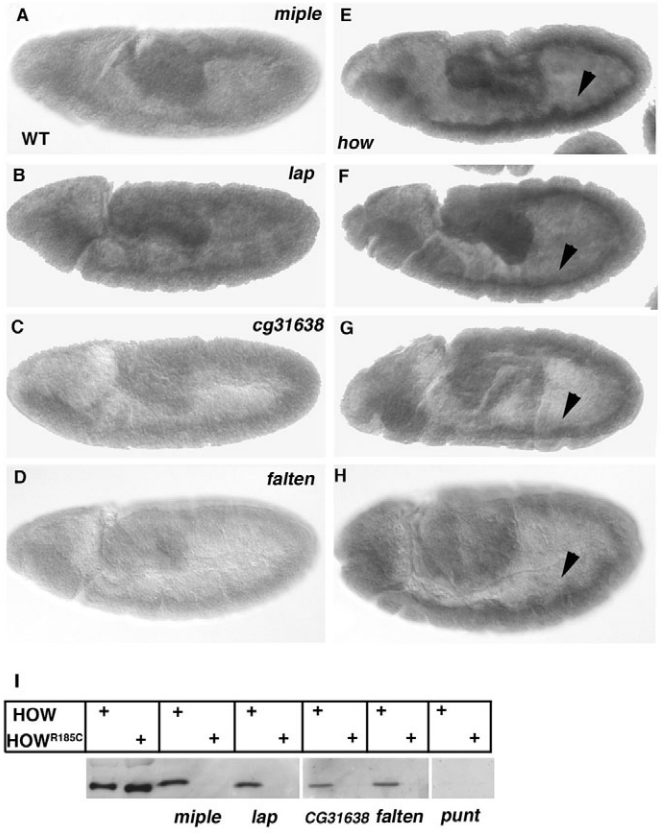

Fig. 3. The four mRNA targets of HOW are elevated in the mesoderm of *how* germline clone embryos and exhibit direct binding to HOW. Whole-mount wild-type (**A-D**) or *how* germline clone (**E-H**) embryos hybridized with each of the four mRNAs identified in the microarray screen: *miple* (A,E), *lap* (B,F), *CG31638* (C,G) and *falten* (D,H). Arrowheads in E-H indicate the specific increase in the levels of the distinct mRNAs in the mesoderm of *how* germline clone embryos. (**I**) HOW-RNA binding assay with in vitro-transcribed RNA of the 3′ UTRs of *miple*, *lap*, *CG31638*, *falten* and *punt*. HOW^{R185C} is a form of HOW that is mutated in the KH RNA-binding domain and served as a control for non-specific binding. The first two lanes on the left represent total HOW protein levels. Each binding experiment shown is representative of three repetitions.

Consistent with a defect in the even spreading of the mesoderm, we also detected loss of pericardial and cardiac cells in 1-2 segments in the embryos overexpressing *miple* at later developmental stages (Fig. 4B,C). The somatic musculature also showed lack of muscles in 1-2 segments, especially at the dorsal aspects of the embryo (Fig. 4D). Double-labeling with both EVE and MEF2 antibodies (as described above) showed that, in regions of aberrant distribution of EVE-positive cells, we often detected abnormal arrangement of MEF2-positive cells (Fig. 4E,F), supporting a defect in dorsal spreading. However, cells that did migrate dorsally expressed EVE, indicating that the cells were capable of responding to patterning signals. We believe that these phenotypes originated during the early stages of mesoderm spreading when the *twist-gal4* driver is expressed at high levels. However, we cannot exclude the possibility that residual expression of Miple by *twist-gal4* is maintained at later stages. Although the *miple* construct was tagged with hemagglutinin (HA), we could not detect the overexpressed Miple-HA by staining with anti-HA antibody. To verify that *UAS-miple-HA* is indeed expressed following the expression of the *twist-gal4* driver, we performed western analysis with embryos overexpressing HA-tagged Miple, using anti-HA antibodies. Only extracts originating from embryos carrying both *UAS-miple-HA* and *twist-gal4* reacted with the anti-HA antibodies (Fig. 4G). We also tested the activity of the putative heparin-binding domain in Miple by incubating the embryo extract with heparin beads, followed by elution with high salt buffer. Western analysis showed that Miple is indeed a heparin-binding protein, because it binds and can be eluted from a heparin column (Fig. 4G).

The 3′ UTR of *miple* contains a single site for HOW binding (at position 800 after the stop codon). This site was mutated from ACUAA into ACGGA, fused to *GFP* and inserted into a pUAST vector suitable for expression in SR$^+$ cells. Similarly, a wild-type 3′ UTR was fused to *GFP* and subcloned into the pUAST vector. SR$^+$ cells were transfected with *GFP-miple* 3′ UTR or with *GFP-miple* 3′ UTR* (mutated) together with HOW(L), and the GFP levels were monitored by western analysis following 2 days of transfection. Consistently, the GFP levels were reduced when the wild-type *GFP-miple* 3′ UTR but not mutated 3′ UTR was present (a representative western blot of three experiments is shown in Fig. 4H). These results favor a direct effect of HOW on *miple* levels via its binding to the HRE at the 3′ UTR of *miple* mRNA.

In summary, based on microarray data, we identified three potential targets recognized by HOW that, when overexpressed in the mesoderm without their 3′ UTRs, led to mesoderm spreading defects that mimic the *how* mutant phenotype.

Overexpression of Miple activates the MAPK pathway in the migrating mesoderm

Mesoderm spreading in the *Drosophila* embryo depends on the activity of the FGF receptor, HTL, and its two FGF8-like ligands, Thisbe and Pyramus (Gryzik and Muller, 2004; Stathopoulos et al., 2004). In wild-type embryos at stages 7-9, HTL-dependent activation of MAPK, as visualized by staining with anti-dpERK antibody, is restricted to the most dorsal mesodermal cells (Gabay et al., 1997) (and Fig. 5B,D). The basis for this local MAPK activation is not clear. To understand the relationship between Miple expression and MAPK activation, Miple was overexpressed in the mesoderm by the *twist-gal4* driver. Overexpression led to uneven dpERK staining scattered over many mesodermal cells, independent

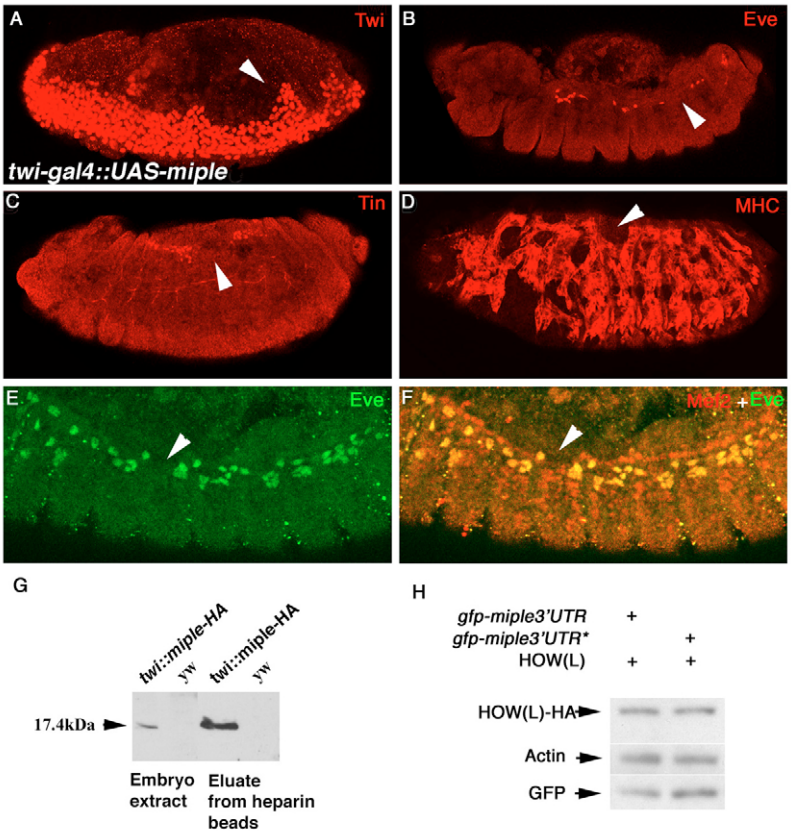

Fig. 4. Overexpression of Miple leads to mesoderm spreading defects. Embryos overexpressing Miple-HA under the *twist-gal4* driver labeled for Twist (TWI; **A**), EVE (**B**), Tinman (TIN; **C**), or Myosin heavy chain (MHC; **D**). (**E**,**F**) Higher magnification of an embryo overexpressing Miple-HA double-labeled for EVE (E) and MEF2 (F is the merged image of both). Arrowheads indicate uneven mesoderm distribution (A), segments lacking EVE cells (B), heart cells (C), dorsal muscles (D), or a region in which both cardioblast and EVE-positive pericardial cells are missing or mislocalized (E,F). (**G**) Western analysis of the embryos overexpressing Miple-HA driven by the *twist-gal4* driver indicates a specific HA-positive band. Miple-HA from these embryos bound and eluted specifically from a Sepharose-heparin column (third lane from left). (**H**) Western blot of SR^+ cell extract with anti-GFP, -Actin and -HA. The cells were transfected with *HOW(L)-HA* together with either *GFP* fused to wild-type *miple* 3′ UTR (*gfp-miple* 3′ UTR) or to *miple* 3′UTR mutated at the HOW-binding site (*gfp-miple* 3′ UTR*).

of their position along the dorsoventral axis (Fig. 5F-H). This scattered activation might form the basis for the defects in mesoderm spreading observed in the embryos overexpressing Miple. Importantly, a scattered MAPK activation was also detected in *how* germline clone embryos (Fig. 5J-L). This effect might stem from the high expression levels of *miple* in these embryos.

The putative ability of Miple to activate the HTL-dependent MAPK was further demonstrated by the elevation of EVE-expressing cells in the dorsal mesoderm following overexpression of Miple driven by the *mef2-gal4* driver (Fig. 6). This driver is highly expressed throughout mesoderm development. Approximately three to four EVE-positive cells were detected in each segment of the dorsal mesoderm of wild-type embryos at stage 11 (Michelson et al., 1998) (Fig. 6A). This number reflects the sum of the activation of both the EGFR and HTL signaling pathways. Higher activation of the pathway (e.g. following overexpression of activated RAS, activated HTL or activated EGFR, or in *argos* mutants) induces elevated numbers of EVE-expressing cells in the dorsal mesoderm, driven by *mef2-gal4* (Carmena et al., 1998).

We detected five to six EVE-positive cells in most of the embryo segments in embryos overexpressing Miple driven by the *mef-2-gal4* driver (Fig. 6B). This elevation in the number of the EVE-

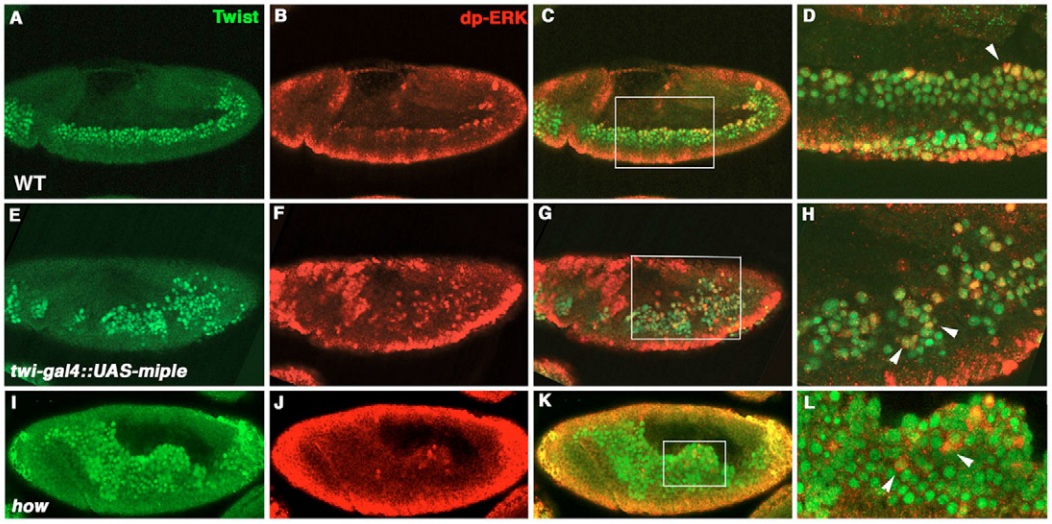

Fig. 5. Ectopic MAPK activation in embryos overexpressing Miple. Whole-mount wild type (WT; **A-D**), embryos overexpressing Miple under the *twist-gal4* driver (**E-H**) or *how* germline clone embryos (**I-L**) double labeled for Twist (green, A,E,I) and dpERK (red, B,F,J), and their merged images (C,G,K), are shown. (D,H,L) Single sections of the framed areas of the embryos shown in C,G,K at higher magnification. Arrowheads indicate the most dorsal MAPK-positive cells (D) or the scattered MAPK-positive cells (H,L).

positive cells is a further indication of the ability of Miple to activate the MAPK pathway in the dorsal mesodermal cells. To further test the possibility that MAPK activation is mediated by the HTL receptor, we examined the ability of Miple to induce the production of a higher number of EVE-positive cells in *htl* mutant embryos. In *htl* mutants there was a complete loss of the EVE-positive cell clusters, and overexpression of Miple by the *mef2-gal4* driver did not restore EVE expression (Fig. 6C). This result is consistent with the possibility that Miple-dependent elevation of the dpERK, as well as the elevation in the number of EVE cells, is mediated by the HTL receptor (Gryzik and Muller, 2004). However, we cannot exclude the possibility that the *htl* mutant cells do not express EVE, due to their failure to reach dorsal positioning.

DISCUSSION

Previous analysis of *how* germline clones suggested that HOW is essential for correct mesoderm spreading over the ectoderm (Nabel-Rosen et al., 2005), a process that is required for the spatial patterning of the mesoderm layer by ectoderm-derived signals. This phenotype could not result from the earlier effect of extra cell division during gastrulation detected in the *how* germline clone embryos, because mesoderm invagination and gastrulation were eventually completed in these embryos. Furthermore, mesoderm spreading was unaffected in *tribbles* (*trbl*) mutant embryos, which exhibit a similar defect of extra cell divisions during gastrulation that leads to delayed and unsynchronized mesoderm invagination (T.V., unpublished data).

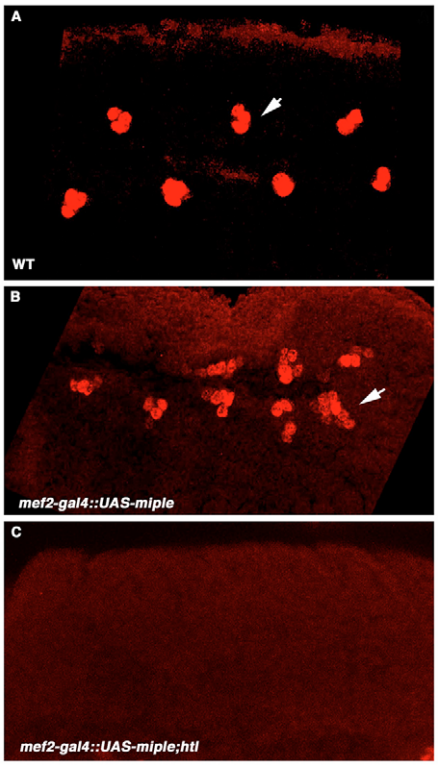

Fig. 6. Increased number of EVE-positive cells in embryos overexpressing Miple appears to depend on HTL. Wild-type embryo (WT; **A**), embryo overexpressing Miple under the *mef2-gal4* driver (**B**), or a *htl* mutant embryo overexpressing Miple under the *mef2-gal4* driver (**C**) were stained with anti-EVE. The arrow in A shows three EVE-positive cells in the dorsal mesoderm, whereas the arrow in B indicates nine EVE-positive cells.

HOW regulates the mRNA levels of maternally contributed and zygotic genes in the mesoderm

Regulation of mesoderm-specific mRNA levels by HOW might contribute to the spatial and temporal control of gene expression during mesoderm spreading. The genome analysis that we have performed was designed to identify mRNAs whose levels might be directly controlled by the repressor isoform HOW(L) in the mesoderm. Such targets should be normally repressed to enable even spreading of the mesoderm. Three out of the four HOW targets identified in this screen, namely *falten*, *CG31638* and *LAP* (CG2520) are contributed maternally (in situ results, H.T.-K. and T.V., unpublished results), and therefore HOW(L)-dependent repression in the mesoderm might be essential for reducing their levels in this tissue to enable proper mesoderm spreading. This scenario is supported by the defective mesoderm spreading induced by overexpression of *falten* and *CG31638*. Miple does not appear to be maternally contributed according to expression data [from Berkeley *Drosophila* Genome Project (BDGP)] and in situ analysis. It is not clear, however, which transcription factor is responsible for *miple* induction. Because *miple* mRNA was detected in mesoderm derivatives at stage 11, it might be induced by mesoderm-specific transcription factors such as Twist, MEF2 and/or Tinman, which are expressed in the mesoderm during spreading. In that case, to abrogate the effects of Miple, it would be necessary to block *miple* expression during mesoderm spreading. Our data suggest that this is the role of HOW(L), because in its absence, *miple* mRNA is significantly elevated in the mesoderm. Thus, HOW(L) in the mesoderm of gastrulating embryos is necessary to reduce maternal mRNA expression and, in addition, to reduce the levels of gene products whose expression is not compatible with early mesoderm development, but might be required shortly after the process of mesoderm spreading has been completed. Thus, HOW(L) is essential to enable temporal morphogenetic processes in the mesoderm during its spreading over the ectoderm (see summary in Fig. 7).

Possible involvement of Miple in HTL-dependent MAPK activation

Miple was further analyzed because its vertebrate homologs, midkine and pleiotropin, are involved in cell migration and are associated with receptor tyrosine kinase (RTK) signaling (Stoica et al., 2002). Therefore, its downregulation by HOW(L) might

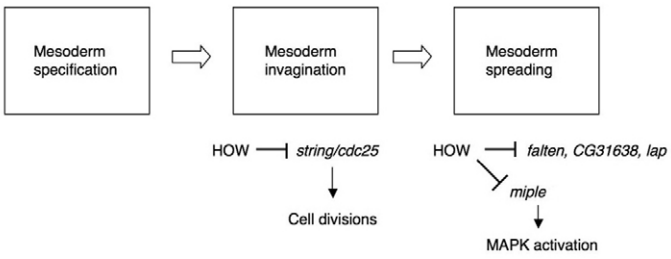

Fig. 7. Summary of HOW functions in early mesoderm development. Following mesoderm specification, levels of HOW(L) are increased in the presumptive mesoderm in order to inhibit the levels of *string* (*cdc25*) mRNA and to therefore arrest cell divisions during mesoderm invagination. During mesoderm spreading, HOW is essential to repress the levels of a set of maternal mRNAs (*falten*, *CG31638* and *lap*), as well as *miple*, the high levels of which might induce ectopic MAPK activation in the mesoderm during spreading.

contribute to the restricted dorsal activation of the HTL-dependent signaling during mesoderm spreading. Moreover, the putative heparin-binding motif of Miple could affect the affinity of the HTL ligands to the HTL receptor, thereby modulating the strength of HTL-dependent signaling.

Indeed, our findings suggest that downregulation of Miple levels in the mesoderm is essential for correct mesoderm spreading, because Miple overexpression led to impaired mesoderm spreading. The disordered pattern of MAPK phosphorylation (detected by anti-dpERK antibody) observed following Miple overexpression might be the primary cause for the mesoderm spreading defect. In wild-type embryos, MAPK activation was detected only at the most dorsal cells of the spreading mesodermal cells. The mechanism by which this spatial MAPK activation is achieved is not clear. It has been suggested that MAPK activation takes place only in cells that directly contact the ectoderm (Wilson et al., 2005). In that case, Miple might trigger prolonged mesoderm-ectoderm cell contacts and this could delay mesoderm spreading. Indirect evidence, especially the observation that overexpression of an activated form of HTL does not lead to an ectopic dpERK signal in the entire mesoderm, led to the suggestion that a constitutive inhibitory input of MAPK activation is present in mesoderm cells (Wilson et al., 2005). This inhibitory activity was suggested to be overcome only in cells that form close contact with the ectoderm. It is unlikely that the role of Miple is to counteract this inhibitory signal, because overexpression of Miple has an effect not only on MAPK activation in early mesoderm spreading but also on the late HTL-dependent signaling in the dorsal EVE-positive cells, in which this inhibitory signal has not been implicated. We therefore favor the possibility that Miple enhances HTL signaling, and that this enhancement is reflected by MAPK activation in both early and later stages of mesoderm development.

The elevation of the dpERK signal detected following overexpression of Miple might be mediated by HTL activation, because no other RTK has been shown to be expressed in the mesoderm at the stage of gastrulation. Although the increased number of EVE-expressing cells detected in the dorsal mesoderm clusters following overexpression of Miple is eliminated in embryos lacking active HTL receptor, we cannot exclude the possibility that the lack of EVE-positive cells in the dorsal mesoderm might stem from the failure of the *htl* mutant mesoderm cells to reach the most dorsal locations.

In vertebrates, midkine and pleiotrophin have been identified by phage display as potential high-affinity ligands for the human receptor tyrosine kinase ALK (Stoica et al., 2002). Although we do not exclude the possible role of Miple in ALK-dependent signaling, ALK is not expressed in the early stages of mesoderm spreading, and does not overlap with the EVE-expressing clusters (Lin et al., 1999); thus, it is unlikely to affect the increased number of EVE-expressing cells. Receptor tyrosine phosphatase-zeta has been implicated as a putative pleiotrophin receptor (Milev et al., 1998). If a similar receptor exists in *Drosophila*, it might respond to Miple overexpression by altering MAPK levels.

It is possible that the heparin-binding domain of Miple enhances the activity of the HTL ligands. In vertebrates, heparin-containing proteins act as co-ligands to FGFs by inducing their dimerization (Ornitz, 2000; Thisse and Thisse, 2005). We confirmed that Miple is a heparin-binding protein, because it binds specifically to a heparin column. The contribution of heparan sulfate proteoglycans to proper mesoderm spreading in *Drosophila* had been demonstrated by the requirement of two enzymes, Sugarless and Sulfateless, for this process (Lin et al., 1999). Moreover, a genetic interaction between mutations in each of these enzymes and the two FGF receptors HTL and Breathless (BTL) was demonstrated (Lin et al., 1999). Overexpression of Miple during mesoderm spreading might, on the one hand, compete with endogenous heparan sulfate proteoglycan for Thisbe and Pyramus binding, and thus could inhibit their ability to activate the HTL-dependent signaling. On the other hand, Miple might also activate the HTL pathway by replacing the endogenous heparan sulfate proteoglycan that is normally involved in activation of the FGF8-like ligands. These dual activities might interfere with the normal dorsal-restricted MAPK activation in the mesoderm.

In wild-type embryos, *miple* is downregulated by HOW(L) in the mesoderm; however, its mRNA expression is detected at later developmental stages, including in the ventral midline and in the brain (Englund et al., 2006). In midline glial cells, a second FGF receptor, Breathless, has been implicated in the promotion of cell migration at stages 12-13 of embryonic development (Klambt et al., 1992). At this stage, Miple might contribute to the spatial and temporal control of Breathless activation. Such a scenario must be tested directly in *miple* mutant embryos.

Although the mesoderm spreading phenotype of *how* germline clone embryos is not fully penetrant and is detected in only a few segments, the contribution of HOW activity is crucial because of the secondary effect that non-homogenous mesoderm spreading exhibits on the development of the heart and dorsal somatic mesoderm. HOW(L) appears to function in the mesoderm as a safety mechanism to prevent mis-expression of either maternally contributed genes or genes whose early transcriptional activation in the mesoderm might interfere with the normal development of the mesoderm. An example of similar repressive activity of HOW(L) is its activity in the reduction of *string* levels in the gastrulating embryo to prevent premature cell division during mesoderm invagination (Nabel-Rosen et al., 2005).

In summary, this study reveals the crucial function of the STAR family member HOW(L) in enabling proper mesoderm development via the repression of specific mRNAs provided either maternally, or expressed prematurely in a specific tissue. HOW(L) and its vertebrate homolog, QKI5, are expressed in wide range of tissues during early developmental stages, and might function in these tissues in a similar fashion to enable proper embryonic and tissue development.

We thank P. Fisher, M. Frasch, S. Roth, R. Palmer, A. Müller and the Bloomington Stock Center for various antibodies and fly lines. We thank A. Stark and A. Feiglin for the help with 3′ UTR alignments; G. Melen for the help in the microarray and in situ protocol; S. Carmon for the anti-dpERK staining; the Weizmann microarray unit; and B. Shilo, E. Schejter, S. Shwarzbaum and members of the Volk lab for critical reading of the manuscript. This study was supported by a grant from the Israel Science Fund (ISF).

References

Baehrecke, E. H. (1997). *who* encodes a KH RNA binding protein that functions in muscle development. *Development* **124**, 1323-1332.

Baylies, M. K., Bate, M. and Ruiz Gomez, M. (1998). Myogenesis: a view from Drosophila. *Cell* **93**, 921-927.

Beiman, M., Shilo, B. Z. and Volk, T. (1996). Heartless, a Drosophila FGF receptor homolog, is essential for cell migration and establishment of several mesodermal lineages. *Genes Dev.* **10**, 2993-3002.

Carmena, A., Gisselbrecht, S., Harrison, J., Jimenez, F. and Michelson, A. M. (1998). Combinatorial signaling codes for the progressive determination of cell fates in the Drosophila embryonic mesoderm. *Genes Dev.* **12**, 3910-3922.

Crittenden, S. L., Bernstein, D. S., Bachorik, J. L., Thompson, B. E., Gallegos, M., Petcherski, A. G., Moulder, G., Barstead, R., Wickens, M. and Kimble, J. (2002). A conserved RNA-binding protein controls germline stem cells in Caenorhabditis elegans. *Nature* **417**, 660-663.

Crittenden, S. L., Eckmann, C. R., Wang, L., Bernstein, D. S., Wickens, M. and Kimble, J. (2003). Regulation of the mitosis/meiosis decision in the

Caenorhabditis elegans germline. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **358**, 1359-1362.
Ebersole, T. A., Chen, Q., Justice, M. J. and Artzt, K. (1996). The quaking gene product necessary in embryogenesis and myelination combines features of RNA binding and signal transduction proteins. *Nat. Genet.* **12**, 260-265.
Edenfeld, G., Volohonsky, G., Krukkert, K., Naffin, E., Lammel, U., Grimm, A., Engelen, D., Reuveny, A., Volk, T. and Klambt, C. (2006). The splicing factor crooked neck associates with the RNA-binding protein HOW to control glial cell maturation in Drosophila. *Neuron* **52**, 969-980.
Englund, C., Birve, A., Falileeva, L., Grabbe, C. and Palmer, R. H. (2006). Miple1 and miple2 encode a family of MK/PTN homologues in Drosophila melanogaster. *Dev. Genes Evol.* **216**, 10-18.
Frasch, M. (1999). Controls in patterning and diversification of somatic muscles during *Drosophila* embryogenesis. *Curr. Opin. Genet. Dev.* **9**, 522-529.
Gabay, L., Seger, R. and Shilo, B. Z. (1997). MAP kinase in situ activation atlas during Drosophila embryogenesis. *Development* **124**, 3535-3541.
Gisselbrecht, S., Skeath, J. B., Doe, C. Q. and Michelson, A. M. (1996). heartless encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the Drosophila embryo. *Genes Dev.* **10**, 3003-3017.
Gryzik, T. and Muller, H. A. (2004). FGF8-like1 and FGF8-like2 encode putative ligands of the FGF receptor Htl and are required for mesoderm migration in the Drosophila gastrula. *Curr. Biol.* **14**, 659-667.
Hansen, D. and Schedl, T. (2006). The regulatory network controlling the proliferation-meiotic entry decision in the Caenorhabditis elegans germ line. *Curr. Top. Dev. Biol.* **76**, 185-215.
Hardy, R. J. (1998). Molecular defects in the dysmyelinating mutant quaking. *J. Neurosci. Res.* **51**, 417-422.
Israeli, D., Nir, R. and Volk, T. (2007). Dissection of the target specificity of the RNA-binding protein HOW reveals dpp mRNA as a novel HOW target. *Development* **134**, 2107-2114.
Klambt, C., Glazer, L. and Shilo, B. Z. (1992). breathless, a Drosophila FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. *Genes Dev.* **6**, 1668-1678.
Larocque, D. and Richard, S. (2005). QUAKING KH domain proteins as regulators of glial cell fate and myelination. *RNA Biol.* **2**, 37-40.
Lin, X., Buff, E. M., Perrimon, N. and Michelson, A. M. (1999). Heparan sulfate proteoglycans are essential for FGF receptor signaling during Drosophila embryonic development. *Development* **126**, 3715-3723.
Melen, G. J., Levy, S., Barkai, N. and Shilo, B. Z. (2005). Threshold responses to morphogen gradients by zero-order ultrasensitivity. *Mol. Syst. Biol.* **1**, doi:10.1038/msb4100036.
Michelson, A. M., Gisselbrecht, S., Zhou, Y., Baek, K. H. and Buff, E. M. (1998). Dual functions of the heartless fibroblast growth factor receptor in development of the Drosophila embryonic mesoderm. *Dev. Genet.* **22**, 212-229.
Milev, P., Chiba, A., Haring, M., Rauvala, H., Schachner, M., Ranscht, B., Margolis, R. K. and Margolis, R. U. (1998). High affinity binding and overlapping localization of neurocan and phosphacan/protein-tyrosine phosphatase-zeta/beta with tenascin-R, amphoterin, and the heparin-binding growth-associated molecule. *J. Biol. Chem.* **273**, 6998-7005.
Nabel-Rosen, H., Dorevitch, N., Reuveny, A. and Volk, T. (1999). The balance between two isoforms of the Drosophila RNA-binding protein how controls tendon cell differentiation. *Mol. Cell* **4**, 573-584.
Nabel-Rosen, H., Volohonsky, G., Reuveny, A., Zaidel-Bar, R. and Volk, T. (2002). Two isoforms of the Drosophila RNA binding protein, how, act in opposing directions to regulate tendon cell differentiation. *Dev. Cell* **2**, 183-193.
Nabel-Rosen, H., Toledano-Katchalski, H., Volohonsky, G. and Volk, T. (2005). Cell divisions in the *Drosophila* embryonic mesoderm are repressed via posttranscriptional regulation of string/cdc25 by HOW. *Curr. Biol.* **15**, 295-302.
Ornitz, D. M. (2000). FGFs, heparan sulfate and FGFRs: complex interactions essential for development. *BioEssays* **22**, 108-112.
Prout, M., Damania, Z., Soong, J., Fristrom, D. and Fristrom, J. W. (1997). Autosomal mutations affecting adhesion between wing surfaces in Drosophila melanogaster. *Genetics* **146**, 275-285.
Shishido, E., Ono, N., Kojima, T. and Saigo, K. (1997). Requirements of DFR1/Heartless, a mesoderm-specific *Drosophila* FGF-receptor, for the formation of heart, visceral and somatic muscles, and ensheathing of longitudinal axon tracts in CNS. *Development* **124**, 2119-2128.
Stark, A., Brennecke, J., Bushati, N., Russell, R. B. and Cohen, S. M. (2005). Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3′UTR evolution. *Cell* **123**, 1133-1146.
Stathopoulos, A., Tam, B., Ronshaugen, M., Frasch, M. and Levine, M. (2004). pyramus and thisbe: FGF genes that pattern the mesoderm of Drosophila embryos. *Genes Dev.* **18**, 687-699.
Stoica, G. E., Kuo, A., Powers, C., Bowden, E. T., Sale, E. B., Riegel, A. T. and Wellstein, A. (2002). Midkine binds to anaplastic lymphoma kinase (ALK) and acts as a growth factor for different cell types. *J. Biol. Chem.* **277**, 35990-35998.
Thisse, B. and Thisse, C. (2005). Functions and regulations of fibroblast growth factor signaling during embryonic development. *Dev. Biol.* **287**, 390-402.
Vernet, C. and Artzt, K. (1997). STAR, a gene family involved in signal transduction and activation of RNA. *Trends Genet.* **13**, 479-484.
Volohonsky, G., Edenfeld, G., Klambt, C. and Volk, T. (2007). Muscle-dependent maturation of tendon cells is induced by post-transcriptional regulation of stripeA. *Development* **134**, 347-356.
Wilson, R. and Leptin, M. (2000). Fibroblast growth factor receptor-dependent morphogenesis of the Drosophila mesoderm. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **355**, 891-895.
Wilson, R., Battersby, A., Csiszar, A., Vogelsang, E. and Leptin, M. (2004). A functional domain of Dof that is required for fibroblast growth factor signaling. *Mol. Cell. Biol.* **24**, 2263-2276.
Wilson, R., Vogelsang, E. and Leptin, M. (2005). FGF signalling and the mechanism of mesoderm spreading in *Drosophila* embryos. *Development* **132**, 491-501.
Zaffran, S., Astier, M., Gratecos, D. and Semeriva, M. (1997). The held out wings (how) *Drosophila* gene encodes a putative RNA-binding protein involved in the control of muscular and cardiac activity. *Development* **124**, 2087-2098.