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Drosophila Past1 is involved in endocytosis and is required for germline development and survival of the adult fly

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Summary

Endocytosis, which is a key process in eukaryotic cells, has a central role in maintaining cellular homeostasis, nutrient uptake, development and downregulation of signal transduction. This complex process depends on several protein-protein interactions mediated by specific modules. One such module is the EH domain. The EH-domain-containing proteins comprise a family that includes four vertebrate members (EHD1-EHD4) and one *Drosophila* ortholog, *Past1*. We used *Drosophila* as a model to understand the physiological role of this family of proteins. We observed that the two predicted *Past1* transcripts are differentially expressed both temporally and spatially during the life cycle of the fly. Endogenous Past1 as well as Past1A and Past1B, expressed from plasmids, were localized mainly to the membrane of *Drosophila*-derived cells. We generated mutants in the *Past1* gene by excising a P-element inserted in it. The *Past1* mutants reached adulthood but died precociously. They were temperature sensitive and infertile because of lesions in the reproductive system. Garland cells that originated from *Past1* mutants exhibited a marked decrease in their ability to endocytose fluorescently labeled avidin. Genetic interaction was found between *Past1* and members of the Notch signaling pathway, suggesting a role for Past1 in this developmentally crucial signaling pathway.

Key words: *Past1*, *Drosophila*, EHD, EH domain, Endocytosis

Introduction

Endocytosis in eukaryotes mediates a variety of key cellular processes, such as maintenance of homeostasis, development, uptake of nutrients and downregulation of signal transduction (Le Roy and Wrana, 2005; Mukherjee et al., 1997), which is highly regulated by a complex network of interacting proteins. One of the protein classes involved in endocytosis is the family of Eps15 homology (EH)-domain-containing proteins (Confalonieri and Di Fiore, 2002). It includes proteins such as Eps15, intersectin and the evolutionarily conserved EHD subfamily. The EHD proteins contain an N-terminal nucleotide-binding domain, a central coiled-coil module and a single C-terminal EH domain (Lee et al., 2005). There are four members of the EHD family in mammals, EHD1-EHD4 (Mintz et al., 1999; Pohl et al., 2000). EHD1 is localized mainly in the endosomal recycling compartment (ERC) (Mintz et al., 1999; Lin et al., 2001). However, it recycles from the plasma membrane, through coated pits, coated vesicles and early endosomes, to the recycling compartment (Rapaport et al., 2006). EHD2 resides in the plasma membrane (Blume et al., 2007; Daumke et al., 2007; George et al., 2007; Guilherme et al., 2004). EHD3 resides within the tubular structures of the recycling compartment (Galperin et al., 2002) or early endosomes (George et al., 2007; Naslavsky and Caplan, 2005), whereas EHD4 appears to be located in endosomal membranes (Blume et al., 2007; Shao et al., 2002; Sharma et al., 2008).

In mammals, the existence of several members in a protein family frequently constitutes a major hurdle for genetic and phenotypic analysis of their roles, because they often have partially redundant functions. Indeed, the EHD1-knockout mice we have generated, do not display any obvious mutant phenotype (Rapaport et al., 2006). Lower organisms, such as nematodes and fruit flies, constitute an ideal alternative because they often contain single members of the family and are highly amenable to genetic analysis. *Caenorhabditis elegans* and *Drosophila melanogaster* each has a single EHD ortholog termed *rme-1* and *Past1*, respectively (Grant et al., 2001; Smith et al., 2004). In the worm, individuals carrying either the G65R substitution (a mutation in the P-loop of the N-terminal domain) or the G429R mutation (in a residue close to the C-terminal EH domain) failed to recycle yolk receptors, which accumulated in endosomal compartments (Grant et al., 2001). A recent report demonstrated that the single *Drosophila* EHD ortholog *Past1* is expressed ubiquitously during early embryogenesis, exhibits both plasma-membrane-associated and punctate cytosolic staining, and is capable of binding, in vitro, to the adaptor protein Numb (Smith et al., 2004). Numb is a conserved membrane-associated protein that antagonizes Notch signaling. It binds to the intracellular domain of Notch and to α-adaptin. Numb-mediated inhibition of Notch appears to require α-adaptin function, suggesting that Numb is directly involved in targeting Notch for endocytosis (Berdnik et al., 2002).

The importance of receptor-mediated endocytosis has been demonstrated in Notch receptor signaling in the fly. In the sending cell, ligand endocytosis is mandatory for its presentation as an active

ligand that binds Notch on the plasma membrane of the receiving cell (Le Borgne, 2006; Le Borgne et al., 2005). In the receiving cell, Notch endocytosis leads to the activation of the cleaved Notch receptor and might lead to downregulation of the non-cleaved form of the receptor (Le Borgne, 2006; Le Borgne et al., 2005; Wilkin and Baron, 2005). After binding its ligand (Serrate or Delta in *Drosophila*), Notch undergoes two successive proteolytic cleavages. The first metalloprotease-dependent S2 cleavage generates an activated membrane-bound form, which is further processed by γ-secretase to release the Notch intracellular domain (Kopan, 2002). The intracellular domain is translocated into the nucleus, where, together with Suppressor of Hairless, it constitutes a transcription factor. Hairless sequesters Suppressor of Hairless and inhibits its DNA binding, thereby downregulating the function of the Notch Suppressor of Hairless complex as a transcriptional activator (Lecourtois and Schweisguth, 1997).

In the present study, we characterized the *Drosophila Past1* gene and its mutants. We observed that the two *Past1* gene transcripts, predicted by FlyBase (Drysdale and Crosby, 2005) (Release 5.6), are differentially expressed both temporally and spatially during the life cycle of the fly and the corresponding proteins are localized to the plasma membrane of cultured *Drosophila* cells. The *Past1* mutants we generated reached adulthood, but died precociously. They were temperature sensitive and infertile. Garland cells from homozygous mutant larvae exhibited a marked decrease in their ability to endocytose fluorescently labeled avidin. We showed a genetic interaction between *Past1* and both *Notch* and its repressor *Hairless*, suggesting a role for Past1 in the control of Notch signaling. Taken together, our results indicate that the *Drosophila* Past1 is involved in endocytosis and is required for germline development and survival of the adult fly.

Results

Structure and expression of *Past1*

Four EHD homologs exist in vertebrates, which regulate different stages of endocytosis (Naslavsky and Caplan, 2005; Blume et al., 2007). There is only one *Drosophila* EHD homolog (CG6148), designated *Past1* (putative achaete scute target 1), because it was originally cloned as a putative target of achaete scute (Drysdale and Crosby, 2005).

The *Drosophila* database predicts two *Past1* mRNA transcripts: RNA-A (2610 bp) and RNA-B (2679 bp), which encode two nearly identical proteins of 64 kDa (Past1A has 540 amino acids whereas Past1B has 6 amino acids fewer at the N-terminus of the protein) (see Fig. 1A) (Drysdale and Crosby, 2005). The predicted structure of the *Drosophila* Past1 protein is similar to that of the other members of the mammalian EHD family (Mintz et al., 1999), with an N-terminal domain, containing several conserved nucleotide-binding motifs (P-loop, NKxD, DxxG), a central coiled-coil region and a C-terminus containing an EH domain. Phylogenetic analysis indicates that of all the mammalian orthologs, Past1 is evolutionary closest to EHD2 (Fig. 1B) with 70% sequence homology at the protein level.

To explore the possibility of differential expression of the two *Drosophila* EHD (*Past1*) transcripts, RT-PCR was performed on RNA extracted from successive stages of development, using 5′

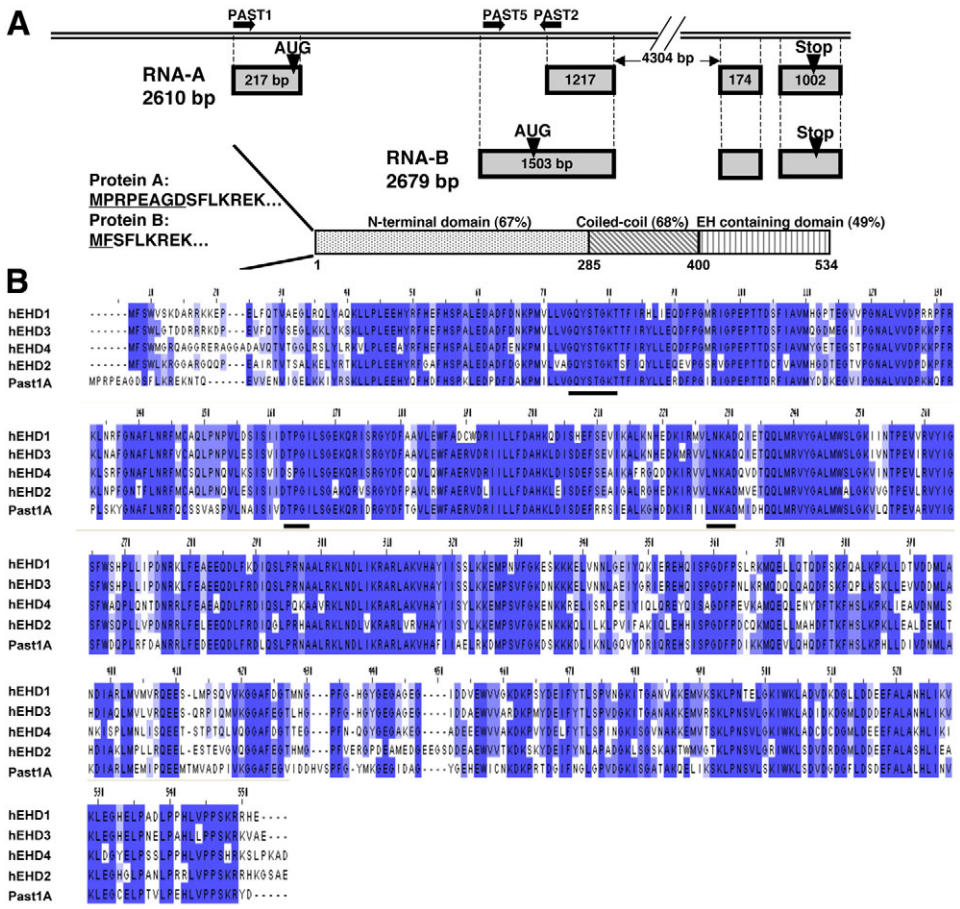


Fig. 1. Gene structure of *Past1* in wild-type *Drosophila* and homology with other members of the EHD family. (A) Schematic diagram of the *Past1* gene (not to scale) and its transcripts: RNA-A and RNA-B. Exons are depicted as gray-filled boxes with their respective nucleotide length denoted within. Primers used for PCR are depicted as arrows above the gene scheme. The N-terminal amino acid sequence of each transcript is specified below the scheme. The difference between the two predicted Past1 proteins is underlined. An illustration of the functional domains of the Past1 proteins and their percent identity to the human EHD2 protein domains is also shown below the gene scheme, with the number of amino acids in each domain. (B) Multiple alignment of the predicted amino acid sequences of the human EHDs and the *Drosophila* Past1A and Past1B. Accession numbers are as follows: hEHD1 (NP_006786); hEHD2 (NP_055416), hEHD3 (NP_055415), hEHD4 (NP_644670), Past1A (NP_731737), Past1B (NP_524332). Identical amino acids are shaded in dark blue, similar amino acids are shaded in light blue. Sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw) and BoxShade software (http://www.ch.embnet.org/software/BOX_form.html). Nucleotide-binding motifs (P-loop; DxxG; NKxD) are underlined.

primers specific for each transcript and a common 3′ primer (PAST1 for RNA-A, PAST5 for RNA-B and PAST2 for both) (Fig. 1A). The results indicated that although RNA-B is expressed throughout development as well as in adults in both sexes, RNA-A is expressed only from the third larval stage onwards, and only in males (Fig. 2A). In males, the expression of RNA-A is restricted to the testes (Fig. 2B). Since *tudor* males, which lack germ cells (Boswell and Mahowald, 1985), do not express RNA-A (Fig. 2C), this RNA species is germ-cell specific.

Western blot analysis performed on lysates of larvae, adult males and females as well as cultured *Drosophila* cells, using polyclonal anti-recombinant-Past1 serum (for details see Materials and Methods), indicated that all contain a Past1 protein (Fig. 2D). Taken together, these results show that Past1 is expressed throughout the life cycle of the fly. However, the two transcripts have a different pattern of expression.

Localization of Past1

Immunofluorescence studies were used to unravel the intracellular localization of endogenous Past1 using anti-Past1 serum (see Materials and Methods). In *Drosophila*-derived SR+ Schneider cells there was mainly plasma-membrane staining of Past1, as detected by colocalization with myristylated RFP, a marker that stains primarily the plasma membrane (Fig. 3). There was little colocalization with GFP-rab5, a marker for early endosomes, or GFP-rab11, a marker for the ERC (Mohrmann and van der Sluijs, 1999). In the same cells transfected with UAS-GFP-Past1A or UAS-GFP-Past1B and the transcription activator actin-GAL4 vector, colocalization was detected between both GFP-Past1 isoforms and myristylated RFP (data not shown). Thus, Past1A and Past1B appear to associate mainly with the plasma membrane.

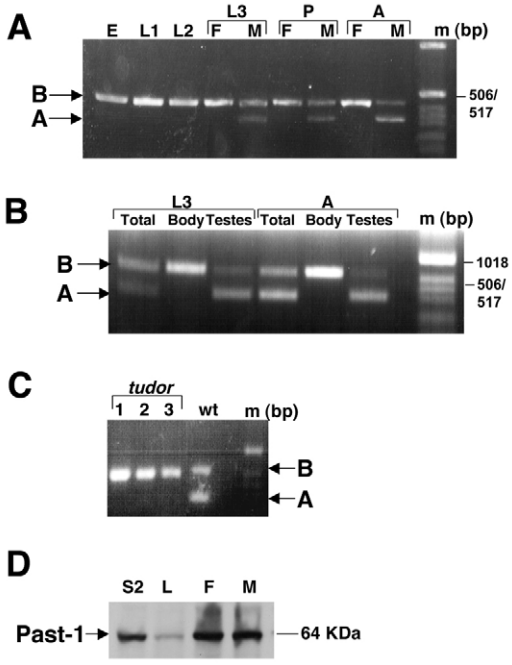


Fig. 2. *Past1* gene expression in wild-type *Drosophila*. (A) RNA from embryos (E), larvae from instars 1-3 (L1-3), pupae (P) and adult flies (A), both males (M) and females (F), was amplified using 5′ primers specific for each transcript (primer PAST1 for RNA-A and primer PAST5 for RNA-B), and a common 3′ primer (primer PAST2). PCR products were separated through a 1.2% agarose gel and visualized by ethidium bromide staining. (B) RNA from total body (Total), body excluding testes (Body), and testes from male third instar larvae (L3) and adults (A) was amplified and separated as described in A. (C) RNA from one wild-type adult male (wt) and three individual *tudor* mutant males (1-3) was amplified and separated as in A. (D) Lysates from S2 Schneider cells, larvae (L), adult female (F) and male (M) flies were separated by 10% SDS-PAGE and the corresponding immunoblot was reacted with rabbit anti-Past1 serum. Detection was performed with HRP-conjugated goat anti-rabbit antibodies.

Characterization of *Past1* mutants

To elucidate the physiological role of Past1, we generated *Drosophila* mutants by mobilizing a homozygous viable P transposable element insert (EY01852), located in the first intron of RNA-A and within the 5′ UTR of RNA-B (Fig. 4A). Imprecise excision of the P-element produced four independent lines that harbored deletions in the *Past1* gene. The excision of the P-element and the extent of each deletion were verified by PCR and sequencing. As seen in Fig. 4B, line $Past1^{60\text{-}4}$ carries a deletion of 1463 bp 3′ to the P-element insertion site, disrupting the second exon of RNA-A and the first exon of RNA-B. Line $Past1^{88\text{-}1}$ harbors a deletion of 776 bp 5′ to the insertion site, affecting the first exon of RNA-A and the 5′UTR of RNA-B (in this line 55 nucleotides from the 3′ end of the P-element are retained) (see Fig. 4B). Lines $Past1^{55\text{-}1}$ and $Past1^{110\text{-}1}$ have 1150 bp and 1741 bp deletions, respectively, spanning both sides of the insertion site and affecting both transcripts (Fig. 4B).

Mutants homozygous for the deletions in all four lines developed at a rate ~20% slower than their heterozygous siblings. They reached adulthood but died precociously, three to five days after eclosion, whereas their siblings or wild-type control flies lived for over a month. This phenotype was not complemented by the chromosomal deletion Df(3R)kar-Sz37, which removes polytene bands 87C5-87D14 including band 87C6, where *Past1* resides. There was no

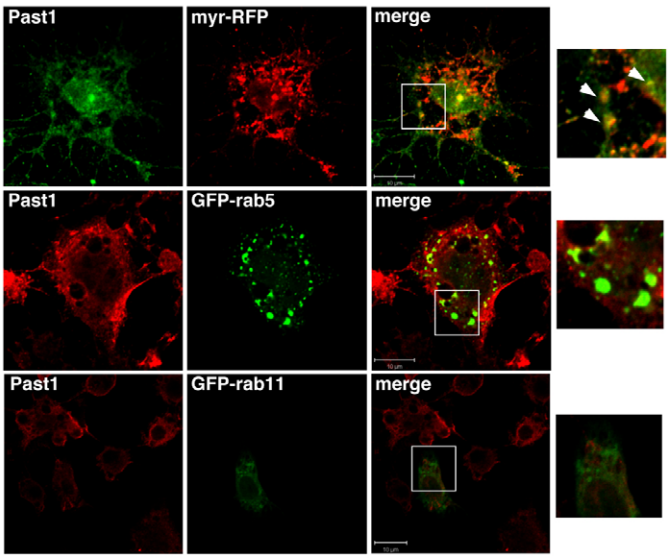


Fig. 3. Intracellular localization of Past1. SR+ cells, grown on coverslips in 24-well plates, were transfected for 48 hours with 1 μg UAS-GFP-rab5, or UAS-GFP-rab11 or UAS-myr-RFP and actin-GAL4 and fixed. The cells were stained with anti-Past1 antibodies and interacted with Cy-3 secondary goat anti-rabbit antibodies when the marker was coupled to GFP, or Cy-2-goat anti rabbit antibodies for RFP. The cells were mounted and scanned using the LSM Meta confocal microscope. The boxes indicate areas enlarged in the panels on the right. Arrows indicate plasma-membrane localization of Past1. Scale bars: 10 μm.

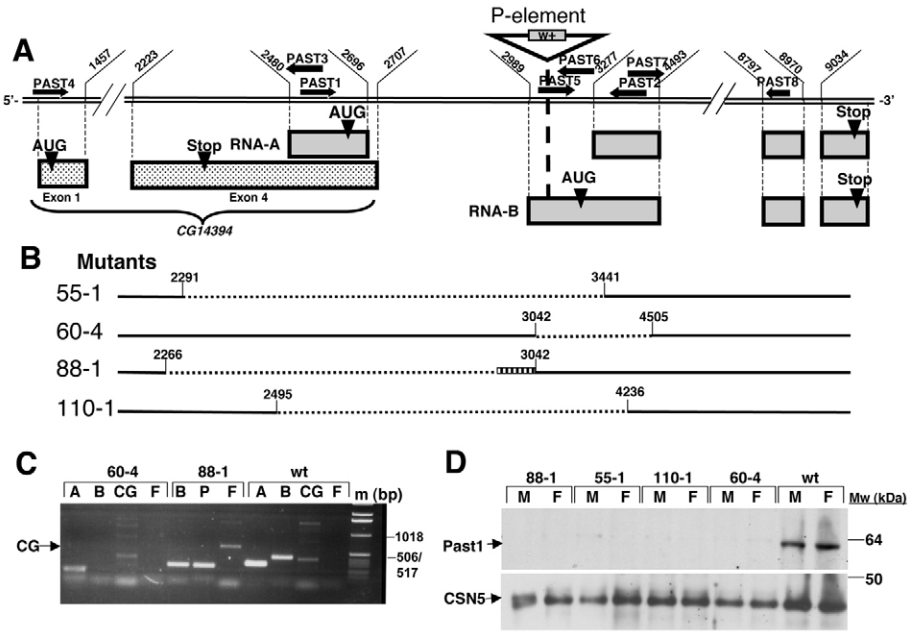


Fig. 4. Analysis of *Past1* mutants. (A) Schematic diagram of the *Past1* gene and its transcripts and transcript of *CG14394*, depicted as dot-filled boxes (according to FlyBase R5.6, March 2008). Nucleotide numbers above the scheme indicate the position of the exons. Primers used for PCR are shown as arrows above the gene scheme. The site of the P-element insertion is depicted by a thick dashed line. (B) Schematic diagram of the deletions in the four mutants. The names of the mutants appear on the left. The dotted lines represent the extent of the deleted region. The dashed box in line $Past1^{88\text{-}1}$ represents the residual 55 nucleotides from the 3′ end of the P-element. (C) RT-PCR analysis of *Past1* expression in the deletion mutants. Wild-type male (wt) cDNA was amplified with primers PAST1 and PAST2 for RNA-A (A), primers PAST5 and PAST2 for RNA-B (B), primers PAST4 and PAST3 for the *CG14394* transcript (CG), and primers PAST4 and PAST2 for the fused transcript of *Past1* and *CG14394* (F). cDNA of male mutant $Past1^{88\text{-}1}$ (88-1) was amplified using primers PAST6 and PAST2 for *Past1* RNA-B (B), primers PAST7 and PAST8 for RNA-(A+B) (P) and primers PAST4 and PAST2 for the fused transcript of both genes (F). cDNA of male mutant $Past1^{60\text{-}4}$ (60-4) was amplified using primers PAST1 and PAST8 for *Past1* RNA-A (A), primers 1B and 3A for RNA-B (B), primers PAST4 and PAST3 for *CG14394* RNA (CG) and primers PAST4 and PAST8 for the fused transcript (F). (D) Lysates from normal adult male (M) and female (F) flies (wt) and from the four deletion mutants, $Past1^{60\text{-}4}$ (60-4), $Past1^{110\text{-}1}$ (110-1), $Past1^{55\text{-}1}$ (55-1) and $Past1^{88\text{-}1}$ (88-1), were separated by 8% SDS-PAGE and the corresponding immunoblot was reacted with rabbit anti-Past1 serum. Membranes were reblotted with rabbit anti-CSN5 antibodies (Freilich et al., 1999) to determine total protein level. Detection was performed with HRP-conjugated goat anti-rabbit antibodies.

complementation between the different mutants, which is to be expected if they affect the same gene.

Expression of *Past1* RNA in the mutant flies was analyzed by RT-PCR, using primers flanking the genomic deletion in each mutant line and sequencing the resultant PCR products. In each line, we found transcripts corresponding to the mutant gene. In line $Past1^{60\text{-}4}$, only RNA-A was expressed (Fig. 4C) and only in males (not shown). However, this RNA had a frame shift, which precluded the production of a corresponding protein. Interestingly, in the mutant lines $Past1^{55\text{-}1}$, $Past1^{88\text{-}1}$ and $Past1^{110\text{-}1}$ (but not in line $Past1^{60\text{-}4}$ or the wild type) the *Past1* transcript was fused to RNA expressed from the upstream gene (*CG14394*), which is an unstudied gene (Fig. 4A,C). Since the open reading frame derived from RNA-B in mutant line $Past1^{88\text{-}1}$ remained intact, the possibility existed that Past1 protein is expressed in this mutant, provided there is an internal ribosome entry site (IRES) between the *CG14394* and *Past1*

Table 1. Temperature sensitivity of *Past1* mutants

A

Mutant	Proportion of homozygotes at 25°C*	Proportion of homozygotes at 29°C*
60-4	16%	2%
110-1	24%	9.5%

B

Temperature	Genotype of 60-4 mutants	% Larvae surviving to pupae	% Pupae surviving to adulthood
25°C	Heterozygous	100	92
25°C	Homozygous	100	89
29°C	Heterozygous	88	86
29°C	Homozygous	96	46

The proportion of homozygotes in the population of two mutant lines grown at 25°C or 29°C is shown in A. The difference between the survival rate at the two temperatures was found to be statistically significant ($P<0.001$). *Expected proportion of homozygotes in the population is 33%. The proportion of larvae surviving to pupae and of pupae surviving to adulthood from homozygous mutants and heterozygotes of line 60-4 grown at 25°C or 29°C is shown in B. The difference between pupal survival to adulthood of heterozygous and homozygous mutants at 29°C was found to be statistically significant ($P<0.000004$).

ORFs in the fused RNA. Similarly, the sequence of the ORF derived from the fused transcript in mutant *Past1*$^{55\text{-}1}$ suggested that a chimeric protein might be produced that lacks the 56 N-terminal amino acids of Past1. In the *Past1*$^{110\text{-}1}$ mutant, the sequence of the fused RNA harbors stop codons in all three reading frames, precluding translation of a protein from this transcript. It is worth noting that the mutant line *Past1*$^{60\text{-}4}$ has a normal sequence of *CG14394* whereas in *Past1*$^{110\text{-}1}$ its ORF is not affected.

To examine whether the mutants indeed produce Past1 protein, western blot analysis was performed on lysates of adult flies from the wild type and the four mutant lines. As evident from Fig. 4D, none of the mutants produced Past1 protein. This suggests that no functional IRES exists in the transcript of mutant *Past1*$^{88\text{-}1}$, and that if a chimeric protein is produced in line *Past1*$^{55\text{-}1}$ it is highly unstable.

Phenotype of *Past1* mutants

A characteristic of several endocytic mutants in *Drosophila* (e.g. *shibire*, *stoned*, *comatose*) is their temperature sensitivity (Poodry and Edgar, 1979; Grigliatti et al., 1973; Siddiqi and Benzer, 1976). To examine whether the survival of the *Past1* mutants is affected by temperature, the balanced strains of the *Past1*$^{60\text{-}4}$ and *Past1*$^{110\text{-}1}$ mutants, which contain both homozygotes and heterozygotes, were grown throughout their lifetime at either 25°C or 29°C. The number of homozygous and heterozygous offspring in each strain was counted daily. As evident from the results (Table 1A), the proportion of the homozygotes in the population of each mutant strain tested was lower than the expected ratio of 33%. In both lines, the proportion of the homozygous mutants was significantly lower at 29°C than 25°C, regardless of the sex of the flies. For example, the proportion of *Past1*$^{60\text{-}4}$ homozygotes was 2% at 29°C versus 16% at 25°C (Table 1A). Survival appeared to be impaired at the high temperature even before adulthood, because monitoring the development of *Past1*$^{60\text{-}4}$ homozygotes from the first larval stage onwards (see Materials and Methods) revealed that only about 50% of the homozygotes that reached the pupal stage succeeded to eclose (Table 1B). The fact that in the *Past1*$^{60\text{-}4}$ strain itself, only 2% reached adulthood probably reflects strong competition with their heterozygous siblings in the relatively crowded bottles (see Materials and Methods). Similar results were observed for the transheterozygotes *Past1*$^{60\text{-}4}$/*Past1*$^{110\text{-}1}$ generated by crossing the two mutant strains to each other (data not shown). These findings led us to conclude that the *Past1* mutants are sensitive to high temperature. The fact that all mutant lines presented the same phenotype argues that it was due to the absence of Past1 (and not CG14394).

The fertility of *Past1* mutants was compromised as demonstrated by the reduced number of offspring of single pair-matings of females and males from two mutant lines, *Past1*$^{60\text{-}4}$ and *Past1*$^{110\text{-}1}$, with wild-type mates (Fig. 5A). For example, the mean number of offspring from *Past1*$^{60\text{-}4}$ females was about 40 times lower than that obtained from the control females.

To test whether the partial sterility of the *Past1* mutants was due, at least in part, to defects in their reproductive system, we examined their testes and ovaries. The testes of the *Past1* mutants (e.g. *Past1*$^{60\text{-}4}$/*Past1*$^{110\text{-}1}$) were much thinner than those of the wild type, and contained significantly fewer bundles of elongating and elongated cysts (Fig. 5B). Nevertheless, we could not detect any gross defects in *Past1* mutant pre-meiotic spermatocytes and post-meiotic round spermatids. During spermatid terminal differentiation, a process known as spermatid individualization takes place. It involves extrusion of the bulk cytoplasm of the elongated spermatids into a waste bag in an apoptosis-like process that results in highly compact, viable sperm (Arama et al., 2003). The majority of mutations that affect male fertility cause defects during the spermatid individualization process (Wakimoto et al., 2004). Therefore, we examined the *Past1* mutant elongated spermatids during their terminal differentiation process. For this, we stained wild-type and mutant testes for active caspase-3, which is expressed in the

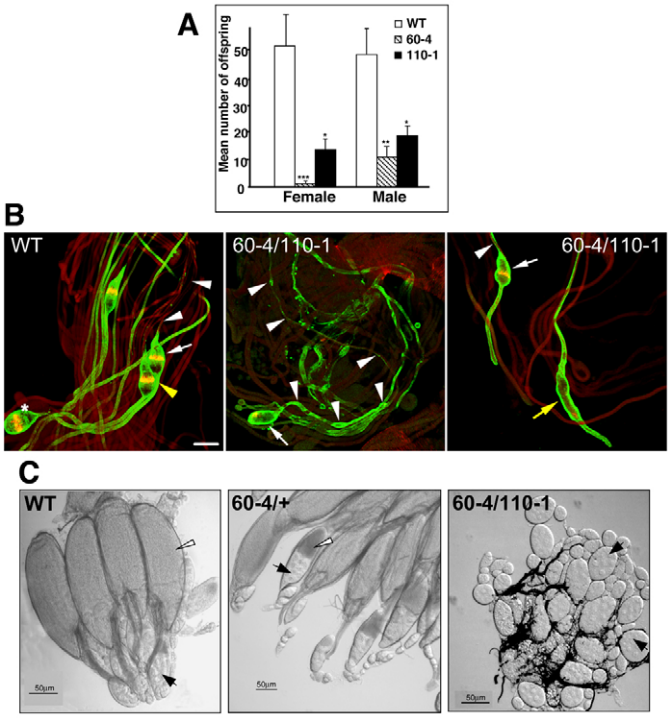


Fig. 5. Fertility of *Past1* mutants is compromised. (A) Histogram depicting the mean ± s.e. of the number of offspring counted from single pair of females or males of the following three genotypes to wild-type mates: wild-type control (wt, open bars n=13 females and n=14 males); homozygous mutant *Past1*$^{60\text{-}4}$ (dashed bars n=13 females and n=16 males); homozygous mutant *Past1*$^{110\text{-}1}$ (black bars n=16 females and n=17 males). *P<0.05; **P<0.01; ***P<0.005 compared with control. (B) Squashed testes from wild-type and transheterozygous *Past-1*$^{60\text{-}4}$/*Past-1*$^{110\text{-}1}$ mutants were stained with anti-effector caspase antibody (CM1) to label the cytoplasm (green) and phalloidin to bind F-actin, which constitutes the individualization complex (orange/yellow). In the wild type, the spermatid extruded cytoplasm, which was removed through the caudal movement of the individualization complex (from top to bottom) is contained within the oval-shaped cystic bulge (white arrow), which eventually becomes a waste bag (asterisk). Importantly, CM1 staining is absent from the post-individualized portion of the spermatids (white arrowheads above the cystic bulge), whereas it is still apparent in the pre-individualized portion (yellow arrowhead). In *Past1* transheterozygotes the cystic bulges and waste bags are frequently reduced in size (white arrows) or appear flat (yellow arrow) because of a failure in the appropriate collection of the cytoplasm of the spermatids. The retained cytoplasm is clearly visualized as a 'trail' of residual cytoplasm (marked with the green CM1 staining) along the entire length of the post-individualized portion of the spermatids (white arrowheads following the 'cytoplasmic trails' of one spermatid bundle). Note that in wild-type testes, 'cytoplasmic trails' do not exist in the post-individualized portion of the spermatids. Scale bar: 50 µm. (C) Ovaries from wild-type (WT), heterozygous, *Past1*$^{60\text{-}4}$/+ and transheterozygous (*Past1*$^{60\text{-}4}$/*Past1*$^{110\text{-}1}$) females, photographed under a light microscope. Although egg chambers are fully developed and contain yolk (white arrowhead) in wild-type and heterozygous ovaries, mutant ovaries have egg chambers that have reached only stage 8 and all lack yolk. Arrows indicate nurse cells.

cytoplasm of individualizing spermatids and with Phalloidin, which binds to actin cones in the individualization complex (Fig. 5B). *Past1* mutants displayed defects during the spermatid individualization process. More specifically, although an individualization complex appeared to assemble normally in the vicinity of the nuclei, *Past1* mutant spermatids failed to extrude their entire cytoplasm into a waste bag, leaving trails of cytoplasm in what should have been the post-individualized region of the spermatids (marked by active caspase-3) (Fig. 5B). Consequently, *Past1* cystic bulges and waste bags were reduced in size or appeared flat (Fig. 5B). Additionally, although the nuclei of the wild-type spermatids bundled at one edge (Arama et al., 2003), the nuclei of some of the *Past1* mutant spermatids appeared scattered (data not shown). According to these results, semi-sterility of the *Past1* mutant males is expected.

The ovaries of mature mated homozygous mutants as well as transheterozygous females were highly abnormal compared with ovaries of mated wild-type and heterozygous females of the same age (Fig. 5C; Table 2). They were much smaller, less organized and contained fewer well-differentiated oocytes (stages 12-14) than those in the control. For example $Past1^{110\text{-}1}$ homozygotes contained on average 5.5 egg chambers at stage 9-11 and only 1.6 fully developed chambers (stage 12-14), whereas the control heterozygous siblings contained 7.7 egg chambers and 9.8 fully developed chambers. Transheterozygotes displayed a similar, although less severe, abnormality. In addition, whereas yolk deposition into the oocyte was evident in all egg chambers beyond stage 8 during the normal oogenesis seen in heterozygotes, in the *Past1* mutants, all oocytes from stage 9 onwards were devoid of yolk. This indicates that the process of oogenesis is highly impaired in *Past1* mutants. Taken together, our results imply that *Past1* is required for the development of germ cells in *Drosophila*.

Genetic interaction

To gain insight into the role of Past1 in *Drosophila* development, we performed crosses between *Past1* mutants and mutants of the Notch signaling pathway. The Notch signaling pathway, extensively studied in *Drosophila*, is pivotal for many developmental processes and is regulated by endocytosis (Le Borgne et al., 2005; Le Borgne, 2006; Wilkin and Baron, 2005). We found a genetic interaction between *Past1* and *Notch* in flies carrying either the $Past1^{60\text{-}4}$ or $Past1^{110\text{-}1}$ homozygous mutation in addition to the *Notch* $N^{md\text{-}1}$ allele (hemizygous males and homozygous females). The wing scalloping of $N^{md\text{-}1}$ is thought to be due to reduced Notch signaling (Royet et al., 1998). The $N^{md\text{-}1}$; $Past1^{-/-}$ ($Past1^{60\text{-}4}$ or $Past1^{110\text{-}1}$ homozygotes) flies reached adulthood at a very low frequency, died after 1 day and displayed a more severe wing notching phenotype than the $N^{md\text{-}1}$ mutant alone (Table 3A), although wings of *Past1* homozygous mutants were completely normal. Genetic interaction was also found between *Past1* and *Hairless* mutants. Hairless is a repressor of the Notch signaling pathway. Its binding to Suppressor of Hairless inhibits the DNA binding and transcription activation by the Notch Suppressor of Hairless complex. Double heterozygotes of the dominant loss-of-function mutant H^2 and either $Past1^{60\text{-}4}$ or $Past1^{110\text{-}1}$ showed a less defective wing venation phenotype than the H^2 mutant alone (Table 3B). The occurrence of shortened L2 and L4 longitudinal veins was much lower (6%) in wings of the double heterozygotes than in wings of H^2 heterozygotes (36%) (Table 3B). *Past1* heterozygotes had normal wing vein venation. These results implicate Past1 in the control of developmental processes associated with the Notch signaling pathway.

Table 2. Ovarian defects in *Past1* mutants

Genotype	Number of ovaries examined	Stage 9-11	Stage 12-14
$Past1^{110\text{-}1}/Past1^{110\text{-}1}$	14	5.5±4.45 No yolk	1.64±1.33 No yolk
$Past1^{60\text{-}4}/Past1^{110\text{-}1}$	15	4.26 ±2.37 No yolk	5.53±3.31 No yolk
$Past1^{-}/Past1^{+}$	11	7.72 ±2.3 Full yolk	9.8±3.78 Full yolk

Ovaries were dissected from 5-day-old mated females and the number of egg chambers at the different stages of oogenesis was recorded, as well as the state of yolk deposition. Values are mean ± s.d.

Role of Past1 in endocytosis

The *Drosophila* Past1 has four mammalian homologs, EHD1-EHD4. EHD1, the most studied EHD, is localized mainly to the ERC and regulates recycling. Expression of its dominant-negative mutants in tissue culture cells, its RNAi, or its absence in animal models led to attenuated recycling (George et al., 2007; Naslavsky et al., 2004; Rapaport et al., 2006). EHD2 is a plasma-membrane protein that controls actin cytoskeleton reorganization. Its overexpression results in a marked decrease of internalization (Guilherme et al., 2004) (S.B. and M.H., unpublished). EHD3 regulates trafficking from the early endosomes to the ERC (Naslavsky et al., 2006) whereas EHD4 has been shown recently to control trafficking at the early endosomes and regulates exit of cargo toward the recycling compartment and the late endocytic pathway (Sharma et al., 2008).

To test whether Past1 has a role in endocytosis, we assessed the ability of normal and mutant Garland cells to endocytose fluorescently labeled avidin. Larval Garland cells are similar to nephrocytes, have a rapid rate of fluid phase endocytosis, and are an established system for the study of endocytic dynamics (Chang et al., 2002; Dermaut et al., 2005; Kosaka and Ikeda, 1983). We first tested the ability of normal Garland cells to endocytose Texas-red-labeled avidin. We did so by incubating wild-type Garland cells with Texas-red-avidin, after which the cells were transferred onto ice and incubated for 5 minutes with fluorescein-labeled avidin, to allow its binding to the membrane. As shown in Fig. 6 and Table 4, fluorescein-labeled avidin marked the membrane whereas Texas-red-avidin staining was seen inside the cells. We then tested the ability of *Past1* mutant Garland cells to endocytose fluorescently labeled avidin. As shown in Fig. 6 and Table 4, avidin was readily endocytosed by all wild-type Garland cells. However, Garland cells isolated from *Past1* mutant larvae displayed attenuated endocytosis. Mutant Garland cells could be divided into three groups: cells that endocytosed avidin similarly to wild-type cells; cells that presented reduced endocytosis of avidin, and cells that did not display any endocytosis. Our results strongly suggest that Past1 is directly involved in endocytosis.

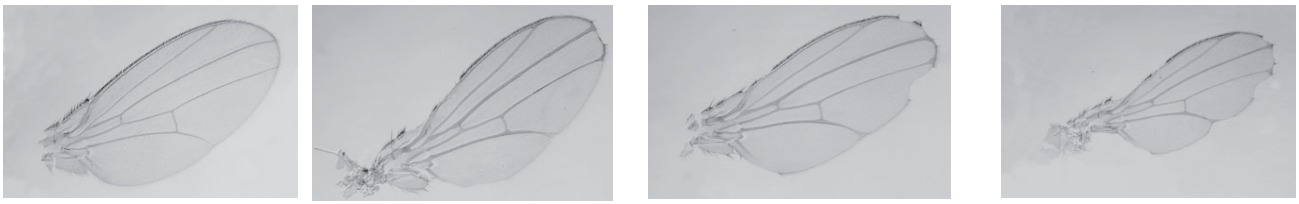
Discussion

We used *Drosophila* as a model to study the EHD homolog, *Past1*. Two transcripts, encoding two nearly identical proteins, are transcribed from the *Past1* gene. One transcript is expressed throughout development, as well as in adults, in both sexes; the other transcript is expressed only from the third larval stage onwards, and only in the male testes. The differential expression of these two transcripts suggests that they are under distinct control. Although the regulatory regions of the gene remain to be identified,

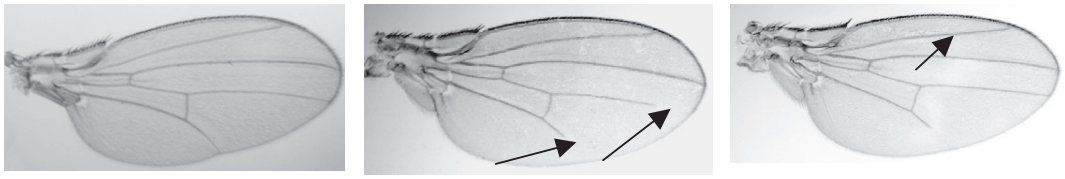
Table 3. *Past1* genetically interacts with *Notch* and *Hairless*

A

Wild type | N^{nd-1} | N^{nd-1};$Past1^{60-4}$/$Past1^{60-4}$ | N^{nd-1};$Past1^{1101}$/$Past1^{110-1}$



B



Genotype	Normal	L5 and (L4 or L2)	L5
$Past1^{60-4}$/+(*n*=100)	*n*=100 (100%)	0	0
H^2/+(*n*=140)	0	*n*=50 (36%)	*n*=90 (64%)
H^2 +/+ $Past1^{60-4}$(*n*=173)	0	*n*=10 (6%)	*n*=163 (94%)

Wing preparations of a wild-type wing, a *Notch* (N^{nd-1}) male wing and a double mutant male wings (N^{nd-1};$Past1^{60-4}$/$Past1^{60-4}$ or: N^{nd-1};$Past1^{110-1}$/$Past1^{110-1}$). Wings of homozygous and transheterozygous *Past1* mutants are normal, as in wild-type flies, shown in part A. The number of wings counted in the population of *Hairless* heterozygotes (H^2/+) and *Hairless* $Past1^{60-4}$ double heterozygotes (H^2+/+$Past1^{60-4}$) that have wild-type wings ($Past1^{60-4}$/+) or defective L5, and (L2 or L4) wing veins (H^2,+) or only a defective L5 wing vein (H^2+/+$Past1^{60-4}$) are shown in part B. An example of the three classes of wing venation observed is presented at each column title. *n*=number of wings counted.

there is probably a separate promoter for each transcript. Since they do not share an upstream region, alternative splicing cannot account for the two transcripts (our unpublished results). The fact that Past1A is expressed in testes only from the third larval stage onwards, suggests that this protein must be precluded from these cells in early developmental stages.

Localization of endogenous Past1, as well as of the two Past1 proteins expressed from plasmids, to the plasma membrane in Schneider cells, suggests a role in early stages of endocytosis, similar to EHD2 (Guilherme et al., 2004) (S.B. and M.H., unpublished). Moreover, there is not only functional resemblance between Past1 and EHD2, but also structural homology, as suggested by their evolutionary conservation (Fig. 1B).

Results of endocytosis assays, in which we followed the fate of fluorescently labeled avidin in larval garland cells, showed a remarkable attenuation in internalization of the marker in *Past1* mutant Garland cells. It is tempting to postulate that the higher the proportion of cells defective in endocytosis, the lower the likelihood of the larvae to survive. Indeed, we observed varying degrees of viability among larvae with the same *Past1* mutation. These results strongly suggest that Past1 is involved in endocytosis.

The mutants that we generated did not express Past1 protein and exhibited temperature sensitivity, infertility and death at early adulthood. All *Past1* mutants expressed abortive transcripts. Our results indicate that the *Past1* RNA expressed from the $Past1^{55-1}$, $Past1^{88-1}$ and $Past1^{110-1}$ mutants, initiated from the promoter of the upstream *CG14394* gene. Temperature sensitivity characterizes endocytic mutants of two types: missense and null mutants. Missense mutations cause the mutant protein to lose its correct structure and/or function (for example *shibire*) at high temperature (Grant et al., 1998). The absence of a protein because of a null mutation such as *Dap160* or *Past1* causes the complex in which it is normally involved, to become less stable or unstable, especially at high temperature (Koh et al., 2004).

Past1 mutants exhibited impaired gonads and a significant reduction in fertility. Mutant male sperm differentiation is derailed at the individualization step, and mutant females have ovaries that contain yolkless egg chambers. Yolk is produced in the nurse cells and is transferred into the oocyte at stage 8 onwards. The lack of yolk in a large number of the mutant oocytes suggests that Past1 is required for yolk trafficking from the nurse cells into the oocytes. Interestingly, other fly mutants defective in endocytosis also display male or female sterility, and the latter is associated with abnormal oogenesis, e.g. *yolkless*, *quitPK61*, *stillPS34*, *Merlin* and *clathrin-heavy-chain 4* (Bazinet et al., 1993; DiMario and Mahowald, 1987; Dorogova et al., 2008; Gutzeit and Arendt, 1994), and mutants in the *C. elegans* EHD ortholog *rme-1* failed to recycle yolk receptors (Grant et al., 2001). These results highlight the importance of Past1, and endocytosis, in development and maturation of the germlines.

The transition from the third instar larva to pupa is the stage at which the primordial germ cells are selected to become germline stem cells (Gilboa and Lehmann, 2004). This might be the trigger for expression of the testis-specific RNA-A transcript. The fact that both male and female germ cell development are impaired in the absence of *Past1* expression strongly implies that both Past1 proteins have a role in early germ cell differentiation.

The possible involvement of Past1 in Notch signaling led us to test genetic interactions between *Past1* mutants and mutants in the

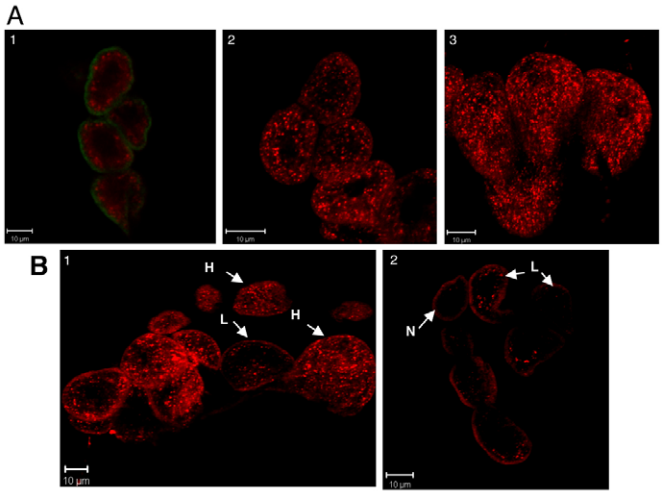


Fig. 6. Endocytosis of Texas-red-avidin by normal and mutant Garland cells. Garland cells were dissected from third instar wild-type (A) or $Past^{110\text{-}1}/Past^{110\text{-}1}$ larvae (B) and incubated with 10 µg/ml Texas-red-conjugated avidin. Cells were either fixed with 4% paraformaldehyde (in the case of panels 2 and 3 in A and panels 1 and 2 in B) or transferred to ice and incubated for 5 minutes with fluorescein-conjugated avidin (panel 1 in A) before fixation. The cells were transferred onto glass slides, which were mounted with galvanol reagent and covered with coverslips. The cells were visualized with an LSM Meta confocal microscope. H, high internalization level; L, low internalization level; N, no internalization (see Table 4). Scale bars: 10 µm.

Notch signaling pathway. Mutated *Past1* in flies carrying the *Notch* $N^{md\text{-}1}$ allele caused an enhanced wing notching phenotype compared with that of the $N^{md\text{-}1}$ mutants alone. Additionally, reduced expression of *Past1* ameliorated the venation defects of the haploinsufficient H^2 mutant in the *Hairless* gene.

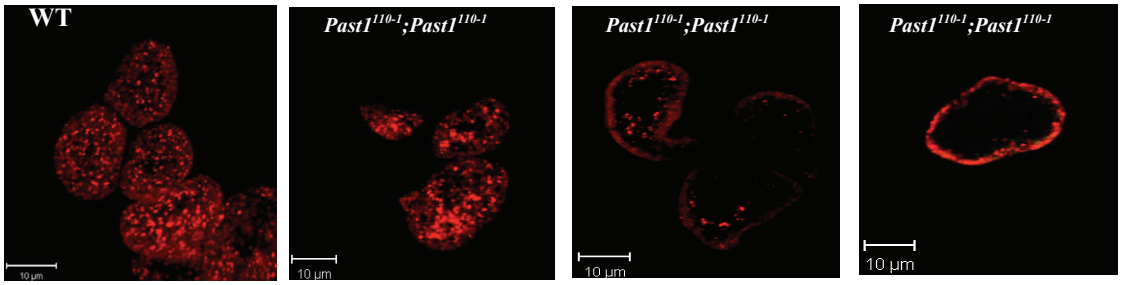
Notch has an inductive role in the development of the wing margin (Portin, 2002). Notch signaling between the dorsal and ventral compartments of the future wing specifies the wing margin – a line of cells that organizes the outgrowth of the wing. Loss of Notch signaling eliminates the wing margin and wing tissue, producing notched wings (Lai, 2004). Hairless is known to sequester Suppressor of Hairless and thus inhibit its ability for DNA binding and transcription activation together with Notch. Our results show that the *Notch* $N^{md\text{-}1}$ phenotype was augmented by the absence of Past1 and the severity of the *Hairless* H^2 phenotype was diminished by the reduction of *Past1* expression. These observations strongly suggest that Notch and Past1 function synergistically, whereas Hairless and Past1 are antagonistic, suggesting that Past1 promotes Notch signaling.

It is well documented that endocytosis has a crucial role in Notch signaling, both in the sending cells and in the receiving cells (Emery et al., 2005; Le Borgne, 2006; Polo and Di Fiore, 2006). In the sending cell, endocytosis of the ligand (Delta or Serrate in *Drosophila*) is required for its activation, whereas in the receiving cell, endocytosis is central to activation of the cleaved Notch receptor or downregulation of the membranous, non-cleaved form (Le Borgne, 2006; Wilkin and Baron, 2005). The fact that Past1 synergizes with Notch in its effect on the wing margin suggests that Past1 is required as an activator of Notch signaling. We suggest that in the absence of Past1 in the sending cell there is less active ligand, leading to less Notch activation. The antagonistic effect of Past1 on Hairless, presumably due to abrogation of ligand-dependent Notch activation in the sending cell, leads to partial restoration of vein formation. It is still possible that Notch undergoes Past1-regulated recycling in the receiving cell.

Notch signaling has an important role during oogenesis as well as during spermatogenesis (Polesello and Tapon, 2007; Shcherbata et al., 2004; Song et al., 2007; Xu et al., 1992). This can explain the sterility of both sexes in the *Past1* mutant flies.

In conclusion, lack of Past1 renders flies partially infertile and temperature sensitive, and shortens their life expectancy. Furthermore, *Past1* mutants enhance the effect of the *Notch* mutation on wing scalloping and ameliorate the effect of the *Hairless* mutation on wing vein formation, implicating Past1 in the control of endocytosis of Notch ligands in a key developmental signaling pathway. Our results provide the first evidence for a physiological role of Past1 proteins, which are members of the EHD protein family, in development.

Table 4. Endocytosis of Texas-red-avidin by normal and mutant Garland cells



	High labeling	High labeling	Low labeling	No labeling
Number of cells	42	94	33	33
% of cells	100	59	20.5	20.5
Fluorescence intensity	569455	531897	198432	104122

Garland cells were dissected from third instar wild-type or $Past^{110\text{-}1}/Past^{110\text{-}1}$ larvae and incubated with 10 µg/ml Texas-red-conjugated avidin. *Z* slices were obtained for all the cells and the section with the largest diameter was selected for analysis using the ImageJ program. Fluorescence intensity was calculated in 10 cells and the mean (in arbitrary units) is presented. Shown are normal cells that exemplified high labeling and mutant garland cells exemplifying high labeling, low labeling or no labeling with Texas-red-avidin.

Materials and Methods

Cells

Drosophila Schneider SR+ cells were cultured in Schneider medium (Beit Haemek, Israel) supplemented with 10% fetal calf serum at 25°C.

Transfections and immunofluorescence

For transfection, plasmid DNA was introduced into *Drosophila* Schneider SR+ cells, grown on coverslips, using the ESCORT IV transfection reagent (Sigma). Actin-GAL4 expression vector was a kind gift from T. Volk at the Weizmann Institute of Science, Rehovot, Israel. UAS-GFP-rab5 and UAS-GFP-rab11 expression vectors were a kind gift from B. Shilo also at the Weizmann Institute of Science. UAS-myr-RFP was a kind gift from Henry Chang, Yale University School of Medicine, New Haven, CT (Chang et al., 2002). GFP-EHD1 was previously described (Mintz et al., 1999).

For immunofluorescence studies, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 18 hours after transfection. Following incubation with 20% normal goat serum and 1% BSA in PBS, to block nonspecific interactions, the cells were incubated with the appropriate antibodies. Following washing in PBS, the cells were incubated with secondary antibodies and, after several additional PBS washes, the coverslips were mounted on glass slides with galvanol. The images were obtained using either an LSM510 confocal microscope or an LSM510 Meta confocal microscope (Carl Zeiss, Germany).

RNA extraction, reverse transcription and PCR

For RNA extraction, embryos, larvae, pupae, adult flies or testes from larvae and adults, were collected, frozen in liquid nitrogen and then homogenized in TRI reagent solution (MRC, Cincinnati, OH). The extraction was performed according to the manufacturer's recommendations. 1 μg RNA was reverse transcribed, using M-MLV reverse transcriptase kit (Promega, Madison, WI), in the presence of random hexamer primers and RNAsin (Promega). The resulting cDNA was used to amplify *Past1* transcripts with Ready Mix *Taq* polymerase kit (Bio-Lab, Jerusalem, Israel) and the following primers: PAST1, 5′-GCTAGCCGCGTTTTAGTG-3′; PAST2, 5′-CTTCGCATCGAAGTCTGGATCCT-3′; PAST3, 5′-GTGGCAAGCAAGTGTA-3′; PAST4, 5′-GGGCATGCTGGACATCGC-3′; PAST5, 5′-GGCGTGGTCACGTT-GATTG-3′; PAST6, 5′-CGCCTCCGTGACATCCG-3′; PAST7, 5′-CCAGGTATA-CGATCGCATCCAG-3′; PAST8, 5′-CATGGTCGTCGATGACTCC-3′. The positions of the primers used are shown in Fig. 1A and Fig. 4A. Thermal cycling consisted of 94°C for 10 minutes, followed by 30 cycles of denaturation (94°C, 1 minute), annealing (56°C, 1 minute), extension (72°C, 1 minute), and a final extension at 72°C for 10 minutes.

Western blot analysis

For each preparation, 10 flies (or 5×10^6 S2 Schneider cells) were homogenized in RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin and 0.1 mM phenylmethylsulfonylfluoride, all from Sigma). 100 μg protein was separated by 8% or 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). After blocking in 5% skimmed milk and 0.1% Tween 20 in Tris-buffered saline (TBS) at 4°C overnight, the membranes were reacted overnight with rabbit anti-Past1 serum diluted 1:1000. Following washes in TBST (TBS containing 0.1% Tween 20), the membranes were reacted with HRP-conjugated goat anti rabbit antibodies for 1 hour (diluted 1:10,000; Jackson ImmunoResearch, West Grove, PA). After additional washes, proteins were detected using Western blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA).

Antibody preparation

A *Bam*HI-*Sal*I fragment of the ORF of *Past1* (lacking sequences encoding 54 amino acids from the N-terminus and 44 amino acids from the C-terminus of the protein), was cloned into the pET29b expression vector (Novagen, San Diego, CA) at its *Bam*HI-*Xho*I sites. Clones of transformed *E. coli* (strain DE3), after IPTG induction at 30°C, were lysed and extracts were analyzed on 10% SDS-PAGE. Extracts from a highly expressing clone were purified using the QIAexpressionist Ni-NTA protein purification system according to the manufacturer's recommendations (Qiagen, Hilden, Germany). Polyclonal anti-Past1 antibodies were prepared by immunizing rabbits with five injections of 0.5-1 mg purified protein at 4-week intervals. Animals were bled 10 days after each boost. Serum was prepared from the blood and stored at –80°C.

Fly strains

Canton-S flies (CS) served as a wild-type control. Strains were maintained and crosses were made on cornmeal molasses medium at 25°C, taking special care to avoid overcrowding. Strain EY01852, containing a P-element in the *Past1* gene, was obtained from the Bloomington Stock Center (strain #15082). A strain carrying the stable source of P-transposase (Δ2,3) was used to mobilize the P-element. The resulting mutants were balanced over the TM3, *Ser* act-GFP balancer, to allow identification of homozygous mutants at the larval stage. A strain containing a chromosomal deletion that removes *Past1* and adjacent genes [Df(3R)kar-Sz37, Bloomington #6306] was used to confirm the lesions generated in the *Past1* gene by the mobilization of the P-element. The *Notch* allele $N^{md\text{-}1}$ (strain #2209) and the *Hairless* H^2 allele (strain #517) are from Bloomington.

Generation of *Past1* mutants

The P-element in the EY01852 strain carries the dominant w^+ eye color marker. Reciprocal crosses of this strain and a strain carrying the Δ2,3 stable source of transposase were conducted. Imprecise excisions of the P-element were verified in F2 by PCR amplification and sequencing.

Temperature sensitivity

For survival measurements, 25 males and 25 females from balanced *Past1* mutant lines were allowed to mate and lay eggs at 25°C or 29°C for 7 days, after which they were discarded. The resulting adult offspring were separated into homozygotes and heterozygotes, according to the *Stubble* marker on the TM3, *Sb* balancer, and were counted daily for a week after they had started to eclose. The experiment was repeated at least four times. For assessing survival rate during development, first instar larvae from the *Past1* mutant strain were collected and separated into homozygotes and heterozygotes, according to the GFP marker on the TM3, *Ser* act-GFP balancer. Larval development was monitored daily and the number of survivors in every developmental stage was counted. At least 20 homozygous larvae were collected. The experiment was repeated twice.

Fertility test

To assay female fertility, a virgin *Past1* mutant female and a virgin Canton-S male (or a pair of Canton-S male and female as control), collected within 8 hours of eclosion, were placed together in a culture vial for 3 days, at which time the pair was removed from the vial. The vial was cultured for an additional 17 days, and the number of offspring that eclosed from the vial during that time was counted. To assay male fertility, a virgin Canton-S female and a virgin *Past1* mutant male were placed together, following the same procedure that was used for female fertility. The number of offspring produced per vial was counted until 20 days after mating for vials in which the parental pair survived the 3-day breeding period. The data was analyzed statistically: Two outliers, were eliminated using the Grubbs test (α=0.01) (Grubbs, 1969), and were not included in the mean. The mean rank of each experimental group was compared with the mean rank of Canton-S using the nonparametric Dunn test (Dunn, 1964).

Fly tissue preparation and staining

Testes of young adult males were dissected, fixed and stained as described (Arama et al., 2003) with anti-active mouse caspase-3 (Cell Signaling, Danvers, MA), TRITC-phalloidin (Sigma) and DAPI (Vector Laboratories, Burlingame, CA). Ovaries of 3-day-old mated adult females were dissected, separated from extra-ovarian tissue, spread on a microscope slide and visualized under a light microscope. Wings were dissected from young adults, mounted in Hoyer's medium and visualized under a light microscope.

Endocytic assay in Garland cells

Garland cells were dissected from third instar larvae and incubated with 10 μg/ml of Texas-red-conjugated avidin (A-820, Molecular Probes) in PBS for 1 minute at 25°C, in six-well plates. The cells were washed with PBS, chased for 20 minutes in PBS at 25°C and fixed with 4% paraformaldehyde. The cells were transferred onto glass slides, which were mounted with gavanol reagent and covered with coverslips. Cells were visualized with a LSM510 Meta confocal microscope. The ImageJ program was used to quantify the fluorescence intensity of individual cells.

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