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An *EP* overexpression screen for genetic modifiers of Notch pathway function in *Drosophila melanogaster*

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Summary

The Notch pathway comprises a signal transduction cascade required for the proper formation of multiple tissues during metazoan development. Originally described in *Drosophila* for its role in nervous system formation, the pathway has attracted much wider interest owing to its fundamental roles in a range of developmental and disease-related processes. Despite extensive analysis, Notch signaling is not completely understood and it appears that additional components of the pathway remain to be identified and characterized. Here, we describe a novel genetic strategy to screen for additional Notch pathway genes. The strategy combines partial loss of function for pathway activity with *Enhancer-promoter* (*EP*)-induced overexpression of random loci across the dorsoventral wing margin. Mastermind (Mam) is a nuclear component of the Notch signaling cascade. Using a *GAL4-UAS*-driven dominant-negative form of Mam, we created a genotype that exhibits a completely penetrant dominant wing-nicking phenotype. This phenotype was assayed for enhancement or suppression after outcrossing to several thousand *EP* lines. The screen identified known components or modifiers of Notch pathway function, as well as several potential new components. Our results suggest that a genetic screen that combines partial loss of function with random gene overexpression might be a useful strategy in the analysis of developmental pathways.

1. Introduction

The Notch pathway is one of a small group of signaling systems that regulate metazoan development (Gerhart, 1999; Barolo & Posakony, 2002). These systems share the general feature of receiving an extrinsic signal and transmitting an intracellular signal involving multiple components, and they also exhibit common principles involving specific activation and repression (Barolo & Posakony, 2002). Notch functions during numerous cellular interactions within vertebrates and invertebrates. The pathway is composed of membrane receptors (*Drosophila* Notch, *Caenorhabditis elegans* Glp-1 and Lin-12, and mammalian Notch1–Notch4), ligands of the Delta/Serrate class and multiple cytoplasmic and nuclear proteins that are involved in signal transmission and the regulation of target gene expression. Notch pathway genes, originally designated as neurogenic loci, were first associated with lateral inhibition of neuroblast formation in the *Drosophila* central nervous system (CNS) (Lehman *et al*., 1983). Subsequently the pathway was shown to act at other stages of CNS formation (Skeath & Doe, 1998; Buescher *et al*., 1998; Schuldt & Brand, 1999; Van Der Bor & Giangrande, 2001), as well as within a wide spectrum of tissues throughout *Drosophila*/invertebrate development (Hartenstein *et al*., 1992; Artavanis-Tsakonas *et al*., 1999; Weinmaster, 1997) and vertebrate/mammalian development (Gridley, 1997; Mumm & Kopan, 2000). Moreover, as additional activities of the Notch pathway were characterized, it became apparent that a much wider array of gene products was involved in its function (Baron *et al*., 2002). In various contexts, Notch mediates inductive cell interactions, cell proliferation and apoptosis (Artavanis-Tsakonas *et al*., 1999). Evolutionarily conserved components of the Notch pathway have been implicated in human diseases and dysmorphic syndromes (Gridley, 1997, 2003), including Alzheimer's disease (Haas, 1997; Ye *et al*., 1999),

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Alagille syndrome (McCright *et al*., 2002), spondylocostal dysostosis (Gridley, 2003) and CADASIL (Villa *et al*., 2001), and two mammalian *Notch* genes were first characterized as oncogenes. The human *Notch1* locus was associated with a translocation present in T lymphoblastic leukemia (Ellisen *et al*., 1991); excessive activity of Notch1, Notch2 or Notch3 predisposes to T-cell leukemia (Aster & Pear, 2001). Moreover, the mouse *Notch4/int-3* locus is a Mouse Mammary Tumor Virus (MMTV) integration site, and thus associated with mammary tumors (Van Leeuwen & Nusse, 1995). It is now hypothesized that dysregulation of Notch signaling is associated with additional human neoplasms, including those of the cervix, lung, skin and salivary gland (Maillard & Pear, 2003; Tonon *et al*., 2003).

Despite intensive study, the genetic and biochemical details of Notch signaling are only partially understood and it appears that a more complete description will require the identification of additional pathway components. For example, genetic screens (Levitan & Greenwald, 1995; Verheyen *et al*., 1996; Yedvobnick *et al*., 2001; Jarriault & Greenwald, 2002) and biochemical strategies (Royet *et al*., 1998; Hubbard *et al*., 1996; Lamar *et al*., 2001; Negerie *et al*., 2002) continue to uncover loci involved in Notch function, strongly suggesting that others remain undiscovered. We described previously a *Drosophila* GAL4 upstream activation sequence (*GAL4-UAS*) (Brand & Perrimon, 1993) genetic system that has been used to depress the Notch pathway in a tissue-specific manner, leading to typical loss of function phenotypes in imaginal tissues (Helms *et al*., 1999; Yedvobnick *et al*., 2001) or embryos (Yedvobnick *et al*., 2004). This was accomplished through overexpression of truncated forms of Mastermind (Mam), an essential nuclear component (Smoller *et al*., 1990; Bettler *et al*., 1996) of the Notch activation complex (Petcherski & Kimble, 2000; Wu *et al*., 2000; Kitagawa *et al*., 2001; Fryer *et al*., 2002).

In this study, we genetically combined a *GAL4-UAS*-driven dominant-negative Mam truncation with a collection of *Enhancer-promoter* (*EP*) overexpression lines (Rorth, 1996). The truncation we used, named MamH, terminates shortly after the basic Mam domain that mediates a physical association with nuclear Notch. MamH protein presumably competes with wild-type Mam for Notch binding, leading to depressions in Notch target gene expression (Helms *et al*., 1999; Yedvobnick *et al*., 2001). Aside from targeting redundant functions that might be missed during screens for loss-of-function mutations, *EP* overexpression of genes can be a useful tool in genetic modifier screening (Rorth *et al*., 1998). Within the present screen, the *GAL4-UAS* system simultaneously expresses the dominant-negative Mam construct and a random set of genes downstream of the *EP* promoter. F1 progeny are then scored for enhancement or suppression of the standard dominant-negative phenotype. Our results indicate that a genetic screen that combines Notch pathway loss of function with overexpression of random loci is capable of uncovering new pathway components.

2. Methods

(i) *Mam truncation constructs*

Construction of Mam truncations in pUAST is described in Helms *et al*. (1999). *UAS-MamH* terminates at nucleotide 1489 of cDNA B4 (Smoller *et al*., 1990), Mam residue 245, which is 55 residues C-terminal of the basic charge cluster.

(ii) Drosophila *strains*

Wing margin (*C96-GAL4*) and proneural (*309-GAL4*) driver lines, and *UAS-*N^{actW}, *UAS-Hairless* and *UAS-Delta* have been described previously (Helms *et al*., 1999). The collection of approximately 2300 *EP* lines (Rorth *et al*., 1996) was obtained from Exelixis (http://flystation.exelixis.com/). The nd^{1} line was obtained from the Artavanis lab (Massachusetts General Hospital Cancer Centre, Charleston, MA) and Dl^{BX9}/*TM6c* was obtained from the Muskavitch lab (Boston College, Chestnut Hill, MA). The $fringe^{13}$/*TM3 Sb* strain was obtained from K. Irvine (Rutgers University, Waksman Institute, Piscataway, NJ). The remaining strains were obtained from the Bloomington Stock Center.

(iii) *Genetic screen*

All crosses were performed at 25 °C. The *C96-GAL4* driver line expresses across the dorsoventral wing margin. This transgene was recombined onto a chromosome containing the *UAS-MamH* transgene and the recombinant chromosome balanced over *TM3 Sb* (Helms *et al*., 1999). The recombinant chromosome (*C96-MamH*) elicits a completely penetrant, dominant wing nicking phenotype (Fig. 1). Flies from the *C96-MamH/TM3 Sb* strain were mated to individual *EP* insert lines and the offspring were scored for enhanced or suppressed wings. Vials showing modified offspring were retested by selecting w^{+} (*EP*) and *Sb* F1 males and crossing them once again to *C96-MamH/TM3 Sb* flies. The sequence of *EP* insertion sites and their distance and orientation relative to transcription units were obtained from FlyBase. *EP* lines producing wing modification of *C96-MamH* were subsequently mated to a *C96-GAL4* strain to examine overexpression effects in the absence of the Mam truncation. Phenotypic interactions resulting from background modifiers in the *EP* strains were investigated by crossing the *EP* lines to other Notch pathway mutations exhibiting wing phenotypes. Potential loss-of-function effects

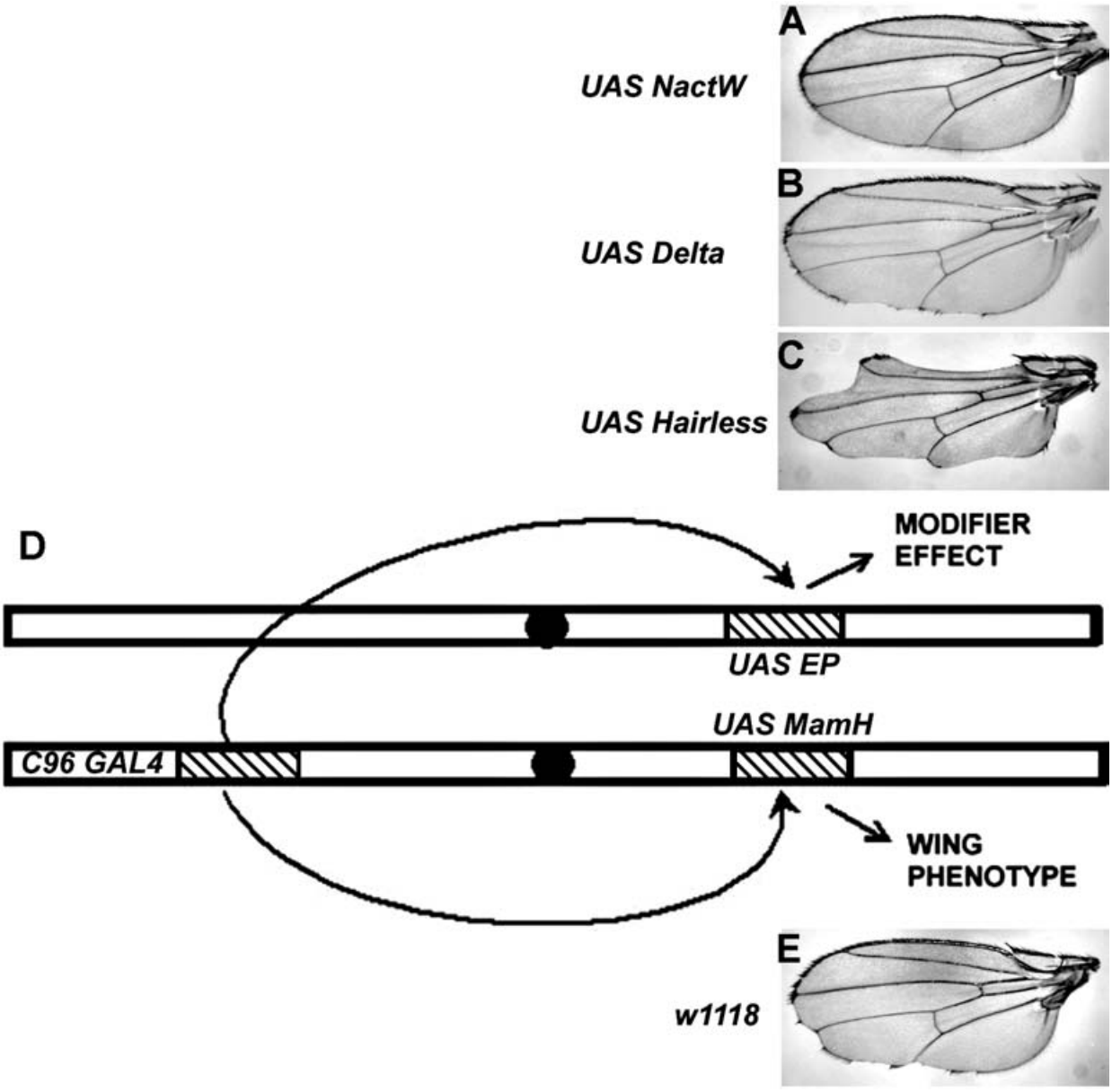

Fig. 1. The *EP*-overexpression/modifier screen. The standard transheterozygous genotype used in the screen is shown in (D). The bottom chromosome depicts the recombinant *C96-MamH* chromosome, which contains the *C96-GAL4* driver and *UAS-MamH* transgenes. The positions of these transgenes on the third chromosome are not accurately presented. The top chromosome depicts a single *EP* insertion. In such genotypes, GAL4 protein is produced across the dorsal-ventral wing margin through *C96* promotion (Helms *et al*., 1999). GAL4 protein simultaneously activates the *UAS-MamH* truncation transgene and other genes downstream of the *EP* element (arrows). The standard *C96-MamH* dominant wing phenotype (E) is assayed for enhancement or suppression. Transheterozygous genotypes containing transgenes with *UAS*-regulated forms of activated Notch (N^{actW}) or wild type *Delta* (A, B) show suppression of the *C96-MamH* wing nicking phenotype. In contrast, transheterozygotes containing *UAS-Hairless* show enhancement (C).

at the sites of *EP* insertion were tested by use of chromosomal deficiencies that spanned the insertion site or a loss-of-function allele of the relevant gene.

(iv) *Mounting of wings and notums*

Wings were dehydrated in isopropanol and mounted in Euparol. Notums were positioned on slides and photographed directly under a dissecting microscope.

3. Results

(i) EP *modifier screen*

The w^+ *EP P* element (Rorth, 1996) contains *UAS* (*E*) sequences immediately 5′ to the *hsp70* promoter (*P*). When the *EP* element is mobilized, in addition to insertional inactivation of loci, it can activate downstream genes when GAL4 is present. In screens, the w^+ *EP P* element is transposed to random genomic

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sites and the new site of residence of the element is tested for phenotypic effects when driven by an *Enhancer-GAL4* construct. Therefore, using a tissue-specific *GAL4* driver, the genome can be screened for loci that produce phenotypes when overexpressed (Abdelilah-Seyfried *et al*., 2000). Alternatively, when combined with a pre-existing mutant phenotype, the screen can be used to identify loci that bypass, suppress or enhance the mutant phenotype (Rorth *et al*., 1998; Kazemi-Esfarjani & Benzer, 2000). In the present screen, the *C96-GAL4* element present on the same chromosome as *UAS-MamH* (*C96-MamH*) can simultaneously create the mutant phenotype and drive the random *EP* insertion and its downstream gene (Fig. 1D). The *C96-MamH* chromosome elicits a 100% penetrant dominant wing-nicking phenotype when outcrossed to w^{1118}, as shown in Fig. 1E. Genotypes that also contain an *EP P* element hop can be assayed for changes in wing phenotype. This approach was validated in genotypes containing the *C96-MamH* truncation chromosome and *UAS*-regulated Notch pathway loci that function as positive or negative effectors. For example, overexpression of the Delta ligand or an activated form of Notch is expected to elevate pathway signaling, and we observed suppression of the *C96-MamH* wing phenotype in these genotypes (Fig. 1A, B). By contrast, overexpression of Hairless, which tethers the Su(H) repression complex to promoters and negatively regulates the pathway (Barolo & Posakony, 2002) enhances *C96-MamH* (Fig. 1C). Therefore the nicked-wing phenotype produced in the *C96-MamH* genotype is sensitive to alterations in Notch pathway signaling level, and should be capable of detecting additional pathway components.

(ii) *Screening the Rorth* EP *collection*

We tested the *EP* strategy by screening the Rorth (1996) collection. This collection contains approximately 2300 *EP* inserts. Each of the lines was mated with the *C96-MamH*/*TM3 Sb* strain and the non-*Sb* progeny were scored for alteration of the *C96-MamH*/+ wing phenotype. Several modifier lines were obtained and the data is summarized in Figs 2, 3 and Table 1. Unless stated otherwise, the *EP* inserts are oriented to overexpress the most proximal locus. Three enhancers are inserted in or upstream of known loci associated with the Notch pathway or a target locus of Notch activation. One of these, *EP 3082*, inserted upstream of the *fringe* gene. Fringe is a glycosyltransferase that modifies the Notch protein and alters its responsiveness to Delta and Serrate (Moloney *et al*., 2000). *EP 3082* is associated with a minor distal wing nick when combined with *C96-GAL4* (Fig. 2B2), but it produces a severe synergistic phenotype in combination with the *C96-MamH* truncation (Fig. 2B1). *EP 0509* is inserted upstream of the *kekkon-1* (*kek1*) locus, which encodes a component of epidermal growth factor (EGF) signaling. Kek1 is a transmembrane protein that negatively regulates the EGF receptor (Ghiglione *et al*., 1999). The EGF pathway functions at the dorsoventral wing margin to activate Vestigial expression, and reduced EGF receptor activity leads to nicked wings (Nagaraj *et al*., 1999). Therefore, overexpression of Kek1 is expected to synergize with depressions in Notch signaling at the margin. Although we observe a minor wing-nick effect with the *EP 0509* and *C96-GAL4* combination (Fig. 2C2), an enhanced and highly penetrant phenotype derives from the combination with the Mam truncation (Fig. 2C1). *EP 2127* inserts into the first intron of the *RpL19* gene, which encodes ribosomal protein L19. *EP 2127* enhances the *C96-MamH* phenotype, but no phenotype is observed in combination with *C96-GAL4* (Fig. 2D1, D2). *EP 2127* is oriented opposite to the normal transcription of *RpL19* and is probably associated with loss of function. We corroborated this by testing a deficiency for the *RpL19* region for interaction with *C96-MamH* and observed enhancement (Fig. 2D5). Mutation of this locus was previously isolated in a screen for Delta modifiers (Klein and Campos-Ortega, 1992). Given the structural role of *RpL19* in the ribosome, *EP 2127* is probably a nonspecific modifier. However, the targeting of *fringe* and *kek1* provide validation that the *EP* screen is capable of uncovering more relevant loci.

Several other strong and highly penetrant interactions were identified in the collection (Fig. 3). Two of these, *EP 2371* and *EP 0684*, targeted genes that encode the Domino and Escargot transcription factors, respectively. Mutations of *domino* were isolated in a screen for disorders in hematopoiesis, a process that also requires the Notch pathway (Duvic *et al*., 2002). *Domino* encodes two proteins of the SW12/SNF2 class of DNA-dependent ATPases that provide a repressed structure to chromatin (Ruhf *et al*., 2001). The proteins are widely expressed during development, including all imaginal discs and the CNS. Genotypes containing *EP 2371* (Domino) and *C96-MamH* exhibit a strong wing enhancement (Fig. 3B1), whereas no phenotype is observed in the control *C96-GAL4* plus *EP 2371* class (Fig. 3B2). Escargot is a zinc-finger transcription factor that functions redundantly with Snail protein during embryonic wing disc formation and neuroblast asymmetric division (Fuse *et al*., 1996; Cai *et al*., 2001). Overexpression of Escargot alone produces a strong phenotype; genotypes carrying *EP 0684* and *C96-GAL4* show a striking distal wing curvature phenotype (Fig. 3C2). The wing is also wider across its anterior-posterior dimension. *C96-MamH* combined with *EP 0684* suppresses the distal curvature phenotype and enhances loss of wing blade material from distal regions (Fig. 3C1). *EP 3375*

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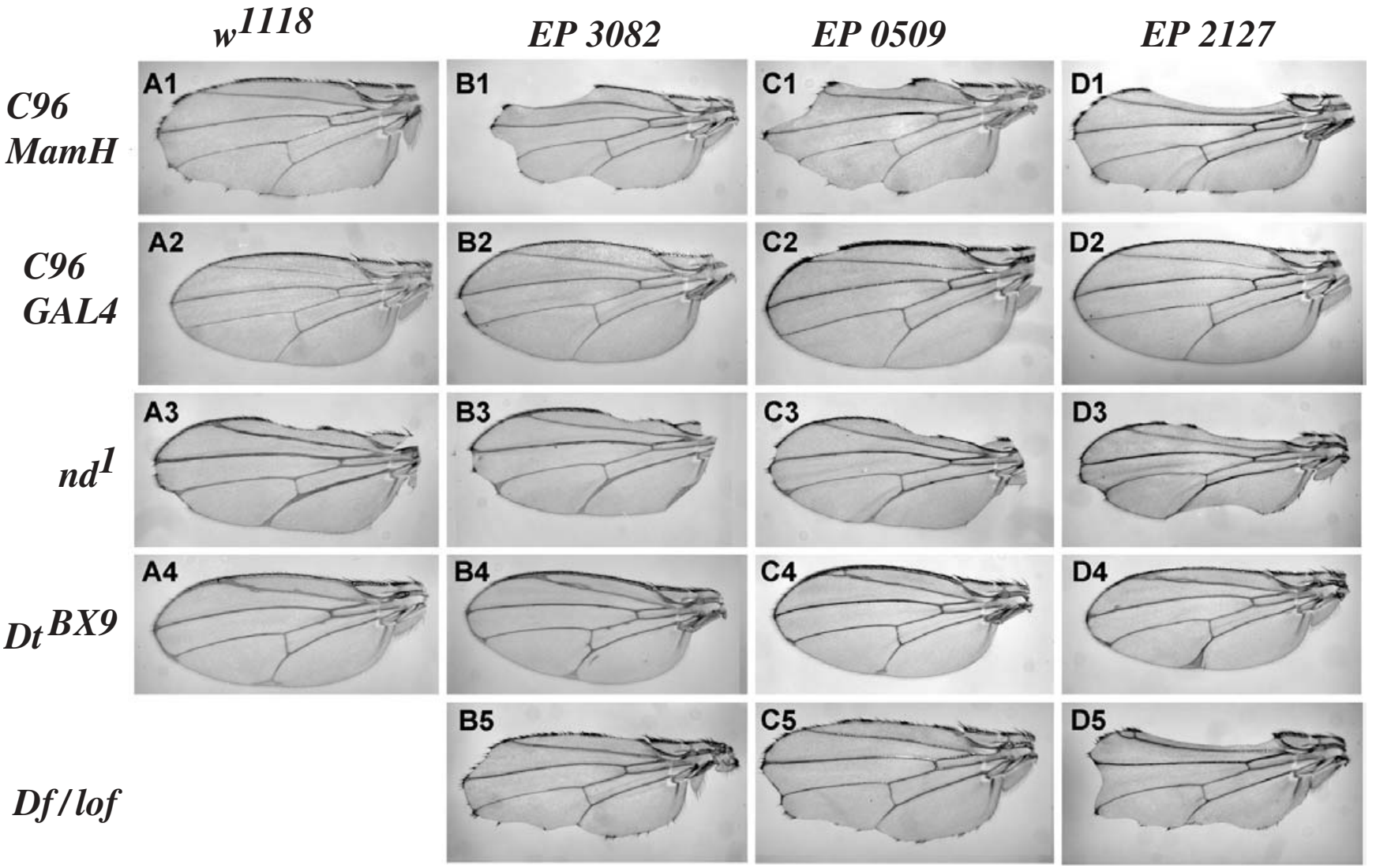

Fig. 2. *EP* line modifiers include previously identified loci that interact with the Notch pathway or a pathway target. Panels show wings prepared from genotypes containing *C96-MamH* (row 1), *C96-GAL4* (row 2), nd^{1} (row 3), Dl^{BX9} (row 4) or chromosomal deficiencies spanning the insertion site of *EP* elements, or a loss of function mutation for the insertion locus (row 5). Strains in rows 1–4 were mated with w^{1118} (column A) or *EP* lines *EP 3082*, *fringe* (column B), *EP 0509*, *kekkon 1* (column C) and *EP 2127 RpL19* (column D). In the case of deficiency/loss-of-function (lof) mutations, row 5 shows transheterozgotes with the *C96-MamH* chromosome: B5, fng^{13}; C5, *Df(2L)prd1.7*; D5, *Df(2R)M60E*. All genotypes are transheterozygous except for the hemizygous nd^{1}/Y males.

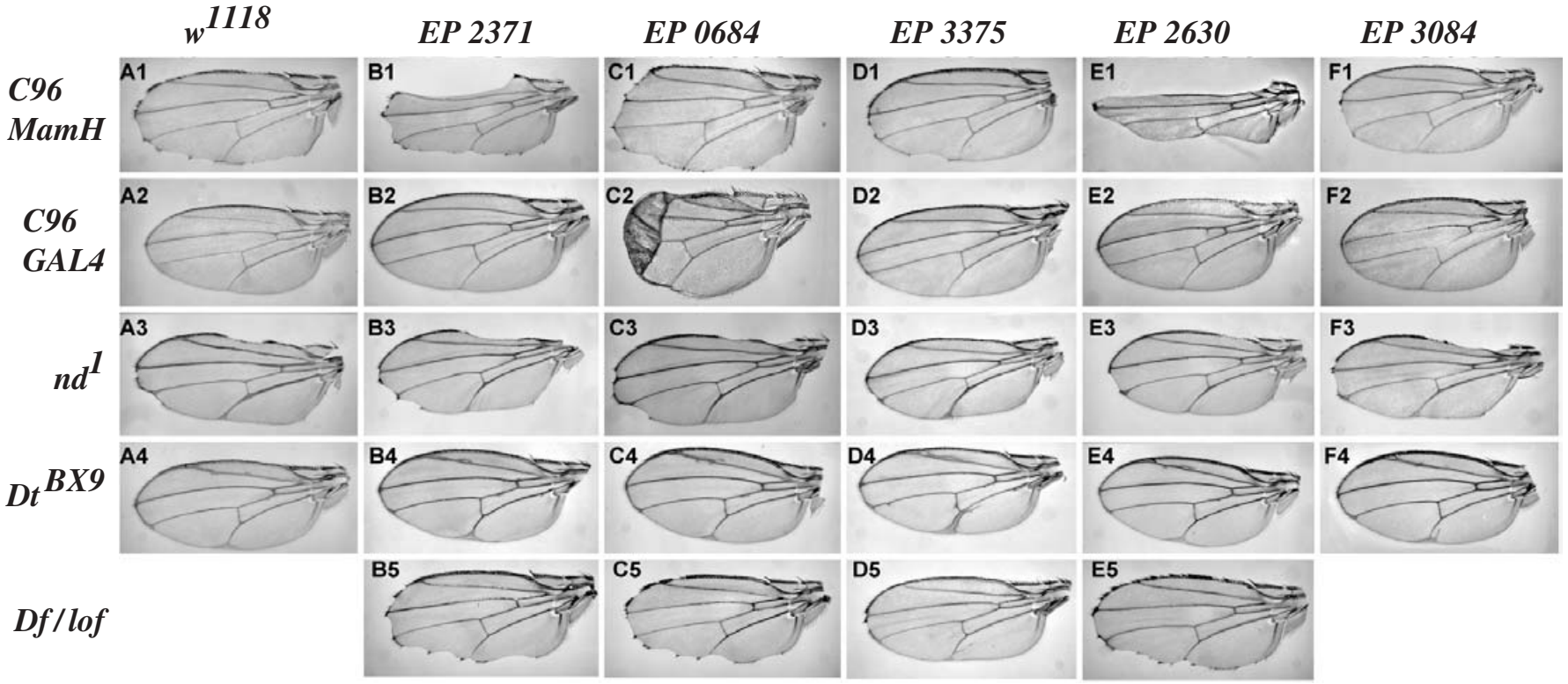

Fig. 3. Additional *EP* line modifiers include novel loci. Wings are prepared from genotypes containing *C96-MamH* (row 1), *C96-GAL4* (row 2), nd^{1} (row 3), Dl^{BX9} (row 4) or chromosomal deficiencies spanning the insertion site of *EP* elements, or a loss of function mutation for the insertion locus (row 5). Strains in rows 1–4 were mated with w^{1118} (column A) or *EP* lines *EP 2371*, *domino* (column B), *EP 0684*, *escargot* (column C), *EP 3375*, *pap* (column D), *EP 2630* (column E) and *EP 3084* (column F). In the case of deficiency/loss-of-function (lof) mutations, row 5 shows transheterozgotes with the *C96-MamH* chromosome: B5, *Df(2R)Pu-D17*; C5, *Df(2L)TE35BC-8*; D5, pap^{rK760}; E5, *Df(2L)N22-5*. All genotypes are transheterozygous except for the hemizygous nd^{1}/Y males.

Table 1. *Summary of* EP *modifiers*

EP line[a]	Cyto site	Gene	Function	*C96-GAL4*	*nd*^{1}	*Dl*	*Df/lof*	*309-MamH*
3082 (E)	78A1	*fringe*	Glycosyltransfrase	WE	NE	NE	E	S
0509 (E)	33F4	*kekkon-1*	Tyrosine phosphatase	WE	NE	NE	NE	E
2127 (E)	60E10	*RpL19*	Ribosomal protein	NE	E	E	E	E
2371 (E)	57D11	*domino*	Transcription	NE	NE	NE	NE	E
0684 (E)	35C4	*escargot*	Transcription	SE	NE	NE	NE	SE
3375 (S)	78A5	*poils aux pattes*	Transcription	NE	S	E	S	S
2630 (E)	30A2	Unknown	Unknown	NE	NE	NE	NE	E
3084 (S)	Ch3 hetero	Unknown	Unknown	NE	NE	NE	?	S

[a] The interaction of each *EP* line with the *C96-MamH* phenotype is designated as enhancer (E) or suppressor (S).
Abreviations: NE, no effect; SE, strong effect; WE, weak effect. These designations also apply to *nd*^{1}, *Dl*, *Df/lof* and *309-MamH* columns.

is a strong suppressor of *C96-MamH* that is not associated with any phenotype in combination with *C96-GAL4* (Fig. 3D1, D2). *EP 3375* inserts within the second exon of the *poils aux pattes* (*pap*) locus, oriented opposite to its transcription, and is probably associated with a loss of function. Consistent with this idea, the *pap*^{rK760} mutation also suppresses the *C96-MamH* phenotype (Fig. 3D5). The *pap* locus encodes a protein related to human TRAP 240, a component of the Mediator transcription complex (Boube *et al*., 2000).

Two other sites of *EP* insertion might affect uncharacterized genes. *EP 2630* inserts within the first intron of the *taiman* locus but is oriented to drive expression opposite to transcription of *taiman*. *EP 2630* shows a strong and completely penetrant enhanced phenotype in combination with *C96-MamH*, producing extensive loss of wing blade material (Fig. 3E1). To determine whether *EP 2630* produces the wing enhancement through inactivation of *taiman*, we combined the *C96-Mam* truncation with several mutations of *taiman* (data not shown), as well as a deficiency for the *taiman* region, *Df(2L)N22-14*. No enhancement was observed in these genotypes, indicating that loss of function for *taiman* is not responsible for the phenotypic interaction (Fig. 3E5). Additionally, the combination of *EP 2630* with *C96-GAL4* does not elicit a detectable phenotype (Fig. 3E2). *EP 2630* could be driving an uncharacterized sequence within the first intron or upstream of *taiman* that is responsible for the interaction with *C96-MamH*. *EP 3084* is a strong suppressor of the *C96-MamH* phenotype that is not associated with a phenotype in combination with *C96-GAL4* (Fig. 3F1, F2). The insertion site of *EP 3084* is within repeated sequence of chromosome 3 heterochromatin and we have not been able to identify the sequences responsible for the interaction.

(iii) *Secondary characterization of* EP *lines*

EP lines can modify the *C96-MamH* wing phenotype for reasons unrelated to overexpression. As discussed above, an *EP* insert may inactivate a locus. *EP* inactivation of a locus should elicit a modifier phenotype that resembles a deficiency or loss-of-function mutation interaction for that region (Yedvobnick *et al*., 2001), as we observe for *EP 2127* and *EP 3375*. Our analysis suggests that modifier *EP* lines oriented to drive a downstream locus do not act through insertional inactivation. As shown in Figs 2 and 3 (row 5), mutations or deficiencies that span the site of *EP* insertion for lines *0509*, *2371*, *0684* and *2630* do not enhance the *C96-MamH* phenotype. Although we do observe some enhancement with loss of *fringe* function (Fig. 2B5), the effect is very different from the overexpression effect of *EP 3082* (Fig. 2B1).

An interaction apparently caused by an *EP* might actually derive from a background mutation induced elsewhere in the genome. Background mutations can be expected to modify other Notch pathway wing mutations and would not act through the *GAL4* system. Therefore, we tested *EP* lines for genetic interactions in males hemizygous for the *nd*^{1} allele of *Notch*, and also in flies heterozygous for the dominant *Delta*^{BX9} allele. The *nd*^{1} wings exhibit minor nicks along the margin and wing vein thickening. Phenotypes were compared with those observed after outcrosses of *nd*^{1} and *Delta*^{BX9} to the control *w*^{1118} strain. *EP* lines oriented to overexpress genes did not exhibit interactions (Figs 2 and 3, rows 3 and 4), but those associated with insertional inactivation showed *nd*^{1} enhancement (*EP 2127*, Fig. 2D3) or *nd*^{1} suppression (*EP 3375*, Fig. 3D3). An enhancement of the *Delta*^{BX9} phenotype was detected for *EP 2127* (Fig. 2D4). However, the suppressor *EP 3375* also exhibited enhancement of *Delta*^{BX9} (Fig. 3D4).

Finally, *EP* wing modifiers might elicit similar effects within other contexts of Notch function. Therefore, *EP* lines were mated to the *309-MamH/Cy* strain to investigate interaction in a different tissue (Fig. 4, Table 2). This strain contains a recombinant chromosome that drives the *UAS-MamH* truncation within proneural clusters on the notum. Notums from *309-MamH* flies contain additional macrochaetes

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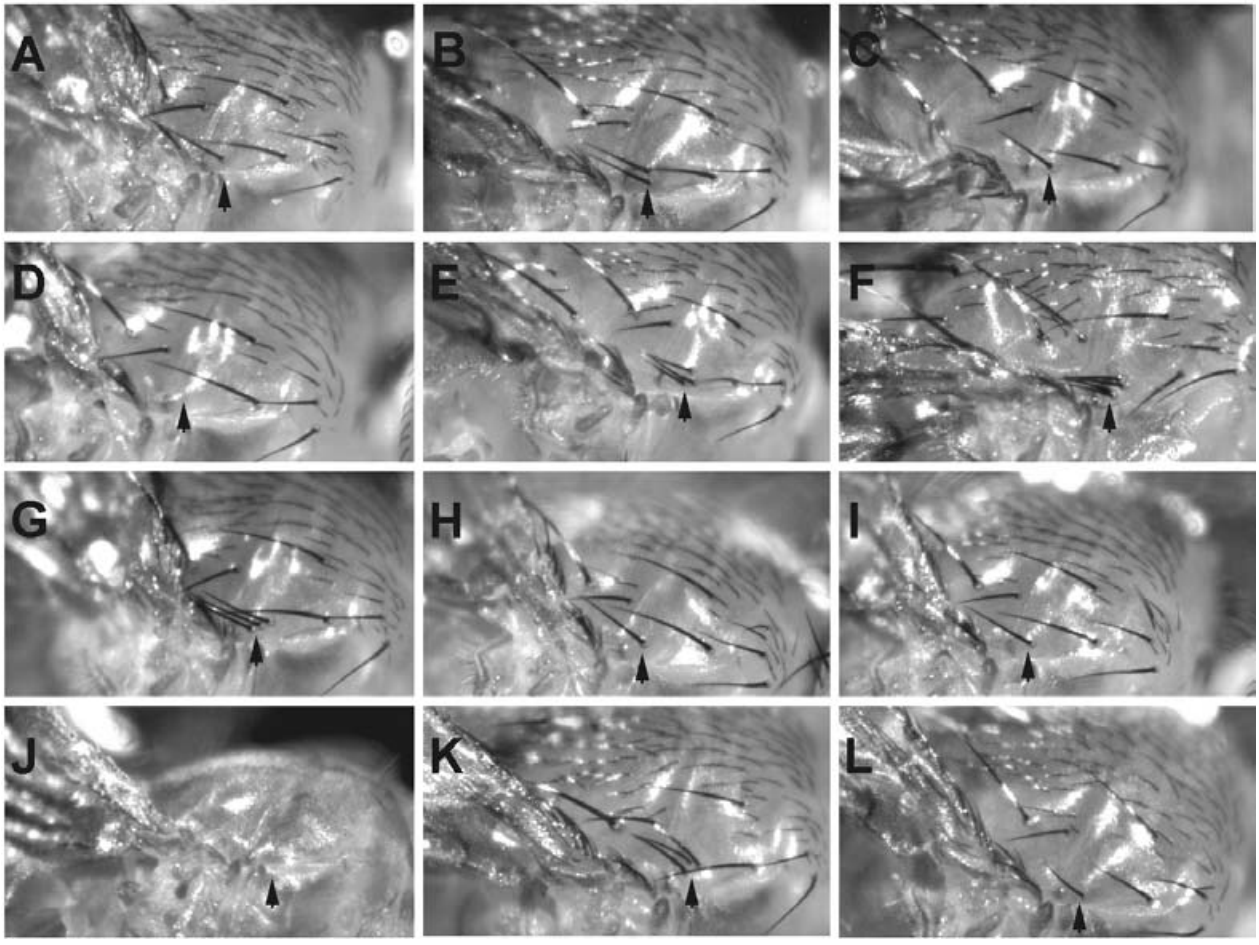

Fig. 4. *EP* modifier effects on bristle number. *EP* lines were crossed with the *309-MamH*/*Cy* strain and the offspring were scored for changes in posterior notopleural (pn) bristle number (arrow designates the pn bristle site). Except where noted, control crosses of *EP* lines to *309-GAL4* did not produce effects. Quantitation of bristle effects is presented in Table 2. (A) In control w^{1118} notums, there is a single pn bristle at 100% of the sites. (B) In *309-MamH*/w^{1118} or *309-MamH*/*Cy* notums, a duplication of the pn bristle is observed 75–80% of the time. (C) *EP 3082* suppresses the *309-MamH* duplication; a single np is observed in 89% of sites. (D) *EP 3082* driven by *309-GAL4* is associated with loss of the pn bristle 24% of the time and a single bristle is observed in the remaining sites. (E) *EP 2127* enhances the *309-MamH* phenotype, with 97% of sites showing multiple bristles and 39% show three or four bristles. (F) *EP 0509* enhances the *309-MamH* phenotype because multiple np bristles are observed at every site. Clusters of three or more bristles are observed at 86% of the sites. (G) *EP 2371* enhances the *309-MamH* phenotypes; multiple np bristles are observed at 99% of the sites. (H) *EP 3375* (*pap* allele) suppresses the *309-MamH* phenotype; single np bristles are observed at 49% of the sites. (I) *P{PZ}pap*^{rK760} strongly suppresses the *309-MamH* phenotype; single np bristles are observed at 95% of sites. (J) *EP 0684* eliminates bristle formation in combination with *309-MamH*. Identical effects are observed when *EP 0684* is driven by *309-GAL4* (data not shown). (K) *EP 2630* enhances the *309-MamH* phenotype because multiple np bristles are observed at 97% of the sites. Clusters of three or more bristles are observed at 43% of the sites. (L) *EP 3084* suppresses the *309-MamH* phenotype; single np bristles are observed at 83% of the sites.

at several sites (Helms *et al*., 1999); the posterior notopleural site is particularly sensitive. For example, compared with control w^{1118} notums, in which we never observe a duplication of the posterior notopleural bristle, the *309-MamH* strain exhibits duplication at 79% of the sites (Fig. 4A, B). *EP* line, *3082* (*fringe*) suppresses the *309-MamH*-associated bristle duplication (89% single bristle, Fig. 4C). Furthermore, genotypes in which *EP 3082* is driven by *309-GAL4* alone exhibit loss of the posterior notopleural bristle 24% of the time (Fig. 4D). Similar bristle effects have been reported previously after Fringe overexpression (Klein & Martinez-Arias, 1998). The *EP 2127* line (*RpL19*) showed an enhancement of the bristle duplication phenotype, with 97% of the sites showing multiple bristles (Fig. 4E). *EP 0509* (*kek1*) enhanced the *309-MamH* phenotype, because 100% of the sites exhibited additional posterior notopleural bristles and, in many cases, clusters of bristles were evident (Fig. 4F). A similar but somewhat weaker enhancement was observed with *EP 2371* (*domino*) (Fig. 4G). The *EP 3375* (*pap*) wing suppressor also suppressed the bristle duplication phenotypes to 51% of the sites (Fig. 4H). However, a nearly complete suppression

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Table 2. EP *modifier interaction with* 309-MamH*, showing the percentages of posterior notopleural bristle sites with 0, 1, 2 or more bristles*

	Posterior notopleural bristle number				
Strain	0	1	2	>2	N^a
w^{1118}	0	100	0	0	500
309-MamH	0	21	70	9	140
EP 3082	0	89	11	0	54
EP 0509	0	0	14	86	71
EP 2127	0	3	58	39	104
EP 2371	0	1·5	75	23·5	68
EP 0684[b]	–	–	–	–	20
EP 3375	0	49	43	8	127
pap^{rK760}	0	95	5	0	94
EP 2630	0	3	54	43	54
EP 3084	0	83	17	0	121

The *309-MamH* value is in transheterozygotes with *Cy* Balancer chromosome. The values for *EP* lines and the pap^{rK760} allele represent genotypic combination with *309-MamH* chromosome.

[a] *N* is the number of posterior notopleural sites scored.

[b] Transheterozygotes for *EP 0684* and *309-MamH* or *309-GAL4* eliminated nearly 100% of all notum bristles, so the posterior notopleural site could not be scored in these genotypes.

(95%) was obtained after crossing the *309-MamH* strain to flies carrying the pap^{rK760} allele (Fig. 4I). Crosses of *EP 0684* to either *309-MamH* (Fig. 4J) or *309-GAL4* strains (data not shown) eliminated nearly all bristles. The *EP 2630* wing enhancer exhibited strong enhancement (97% multiple bristles, Fig. 4K), whereas the *EP 3084* wing suppressor exhibited suppression (17% multiple bristles, Fig. 4L). *EP* lines were also mated to the *309-GAL4* control strain to look for effects of *EP* overexpression alone and, except for lines *3082* and *0684*, no effects on bristle number were observed.

4. Discussion

(i) EP-*based overexpression modifier screen*

In this study we combined depression of Notch signaling with overexpression of random loci to screen the genome for additional Notch pathway components. The rationale for this approach is the ability to identify pathway components encoded by redundant loci, which may be refractory to typical loss-of-function screens. Additionally, the screens can be targeted to specific tissues by using appropriate *GAL4* drivers and might be used to bypass early lethal effects of mutations in genes that act at multiple stages. Finally, candidate modifiers are rapidly identified through inverse PCR. The strategy is based on the observation that *GAL4-UAS*-directed expression of Mam truncations in imaginal tissues (Helms *et al*., 1999; Yedvobnick *et al*., 2001) or during embryogenesis (Yedvobnick *et al*., 2004) produces dominant negative effects on Notch signaling. Dominant Mam truncations retain the polypeptide's basic charge cluster (Smoller *et al*., 1990), which is required for physical association with the intracellular domain of Notch and complex formation with Su(H) protein. However, they lack more C-terminal regions of the polypeptide that function in gene activation (Helms *et al*., 1999; Petcherski & Kimble, 2000; Wu *et al*., 2000; Kitagawa *et al*., 2001; Fryer *et al*., 2002). Using a recombinant *C96-GAL4 + UAS-MamH* (*C96-MamH*) chromosome, we tested single *EP* insertion transheterozygotes for modification of truncation-induced wing phenotypes. In these genotypes, the *C96-GAL4* transgene drives both the Mam truncation and sequences downstream of the *EP* element (Fig. 1). This strategy can detect either suppressed or enhanced wing phenotypes, as shown with *UAS* strains expressing activated *Notch*, *Delta* or *Hairless* transgenes (Fig. 1). Furthermore, *EP* candidates are readily tested for overexpression phenotypes in the absence of the Mam truncation by outcrossing the *EP* line with the appropriate *GAL4* driver strain. Potential loss-of-function effects associated with *EP* insertion and background mutations within *EP* lines that can modify Notch pathway function can also be distinguished through test crosses to relevant mutants (Figs 2–4, Table 1).

We identified *EP* inserts in or near loci previously associated with the Notch pathway or a target of the pathway (Fig. 2), and other inserts that might represent new pathway components (Fig. 3). The *EP 3082* and *EP 0509* enhancers insert upstream of the *fringe* and *kekkon-1* genes, respectively. Fringe modifies Notch receptor activity at the wing margin through its glycosytransferase activity (Moloney *et al*., 2000), whereas Kek1 is involved in negative regulation of the EGF receptor. EGF signaling is involved in Vestigial expression in the wing (Nagaraj *et al*., 1999). Thus, in neither case is it surprising that overexpression impacts Notch pathway processes. However, it is noteworthy that, in each case, simple overexpression produces a very minor phenotype that synergizes with the *C96-MamH*-associated phenotype (Fig. 2). This highlights the likelihood that a combined screen that uses overexpression in conjunction with loss-of-function will be useful in uncovering new loci. This conclusion is further supported by other *EP* strains that exhibit no phenotype upon outcrossing to *GAL4* drivers, yet strongly enhance the *C96-MamH* phenotype (see below). In the case of *EP 2127*, the insert appears to disrupt function of the *RpL19* locus, previously identified as a Notch pathway modifier (Klein & Campos-Ortega, 1992). Thus, the *EP* screen can also detect modifiers through insertional inactivation of dosage-sensitive loci.

The *EP 2371* enhancer is inserted upstream of the *domino* locus, which encodes SW12/SNF2-related proteins that are involved in gene repression (Ruhf *et al*., 2001). Because Mam truncation expression reduces Notch target expression (Helms *et al*., 1999; Yedvobnick *et al*., 2001), one hypothesis to explain the *EP 2371* genetic enhancement effect is that overexpression of Domino further downregulates the pathway or its targets. However, it is presently unknown whether Domino normally functions to repress the Notch pathway or its targets at the wing margin. *EP 0684* inserts upstream of *escargot*, and we observed a strong wing phenotype when this line was crossed to the control *C96-GAL4* strain. The phenotype appears to derive, in part, from additional growth in the distal wing blade, and this effect is blocked by coexpression of the *MamH* truncation. Strong overexpression effects with other *GAL4* constructs driving Escargot have been observed (Rorth *et al*., 1998), suggesting that multiple tissues are sensitive to elevated levels of this protein. It is unclear why the *MamH* truncation diminishes the overexpression effect of Escargot, but the Escargot phenotype might require normal levels of Mam function. In this regard, it is interesting that Mam and Escargot are involved in both wing development and asymmetric cell division in the CNS (Fuse *et al*., 1996; Cai *et al*., 2001). *EP 3375* inserts within the second exon of the *pap* locus, which encodes a *Drosophila* homolog of the TRAP240 protein, and this results in a suppressor phenotype. TRAP240 forms part of a large transcription complex linking specific factors to the RNA polymerase II core complex (Boube *et al*., 2000). The *EP 3375* insert appears to produce a loss-of-function mutation in *pap*, because it behaves similarly to other *pap* mutations. The dosage-sensitive interaction observed between *pap* mutations and the *MamH* truncation effect in wing and notum could reflect a negative regulatory role for the Pap protein in the nuclear arm of the Notch pathway.

The modifier effects for two of the identified *EP* inserts could not be associated with a gene. *EP 2630* is the strongest enhancer we found and it is inserted within the first intron of the *taiman* locus, which encodes a protein related to AIB1, a steroid receptor coactivator (Bai *et al*., 2000). *EP 2630* is oriented to drive expression opposite to transcription of *taiman*, suggesting that the enhancement could be due to loss of function for *taiman*. However, deficiencies for the *taiman* locus do not enhance the *C96-MamH* wing phenotype (Fig. 3E5), arguing that loss of *taiman* function does not underlie the effect. Additionally, the *EP 2630* strain does not enhance nd^{1} or Dl^{BX9} mutations, indicating that the effect is not due to a background mutation in the *EP 2630* strain. Finally, w^{-} revertants of the *EP 2630* element no longer enhance *C96-MamH* wings (data not shown). The basis for the strong enhancement of *EP 2630* could derive from overexpression of a locus within the first intron of *taiman*, or possibly upstream of *taiman*. Interestingly, control genotypes containing *C96-GAL4* and *EP 2630* do not exhibit any wing phenotypes. Therefore, if *EP 2630* enhancement requires overexpression of a locus, its effect is only apparent in the sensitized genotype produced through Mam truncation coexpression.

Finally, our assay for the effects of *EP* inserts in a different context of Notch function, proneural clusters on the notum, was very consistent with the wing margin data. In a genotype that produces additional bristles (*309-MamH*), we observed enhancement or suppression of the phenotype in transheterozygotes with *EPs* (Fig. 4, Table 2). The notable exception was *EP 3082*, which drives the Fringe protein, in which we observed enhancement in the wing assay (Fig. 2) but suppression in the bristle assay (Fig. 4). An explanation for this derives from the effect of driving *EP 3082* in the notum with *309-GAL4* alone, where loss of bristles is observed (Fig. 4D). In an earlier report, Klein and Martinez-Arias (1998) showed that overexpression of Fringe could lead to loss of bristles through lack of sensory organ precursor formation. Thus, it appears that the effect of Fringe overexpression is epistatic to that of *MamH* truncation expression during the formation of sensory organ precursors.

In conclusion, we have used a genetic system in which *GAL4-UAS* drives the expression of a Mam truncation in combination with random *EP* inserts. The system was used to screen for loci involved with Notch pathway function, and several candidates were obtained. Because of hot spots for insertion, screening with a *P*-element-based strategy requires many transpositions (Spradling *et al*., 1999). Consequently, we are presently generating a collection of new *EP* insertions to screen for additional pathway components.

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