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The Hem protein mediates neuronal migration by inhibiting WAVE degradation and functions opposite of Abelson tyrosine kinase

Zengrong Zhu and **Krishna Moorthi Bhat***
Department of Neuroscience and Cell Biology, University of Texas Medical Branch School of Medicine, Galveston, Texas 77598

Abstract

In the nervous system, neurons form in different regions, then they migrate and occupy specific positions. We have previously shown that RP2/sib, a well-studied neuronal pair in the *Drosophila* ventral nerve cord (VNC), has a complex migration route. Here, we show that the Hem protein, via the WAVE complex, regulates migration of GMC-1 and its progeny RP2 neuron. In *Hem* or *WAVE* mutants, RP2 neuron either abnormally migrates, crossing the midline from one hemisegment to the contralateral hemisegment, or does not migrate at al and fail to send out its axon projection. We report that Hem regulates neuronal migration through stabilizing WAVE. Since Hem and WAVE normally form a complex, our data argues that in the absence of Hem, WAVE, which is presumably no longer in a complex, becomes susceptible to degradation. We also find that Abelson Tyrosine kinase affects RP2 migration in a similar manner as Hem and WAVE, and appears to operate via WAVE. However, while Abl negatively regulates the levels of WAVE, it regulates migration via regulating the activity of WAVE. Our results also show that during the degradation of WAVE, Hem function is opposite to that of and downstream of Abl.

Keywords

Drosophila; Hem; Abl; WAVE; Neuron; Migration

Introduction

Cell migration is one of the most fundamental phenomena in biology displayed in simple organisms like yeast, to complex organisms like humans. It is involved in many biological processes including chemotaxis, tissue repair, immune responses and development (Keller, 2005; Luster et al, 2005; Vicente-Manzanares et al, 2005). Improper cell migration has been observed in many pathological situations (Ridley et al, 2003). In the nervous system, neurons and their precursor cells are formed in different regions, but they migrate and occupy specific positions in the mature nervous system. Their position is thought to be crucial in establishing proper synaptic connectivity. Neurons can migrate a few cell-lengths to several thousand cell-lengths - often taking very complex routes - by responding the internal of external signals (Wong et al, 2002). Perturbations in neuronal migration are

*Corresponding author kmbhat@utmb.edu, Phone: 409-747-2214.

known to cause neurodevelopmental defects such as smooth brain disease and mental retardation (Ghashghaei et al, 2007).

Though widely used as a model system to study development of the CNS, very little is known about neuronal migration in *Drosophila*. In *Drosophila*, the ventral nerve cord consists of segmentally repeated units, divided by the midline. Thus, each hemisegment contains ~320 neurons and ~ 30 glia. Neurons are generated from neuroblasts (NBs). A NB repeatedly divides to self-renew and produces a series of ganglion-mother-cells (GMCs). Each GMC divides asymmetrically to produce two neurons (or glia or a neuron and a glia) of different identities. The only description of an active neuronal migration in the *Drosophila* VNC comes from the NB4-2®GMC-1®RP2/sib lineage (Bhat, 2007). This is a typical and well-studied lineage formed from NB4-2, one of ~30 NBs in a hemisegment. It generates its first GMC, GMC-1, which divides asymmetrically into an RP2 motor neuron and its sibling cell known as sib (reviewed in Gaziova and Bhat, 2007). Our recent work on the migration has shown that GMC-1àRP2/sib undergo a complex 3-step migration and Wingless (Wg) signaling is necessary for the step 2 and step 3 migrations (Bhat, 2007). Recent work has shown that there is an active migration process going on in the optic lobe (Morante et al., 2011; Hasegawa et al., 2011). For example, medulla cortex cells in the optic lobe oerform two patterns of cell migrations to acquire their final position. First, neurons move to become arranged in columns below each neuroblast. Then, These neurons migrate laterally, intermingling with each other to reach their retinotopic position in the adult optic lobe. That these migratiosn are an active process is indicated by the involvement of *eyeless*, a Pax6 transcription factor (Morante et al., 2011). However, much remains to be elucidated to understand the underlying mechanisms responsible for complex migration patterns that occur during neurogenesis.

Polymerization of actin filaments (F-actin) to form filopodia and/or lamellipodia and depolymerization in the leading edge plays key roles in the regulation of cell migration (Raftopoulou and Hall, 2004). The Arp2/3 complex induces polymerization by promoting nucleation and by binding to the side of F-actin to elongate branched filaments. The Arp2/3 complex is activated by WASp family members, WASp and WAVE (Machesky and Insall, 1998; Symons et al, 1996; Suetsugu et al, 1999). However, WASp and WAVE are activated independently (Takenawa and Miki, 2001). WASp is autoinhibited, and could be directly activated by Cdc42. WAVE is not autoinhibited but forms a WAVE-complex with four other molecules: Abelson interactor (Abi), Hem, Sra-1 and HSPC300 (Higgs and Pollard, 2000; Ismail et al, 2009; Kim et al, 2000; Martinez-Quiles et al, 2001; Padrick et al, 2008; Rohatgi et al, 2001). Once activated by another GTPase, Rac, WAVE promotes actin polymerization through Arp2/3 (Miki et al, 2000; Eden et al, 2002). The exact mechanism by which WAVE is activated remains controversial. There is evidence suggesting that Rac activity causes dissociation of the WAVE complex and release of the WAVE-HSPC300 and the activation of WAVE (Eden et al, 2002). Other studies, however suggests that after Rac activation, WAVE complex is translocated to the membrane, where Abi recruits Ableson-tyrosine- kinase (Abl) into this complex, which then activates the WAVE complex (Leng et al, 2005).

The Hem protein (or Kette/dhem2/Hem-2/Nap1/Nap125) belongs to a highly conserved family, from invertebrates to mammals (Baumgartner et al, 1995). In humans, there two genes of the Hem family, *Hem1* (NCKAP1L) and *Hem2* (NCKAP1). Both *Hem1* and *Hem2* in humans generate two isoforms each. In Drosophila, however, there is only one *Hem* gene, which is homologous to the human *Hem2*. All the members of Hem family are predominantly expressed in the nervous system, where as the *Hem-1* gene in humans is predominantly expressed in the hematopoietic cells (Weiner et al, 2006). On the other hand, in *C. elegans*, the Hem-2 protein, known as GEX-3 (and Hem in Drosophila) is expressed

everywhere, and has an essential function in the migration of epithelial cells in embryos (Soto et al. 2002). In *Drosophila*, Hem is maternally expressed during early embryogenesis but then becomes specifically expressed in the brain and the VNC. Hem has six transmembrane domains, however, in *Drosophila* S2 cells, most of Hem is in the cytosol and only very little is present in the membrane (Bogdan and Klambt, 2003).

Hem is involved in processes such as apoptosis, formation and maturation of neuromuscular junction, axon-pathfinding, neuronal-differentiation, myoblast fusion etc (Hummel et al, 2000; Nakao et al, 2008; Schenck et al, 2004; Schroter et al, 2004; Suzuki et al, 2000; Yokota et al, 2007). These studies suggest that Hem dynamically regulates polymerization of F-actin. Hem binds to the first Src homology 3 (SH3) domain of Nck/Dock, an adaptor molecule containing one SH2 domain and three SH3 domains and links several receptor tyrosine kinases to the cytoskeleton (Li et al, 2001). Thus, Hem may link extracellular signals to the cytoskeleton. However, Hem is also part of the WAVE-complex and it may regulate the activity of this complex to promote polymerization of F- actin. It remains controversial how Hem regulates WAVE. Eden *et al* (2002) argued that WAVE is inhibited in the WAVE complex by Hem and PIP121. Upon activation by Rac1 or Nck, the WAVE complex is dissociated, releasing an active WAVE-HSPC300 to mediate actin nucleation (Eden et al, 2002). This conclusion is supported by the findings by Bogdan *et al* (2002) that loss of *Hem* function leads to an excess of F-actin in the cytosol; by reducing the *WAVE* gene dose on the other hand, the *Hem* mutant phenotype can be suppressed (Bogdan and Klambt, 2003). However, Kunda *et al* (2003) showed that Hem protects WAVE from proteasome-mediated degradation in cultured *Drosophila* cells. Furthermore, Schenck *et al* (2004) showed that the axon guidance defects in *WAVE*, *Sra-1* and *Hem* mutants are similar to each other and loss of Hem leads to a reduction in WAVE.

Here, we show that Hem regulates neuronal migration during development of the VNC in *Drosophila* embryos. The loss of Hem activity results in migration defects of RP2/sib cell pairs. Moreover, Hem regulates neuronal migration through WAVE; not by inhibiting the WAVE activity, but by preventing WAVE degradation. Interestingly, we also find that Abl affects RP2 migration in a similar way as Hem and WAVE, and operates via WAVE. In this case, however, while Abl negatively regulates the level of WAVE protein, the regulation of migration by Abl appears to be via regulating the activity of WAVE. Our results also show that the Hem function in regulating the levels of WAVE is downstream of Abl.

Materials and Methods

Fly strains, genetics

All flies and crosses are kept/done at 22 °C unless otherwise indicated. The following strains were used: *Hem^{J4-48}*, *Hem^{C3-20}*, *Df(3L)ED230*, *insc^{22}*, *nb^{796}*, *Abl^{2}*, *Abl1 FRT(w[hs])2A*, *UAS-Abl*, *Df(3L)st-j7*, *SCARΔ37*, *Df(2L)BSC32*, *UAS-WAVE*, *w;p{SCARK13811,W+} FRT40A/CyO*, *sca-Gal4*, *P{GAL4∷VP16-nos.UTR}MVD2*, *Sra-1^{EY06562}*, *Df(3R)Exel6174*, *Abi^{Ey20423}*, *Df(3R)Exel7359*, *HSPC300^{EP506}*, *HSPC300^{G19021}*, *Df(2R)Exel6080*, *Wsp^{1}* and *Wsp^{3}*. Various mutant combinations are generated by standard genetics. To exclude possible maternal modifier effects of balancers (Bhat et al, 2007; Gaziova and Bhat, 2009), homozygous mutant embryos were also tested by out-crossing the balancer-bearing mutants to wild type and back-crossing the non-balancer bearing mutant adults. Staging of embryos was as described by Wieschaus and Nusslein-Volhard (1986).

Generating mosaic animals

Germline clones for WAVE were generated as described by Zallen et al (2002), by heat shocking *hsFLP; SCAR13811FRT40A/ovoD FRT40A* early stage larvae at 37°C for 1-hour

and another heat shock 24-hr later. Adult germline clones carrying females were crossed to *SCARΔ37* males and embryos *(WAVEmat, zyg)* were collected for analysis. Germline clones for *Abl* were generate by heat shocking *hsFLP; Abl1 FRT(w[hs])2A/ovoD FRT(w[hs])2A* early stage larvae at 37°C for 1-hour and another heat shock 24-hr later. Adult germline clone females were crossed to *Abl2* males and embryos (*Ablmat,zyg*) were collected for analysis.

Generating Transgenic animals

To determine the antimorphic properties of the truncated Hem protein in *HemJ4*-48allele *(ΔHemJ4-48)*, we synthesized the *Hem* truncated gene corresponding to the coding fragment of the first 489 amino acids of Hem by PCR. We used the following primers that carried specific restriction sites and a stop codon: *5'*-ATAAGAATGCGGCCGCTAAACTATTGCACGCCTCCCAATACG-3' and 5'-GCTCTAGATTAGTCCAGGCGGAATGGTC-3'. The PCR product was digested with NotI and XbaI and subcloned into NotI/XbaI digested pUAST and the cloned truncated Hem gene was sequenced. Several independent transgenic lines were generated and used for analysis.

Immunohistochemistry

Standard immunostaining procedures were used. The following antibodies were used: Eve (rabbit, 1:2000 dilution), Eve (mouse, 1:5), Zfh1 (mouse, 1:400), 22C10 (mouse, 1:4), LacZ (rabbit, 1:3000 or mouse, 1:400), BP102 (mouse 1:10) Fas II (mouse; 1:5), Sim (rat, 1:200), Wave (guinea pig 1:100). For confocal microscopy, cy5 and FITC-conjugated secondary antibodies were used. For light microscopy, alkaline phosphatase or DAB-conjugated secondary antibodies were used.

Sequencing of the mutant alleles and confirmation of the mutant homozygous identity by single embryo PCR

Mutant embryos were individually selected using the GFP-balancer as well as by using the visible mutant phenotype (that the *Hem* mutant embryos exhibit) under microscope. DNA from these single embryos was isolated and subjected to PCR using primers for the *Hem* gene. The PCR products were sequenced in both strands.

Western-blotting experiments

For each genotype examined, twenty embryos were picked under GFP microscope and homogenized in 40μL of Lysis buffer (0.15 M NaCl, 0.02 M Tris pH 7.5, 0.001M EDTA, 0.001 M MgCl2, 1% Triton-X-100, PIC). After centrifugation, the supernatant (37.5μL) was collected and 12.5μL of 4X Laemmli sample buffer was added. Out of this 50μL, 10μL was subjected for SDS-PAGE and Western analysis. Primary antibodies used were against: WAVE (guinea pig 1:1500), Hem (rabbit 1:1000), and Tubulin (Abcam, rabbit, 1:2000). X-ray films from various Western blotting analyses were scanned and the densities of the signals were determined by using AlphaEaseFC (AlphaInnotech, V6.0). Anti-Tubulin was used as loading control and intensity of the band were normalized against Tubulin signal. Several independent experiments were done and the intensity values followed the same trends with narrow variations in all the experiments.

Phosphatase treatment of embryo extract

Twenty embryos were collected, dechorionated and homogenized in 37.5μL of the Lysis buffer (see above). After centrifugation, supernatant (~37.5μL) was collected and incubated with Lambda protein phosphatase (Lambda PP, 100U) in NEB phosphatase buffer (5μL of 10X) and MnCl2 (5μL of 10 mM solution) in a total final volume of 50μL for 30 min at

30°C. For the control, lambda PP was omitted. Following the incubation, 16.6μL of 4X Laemmli buffer) was added, boiled for 10 min and the samples were run on an 8% separating SDS gel for Western analysis.

Real-time Polymerase chain reaction (PCR)

Embryos were collected, dechorionated and ~100 embryos were selected under microscope and RNA was isolated using RNAqueous Kit (Ambion). Synthesis of cDNA was performed with 1 μg of total RNA in a 20μL reaction using the reagents in the Taqman Reverse Transcription Reagents Kit from ABI (#N8080234). Reaction conditions were as follows: 25°C, 10 minutes, 48°C, 30 minutes and 95 °C, 5 minutes. Primers for real-time PCR were designed and were done by the Molecular Genomic Core facility at UTMB. Real-time PCR (in triplicates) were done using 2μL of cDNA in a total volume of 25μL with SYBR green using the SYBR Green PCR Master Mix (ABI #4364344). RpL32 was used as endogenous control. All PCR assays were performed in the ABI Prism 7500 Sequence Detection System and the conditions are as follows: 50 °C, 2 minutes, 95 °C, 10 minutes, 40 cycles of 95 °C, 15 seconds and 60 °C, 1 minute. Primers used: WAVE (Forward: 5' ACGAAGAAGCCGGATACGG 3', Reverse: 5' GAAGCTGCTCGTAGGTGCTACC 3'), Abl (Forward: GCAATTTCATCGACGACCTCA, Reverse: GACTCTGCTCCAGACTATCGCC). Various p-values were calculated using the student t-test.

Rescue HemJ4-48 phenotype with WAVE

To rescue the migration defects in *HemJ4-48* mutants with *UAS-WAVE, UAS-WAVE* (on the third chromosome) was introduced to the *HemJ4-48* background by recombination. This *UAS-WAVE* was induced using *sca-Gal4* and the embryos were examined using Eve and WAVE antibodies for the rescue of the migration defect.

Results

Hem mutants show a duplication-and-loss of RP2 phenotype

In a previous deficiency screen, we discovered that while in wild type a single RP2 motoneuron is present in each hemisegment (Fig. 1A, arrow), a deficiency that uncovers the *Hem* gene had a unique phenotype: duplication of the RP2 neuron in one hemisegment and missing in the contralateral hemisegment (Fig. 1B, arrows). We mapped this phenotype to mutations in the *Hem* gene. We examined two of the embryonic lethal alleles, $Hem^{J4\text{-}48}$ and $Hem^{C3\text{-}20}$ (Hummel et al., 2000) and both showed this RP2 defect (Fig. 1C and D). This phenotype was also observed in embryos transheterozygous for the two alleles or transheterozygous for the mutant alleles and the *Hem*-deficiency [*(3L)ED230*; Fig. 1E]. The penetrance of the phenotype varied in the two alleles and in the deficiency (Fig. 1G). In $Hem^{J4\text{-}48}$ we found a maximum of 45% of the hemisegment per embryo showing the defect, the average was 13% (total number of hemisegments examined, n=648). In $Hem^{C3\text{-}20}$ the maximum was 20% (4/20 hemisegments) and the average was 8% (n=96). In the *Hem*-deficiency the maximum number of hemisegments affected was 20% (4/20) and the average was 9% (n=114). Based on these results, $Hem^{J4\text{-}48}$ allele is the strongest and $Hem^{C3\text{-}20}$ and the *Hem*-deficiency are of the same strength. This suggests that $Hem^{C3\text{-}20}$ behaves as a null allele, whereas $Hem^{J4\text{-}48}$ behaves as an antimorphic allele. Consistent with this possibility, the penetrance of the defect was lower in embryos that are transheterozygous for $Hem^{J4\text{-}48}$/ *Hem* deficiency (Fig.1G).

We sequenced the *Hem* gene from these two alleles. In $Hem^{C3\text{-}20}$ there was a stop codon at amino-acid 256 in the *Hem* gene, while in $Hem^{J4\text{-}48}$ the stop codon was at 490 (Fig. 1F). These molecular lesions are consistent with what has been previously reported (Hummel et

al, 2000). Thus, the molecular lesion in the Hem^{C3-20} allele is consistent with the possibility that this is a null allele, whereas the lesion in the Hem^{J4-48} allele is consistent with the possibility that it can produce a truncated protein that behaves as a dominant negative protein. The partial penetrance of the phenotype in the *Hem*-deficiency or *Hem* alleles is likely due to the maternal perdurance of the Hem protein in the embryo. Indeed, as shown in Fig. 1H, the wild type maternally deposited protein (~110 kDa) is still present in embryos that are 12-16 hrs old (the truncated Hem in the mutant is not recongnized by the antibody since it is raised against the portion of the protein beyond the truncation)., While the level of Hem protein in *Hem* deficiency is less than in wild type (Fig. 1H, l, compare lanes 1, 3, 4 and 6), in Hem^{J4-48}, the level is much lower than in the deficiency or the wild type (Fig. 1H, compare lanes 1, 2, 4 and 5). This result suggests that the Hem^{J4-48} mutant (truncated) protein down-regulates the maternal wild type Hem protein. Perhaps the truncated Hem makes the maternal wild type Hem more degradable. Alternatively, this may be a maternal effect where the truncated Hem in the mutant suppresses maternal expression of the wild type protein. This is consistent with our previous findings that balancer chromosomes can have a maternal effect that influences the expressivity of a given mutant phenotype (Bhat et al, 2007; Gaziova and Bhat, 2009). In any case, the above result that the level of Hem in Hem^{J4-48} embryos is less than what it is in *Hem* deficiency embryos is consistent with the higher penetrance of the defect in Hem^{J4-48} embryos compared to *Hem* deficiency embryos.

We rarely observed Hem^{J4-48} embryos (~4% of Hem^{J4-48} mutant embryos, n=500) where the RP2 neurons are located in the position of a newly formed GMC-1 (Fig. 1I, see also Fig. 2M, N, P). This suggests that if the Hem function is eliminated at the GMC-stage, the GMC-1 and its progeny may not migrate at all. However, we could not generate germline mosaics for *Hem* because of its location on the chromosome, which is very close to the centromere. But, there is additional evidence in the proceeding sections that suggest that maternal and zygotic null for *Hem* will prevent the GMC-1 and its progeny from undertaking any migration.

The truncated Hem4-48 protein indeed behaves as an antimorphic protein

To determine if the truncated Hem in Hem^{J4-48} does behave as an antimorphic protein and down-regulates maternally deposited wild type Hem (see Fig. 1H), we generated transgenic lines carrying the Hem^{J4-48} truncated Hem (ΔHem^{J4-48}) under the UAS promoter. We expressed this transgene using a GAL4 driver (VP16-nos.UTR). The GAL4 driver was brought from the mother since there is maternal deposition of GAL4 with this driver. In these embryos, we rarely (<1%) found the same migration defect as Hem^{J4-48} embryos, the weak effect is likely due to the high maternal deposition of wild type Hem and the reduction in the levels of Hem by the transgene product was not sufficiently high to have an effect. Therefore, we induced the ΔHem^{J4-48} transgene in the *Hem*-deficiency background and the penetrance of the defect was determined. The penetrance was now increased to ~17% (Fig. 1G and J, see also Fig. 2Q; deficiency alone had ~9% penetrance). Given the above results, we determined if the expression of the truncated protein down-regulates the levels of wild type Hem. Since we could not test this in *Hem*-deficiency background, we examined it in wild type embryos expressing the ΔHem^{J4-48} transgene. While the effect was weak, we consistently observed a slight reduction in the levels of Hem in these embryos (Fig. 1K).

RP2 but not sib aberrantly migrates to the contralateral hemisegment in *Hem* mutants

We followed the development of the GMC-1->RP2/sib lineage in the mutant embryo. One possibility for the duplication-and missing-phenotype is that an RP2 aberrantly crosses the midline and resides next to the RP2 on the contralateral hemisegment. A detailed description of the migration pattern for the GMC-1, RP2 and sib cells has been previously published (Bhat, 2007) and summarized in Fig. 2K. Briefly, the GMC-1, once formed begins its

migration perpendicular to and toward the midline, the Step 1 process of the migration. During Step 1, the GMC-1 divides to generate an RP2 and a sib, and both these cells continue their migration toward the midline. RP2 and sib cells stop Step 1 migration approximately two to three neuroectodermal cells away from the midline (Fig. 2B) and begin their Step 2 migration. In this step, RP2 and sib move in the posterior direction, parallel to the midline. By ~9.5 hours of development, both RP2 and sib cells have crossed the Wg expressing row 5 cells; these cells then stop their posterior migration and reside right posterior to the Wg stripe (Fig. 2C). By 11 hours of development, the sib rotates around the RP2 to reside closer to the midline (Fig. 2D). Soon after, the RP2 (but not the sib) migrates in the anterior direction (Step 3), parallel to the midline, crosses back the Wg row of cells and resides in the location where the RP2 and sib had initially started their posterior migration (Fig. 2E). The Step 3 migration of sib involves the rotational move around an RP2 but then it also follows the RP2, crosses the Wg-positive row of cells and resides slightly posterior and ventral to RP2 (see Bhat, 2007).

In the mutant, GMC-1 is formed in both hemisegments (Fig. 2F) and the GMC-1 normally divides into an RP2 and a sib; these cells also complete their step 1 and step 2 migrations (Fig. 2G and H). However, the RP2 from one of the hemisegments continues to migrate but towards and across the midline (Fig. 2H). By 11 hours of development, the aberrant migration across the midline to the contralateral hemisegment is complete (Fig. 2I). By 13 hours, such RP2 neurons complete step 3 migration together with the resident RP2 neuron and occupy the final position (Fig. 2J). The sib, however, stays in its own hemisegment (Fig. 2I and J) indicating that the aberrant migration is specific to the RP2 neuron and not to its sibling cell. This aberrant migration of RP2 in *Hem* mutants is summarized in Fig. 2L. Furthermore, when we induced the $\Delta Hem^{J4\text{-}48}$ transgene in *Hem* deficiency background using the VP16-GAL4 driver and examined younger stage embryos with Eve staining, we observed the strong migration defect (Fig. 2Q), also seen among $Hem^{J4\text{-}48}$ mutant embryos (Fig. 1J).

As pointed out earlier, among the $Hem^{J4\text{-}48}$ mutant embryos, in a small number of mutant embryos (4% of $Hem^{J4\text{-}48}$ mutant embryos, N=500) RP2 neurons in about 50% of the hemisegments (n= 356 hemisegments) and sib cells in about 20% of the hemisegments fail to migrate. Instead, they stay in the same location as the parent GMC-1 (Fig. 2M and P). We examined such RP2s for their axon projections by staining with Mab22C10/MAPIB antibody. This antibody stains axon projection from neurons such as RP2. As shown in Figure 2O, in wild type an RP2 (arrow) sends its ipsilateral axon projection (long arrow). However, in $Hem^{J4\text{-}48}$ mutants, the non-migrating RP2s had no axon projections although they had strong 22C10 staining in the cell body (Fig. 2P, arrow-with-a-star). These results suggest that proper migration is essential for sending out an axon projection. This result, however, does not exclude the possibility that the Hem-mediated pathway affects the ability of this neuron to generate a growth cone (axonogenesis) independent of the migration.

To obtain additional evidence for the mis-migration of RP2s, we examined embryos that are double mutant for *Hem* and *inscuteable* (*insc*) and *Hem* and *numb*. In *insc* mutants, the GMC-1 symmetrically divides into two RP2s (Fig. 3A; see also Buescher et al, 1998) where as in *numb*, it divides into two sibs (Fig. 3C; Buescher et al, 1998; Wai et al, 1999). Because we have never seen a neurogenic phenotype in *Hem* mutants and also that we have not seen two GMC-1s in *Hem* mutants, we reasoned that if the duplication in *Hem* mutants is due to migration defect, we should see hemisegments with four RP2 neurons in one hemisegment and missing in the contralateral hemisegment in *Hem; insc* mutants. Indeed, hemisegments with four RP2 neurons in one hemisegment and missing in the contralateral hemisegment were observed in $Hem^{J4\text{-}48}$*; insc* double mutants (Fig. 3). But in $Hem^{J4\text{-}48}$*; numb* double mutants, the *numb*-phenotype should be epistatic to *Hem*, with only two sibs per

hemisegment, and it was (Fig. 3D). These results show that the abnormal migration in *Hem* mutants is indeed RP2-specific but not sib-specific.

The aberrant RP2 migration in *Hem* mutants is not due to a midline defects, identity changes or commissural defects

We next examined if the abnormal migration of RP2 in *Hem* mutants is due to a midline defect by looking at the expression of Single-minded (Sim). Sim is expressed in all midline cells (Fig. 4A) and is involved in the specification of midline cells. When *Hem* embryos are double-stained with Sim and Eve antibodies, we observed normal expression of Sim in midline cells (Fig. 4B). We could even observe an RP2 right on the midline in the process of crossing (Fig. 4B), but not its sib.

It was possible that the identity of the RP2 that crosses the midline has changed, which in turn induces it to migrate to the contralateral hemisegment. Therefore, we examined the mutant embryos with Zfh-1, an RP2-specific marker (Fig. 4C; a dividing GMC-1 and an occasional newly formed sib transiently expresses Zfh-1, Gaziova and Bhat, 2009). The abnormally migrated RP2 in *Hem* is still positive for Zfh-1 (Fig. 4D).

We next examined embryos double-stained with Eve and 22C10/MABIB. 22C10 stains the RP2 axonal projection that fasciculates with the Intersegmental Nerve bundle (ISN; Fig. 4E, long-arrow). In *Hem* mutants, the mis-migrated RP2 had a contra-ipsilateral axon projection, fasciculating with the ISN. This type of axon projection by an RP2 has never been documented before, in all those cases where the RP2 neurons are duplicated, both the neurons of the pair projected their axons ipsilaterally on its own hemisegment and never contralaterally. It is unlikely that the mis-migrated RP2 has changed its identity. An RP2 begins to project its axon 9-9.5 hours of age (data not shown). An RP2 in *Hem* mutants begins its abnormal migration ~9 hours of age. It appears likely that once a neuron begins its axonal projection, its growth cone is subjected to pathfinding cues and irrespective of the position of the cell body, the axon, as long as the cues are not affected, will project towards its usual target.

We further tested if the mis-migration of RP2 is tied to an abnormal commissural tract by visualizing the tracts with BP102. BP102 stains the anterior commissure (AC), the posterior commissure (PC) and the longitudinal connectives (LC)(Fig. 4G). An RP2 neuron normally resides in the armpit of AC (Fig. 4G). In *Hem* mutants, we observed RP2 mis- localized to the contralateral hemisegment even when the commissural tracts were more or less normal (Fig. 4H). These results are consistent with the conclusion that the mis-migration of RP2 is an active process.

WAVE/SCAR mediates the Hem-specific migration of RP2 neurons

The WAVE-complex, which activates Actin polymerization, has the following proteins: WAVE, Hem, Abi, Hspc300, Sra-1. In addition to the WAVE-complex, WASp is also part of the Hem-pathway. Therefore, we examined embryos mutant for some of these genes. WASp is located both in the membrane and in the cytoplasm and Hem is thought to activate WASp in the membrane (Bogdan and Klambt, 2003). However, *WASp* mutant embryos had no RP2 migration defects. While WASp is maternally deposited, migration defects were not observed in embryos that are mosaic for *WASp* (Ben-Yaacov et al, 2001). Thus, either its function is redundant or it is not required for migration. We also examined embryos mutant for Abi, Hspc300, Sra-1 using zygotic loss of function mutants. However, we did not find any migration defects in these mutants, which is likely due to maternal deposition of these gene products.

Abi is part of the WAVE-complex (Eden et al, 2002) and in vitro cell culture studies suggests Abi recruits Abl to the WAVE-complex, where Abl mediates WAVE-activation (Leng et al, 2005). When we examined *Abi* mutant embryos, no RP2 migration defect was observed. The absence of migration defect in *Abi* could be due to its maternal deposition. On the other hand, we found that *Abl* mutants have the same RP2 migration defect as *Hem* (Fig. 5B) with ~9% of the hemisegments showing the defect (n=600). This partial penetrance is likely due to the perdurance of the maternal *Abl* gene products. We generated *Abl* mosaic individuals lacking both the maternal and zygotic gene products. As shown in Figure 5C, in these embryos the RP2 neurons remained in the location where its parent GMC-1 forms, indicating that these neurons fail to migrate from the site of formation. This phenotype was nearly fully penetrant. As noted above, the same phenotype is also observed in $Hem^{J4\text{-}48}$ embryos at a low frequency (see Fig. 1I and 2M, N, P).

We next examined if embryos mutant for *WAVE* had any migration defects. The RP2 midline-crossing defect was also observed in embryos mutant for *WAVED37*, which is an excision allele with the entire WAVE sequence excised downstream of the 5' UTR of *WAVE* (Fig. 5D). A maximum of 15% of the hemisegments (n=600) were affected in WAVE mutant embryos. A similar penetrance was also observed in embryos that are homozygous for a deficiency that eliminates *WAVE*. WAVE is also maternally deposited (Zallen et al, 2002). Therefore, mosaic embryos for *WAVE* were generated and examined for the RP2 defects. A weaker *WAVE* allele, *SCARk13811*, was used since stronger alleles fail to yield mosaic embryos. In these mosaic embryos we found the same RP2 migration defect as in *Hem* mutants (Fig. 5E). We also found mosaic embryos where RP2s failed to migrate from their location of formation (Fig. 5F). This phenotype was similar to *Abl* maternal and zygotic mutant embryos (Fig. 5C) as well as a subset of *HemJ4-48* embryos (Fig. 1I and 2N, Q). Consistent with this, we found younger stage mosaic embryos where the GMC-1 failed to perform stage 1 migration. Instead, it remained in its original NB4-2 location and divided into an RP2 and a sib (Fig. 5I). These results indicate that WAVE is necessary for the migration from the GMC-1-stage itself and in a *WAVE* null embryo there will be not be any migration.

WAVE protein level is down regulated in *Hem* mutant embryos

Since loss-of-function for *WAVE* had the same defect as *Hem* mutants, we determined if the level of WAVE is affected in *Hem* embryos by Western analysis. As shown in Figure 6A, in wild type a band of ~80-kDa was observed, which is higher than the previously reported molecular weight of ~68-kDa (Schenck et al, 2004) for WAVE. Therefore, we over-expressed WAVE from a *UAS-WAVE* transgene using the scabrous (sca)-GAL4 driver. As shown in Figure 6A (lane 2), the level of the 80-kDa band was increased by ~1.5 times the wild type in these embryos.; the level of WAVE was significantly reduced in *WAVE*-deficiency embryos compared to wild type (Fig. 6B, lanes 3, 7). Interestingly, with the reduced level, we could resolve the major band into 2 major ones and one minor band (Fig. 6B, lanes 3, 7; also 6C, lanes 1, 2) of the sizes ranging from 73-80 kDa range.

It was possible that WAVE is phosphorylated and these three bands represent different levels of WAVE-phosphorylation. A previous paper mentions that WAVE2 is phosphorylated, moreover, this work was done using tissue culture cells (Lebensohn and Kirschner, 2009) and thus there is no in vivo evidence to show that WAVE is indeed phosphorylated in any organism. Therefore, we treated protein extracts from wild type as well as from embryos over-expressing WAVE with Lambda-phage phosphatase. As shown in Figure 6C, all the three bands in control-treated samples (lanes 1, 2) collapsed to one band of ~73 kDa size with the treatment (lanes 3, 4). This is closer to the predicted size of WAVE from the primary sequence.

We next examined the levels of WAVE in *Hem* mutant embryos. While Hem was unaffected with the over-expression of *WAVE* (Fig. 6C, lanes 2, 4) and unaffected or marginally affected in loss of function for *WAVE* (Fig. 6B, lanes 3, 7), as shown in Figure 6B (lanes 2, 6) and Figure 6D (lane 2), the level of WAVE was greatly reduced in $Hem^{J4\text{-}48}$ embryos. The level was also reduced in *Hem*-deficiency embryos (Fig. 6D, lane 3), although the reduction was slightly lower compared to $Hem^{J4\text{-}48}$. Furthermore, expression of the Δ*HemJ4-48* truncated transgene in wild type background down-regulated WAVE (Fig. 6E, lane 2) compared to wild type (Fig. 6E, lane 1). These results indicate that Hem is necessary for maintaining a high level of WAVE, thus, the migration defect in *Hem* mutants could be due to the reduction in the levels of the WAVE protein.

We further examined if the reduction in the levels of WAVE protein in *Hem* mutant embryos is at the protein level or at the mRNA level by determining the mRNA level of WAVE in *Hem* embryos by quantitative PCR (qPCR). As shown in Figure 6F, a marginal increase in the levels of WAVE-mRNA was observed in *Hem* embryos (which is unlikely to be significant based on student t-test analysis). This result suggests that the reduction in the levels of WAVE in *Hem* is likely due to its enhanced degradation. This decrease seems unlikely due to a reduced translational rate given that the increase in mRNA is marginal, although we cannot entirely rule out this possibility Thus, it appears that Hem protects WAVE from degradation, and in the absence/reduction of Hem, more of WAVE is degraded.

In tissue culture experiments, Abl appears to mediate WAVE-activation in WAVE- complex (Leng et al, 2005). Our phenotypic analysis of *Abl* reveals that *Abl* have the same migration defects as *WAVE* and *Hem* mutants. We determined if the levels of WAVE and Hem are altered in *Abl* embryos. As shown in Figure 6B (lanes 4, 8), the levels of Hem was about the same as in wild type, however the level of WAVE was higher in 12-16 hr old *Abl* embryos (Fig. 6B, lane 8). By qPCR, there was no significant increase in the WAVE mRNA level in *Abl* mutant embryos (Fig. 6F). These results suggest that in *Abl* mutants, either that the WAVE is inactive or that Abl regulates RP2 migration independent of WAVE (see below).

Since Abl is maternally deposited to developing embryos, it was possible that the effect of zygotic loss-of-function for *Abl* on the levels of WAVE is not pronounced at the time of RP2 migration. Therefore, we generated *Abl* maternal and zygotic loss-of-function mutant embryos. We examined the level of WAVE in 6-10 hr old embryos (i.e., during the active migration of RP2 neurons)(Fig. 7A and B). Since these mutant embryos were smaller and become somewhat deformed by 12-16 hours of development (Fig. 7C; this difference in size is not apparent at 6-10-hr age), we also doubled the number for examining the level of WAVE in Western analysis (this small size embryo phenotype indicates that the *Abl* maternal and zygotic loss is significant). As shown in Figure 7A (lanes 3, 4; 6-10-hr old embryos) and Figure 7B (lanes 2, 3; 12-16-hr old embryos) we found that loss of both maternal and zygotic Abl function causes an increase in the levels of WAVE protein compared to wild type (lane 1) or *Abl* zygotic mutant embryos (lane 2).

We examined this further by determining if over-expression of *Abl* from a *UAS-Abl* transgene using the sca-GAL4 driver down regulates WAVE. As pointed out above, in 12-16-hr old embryos, gain-of-function for *Abl* down-regulates WAVE almost by half (Fig. 6D, lane 5), indicating Abl involvement in the regulation of WAVE protein levels. We also determined if the levels are down-regulated at earlier developmental time points, during the migration of RP2. As shown in Figure 7D (lanes 2, 4), the levels of WAVE are reduced in 5-9-hr old embryos compared to wild type (lanes 1, 3). Although *Abl* appears to down regulate WAVE, the effect on WAVE during the time of RP2 migration may not be significant enough to have an impact. This is also consistent with our finding that over-

expression of *Abl* during the migration of RP2 has no effect on its migration (data not shown). However, these data show that Abl does mediate, directly or indirectly, the down regulation of the WAVE protein during development.

Given the opposing effects of *Hem* and *Abl* on WAVE, we determined the epistatic relationship between *Hem* and *Abl* with respect to WAVE protein levels and the migration of RP2. We recombined $Hem^{J4\text{-}48}$ mutation with *Abl2* and examined both the protein levels and migration defect in $Hem^{J4\text{-}48}$, Abl^{2} double-mutant embryos. As shown in Figure 7E, *Hem* was epistatic to *Abl* in terms of WAVE levels as the WAVE level in the double mutant was similar to *Hem* single mutant (compare lane 3 with 2). Since our results suggest that WAVE may not be fully active in *Abl* mutants, and that the level of WAVE is down-regulated in *Hem* mutant embryos, the penetrance of the RP2 migration defect is expected to be enhanced in the double-mutant. Indeed, the frequency of the migration defect was enhanced in these double mutants (Fig. 7F and G). Given that there is maternal wild type Hem and Abl in these embryos, the enhancement was modest.

Expression of WAVE in *Hem* mutant embryos rescues the RP2 migration defect

Since WAVE is down-regulated in *Hem* embryos, we determined if the migration defects are due to this reduced levels of WAVE. We recombined a *UAS-WAVE* transgene into $Hem^{J4\text{-}48}$ chromosome and induced this transgene with the *sca-GAL4* driver. While the level of WAVE is barely detectable in $Hem^{J4\text{-}48}$ embryos (Fig. 8B) compared to wild type (Fig. 8A), as shown in Figure 8C, the expression of *UAS-WAVE* in *Hem* embryos induced a high level of WAVE and completely rescued the RP2 migration defect (*N*, the number of mutant embryos examined, 30). This result shows that the migration defect in *Hem* mutant embryos is caused by the down-regulation of WAVE. Since Hem and WAVE normally form a complex, in the absence of Hem, WAVE, which is perhaps no longer in a complex, appears to be degraded.

Discussion

In this paper, we have shown that mutations in *Hem*, *WAVE* and *Abl* affect the migration of RP2 neurons in the developing embryonic VNC. Depending upon the strengths of the mutations, the cell either fails to migrate from its location of formation or mis-migrates across the midline into contralateral hemisegment. This defect is specific to RP2 but not its sibling cell. Indicating that proper migration is necessary for sending out its axonal projection, no axonal projection can be seen in those hemisegments where the RP2 had failed to migrate at all. Moreover, loss of migration in *Hem* mutants is due to a reduction in the levels of WAVE protein, and that Hem protects WAVE protein from degradation. While the loss-of-function for *Abl* gives the same migration defect as *Hem* or *WAVE*, and Abl mediates RP2 migration via WAVE, the effect of Abl on WAVE appears to be at regulating WAVE activity independent of Hem rather than the levels of WAVE. Our results also show that during the degradation of WAVE, Hem function is opposite to that of Abl and is downstream of Abl. These results provide novel insight into not only the problem of neuronal migration but also the functions of Hem and Abl in regulating WAVE during development.

Hem mediated regulation of neuronal migration

Several studies have suggested that Hem dynamically regulates polymerization of F- actin. Hem can play a crucial role in linking extracellular signals to the cytoskeleton. On the other hand, Hem is also part of the WAVE complex and it may regulate the activity of the WAVE complex to promote polymerization of F-actin. Our result that the migration defect in *Hem*

mutants can be completely rescued by expression of WAVE from a transgene (Fig. 8) indicates that Hem regulates neuronal migration via WAVE.

How Hem regulates WAVE is controversial. Eden *et al* (2002) argued that Hem (together with PIP212) inhibits WAVE in the WAVE complex. Upon activation by Rac1 or Nck, the WAVE complex dissociates, releasing an active WAVE-HSPC300 to mediate actin nucleation (Eden et al, 2002). This conclusion was also supported by the findings by Bogdan *et al* (2002) that loss-of-function for *Hem* leads to an excess of F-actin in the cytosol. Moreover, a reduction in the *WAVE* gene dosage suppressed axon guidance defects in *Hem* mutant embryos (Bogdan and Klambt, 2003). But, *in vitro* studies using *Drosophila* tissue culture cells argue that Hem protects WAVE from proteasome-mediated degradation (Kunda et al, 2003). Our *in vivo* results are consistent with these studies and show that WAVE is protected by Hem and the above alternate model may be incorrect.

Hem regulates RP2 migration via regulating the levels of WAVE

The WAVE protein was first identified in *Dictyostelium discoideum* as a suppressor of mutations in the cAMP receptor (SCAR) (Bear et al, 1998) but it is present in flies to humans. All WAVEs contain a N-terminal WHD/SHD (WAVE/SCAR homologue domain), a central proline-rich region and a C-terminal VCA domain. WAVE protein regulates actin polymerization by mediating the signal of Rac to Arp2/3 in lamellipodia. It is involved in forming branched and cross-linked actin networks. Unlike WASp proteins, which are intrinsically inactive by autoinhibition and activated by directly binding to Cdc42, PIP2 etc (Kim et al, 2000), WAVE appears to be intrinsically active, at least *in vitro* (Eden et al, 2002; Machesky et al, 1999; Miki et al, 2000). However, the majority of WAVE is in the "WAVE complex" with four other proteins: Hem, Sra-1/PIR121/CYFIP, Abi and HSPC300/Brk1.

In the WAVE-complex, direct association between WAVE, Abi and HSPC300 represents the backbone of the complex. Hem binds to Sra-1 forming a sub-complex, which is able to bind to Rac through Sra-1 (Bogdan et al, 2004; Kitamura et al, 1997). The interaction between Abi and Hem is what binds Hem and Sra-1 into the complex (Derivery et al, 2009; Ismail et al, 2009). Hem and Sra-1 are sequentially recruited to the WAVE complex (Derivery et al, 2008). Free subunits and assembly intermediates of the WAVE-complex are usually not detected but supposedly degraded (Derivery and Gautreau, 2010). Also, previous studies suggest that depletion of one component leads to degradation of others (Kunda et al, 2003; Rogers et al, 2003; Schenck et al, 2004). Indeed, our result that in *Hem* mutants, the level of WAVE protein, but not the *WAVE* gene transcription, is drastically reduced supports this contention. Perhaps in the absence of Hem, WAVE complex is either not formed or partially formed, resulting in the degradation of WAVE and phenotypes such as mis-migration of neurons. When the levels of WAVE are supplemented using a WAVE transgene (*UAS-WAVE*), the migration defect in *Hem* mutants is promptly rescued.

While a complete lack of WAVE (or Hem) function causes an arrest in the migration of RP2, a reduction in the levels of WAVE due to a reduction in the levels of Hem causes abnormal migration. For example, the lowest level of WAVE is seen in the Hem allele that has the strongest penetrance. Moreover, since this mis-migration defect is rescued by expressing WAVE from a transgene, we can conclude that this mis-migration is also due to an effect on WAVE. It has been suggested that the WAVE-complex exists cytoplasmically and in membrane-bound forms (Suetsugu et al, 2006; Steffen et al, 2004). Through an interaction with Rac, WAVE gets recruited to the lamellipodia where actin polymerization required for membrane protrusion is initiated and regulated. The integrity of the complex is critical for it's proper localization since removal of either WAVE or Abi prevents its translocation to the leading edge of the lamellipodia (Innocenti et al, 2004). It is possible

that a reduction in the levels of WAVE in *Hem* mutant embryos causes non-translocation of the WAVE complex to the membrane, causing a non/mis-migration of RP2.

Ableson Tyrosine Kinase and WAVE function

Our results show that WAVE protein exists as three different molecular weight forms (Fig. 6). Treatment of the extract with phosphatase collapses these three forms into a single band (Fig. 6C), indicating that WAVE protein is phosphorylated, with varying degrees of phosphorylation to yield different molecular weight species. We carefully examined if there are any changes in the three different forms/their relative contributions in *Hem* and *Abl* mutants. However, we did not find any changes in their relative contributions and the levels of all the forms are reduced in *Hem* mutants. Therefore, it may be that the reduction in all the forms, or that the reduction in one or two of the forms is responsible for the migration defect.

In *Abl* mutants, the level of WAVE is modestly increased, which is the opposite to that of the effect of *Hem* on WAVE. Thus, it seems more likely that the activity of WAVE is affected in *Abl* mutants. Being a protein kinase, it was possible that Abl phosphorylates WAVE, thus affecting either its activity or level. However, no significant changes in the relative levels of the different phosphorylated forms of WAVE were observed in *Abl* mutants. It has been shown *in vitro* that Abl is recruited to WAVE2 by Abi following cell stimulation, triggering the translocation of Abl together with the WAVE complex to the leading edge of the membrane (Leng et al, 2005; Stuart et al, 2006). Thus, Abl might affect WAVE activity, either directly or indirectly, via the translocation of the WAVE complex to the membrane of an actively migrating RP2. It is also possible that *Abl* affects migration in a pathway that does not involve WAVE.

On the other hand, the effect of loss-of-function for *Abl* on WAVE levels is more pronounced in older embryos. These results indicate that Abl directly or indirectly regulates the levels of WAVE. Furthermore, though modest, ectopic expression of Abl does down-regulate WAVE (Fig. 6D and 7D). Interestingly, our results also show that Hem regulation of WAVE levels is downstream of the Abl regulation of WAVE since the *Hem; Abl* double mutants had the same levels of WAVE as *Hem* single mutants. It seems likely that in the absence of Hem, WAVE protein gets degraded, resulting in the loss of migration/abnormal migration. Whereas in *Abl* mutants, the most likely scenario is that the activity of WAVE is affected, resulting in the same migration defect.

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Research highlights

1. Hem, via WAVE, mediates migration of neuronal cells in the ventral nerve cord.
2. In *Hem*, *WAVE* or *Abl*, RP2 neurons abnormally migrate from one hemisegment to the contralateral, or do not migrate at all.
3. Such neurons fail to send out axon projection signifying the importance of migration for connectivity.
4. Hem regulates neuronal migration through stabilizing WAVE.
5. While Abl negatively regulates the levels of WAVE, it regulates migration via regulating the activity of WAVE.

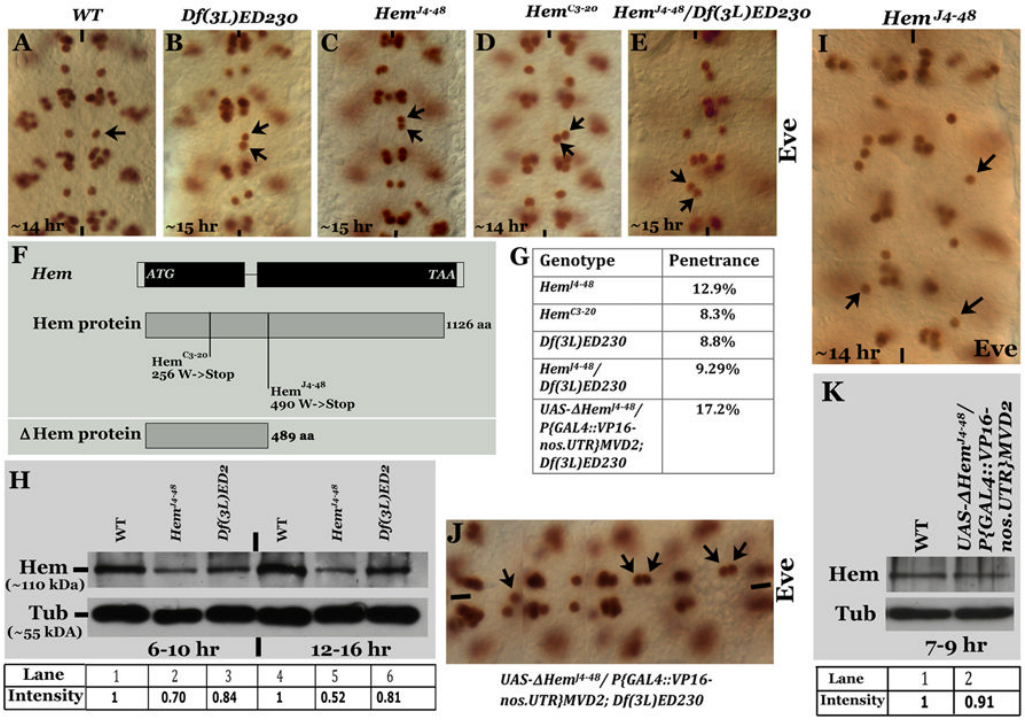

Figure 1. RP2 defect in Hem mutants

Embryos in panels A-E and I-J are stained with anti- Eve, anterior end is up in all Eve-stained panels except in panel J, where the anterior end is to the left. Midline is marked by vertical lines, RP2 is marked by arrow. **Panel A**: Wild type with one RP2 per hemisegment. **Panels B-E**: Different *Hem* mutant alleles. **Panel F**: Truncated Hem protein in the two *Hem* alleles and the truncated Hem produced in the transgenic *UAS*-D*Hem* line. **Panel G**: Table with penetrance of the RP2 defect in different mutants. The number of embryos examined is shown within the bracket. **Panel H**: Western analysis for Hem in wild type (WT), $Hem^{J4\text{-}48}$ and *Hem*-deficiency [Df(3L)ED2] embryos from 6-10 and 12-16-hrs of development. The intensity of the signals for Hem, normalized against the loading control Tubulin, is given below the Western. **Panel I**: $Hem^{J4\text{-}48}$ embryo with RP2s located in the position of its parent GMC-1. **Panel J**: *Hem*-deficiency embryo with the truncated *Hem* expressed from a *UAS*-D*Hem* transgene. This is a composite figure from three different photographs of the same embryo taken in different focal planes. **Panel K**: Western analysis of wild type embryos for wild type Hem expressing the truncated Hem from a *UAS*-D*Hem* transgene. The intensity of the signals for Hem, normalized against the loading control Tubulin, is given below the Western.

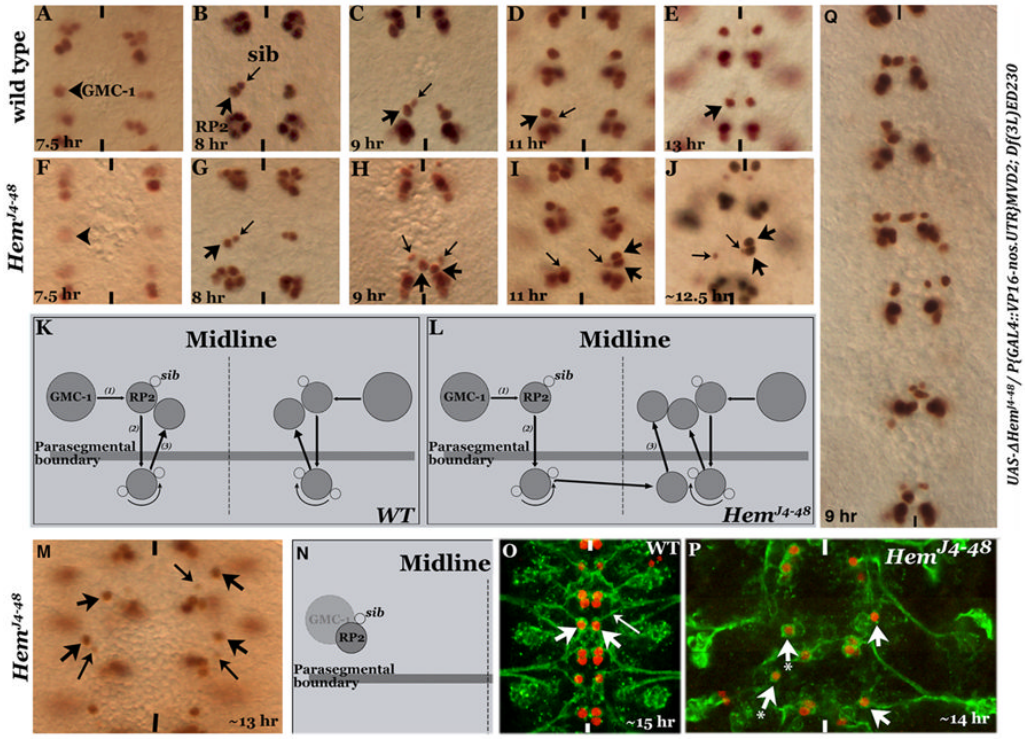

Figure 2. The RP2 defect in Hem mutants is due to aberrant migration

Embryos in panels A-J, M and Q are stained with anti-Eve, and in panels O and P are stained with anti-Eve and 22C10 antibodies. Anterior end is up, midline is marked by vertical lines. **Panels A-E**: Wild type embryos showing the 3-step RP2/sib migration. **Panels F-J**: *Hem* mutant embryos at the corresponding developmental stages. **Panels K and L**: Line drawings depicting RP2/sib migration in wild type and *Hem* mutants. **Panel M**: RP2 fails to migrate and is located at the position of its parent GMC-1. **Panel N**: Line drawing illustrating the migration defect. **Panel O**: Wild type embryo showing axon projection (22C10 positive) from an RP2, fasciculating with ISN. **Panel P**: *Hem* mutant embryo, RP2 fails to migrate from its location of formation; this RP2 has no discernible axonal projection (arrow-with-star). **Panel Q**: *Hem*-deficiency embryo with truncated *Hem* expressed from a *UAS*-D*Hem* transgene.

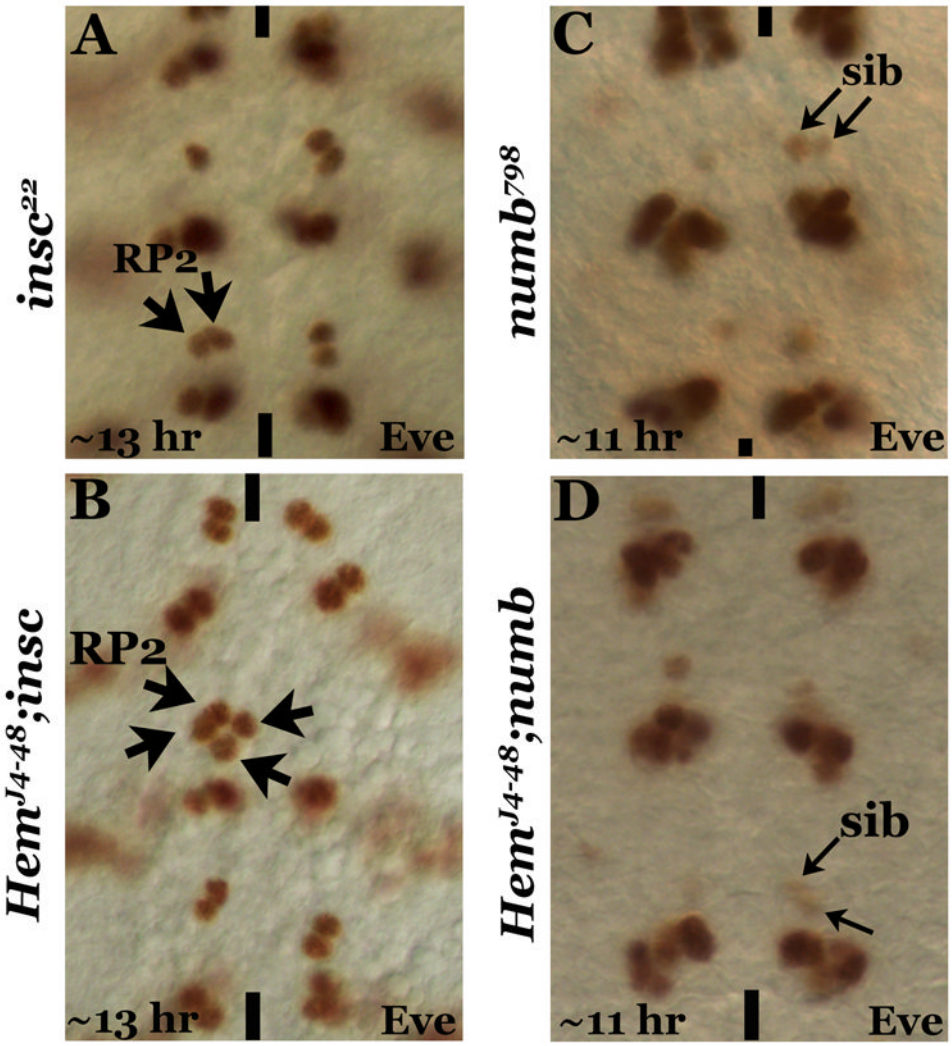

Figure 3. The migration defect is specific to RP2 but not its sib

Embryos are stained with anti-Eve, anterior end is up, midline is marked by vertical lines. **Panel A**: *insc* mutant with the duplication of RP2. **Panel B**: *Hem*$^{J4\text{-}48}$*; insc* double mutant showing four RP2s on one hemisegment and none on the contralateral hemisegment. **Panel C**: *numb* mutant showing the duplication of sib. **Panel D**: *Hem*$^{J4\text{-}48}$*; numb* double mutant showing the *numb*-phenotype without any sib migration across the midline.

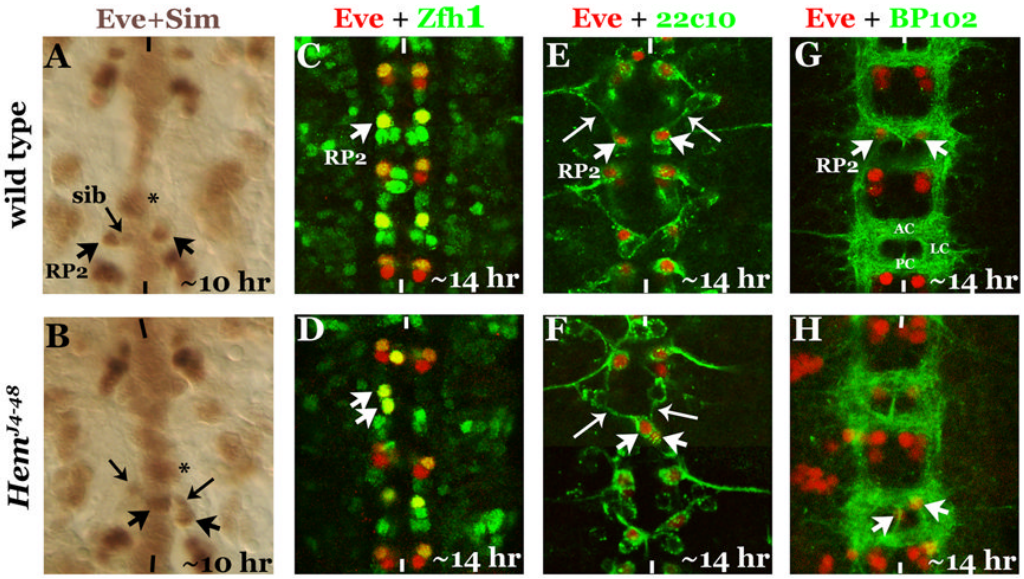

Figure 4. The migration defect in Hem mutants is not related to midline defects, identity changes or commissural defects

Anterior end is up, midline is marked by vertical lines. **Panels A, B**: Eve and Sim double-stained embryos. Star indicates a Sim-positive cell at the midline in the same focal plane as an RP2. The RP2 in the mutant is on the midline, its sib is in its normal location. **Panels C, D**: Embryos are double-stained with Eve and Zfh1. The mis-migrating RP2 expresses both Eve and Zfh1. **Panels E, F**: Embryos are double-stained with Eve and 22C10. **Panel G, H**: Embryos are double-stained with Eve and BP102. Although the commissural tracts in the mutant appear normal, the RP2 has crossed the midline in the mutant.

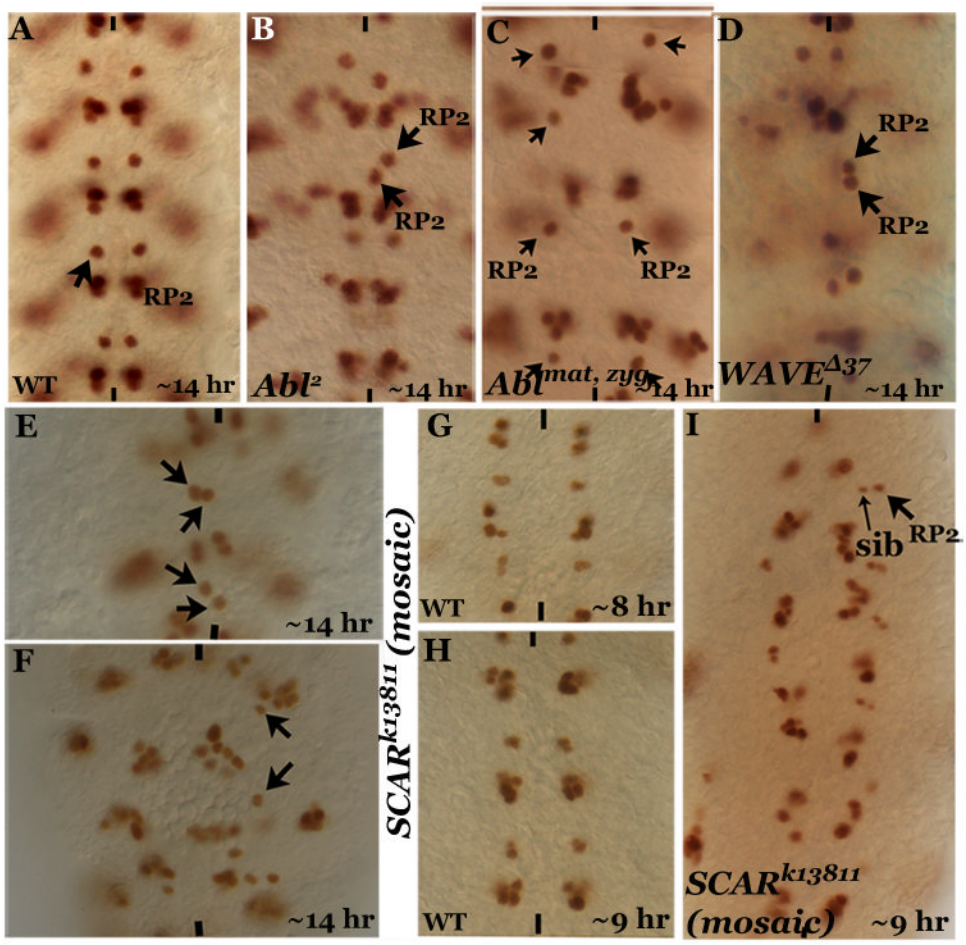

Figure 5. Hem-like RP2 migration defects in embryos mutant for Abl and WAVE

Embryos are stained with anti-Eve, anterior end is up, midline is marked by vertical lines. **Panel A**: Wild type with one RP2 per hemisegment. **Panel B**: *Abl* zygotic mutant showing the RP2 migration defect. **Panel C**: *Abl* maternal and zygotic mutant. Note that the RP2s fail to migrate from their position of formation and are placed farther apart. **Panel D**: WAVE zygotic mutant. **Panels E, F**: WAVE (also known as SCAR) zygotic and maternal mutants showing the abnormal midline crossing defect and failure of migration, respectively. **Panels G, H**: Wild type younger stage embryos. **Panel I**: WAVE zygotic and maternal mutant embryo, younger stage, showing the failure of migration of RP2 in an earlier stage.

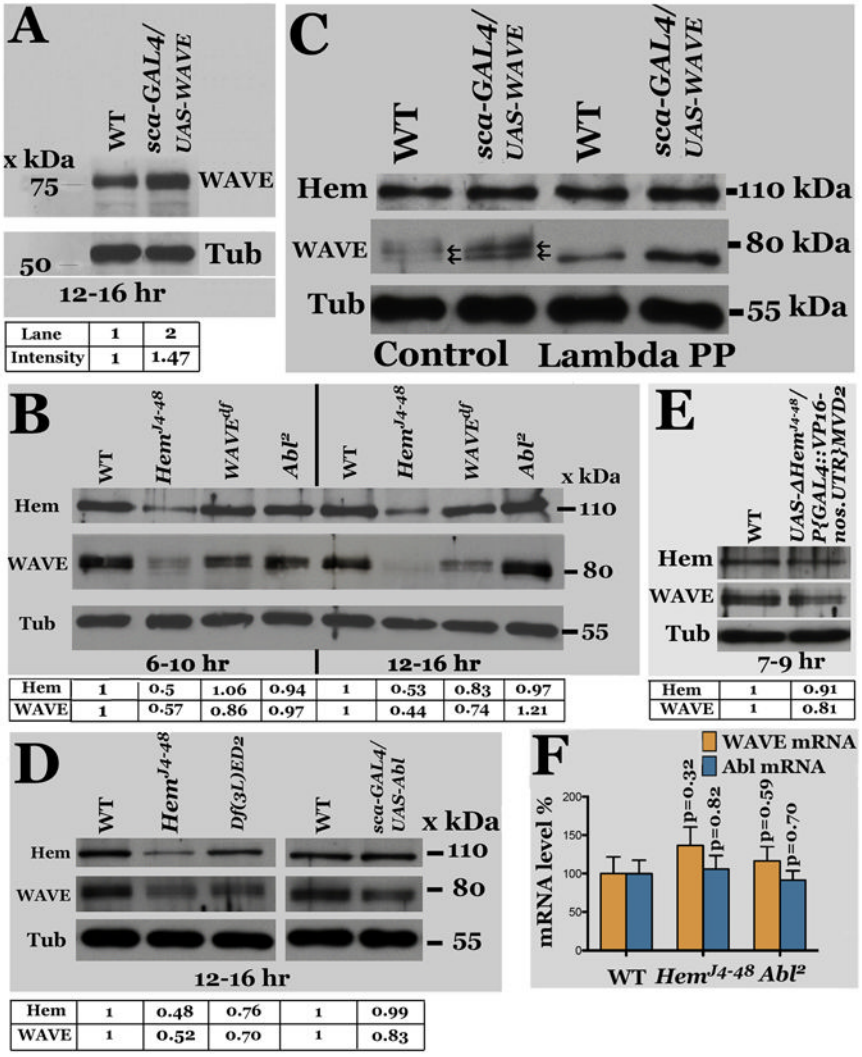

Figure 6. Regulation of WAVE by Hem

Panel A: Western analysis for WAVE using anti- WAVE antibody on extracts from 12-16 hr old wild type and sca-GAL4/UAS-WAVE embryos. **Panel B**: Western analysis for WAVE, Hem and Tubulin (Tub, as loading control) from 6-10 and 12-16-hr old wild type (WT), *HemJ4-48*, WAVE-deficiency, and *Abl2* embryos. **Panel C**: The WAVE protein is phosphorylated. Western analysis of untreated and Lambda phosphatase-treated embryo extracts for Hem and WAVE. **Panel D**: Western analysis of extracts from *Hem* and *Hem*-deficiency embryos for Hem and WAVE. **Panel E**: Western analysis for WAVE and Hem in wild type embryos expressing the truncated *UAS*-D*Hem* transgene. **Panel F**: qPCR for *WAVE* and *Abl* mRNA from 12-16-hr old *Hem* and *Abl* mutant embryos. The error bars indicate standard error, with corresponding p values (student t-test). The intensity of the signals for WAVE and Hem, normalized against the loading control Tubulin, is given below each Western.

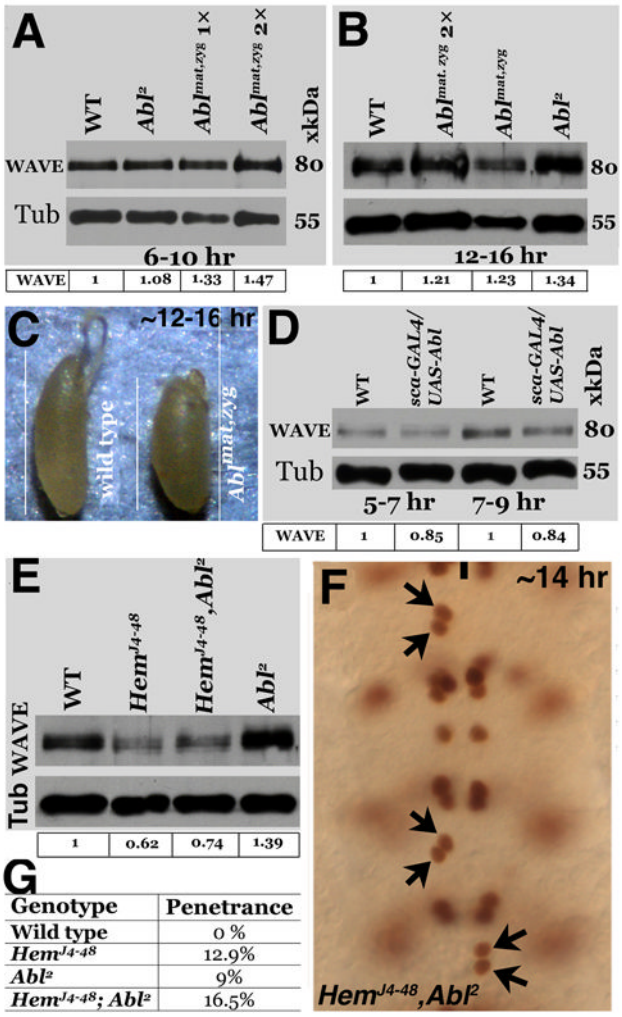

Figure 7. Hem and Abl independently regulate WAVE and the migration of RP2

Panel A: Western analysis of embryo extracts for WAVE. **Panels A and B**: Western analysis of extracts for WAVE from *Abl* zygotic and maternal and zygotic mutant embryos from 6-10- hrs and 12-16-hrs of age. **Panel C**: *Abl* maternal and zygotic mutant embryos are of small size compared to wild type. **Panel D**: Gain-of-function for *Abl* (*UAS-Abl X sca-GAL4*) only marginally reduces the levels of WAVE between 5-9-hrs of age (during the time of RP2 migration). **Panel E**: The levels of WAVE in wild type (lane 1), *Hem* single mutant (lane 2), *Hem, Abl* (zygotic) double mutant (lane 3) and *Abl* zygotic mutant (lane 4). **Panel F**: *Hem, Abl* zygotic double mutant embryo stained with Eve. **Panel G**: Table showing the penetrance of the RP2 mis-migration defect. The intensity of the signals for WAVE, normalized against the loading control Tubulin, is given below each Western.

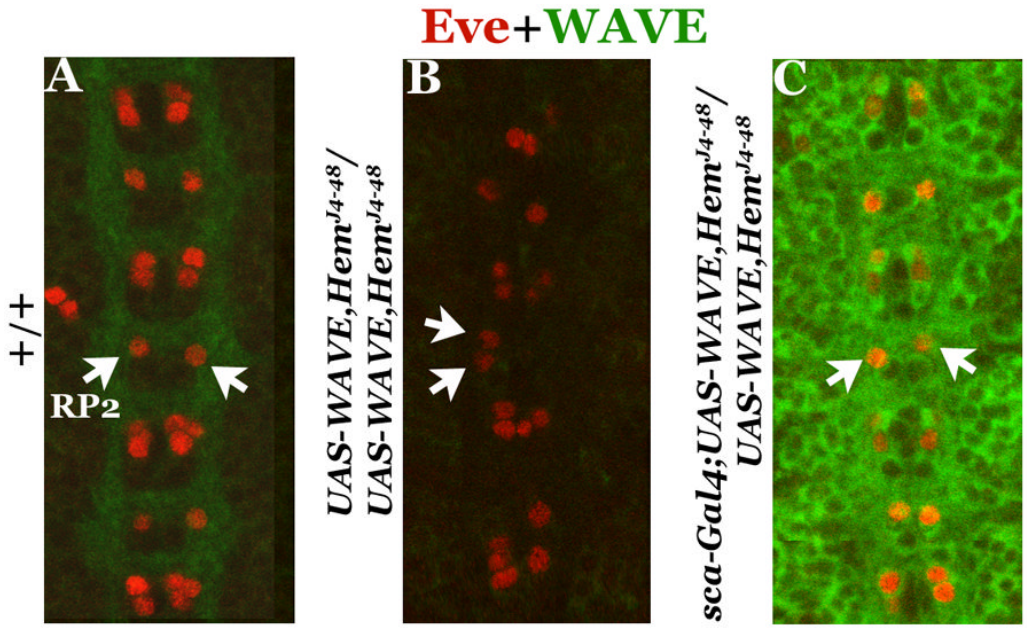

Figure 8. Hem mediates RP2 migration via WAVE

Embryos are double-stained with anti- Eve and anti-WAVE, anterior end is up, midline is indicated by vertical lines. **Panel A**: wild type. **Panel B**: *Hem* mutant that also carries the *UAS-WAVE* transgene (but not induced) on the same chromosome. **Panel C**: *Hem* mutant where the *UAS-WAVE* transgene is induced from the sca-GAL4 driver. Note that the level of WAVE is high and that the RP2 midline crossing migration defect is fully rescued.