A Genetic Screen for Novel Components of the Notch Signaling Pathway During Drosophila Bristle Development

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ABSTRACT

The Notch receptor is the central element in a cell signaling mechanism controlling a broad spectrum of cell fate choices. Genetic modifier screens in Drosophila and subsequent molecular studies have identified several Notch pathway components, but the biochemical nature of signaling is still elusive. Here, we report the results of a genetic modifier screen of the bristle phenotype of a gain-of-function *Notch* allele, *Abruptex*^{16}. *Abruptex* mutations interfere with lateral inhibition/specification events that control the segregation of epidermal and sensory organ precursor lineages, thus inhibiting bristle formation. Mutations that reduce Notch signaling suppress this phenotype. This screen of approximately 50,000 flies led to the identification of a small number of dominant suppressors in seven complementation groups. These include known components in the pathway, *Notch*, *mastermind*, *Delta*, and *Hairless*, as well as two novel mutations. The first, A122, appears to interact with Notch only during bristle development. The other, M285, displays extensive genetic interactions with the Notch pathway elements and appears, in general, capable of suppressing *Notch* gain-of-function phenotypes while enhancing *Notch* loss-of-function phenotypes, suggesting that it plays an important role in Notch signaling.

NOTCH (N) is the receptor for an evolutionarily conserved cell-cell interaction mechanism that controls the implementation of metazoan developmental signals in a broad spectrum of developing tissues. In Drosophila, Notch signaling plays an essential role in many cell fate choices including those during the development of bristles that are mechanosensory organs (reviewed in Muskavitch 1994; Artavanis-Tsakonas *et al.* 1995). Although the molecular details of the signal transduction have not been elucidated, extensive genetic and biochemical studies in Drosophila have identified several components in the pathway (reviewed in Artavanis-Tsakonas *et al.* 1995). Delta (Dl) and Serrate (Ser) are transmembrane ligands for the Notch receptor. *Suppressor of Hairless* [*Su(H)*] encodes a transcription factor that acts as a downstream effector of Notch signaling (Bailey and Posakony 1995; Lecourtois and Schweisguth 1995). Hairless is a negative regulator of Notch signaling, which is thought to act through direct association with Su(H) (Bailey and Posakony 1995; Bang *et al.* 1995; Lyman and Yedvobnick 1995). *Enhancer of split* Complex [*E(spl)*-C] is a downstream target of Notch signaling and encodes a group of basic helix-loop-helix proteins. The *mastermind* (*mam*) locus encodes a nuclear protein whose function is not well understood, but because it displays extensive genetic interactions with Notch pathway elements and has a neurogenic phenotype, it is thought to play an important role in the Notch pathway. While some components of Notch signaling, including *Dl*, *Su(H)*, *mam*, and *E(spl)*-C, seem to be involved in Notch-mediated processes throughout development, others including *Ser*, *H*, *deltex* (*dx*), as well as the genes *numb* and *vestigial* seem to participate only in certain processes (Guo *et al.* 1996; Kim *et al.* 1996).

The bristles of Drosophila are composed of a single bipolar neuron and three different accessory cells, the tormogen, trichogen, and thecogen, all of which are descendants of a single sensory organ precursor (SOP) cell (reviewed in Posakony 1994). The SOP is selected from cells in a proneural cluster that all have the potential to adopt the SOP fate by virtue of proneural gene expression. The selection of the SOP fate from the neural ectoderm as well as the subsequent differentiation of the various cell types within the sensory organ involves lateral inhibition/specification processes that are controlled by Delta-Notch signaling (reviewed in Artavanis-Tsakonas and Simpson 1991; Campuzano and Modolell 1992; Ghysen *et al.* 1993; Skeath and Carroll 1994).

Genetic screens for second-site modifiers are a useful tool for the dissection of various signaling pathways including Notch (Simon *et al.* 1991; Fortini and Artavanis-Tsakonas 1994). To identify novel components in the Notch pathway, we conducted a genetic screen for dominant modifiers of the bristle phenotype associated

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with *Abruptex* (*Ax*) mutations. *Ax* mutations are gain-of-function alleles of the *N* locus involving missense mutations in the EGF-homologous, extracellular portion of the Notch protein (Hartley *et al.* 1987; Kelly *et al.* 1987). These mutations result in what is presumed to be a hyperactive form of the Notch receptor. As judged from the expression of specific cell markers, the *Ax* mutations affect the choice between epidermal and SOP fates, and result in flies with fewer bristles (Palka *et al.* 1990; Heitzler and Simpson 1993; Lyman and Yedvobnick 1995). We therefore expected that suppressors of the *Ax* gain-of-function bristle phenotype would represent mutations that are capable of reducing Notch signaling.

We describe the isolation of dominant suppressors of the Ax^{16} bristle phenotype defining seven complementation groups. Mapping revealed that these genes include the known components in the pathway, *N*, *mam*, *Dl*, and *H*, as well as two novel modifiers of Notch signaling, A122 and M285. M285 appears to play a general role in Notch signaling as it displays extensive genetic interactions with known elements of the Notch pathway. It suppresses gain-of-function phenotypes and enhances loss-of-function phenotypes of *Notch*.

MATERIALS AND METHODS

Genetics: Fly culture and crosses were carried out according to standard procedures at 25° unless otherwise noted. $Su(H)^{T4}$ and the transformant line that carries the genomic region of the *N* locus (Cos479) are described in Fortini and Artavanis-Tsakonas (1994) and Ramos *et al.* (1989), respectively. The transformant line which carries the genomic region of the *H* locus is described in Maier *et al.* (1992), and was kindly provided by A. Preiss. The null allele of *fringe* (*fng*), fng^{80}, is described in Irvine and Wieschaus (1994). All other mutant fly strains are described in Lindsley and Zimm (1992).

For the Ax^{16} bristle screening, $ywAx^{16}$ male flies were fed 25 mm ethyl methanesulfonate (EMS) (Lewis and Bacher 1968) and were mated to $ywAx^{16}$ virgins. All the mutagenized male flies were discarded 5 days after the mutagenesis to ensure that every modifier is independent. All the flies in the next generation were reared at 25° and screened under a dissecting microscope for modifications of the bristle phenotype (see text for the details). Suppressors were backcrossed to the original strain at least three generations without using balancers. Subsequently all the mutants were balanced over either FM6, CyO, TM3, or TM6B balancer chromosome in the Ax^{16} genetic background.

Forty-four macrochaetae (22 on one side) on the head, thorax, and scutellum were scored to compile Table 1 (see Bang *et al.* 1991 or Lindsley and Zimm 1992). Macrochaetae scored on the head were as follows: anterior, medial, and posterior orbital; ocellar; inner and outer vertical; and postvertical. On the thorax and scutellum, macrochaetae scored were upper and lower humeral, presutural, anterior and posterior notopleural, anterior and posterior supraalar, anterior and posterior postalar, anterior and posterior dorsocentral, anterior and posterior scutellar, and anterior and posterior sternopleural.

The lethals were mapped using the following dominant markers: *Star* (*S*), *Sternopleural* (*Sp*), *Bristle* (*Bl*), *Lobe* (*L*), and *brown*-Dominant (bw^D) on the second chromosome.

For mapping genetic interactions, we used chromosomes which carried the following recessive markers: *net*, *black* (*b*), *cinnabar* (*cn*), *bw* in the Ax^{9B2} genetic background on the second chromosome, and *roughoid* (*ru*), *hairy* (*h*), *thread* (*th*), *scarlet* (*st*), *curled* (*cu*), *stripe* (*sr*), $ebony^s$ (e^s), *claret* (*ca*) (rucuca chromosome) either in the Ax^{16} or Dl^{5F102} background on the third chromosome.

Histology: For staining the A101 (*neu*) enhancer trap line (Bellen *et al.* 1989; Huang *et al.* 1991), pupae were removed from the pupal case approximately 20 hr after pupariation, the abdomen was dissected in phosphate-buffered saline (PBS) and most of the fat body was discarded. Carcasses were fixed on ice in 4% paraformaldehyde in PBS for 20 min and washed in PBS. The X-gal staining reaction was developed in 3 mm $K_4[Fe^{II}(CN)_6]$, 3 mm $K_3[Fe^{III}(CN)_6]$, 1 mm $MgCl_2$, 150 mm NaCl, and 0.25% X-gal in phosphate buffer (pH 7.2) containing 0.1% saponin.

Adult fly wings were removed and mounted in Aquamount (BDH Limited), and the video images were assembled in Adobe photoshop.

Adult flies were processed by hydration in an ethanol series, followed by critical point drying and mounting on stubs. Images were obtained by an ISI-SS40 scanning electron microscope.

RESULTS

Notch pathway components suppress the Ax^{16} phenotype: To identify genes capable of modulating Notch signaling, we conducted a genetic screen for modifiers of bristle phenotypes of *Ax*, which are a group of gain-of-function *N* mutations. The Ax^{16} allele, which is associated with a missense mutation in the 29th EGF-repeat of the Notch protein (Kelly *et al.* 1987) was used, because it has a strong phenotype yet good fertility. The bristle phenotype of Ax^{16} consists of missing macrochaetae and a less dense lawn of microchaetae (Figure 1, A and B). In addition, Ax^{16} displays a wing vein phenotype involving a shortened longitudinal vein V (see Figure 4E). These phenotypes are consistent with the gain-of-function nature of the *Ax* mutations because they are suppressed by a deletion of *Notch*, and are the opposite of the bristle and wing vein phenotypes characteristic of loss-of-function mutations, *i.e.*, extra bristles and thickened wing veins (Foster 1975; Portin 1975; De Celis and Garcia-Bellido 1994a,b; Sturtevant and Bier 1995).

Ax mutations are classified into two groups according to their ability to either enhance or suppress the haploinsufficient wing nicking phenotype displayed by *N* mutations (Foster 1975; Portin 1975). Enhancers and suppressors display negative complementation where heteroallelic combinations are pupal lethal (Foster 1975; Portin 1975). According to this criterion, Ax^{16} is an enhancer and is lethal over the suppressor allele Ax^{9B2}. Previous studies have shown that mutations in components of the Notch pathway can suppress the lethality associated with negative complementation of *Ax* alleles. This appears to be a stringent criterion be-

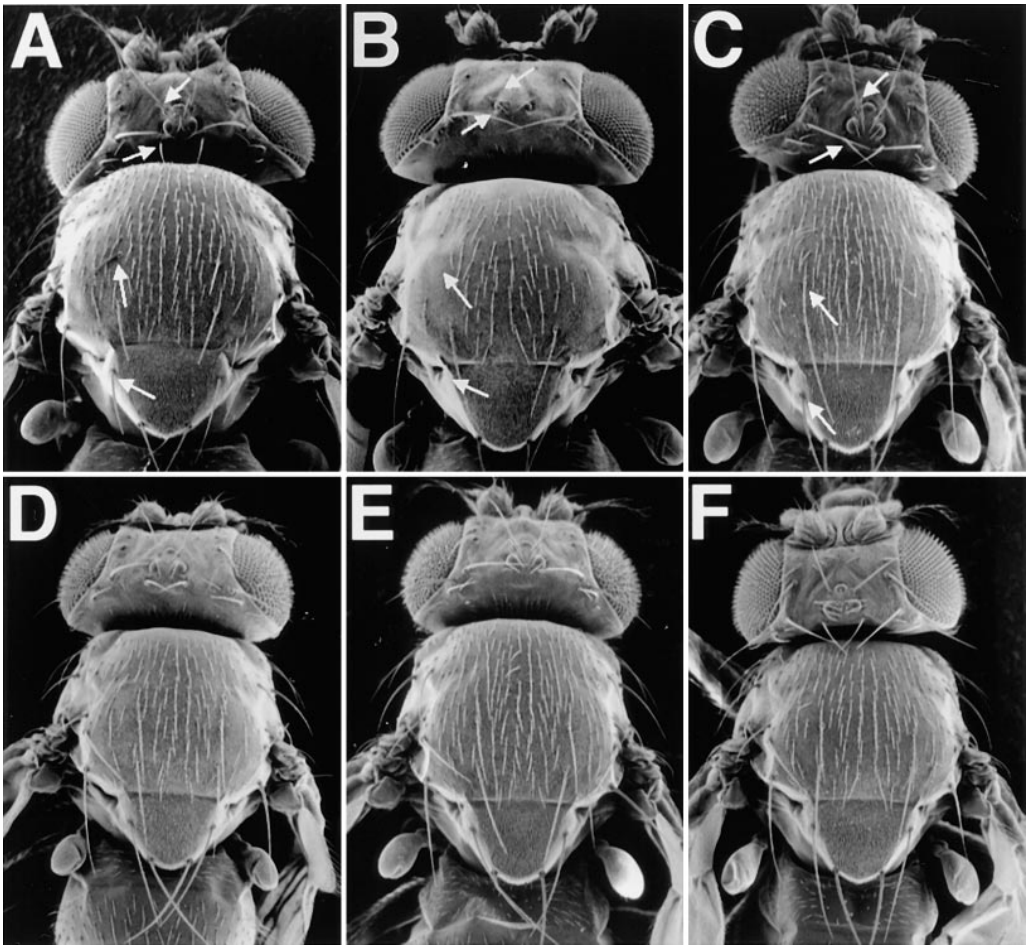

Figure 1.—The Ax^{16} bristle phenotype is dominantly suppressed by mutations in the Notch pathway. Scanning electron microscopy (SEM) images of bristle phenotypes. (A) Wild-type control, *yw*. (B) The original fly strain for the screening, $ywAx^{16}$. (C–E) Ax^{16} flies that are also heterozygous for *N* (C), *mam* (D), or *Dl* (E). (F) An Ax^{16} fly carrying a duplication of *H*. Note that the Ax^{16} bristle phenotype (apparent in B) is dominantly suppressed in C–F. Ocellars and postverticals on the head, anterior dorsocentrals, and anterior scutellars on the notum are the most obvious structures to be affected (arrows in A–C). Arrows in B indicate approximate locations where wild-type bristles should be seen.

cause only *dx*, *mam*, *Su(H)*, and *Dl*, other than *N* itself, are known to rescue the lethality associated with Ax^{E2}/Ax^{9B2} flies (Xu and Artavanis-Tsakonas 1990; Xu *et al.* 1990; Fortini and Artavanis-Tsakonas 1994; Verheyen *et al.* 1996).

We therefore expected that suppressors of the Ax^{16} bristle phenotypes would represent mutations that are capable of reducing Notch signaling activity during bristle development, especially in the cell fate choice between epidermal and SOP cells. For the screen, male flies were treated with EMS and in the next generation both the number of macrochaetae and the density of microchaetae were scored. Approximately 50,000 flies were screened, and 28 strong and 3 weak dominant suppressors of the Ax^{16} bristle phenotype were isolated. The suppressors define seven complementation groups, four of which represent new alleles in the known Notch pathway elements, *N*, *mam*, *Dl*, and *H*.

On the *X* chromosome, six *N* alleles were isolated (Figure 1C), five of which are homozygous lethal and the other is homozygous viable. All of them were also good suppressors for the shortened wing vein phenotype (data not shown). The mutations fail to complement the *N* deletion, N^{54l9} and display phenotypic characteristics typical of loss-of-function *N* alleles. With one exception, which is presumably due to the presence of a secondary lethal mutation, the mutations can be rescued by the cosmid carrying a *N* duplication (Ramos *et al.* 1989), and all the alleles can rescue the lethality associated with the negative complementation between Ax^{16} and Ax^{9B2}. As expected from the fact Ax^{16} belongs to the enhancer group, some of these *N* alleles showed strong wing nicking phenotypes in the Ax^{16} background (data not shown). The results are consistent with the notion that Ax^{16} represents a gain-of-function mutation of *N* in terms of the bristle and wing vein phenotypes, but a loss-of-function mutation of *N* in terms of the wing nicking phenotype.

On the second chromosome, five alleles of *mam* were isolated (Figure 1D). They are homozygous lethal and fail to complement the lethal allele mam^{IL115}. In addition, they show similar wing nicking phenotypes in the genetic background of Ax^{16} (data not shown), and can rescue the lethality of the heteroallelic combination Ax^{E2}/Ax^{9B2}.

On the third chromosome, eight alleles of *Dl* were isolated (Figure 1E), seven of which are homozygous lethal. All the mutations are also good suppressors for the shortened wing vein phenotype (data not shown). They fail to complement the lethal allele Dl^{5F102}, and

can rescue the lethality of the negative complementation Ax^{E2}/Ax^{9B2}. These suppressors have wing vein phenotypes typical of *Dl* mutations in the wild-type background, which can be suppressed by a *Dl* duplication (bxd^{110}). As expected from loss-of-function *Dl* mutations, the lethal alleles show this phenotype in a haplo-insufficient manner, while the viable allele displays it only in a homozygous condition, indicating that it is a hypomorphic mutation. The lethality associated with the viable allele when heterozygous over Dl^{5F102} can be suppressed by Ax^{16}, indicating antagonistic interactions between loss-of-function *Dl* alleles and the gain-of-function *N* allele Ax^{16}.

The second group of suppressors on the third chromosome comprises seven *H* alleles (Figure 1F), six of which are homozygous viable. Meiotic mapping of the suppression of the Ax^{16} bristle phenotype using the rucuca chromosome (see materials and methods) placed all the mutations between *sr* and e^s, consistent with their being *H* alleles. As homozygotes, the six viable alleles show a wing nicking phenotype in the Ax^{16} background (data not shown). All the mutants suppress the bristle and wing vein phenotypes caused by the haploinsufficiency of *H* (H^{B79}), indicating that the alleles isolated in the screen represent duplications of the *H* locus. With the exception of the lethal allele, all other stocks produced occasional revertants. This genetic behavior is consistent with the existence of homozygous viable duplications, which can be lost due to unequal crossing over events. Also, *in situ* hybridization using H DNA as a probe revealed cytological abnormalities of varying degrees in the *H* region in all but one allele. The abnormalities ranged from a subtle distortion of the signal to a large duplication of the surrounding region, in which case we were able to identify a duplication of the 92B to 93E,F region (data not shown). Comparison among parental, revertant, and mutant chromosomes by genomic Southern blot analysis using *H* DNA probe revealed quantitative differences, consistent with the notion that the mutant chromosomes harbor duplications of *H* (data not shown). Last, we found that a transgenic strain carrying the genomic region of the *H* locus is capable of suppressing the Ax^{16} bristle phenotype in the same way as the *H* alleles isolated in the screen (data not shown). Taken together, these data indicate that all the *H* mutations are, surprisingly, duplications of the locus (*H*dp). The genetic behavior of *H*dp is consistent with the notion that the H protein acts as a negative regulator of Notch signaling (Vassin *et al.* 1985; De La Concha *et al.* 1988; Bang *et al.* 1995; Lyman and Yedvobnick 1995), presumably through direct interactions with the Su(H) protein (Brou *et al.* 1994).

The results described above show that the gain-of-function phenotypes associated with the Ax^{16} mutation can be suppressed by the reduction of Notch signaling either by directly reducing the dosage of the ligand Dl, the endogenous Notch receptor, or the presumed downstream effector mam. The same effect is seen when the dosage of the antagonist to Notch signaling, H, is increased. Using the A101 enhancer trap line as a marker for the sensory organ precursor cells, it has been previously shown that the *Ax* bristle phenotypes are due to the failure of differentiation of those cells (Palka *et al.* 1990; Heitzler and Simpson 1993; Lyman and Yedvobnick 1995). We examined the differentiation of SOP cells in the Ax^{16} background using the A101 line. β-*galactosidase* activity was visualized approximately 20 hr after pupariation. As predicted from previous studies, Ax^{16} flies lacked staining in some anterior dorsocentrals, anterior scutellars, and anterior supraalars (Figure 2, A and B). In the presence of the mutations *Dl* (Figure 2C) or *H*dp (Figure 2D), however, staining in these regions was recovered, consistent with our observation of the bristle phenotype. This result corroborates the notion that the abnormal differentiation of SOP cells associated with the Ax^{16} mutation can be restored by reducing Notch signaling.

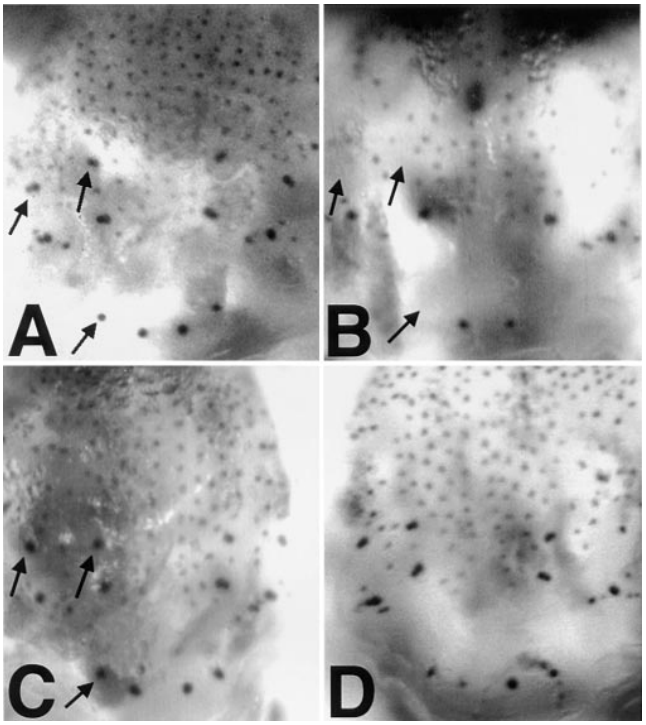

Figure 2.—Abnormal SOP differentiation underlies the Ax^{16} bristle phenotype. X-gal staining of the A101 enhancer trap line was used as a marker for the SOP and the descendant cells. (A) Wild-type control, *yw*. (B) $ywAx^{16}$. (C and D) Ax^{16} flies carrying a heterozygous *Dl* mutation (C) or a duplication of H (D). The darker and broader staining areas represent macrochaetae, and the lighter and smaller staining areas represent microchaetae. Note that staining in some macrochaetae (anterior dorsocentrals, anterior scutellars, and anterior supra alars; arrows in A–C) is missing in B. All the staining, however, is recovered in C and D. Arrows in B indicate approximate locations where wild-type staining should be seen.

***kismet* suppressors:** In addition to the strong suppres-

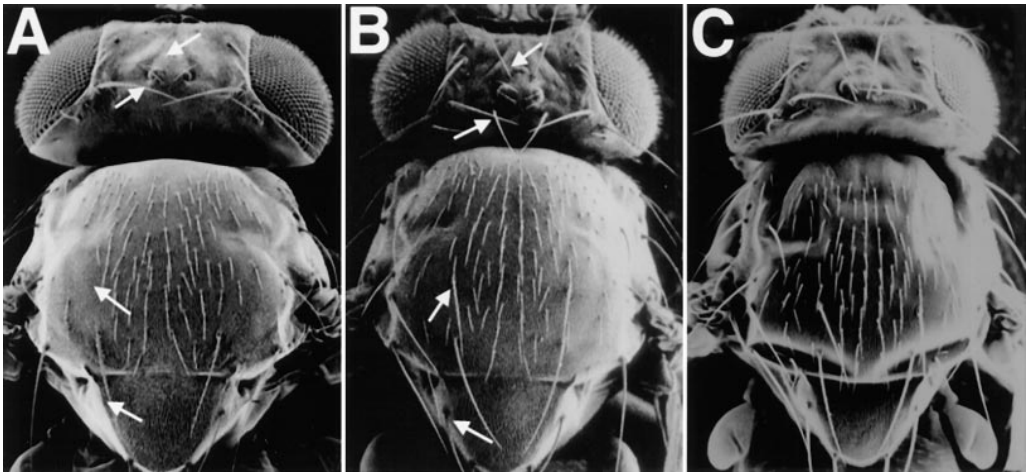

Figure 3.—The Ax^{16} bristle phenotype is dominantly suppressed by the A122 and M285 mutations. Scanning electron microscopy images of bristle phenotypes. The bristle phenotype of $ywAx^{16}$ flies (A) is suppressed when the flies are simultaneously heterozygotes for either A122 (B) or M285 (C) mutations. Ocellars and postverticals on the head, anterior dorsocentrals and anterior scutellars on the notum are the most obvious structures to be affected (arrows in A and B). Arrows in A indicate approximate locations where wild-type bristles should be seen.

sors described above, three alleles of *kismet* (Kennison and Tamkun 1988) were identified as weak suppressors. All the alleles are homozygous lethal, and meiotic mapping of the lethality using multiply marked chromosome *S, Sp, Bl, L, bw*^{D} (see materials and methods) places the mutation to the left of *S.* They fail to complement each other and other *kismet* alleles, which were independently isolated from another modifier screen of the phenotype associated with the expression of constitutively activated forms of the Notch receptor in the eye (Verheyen *et al.* 1996). The alleles from both screens appear to involve loss-of-function *kismet* mutations that could, in a dominant fashion, interact with the Notch pathway.

Two novel suppressors of Ax^{16}: In addition to the known members of the Notch pathway and *kismet*, two complementation groups that do not correspond to known genes were identified as suppressors of the Ax^{16} bristle phenotypes (Figure 3). One, A122, is on the second chromosome (Figure 3B) and the other, M285, is on the third (Figure 3C). We isolated one allele of each. Both are homozygous lethal and neither displays phenotypes as heterozygotes in a wild-type background.

A122 on the second chromosome complements the second chromosome Notch pathway components *mam* (mam^{IL115}) and *Su(H)* [$Su(H)^{T4}$]. The homozygous animals for A122 die as early larvae. The A122 mutation suppresses the Ax^{16} bristle phenotype as efficiently as other known members of the Notch pathway. In fact, A122 is also capable of suppressing the "fewer bristle" phenotype of another *Ax* allele, Ax^{9B2} (data not shown). Although Ax^{9B2} belongs to the suppressor group of *Ax* mutations, this observation is consistent with the notion that all *Ax* alleles represent essentially gain-of-function mutations of *N* in terms of bristle phenotypes. We used this suppression as a marker for the meiotic mapping of A122, because it was easier to monitor than the suppression of Ax^{16} due to the fertility of the flies involved and the penetrance of the suppression. Using the chromosome *net, b, cn, bw* (see materials and methods) in the genetic background of Ax^{9B2}, the suppressor of the Ax^{9B2} bristle phenotype was mapped between *b* and *cn*, close to *cn*. Meiotic mapping of the lethality of A122 using *S, Sp, Bl*, L, *bw*^{D} chromosome (see materials and methods) places the mutation between *Bl* and *L*, closer to *Bl*, suggesting that both phenotypes are the consequences of the same mutation. Although we have not been able to obtain a specific deficiency that uncovers the mutation around this region, taking into account the results of the meiotic mapping described above, we assume that the mutation is located between 40A4-42C.

M285 complements the third chromosome Notch pathway components *Dl* (Dl^{5F102}), *H* (H^{1}), and a deletion of the entire region of *E(spl)*-C including *groucho* [$E(spl)^{8D06}$], as well as the zygotic neurogenic gene neuralized (neu^{12H56}). The homozygous animals for M285 die as late embryos. No gross abnormalities of the nervous system were detected (data not shown). The suppression of the Ax^{9B2} bristle phenotype by M285 was not significant compared to that with A122. However, because M285 enhances the *Dl* wing vein phenotype (see Figure 4, C and D), this phenotype was used for the meiotic mapping of the genetic interactions of M285 using the Dl^{5F102} mutation on the rucuca chromosome (see materials and methods). M285 was placed between *st* and *cu*. Consistent with the meiotic mapping, M285 is lethal over Df(3L)Pc-MK, which covers the region 78A2-78C9. Using 14 other deficiencies around this region (kindly provided by A. Carpenter), the mutation was located in 78A2-78B1. M285 complemented two possible candidate mutations around this region, *fng* (fng^{80}) and *grain* (grn^{7J86}, grn^{7L12}). The deficiencies that uncover the lethality of M285 did not show genetic interactions similar to those of the M285 mutation, suggesting that this allele

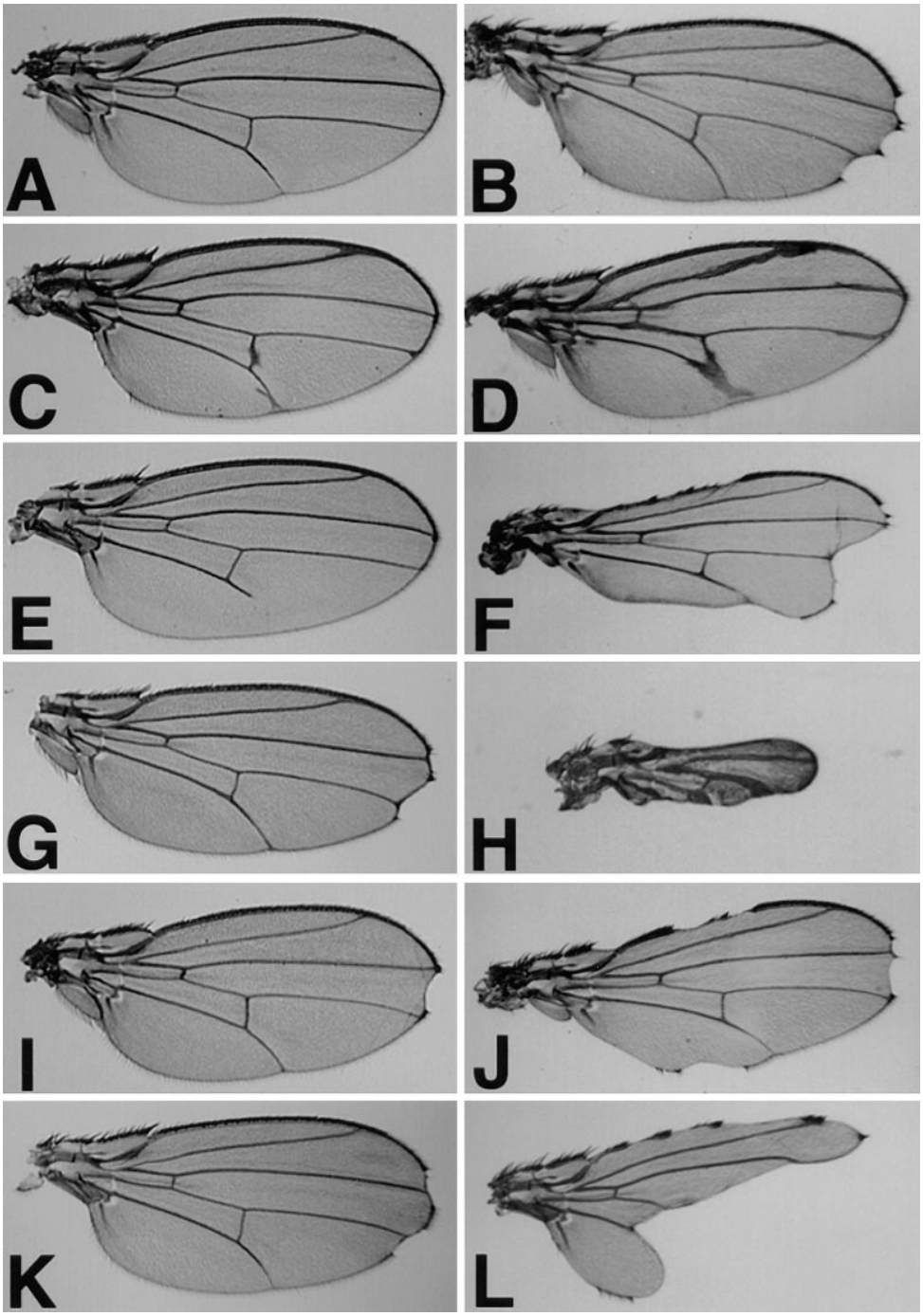

Figure 4.—Genetic interactions of M285 with the Notch pathway components during wing development. (A) Wild-type wing. (B) Wing phenotype of the M285 mutation over the TM3 balancer, M285/TM3. (C–L) Wing phenotypes associated with mutations in the Notch pathway elements are shown in the left column, and double mutants of the same mutations in combination with M285/+ are shown in the right column. (C) Dl^{5F102}/+, (D) Dl^{5F102}/M285, (E) Ax^{16}, (F) Ax^{16}; M285/+, (G) *nd*, (H) *nd*; M285/+, (I) $Su(H)^{T4}$/+, (J) $Su(H)^{T4}$/+; M285/+, (K) *BdG*/+, and (L) *BdG*/M285. Note that *Dl* wing vein phenotype is enhanced by M285 (C and D). M285 is missing portions of wing margin in the Ax^{16} genetic background (E and F). The wing nicking phenotypes of other mutations are also enhanced by M285 (G, H, I, J, K, and L).

represents a gain-of-function mutation. This interpretation is reinforced by the fact that previous genetic screens for Notch signaling have not identified mutations in this region showing similar genetic behavior.

To quantify the effect of the suppression for the Ax^{16} fewer bristle phenotype by the mutations isolated in this screen, we scored the number of macrochaetae for each complementation group but *kismet*, which are weak suppressors. As summarized in Table 1, both A122 and M285 can suppress the phenotype significantly, with M285 being the weaker suppressor. The results are consistent with the observation that A122 can suppress the Ax^{9B2} bristle phenotype better than M285 as described above.

Genetic interactions of M285: Although A122 more efficiently suppresses the *Ax* bristle phenotypes compared to M285 as described in the previous section, we failed to observe any other significant genetic interactions of A122 with the Notch pathway elements (data not shown). In contrast, M285 showed extensive genetic interactions with the Notch pathway components in terms of wing development. Although M285 displays no wing phenotypes as heterozygotes in an otherwise wild-type background (data not shown), it occasionally shows

TABLE 1

Suppression of the Ax^{16} bristle phenotype

Genotype	Average number of macrochaetae
yw	43.8 ± 0.7
ywAx^{16}	32.8 ± 2.5
ywAx^{16}/*N*	43.9 ± 0.2
ywAx^{16}; *mam*/+	40.2 ± 1.8
ywAx^{16}; A122/+	39.2 ± 1.7
ywAx^{16}; *Dl*/+	40.9 ± 1.4
ywAx^{16}; *H*dp/+	38.8 ± 1.7
ywAx^{16}; M285/+	37.8 ± 1.6

To quantify the effect of the modifiers on the Ax^{16} bristle phenotype we scored the number of macrochaetae for each complementation group but *kismet*, which are weak suppressors. One allele from each complementation group was selected and scored in the Ax^{16} genetic background. The number of macrochaetae on the head, thorax, and scutellum was scored among a preselected group of 44 macrochaetae (see materials and methods). The table lists the average number of macrochaetae for each mutant combination and the standard deviation. The *yw* genotype is the wild-type control. Fifty female *N* flies were analyzed. In all other cases 25 male and 25 female flies were checked.

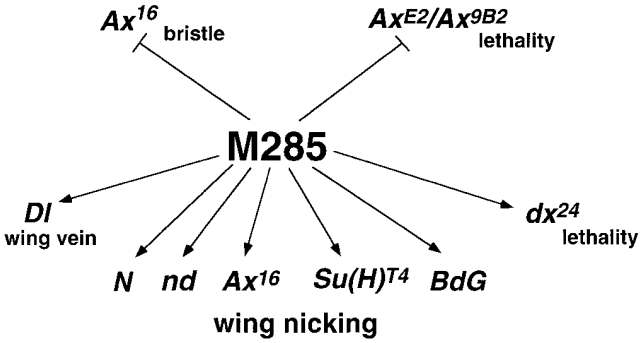

Figure 5.—Summary of significant genetic interactions that M285 showed with mutations in the Notch pathway components. The M285 mutation suppresses the "fewer bristle" phenotype of the hypermorphic *N* allele Ax^{16} and rescues the lethality associated with the heteroallelic combination Ax^{E2}/Ax^{9B2}. The M285 mutation also enhances the *Dl* wing vein phenotype. The wing nicking phenotypes of *N*, the hypomorphic *N* allele *nd*, Ax^{16}, a gain-of-function mutation of *Su(H)* [$Su(H)^{T4}$], and the dominant negative mutation of *Ser*, *BdG* are also enhanced by the M285 mutation. Finally, the M285 mutation results in lethality in the genetic background of *dx* (dx^{SM}, dx^{24}). Alleles are not specified for *N* and *Dl*, because the genetic interactions can be generally observed with loss-of-function mutations of *N* and *Dl*.

wing nicking phenotypes in the presence of the TM3 balancer (Figure 4B), presumably because it carries the *Ser* mutation, which encodes a ligand for the Notch receptor during wing morphogenesis. The TM3 balancer itself seldom shows this phenotype, while the penetrance of the phenotype becomes complete in the genetic background of Ax^{E2} (data not shown). In addition to the enhancement of the wing vein phenotype of *Dl* (Figure 4, C and D), M285 is missing portions of wing margin in the Ax^{16} genetic background (Figure 4, E and F). This phenotype is very similar to loss-of-function *N* mutations in the Ax^{16} genetic background (data not shown). M285 also strongly enhances the wing nicking of the hypomorphic *N* allele, *notchoid* (*nd*) (Figure 4, G and H). The wing phenotypes of a gain-of-function mutation in *Su(H)* [$Su(H)^{T4}$; Fortini and Artavanis-Tsakonas 1994; Figure 4, I and J], the dominant negative mutation of *Ser*, *Beaded Goldshmidt* (*BdG*; Hukriede and Fleming 1997; Figure 4, K and L), and the *N* deletion, N^{54l9} (data not shown) are also enhanced by M285. Finally, the M285 mutation is lethal in the genetic background of *dx* (dx^{SM}, dx^{24}), which encodes a cytoplasmic protein that binds the intracellular domain of the Notch protein (Diederich *et al.* 1994; Matsuno *et al.* 1995). These genetic interactions indicate that the M285 mutation reduces Notch signaling activity during wing development. This conclusion is reinforced by the results

TABLE 2

Effect of the modifiers on the negative complementation Ax^{E2}/Ax^{9B2}

	Ax^{9B2}/Y; */+	Ax^{9B2}/Y; B/+	Ax^{E2}/Ax^{9B2}; */+	Ax^{E2}/Ax^{9B2}; B/+
Control	55	47	0	0
mam	35	29	45	1
A122	48	56	8	0
*H*dp	42	45	56	0
M285	105	90	85	0

The results of the following crosses are compiled: Ax^{E2}/Y; */B × Ax^{9B2}/Ax^{9B2} virgins. All the experiments were done at 25°. As previously shown the lethality associated with Ax^{E2}/Ax^{9B2} was rescued by *mam* (Xu *et al.* 1990). A *mam* allele, which was isolated in our screen, was used for these experiments. A122 failed to show a significant effect on the negative complementation while both the M285 mutation and a duplication of *H* (*H*dp) could significantly rescue the Ax^{E2}/Ax^{9B2} lethality. B is a balancer chromosome. * and + indicate a chromosome with each mutation and a wild-type control chromosome, respectively. The number of adult flies in each genotype was scored within 14 days after the crosses had been done. Each figure represents the number of flies that we scored in each case. Because occasional escapers are found even in the control, expecially on the 14th day, we did not consider the effect of a A122 significant.

summarized in Table 2. We observed that while A122 failed to show a significant effect on the negative complementation Ax^{E2}/Ax^{9B2}, both M285 and *H*dp can effectively rescue the Ax^{E2}/Ax^{9B2} lethality. This observation also suggests that the M285 mutation modulates Notch signaling activity during development in a general way. The significant genetic interactions that M285 showed with mutations in the Notch pathway components are summarized in Figure 5.

DISCUSSION

The use of genetic screens has proven to be a powerful tool in the dissection of developmental pathways. It has been particularly useful for the study of Notch signaling given the unusual sensitivity of normal development to the gene dosage of Notch pathway elements, the very broad expression pattern of the gene products, and their pleiotropic action. Several new components of the pathway have been identified using genetic interactions between two loci as a criterion for placing them in the same pathway (*e.g.*, Brand and Campos-Ortega 1990; Xu *et al.* 1990; Klein and Campos-Ortega 1992; Fortini and Artavanis-Tsakonas 1994; Hing *et al.* 1994; Verheyen *et al.* 1996). Significantly, a given modifier may interact with Notch in a tissue-specific manner, suggesting either the existence of components that are relevant only in a specific developmental context, or reflecting tissue-specific crosstalk between Notch signaling and other signaling mechanisms such as the ras, EGF, wingless pathways (Hing *et al.* 1994; Verheyen *et al.* 1996).

The search for modifiers of the *Ax* bristle phenotype is distinct from the screens carried out so far, thus extending the existing studies. Although the *Ax* mutant protein represents a hyperactive form of the Notch receptor, unlike the ligand-independent, constitutively activated forms of the Notch receptor driven by the *sev* promoter in the eye (Verheyen *et al.* 1996), it is under the control of the endogenous promoter and is ligand dependent (Heitzler and Simpson 1993). In addition, the present screen is the first to use bristles as the phenotypic parameter, even though the involvement of Delta-Notch signaling in lateral inhibition/specification processes controlling the segregation of SOPs from proneural clusters as well as the subsequent specification of sensory organ cell fates is well documented (Hartenstein and Posakony 1990; Parks and Muskavitch 1993). Consistent with the notion that the Ax^{16} bristle phenotype is a reliable marker to search for dominant Notch signaling modifiers, the phenotype is suppressed by lowering the dosage of either *N* or *Dl*. This was also confirmed by the identification of new alleles of the Notch pathway components, *N*, *mam*, *Dl*, and *H*, through the screen, strengthening the potential significance of novel modifiers as modulators of Notch signaling.

Three *kismet* alleles were isolated as weak suppressors of the Ax^{16} bristle phenotype. Interestingly, mutations in *kismet* have been isolated independently as enhancers of the eye phenotype associated with the expression of constitutively activated forms of the Notch receptor (Verheyen *et al.* 1996). *kismet*, which may encode a structural component of chromatin (Kennison and Tamkun 1988), did not display broad genetic interactions with Notch. It was therefore suspected that the identification of these alleles through the eye screen may reflect its effect on the expression of the transgene by perturbing normal chromatin function rather than significant interactions with Notch signaling (Verheyen *et al.* 1996). The fact that we have also isolated such alleles in the bristle screen may be indicative of a link between Notch signaling and *kismet* function; however, further analysis is necessary before such a relationship can be established.

The two novel mutations identified here are effective suppressors of the Ax^{16} bristle phenotype displaying effects similar to mutations in the known Notch pathway elements. They thus seem to result in reduction of Notch signaling, and thereby suppress a gain-of-function bristle phenotype of *Notch*. However, the interpretation of genetic interactions with mutations in the Notch pathway and epistatic relationships must be made with caution. For example, the wing nicking phenotypes of loss-of-function *N* mutations are suppressed by loss-of-function *Dl* mutations (Vassin *et al.* 1985; De La Concha *et al.* 1988; Xu *et al.* 1990). Such complex genetic behavior can be explained by postulating regulatory feedback loops which control the expression of the receptor and the ligands. Indeed, several studies have indicated the existence of such a mechanism (Heitzler and Simpson 1991; Wilkinson *et al.* 1994; Heitzler *et al.* 1996; De Celis and Bray 1997; Huppert *et al.* 1997; Panin *et al.* 1997). In addition, it appears that Su(H) may not be the only effector of Notch signaling (Lecourtois and Schweisguth 1995; Shawber *et al.* 1996; Matsuno *et al.* 1997; Wang *et al.* 1997). Furthermore, the action of effectors on downstream gene activity may differ depending on the developmental context. For instance, CBF1, the mammalian homologue of Su(H), has been shown to act either as a transcriptional repressor or an activator, depending on the presence of the protein EBNA2 (Hsieh and Hayward 1995). Although analogous molecular analyses have not been carried out in Drosophila, genetic analyses raise the possibility that Su(H) may act differentially as well. Gain-of-function *Su(H)* mutations enhance, rather than suppress, the *H* fewer bristle phenotypes (Nash 1970; Ashburner 1982; Fortini and Artavanis-Tsakonas 1994; Schweisguth and Posakony 1994; Verheyen *et al.* 1996). However, gain-of-function *Su(H)* mutations are also associated with a dominant wing nicking phenotype, occasionally in the wild-type background (Figure 5G) and consistently in the Ax^{16} background (our unpublished results), which is a typical

loss-of-function Notch signaling phenotype. Although the gain-of-function *Su(H)* allele, $Su(H)^{T4}$ enhances the *H* bristle phenotype, it suppresses the Ax^{16} bristle phenotype as do the suppressors identified in the present study (our unpublished results). These observations raise the possibility that the mutant protein of Su(H) may act differentially, depending on the developmental context and illustrate the difficulties associated with the interpretation of Notch signaling phenotypes.

A122 appears to affect Notch signaling only during bristle development whereas M285 affects Notch signaling more broadly as summarized in Figure 5. The profile of the genetic interactions we documented with M285 is quite similar to that of mutations in other known components of the Notch pathway, such as *N* itself, *mam*, *Su(H)*, and *H* (data not shown). Particularly, M285 is able to rescue the lethality associated with the negatively complementing combination Ax^{E2}/Ax^{9B2}, an effect so far exclusively seen with mutations in genes directly integrated in the Notch signaling pathway (Xu and Artavanis-Tsakonas 1990; Xu *et al.* 1990; Fortini and Artavanis-Tsakonas 1994; Verheyen *et al.* 1996). Unraveling the function of the M285 locus must await a molecular characterization, especially considering the gain-of-function nature of the mutation. In spite of the difficulties in interpreting genetic interactions between the Notch pathway elements, the genetic behavior of specific alleles such as M285 has so far proven to be a reliable criterion for linking a particular gene with Notch signaling. Accordingly, the gene encoding the M285 mutation is likely to play an important role in the Notch signaling pathway.

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