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Nipped-A, the Tra1/TRRAP Subunit of the *Drosophila* SAGA and Tip60 Complexes, Has Multiple Roles in Notch Signaling during Wing Development

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The Notch receptor controls development by activating transcription of specific target genes in response to extracellular signals. The factors that control assembly of the Notch activator complex on target genes and its ability to activate transcription are not fully known. Here we show, through genetic and molecular analysis, that the *Drosophila* Nipped-A protein is required for activity of Notch and its coactivator protein, mastermind, during wing development. Nipped-A and mastermind also colocalize extensively on salivary gland polytene chromosomes, and reducing Nipped-A activity decreases mastermind binding. Nipped-A is the fly homologue of the yeast Tra1 and human TRRAP proteins and is a key component of both the SAGA and Tip60 (NuA4) chromatin-modifying complexes. We find that, like Nipped-A, the Ada2b component of SAGA and the domino subunit of Tip60 are also required for mastermind function during wing development. Based on these results, we propose that Nipped-A, through the action of the SAGA and Tip60 complexes, facilitates assembly of the Notch activator complex and target gene transcription.

The Notch receptor controls many developmental processes through activation of specific target genes. One of the key paradigms for how Notch controls development is provided by its role in definition of the dorsal-ventral compartment boundary of the developing wing in *Drosophila* (10, 12, 13, 36, 39). Activation of Notch at the dorsal-ventral boundary results in expression of the *vestigial* and *cut* genes, which are required for margin development and wing growth. Notch activates *vestigial* through its boundary enhancer (25), and the vestigial protein activates *cut* through its margin enhancer (20, 23, 37, 42, 49).

Nipped-A mutations were isolated in a genetic screen for factors that regulate activation of *cut* by the wing margin enhancer, and it was found that they reduce Notch activity both at the wing margin and in the developing wing veins (45). Heterozygous *Nipped-A* mutations increase the severity of the mutant wing margin and blade reduction phenotype caused by the weak loss-of-function *Notch* (N^{nd-1}) mutation and decrease the severity of the vein-shortening phenotype caused by a gain-of-function *Notch* mutation (N^{Ax-E2}) (45).

Other genetic data also indicate that *Nipped-A* is important for Notch signaling. Mastermind is a coactivator protein required for transcriptional activation by Notch (18, 26, 43), and heterozygous *Nipped-A* mutations dramatically increase the weak wing-nicking phenotype caused by heterozygous *mastermind* mutations (45). The *vestigial* gene is directly activated by Notch (25), and flies heterozygous for both *Nipped-A* and *vestigial* mutations display wing margin defects (45). The Notch intracellular fragment binds to the Suppressor of Hairless [Su(H)] protein on target genes (17), and a *Nipped-A Su*(*H*) double mutant displays a dominant wing-nicking phenotype (45). Together, the effects that the *Nipped-A* dosage has on the mutant phenotypes displayed by *Notch*, *mastermind*, and *vestigial* mutants indicate that *Nipped-A* encodes a factor critical for Notch activity in the developing wing.

Two *Nipped-A* mutants were recently shown to have point mutations in the gene encoding the *Drosophila* homologue of the yeast Tra1 and mammalian TRRAP proteins (38). Tra1/TRRAP is a key component of the SAGA and Tip60 (NuA4) chromatin-remodeling complexes in yeast, flies, and humans (1, 15, 19, 28, 29, 34, 48, 51).

Tra1/TRRAP is a direct target of transcriptional activators and helps them recruit the SAGA and Tip60 chromatin modification complexes to aid in gene activation (reviewed in reference 7). Mammalian Tra1/TRRAP was first identified as a coactivator that interacts directly with the Myc and E2F activators (33). Tra1/TRRAP is also a target of several other activators in yeast and mammalian cells, including Gal4 (5), E1A (11), VP16 (35), nuclear receptors (52), and p53 (2). Tra1/TRRAP contains an ATM–phosphatidylinositol-3 (PI-3) kinase-like domain near the C terminus that is important for recruitment of histone acetyltransferase (HAT) activity in mammalian cells (41). The C terminus is also critical for interaction of yeast Tra1 with acidic activators (6).

There is evidence that SAGA, which contains Tra1/TRRAP and the Gcn5/PCAF HAT, may be involved in transcriptional activation by the Notch complex (27). Several components of the Notch activator complex are known and functionally identical in worms, flies, and mammals (reviewed in references 30 and 32). Upon binding of ligands such as Serrate or Delta to the extracellular EGF repeats of Notch, an intracellular fragment of Notch (NICD) is proteolytically released, allowing it to enter the nucleus, where it interacts with a DNA-bound CSL [CBF1/Su(H)/Lag-1] protein. NICD helps recruit the master-

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mind coactivator. An N-terminal region of mastermind interacts with both the CSL protein and an ankyrin repeat domain of NICD. The p300/CBP (CREB-binding protein) HAT coactivator is recruited by interactions with both the NICD ankyrin repeats and a specific region in the N-terminal half of mastermind (18, 40). The Gcn5/PCAF HAT is also recruited by the Notch activator complex in cultured mouse cells, which requires the ankyrin repeat region of NICD (27). The NICD ankyrin repeats bind other proteins, such as mastermind and CBP, and thus it is possible that these proteins are also required to recruit Gcn5/PCAF. Because Tra1/TRRAP is the SAGA subunit targeted by several transcriptional activators, it is a distinct possibility that it is required for recruitment of Gcn5/PCAF by the Notch activator complex.

Here we present a molecular genetic analysis of several *Nipped-A* mutations that provides new insights into the roles of the Tra1/TRRAP protein and its complexes in Notch signaling. We find that reducing the *Nipped-A* gene dosage by half reduces both mastermind and Notch activities during wing development and that, surprisingly, certain mutant alleles can replace one copy of wild-type *Nipped-A*. Our data also show that other subunits of the SAGA and Tip60 complexes that contain Nipped-A are required for mastermind and Notch function in wing development and that Nipped-A is required for binding of mastermind to chromosomes. Taken together, the results indicate that Nipped-A plays multiple roles in Notch signaling.

MATERIALS AND METHODS

Northern blot assays. Northern blot assays were performed with formaldehyde-agarose gels and radioactively labeled single-stranded RNA probes as previously described (14). Total RNA was isolated from second-instar larvae with Trizol (Gibco BRL). Ethyl methanesulfonate (EMS)-induced *Nipped-A* mutations were provided by Steve Myster and Mark Peifer. The mutations were backcrossed to the parental isogenic *cn bw* chromosome and balanced over CyO *Kr-GAL4 UAS-GFP* or CyO Df(2R)Kr^{-} Tp(1;2)y^{+}. Homozygous and hemizygous mutants were selected by the lack of green fluorescent protein expression or reduced pigmentation of larval mouthparts. Hemizygous mutants were generated by crossing mutants to the balanced *Nipped-*E^{43} deficiency that deletes *Nipped-A* and neighboring genes (45). Radioactively labeled single-stranded RNA probes were prepared by in vitro transcription with T7 RNA polymerase and a vector prepared by cloning a C-terminal fragment of the Nipped-A coding sequence into pGEM-1.

Sequencing of *Nipped-A* mutations. Total RNA was isolated from second-instar larvae, and 1 μg was used to generate cDNA with random hexamer primers and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's directions. A series of overlapping fragments of Tra1/TRRAP were amplified by PCR, treated with exonuclease I and shrimp alkaline phosphatase (ExoSAP; U.S. Biochemicals), and sent to Retrogen, Inc., to be sequenced with the PCR primers. Sequences were assembled and compared with CodonCode Aligner software (CodonCode Corp.). Mutations in splice sites and the 3′ untranslated region (UTR) were confirmed by sequencing PCR products generated from *cn bw* and mutant genomic DNA.

5′ and 3′ rapid amplification of cDNA ends (RACE). The ends of the Tra1/TRRAP RNA were amplified with the BD SMART RACE cDNA amplification kit according to the manufacturer's directions, and the products were cloned into a TOPO TA pCRII vector (Invitrogen) and sent to Retrogen, Inc., for sequencing.

Effects of *Nipped-A*, *Ada2b*, and *domino* mutations on *Notch* and *mastermind* mutant phenotypes. The dominant effects of the *Nipped-A*, *Ada2b*, and *domino* mutations on the *Notch* and *mastermind* mutant phenotypes were determined by conducting genetic crosses at 25°C as previously described (45). Balanced mutant males were crossed to females that were homozygous N^{nd-1}, homozygous N^{Ax-E2}, or mam^{g2}/CyO Df(2R)Kr^{-} Tp(1;2)y^{+}. Male progeny were scored for the severity of the *Notch* or heterozygous *mastermind* mutant phenotype as described in Results. *Ada2b* mutations were provided by Mattias Mannervik and Thomas Kusch, and the dom^{1} allele of *domino* was provided by Marie-Laure Ruhf and Marie Meister. The dom^{1} mutation is a P element insertion 1 bp downstream of the start of the first intron (47). The dom^{R12} revertant of dom^{1} was generated with P transposase (CyO, HOP2; provided by Ron Blackman) to excise the P element. Reversion was confirmed by restoration of viability and PCR analysis.

Generation of Nipped-A antisera. Two His_6-tagged fragments of Nipped-A encoded by exon 12 (NipA12) and exon 14 (NipA14) were expressed in bacteria with the pMCSG7 vector (50) and purified under denaturing conditions with

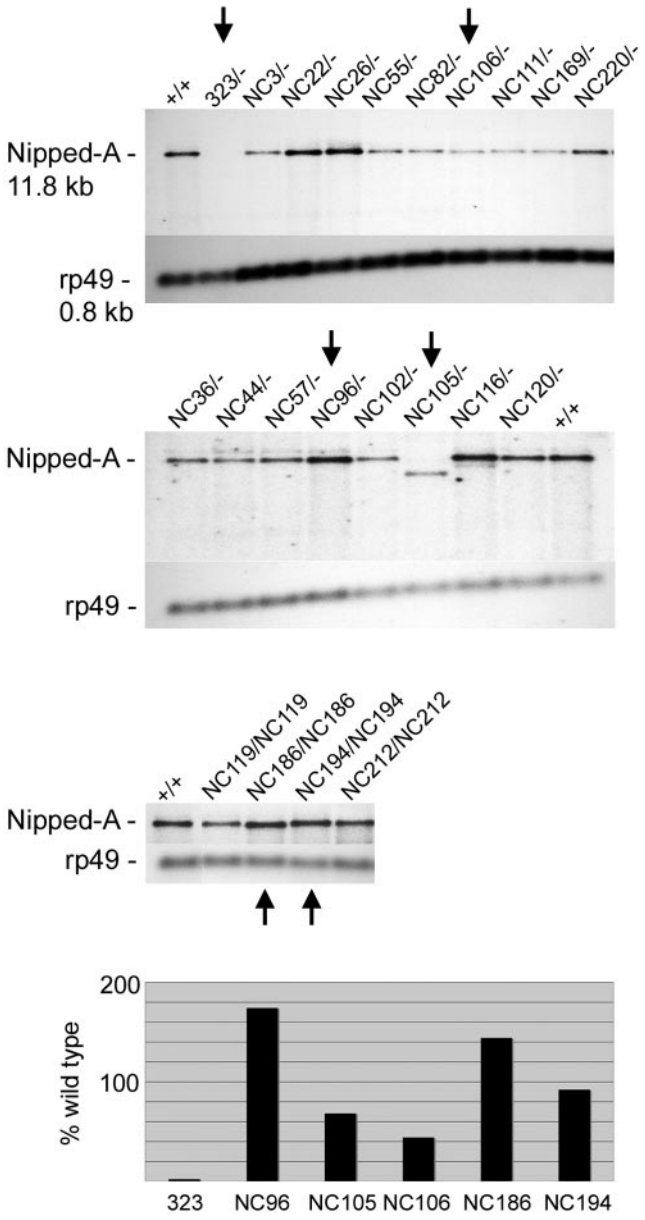

FIG. 1. Tra1/TRRAP transcripts are altered in size and level in *Nipped-A* mutants. The panels show three independent Northern blot assays of total RNA isolated from either hemizygous or homozygous *Nipped-A* mutant second-instar larvae, as indicated above the lanes. The wild-type *Tra1/TRRAP* transcripts are 11.8 kb in size. The Northern blots were stripped and reprobed for *rp49* as a control for gel loading. Arrows indicate mutants used in the genetic analysis of Notch and mastermind function. *Tra1/TRRAP* transcripts in these mutants were quantified by phosphorimager, and the levels were normalized to the amount of *rp49* transcript. The levels of the mutant transcripts, with the amount of wild-type transcript set to 100%, are shown in the bar graph in the bottom panel.

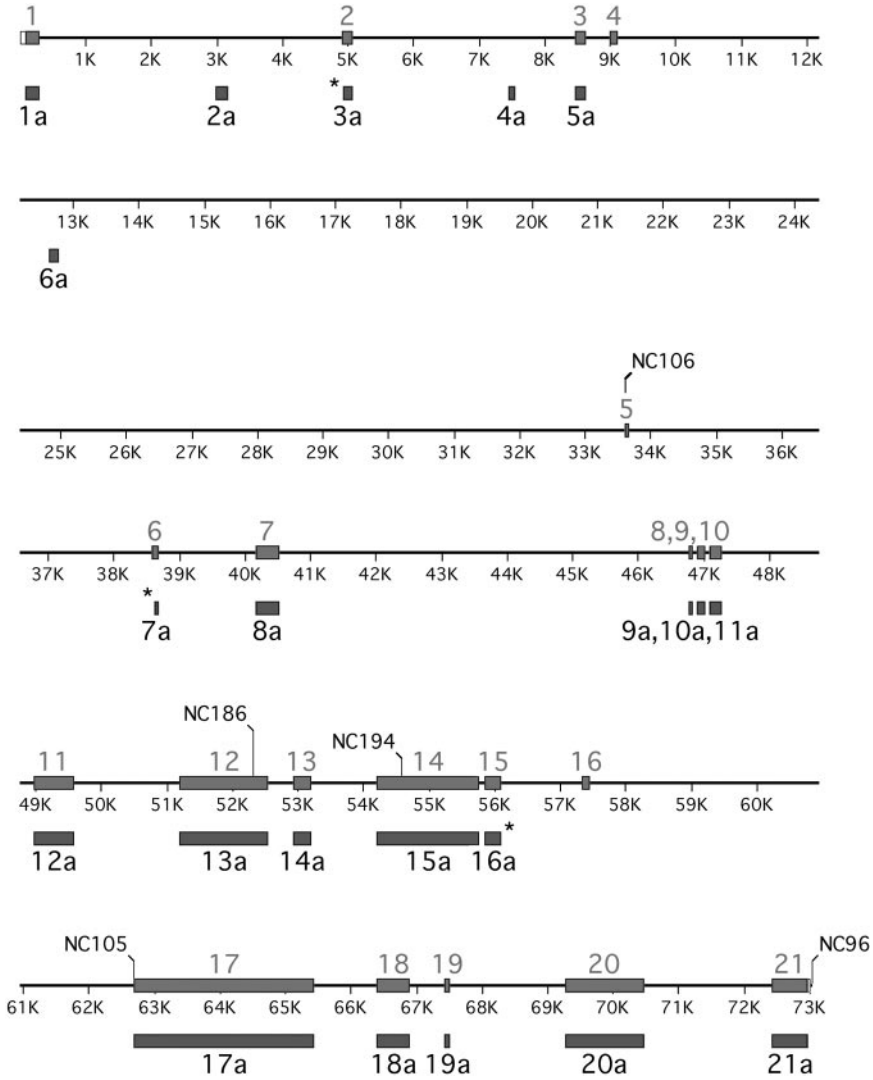

FIG. 2. The *Tra1/TRRAP* gene and locations of *Nipped-A* mutations. Shown is a diagram of the Tra1/TRRAP gene, starting with the most 5′ RACE product and ending with the polyadenylylation site. The numbering is in kilobase pairs. The exons present in the major Tra1/TRRAP transcript are shown and numbered consecutively on the line, and the exons in the previously reported (29) rare transcript are shown below, numbered consecutively (1a, 2a, etc.). While most of the exons that overlap in the major and minor transcripts are identical, the positions of the splice acceptor or donor sites differ in three, as indicated by asterisks. Locations of the *Nipped-A* mutations listed in Table 1 are indicated by vertical lines.

QIAGEN nitrilotriacetic acid beads. Denatured NipA14 protein was eluted from the resin, precipitated with 30% polyethylene glycol, suspended in phosphate-buffered saline (PBS), and used to immunize a rabbit at the Pocono Rabbit Farm and Laboratory (Canadensis, PA). NipA12 protein was washed with PBS on the beads and used to immunize a guinea pig while attached to the beads. The inserts for protein production were generated by PCR from *cn bw* cDNA. The primers for NipA12 were 5′-TACTTCCAATCCAATGCAATGATGGTGGAGCCA CAAGCTTT-3′ and 5′-TTATCCACTTCCAATGCTAATGAGGATCCATAA CTTCAGAAACGG-3′, which amplify sequences encoding amino acids 871 to 1232. The primers for NipA14 were 5′-TACTTCCAATCCAATGCTGAGGCA TCGAGTCCATATCGAG-3′ and 5′-TTATCCACTTCCAATGCTAATTTC CATCCTCCATTCGCAACG-3′, which amplify sequences encoding amino acids 1490 to 1870. NipA12 and NipA14 antibodies were affinity purified from serum with antigen on Immobilon membrane (21).

Western blot assays. Twenty microliters of dechorionated 0- to 12-h-old embryos with 80 μl of PBS was lysed with 100 μl of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer and denatured by boiling for 5 min, and 10 and 20 μl of each sample, containing the denatured protein from ~115 and 230 embryos, was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blots were blocked with PBS containing 1% bovine serum albumin and 1% normal donkey serum. The primary antibody was affinity-purified NipA12 diluted 1:100, and the secondary antibody was horseradish peroxidase-conjugated donkey anti-guinea pig antibody diluted 1:10,000 (Jackson Labs), which was detected with chemiluminescent reagents (Perkin-Elmer).

Immunostaining of larval tissues. Larval tissues were fixed and immunostained as previously described (3), with purified NipA12 (1:50) or NipA14 (1:100) antibody, followed by Cy3-labeded donkey anti-guinea pig (1:400) or fluorescein isothiocyanate-conjugated goat anti-rabbit (1:200) secondary antibody. Samples were mounted in Prolong Antifade (Invitrogen) containing 1 μg of 4′,6′-diamidino-2-phenylindole (DAPI) per ml.

Immunostaining of salivary gland polytene chromosomes. Salivary glands were fixed for 30 s in 2% formaldehyde and then in 45% acetic acid and 2% formaldehyde for 3 min as previously described (31) before storage in 67% glycerol–33% PBS at −20°C. Mouse monoclonal antibody against mastermind, provided by Spyros Artavanis-Tsakonas, and rabbit polyclonal serum against Gcn5, provided by Thomas Kusch and Jerry Workman, were both used at a 1:100 dilution. The NipA12 and NipA14 antisera were used at 1:100 and 1:200 dilutions. Alexa Fluor 488 (Molecular Probes)-conjugated goat anti-mouse and Cy3-labeled goat anti-rabbit (Sigma) antibodies were used at a 1:200 dilution. Fluo-

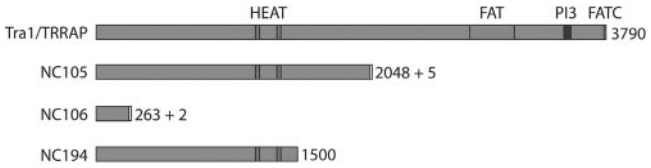

FIG. 3. Tra1/TRRAP proteins encoded by *Nipped-A* mutants. The diagrams show the proteins encoded by the major *Tra1/TRRAP* transcript in the indicated *Nipped-A* mutants (Fig. 2; Table 1). The locations of the PI3 kinase motif, the FAT and FATC motifs associated with phosphatidylinositol-3 kinases, and two HEAT repeats are indicated by boxes. The *Nipped-A*^{NC105} and *Nipped-A*^{NC106} mutations cause frame shifts that add nonnative residues at the ends of the truncated proteins.

rescein isothiocyanate-conjugated goat anti-rabbit antibody (Sigma) was used at 1:100, and Cy3-labeled donkey anti-guinea pig antibody (Jackson Labs) was used at 1:200.

Nucleotide sequence accession number. The sequence of the major *Nipped-A*/*Tra1/TRRAP* transcript determined in this work has been given GenBank accession no. DQ352451.

RESULTS

Identification of *Nipped-A* mutations in the *Tra1/TRRAP* gene. The Tra1/TRRAP protein and the HAT complexes (SAGA and Tip60/NuA4) that contain it have been well characterized biochemically. Little is known, however, about the roles of Tra1/TRRAP in metazoan gene expression, although it has been implicated in signaling pathways that control development. Similar to Notch, *Caenorhabditis elegans* Tra1/TRRAP was recently found to repress ras signaling in vulval development, although the mechanism is unclear (4, 9). *Drosophila Nipped-A* mutations were shown to affect Notch functions in wings (45), and a recent molecular analysis of two EMS-induced *Nipped-A* mutations strongly suggested that *Nipped-A* encodes Tra1/TRRAP (38). We undertook a molecular genetic analysis to confirm that *Nipped-A* is the *Tra1/TRRAP* gene and gain insights into the roles that Tra1/TRRAP complexes play in Notch signaling during wing development.

Northern blot assays of RNAs from several *Nipped-A* mutants provides further evidence that *Nipped-A* encodes Tra1/TRRAP (Fig. 1). A mutant allele generated by γ rays, *Nipped-A*^{323} (45), produces no detectable *Tra1/TRRAP* transcript (lane 2), and an allele generated by EMS mutagenesis, *Nipped-A*^{NC105} (38), produces a transcript shorter than that of the wild type (Fig. 1, lane 17). Several other EMS-generated *Nipped-A* alleles (38) produce wild-type-sized *Tra1/TRRAP* transcripts in greater or lesser amounts (Fig. 1).

We sequenced *Tra1/TRRAP* transcripts by using reverse transcription (RT)-PCR to identify EMS-generated *Nipped-A* mutations. The wild-type transcript in the isogenic *cn bw* chromosome (Fig. 2) used for mutagenesis (38) encodes a Tra1/TRRAP protein of 3,790 residues (Fig. 3). The splicing pattern of this major transcript differs from that of the originally reported mRNA (Fig. 2) (29). The transcript we detect, however, is the major transcript in embryos and second-instar larvae of both *cn bw* and Oregon R. We did not detect the previously reported variant (29) in total RNA from embryos or second-instar larvae by RT-PCR with various primers specific for its unique exons, so we conclude that it is a rare variant.

Nipped-A^{NC105}, which produces a short transcript (Fig. 1), has a mutant splice acceptor site (Table 1) that causes skipping of exon 17 (Fig. 2). The resulting open reading frame encodes a protein lacking much (46%) of the C-terminal half of Tra1/TRRAP, including the putative ATM-PI3 kinase motif (Fig. 3). *Nipped-A*^{NC105} produces nearly 70% of the wild-type level of *Nipped-A* transcript (Fig. 1).

The *Nipped-A*^{NC194} mutation creates a stop codon in exon 14 (Fig. 2; Table 1), truncating the protein after residue 1500 (Fig. 3). The level of *Nipped-A*^{NC194} transcript is nearly the same as that of the wild type (Fig. 1), suggesting that it is not subject to nonsense-mediated decay.

The *Nipped-A*^{NC106} mutation affects the exon 5 splice acceptor site (Fig. 2; Table 1). The lack of splicing causes a frame shift, resulting in a truncated 265-residue protein (Fig. 3). *Nipped-A*^{NC106} produces only 44% of the expected level of transcript (Fig. 1), suggesting that it may be reduced by nonsense-mediated decay.

Nipped-A^{NC96} has a T-to-C nucleotide change in the 3′ UTR (Table 1; Fig. 2), three residues upstream of the polyadenylylation site. This mutant produces more transcript than expected (Fig. 1, bottom panel) but, as described below, is a weak loss-of-function allele. Combined with the Northern blot analysis, identification of point mutations in multiple *Nipped-A* mutants demonstrates conclusively that *Nipped-A* encodes Tra1/TRRAP.

Tra1/TRRAP supports mastermind and Notch function in wing margin and wing vein development. The various *Nipped-A* mutations differ in their effects on Notch signaling (Fig. 4). We measured their dominant effects on the mutant phenotypes displayed by the $N^{md\text{-}1}$ and $N^{Ax\text{-}E2}$ alleles of *Notch* and the heterozygous *mam*^{g2} allele of *mastermind*. The $N^{md\text{-}1}$ hypomorphic mutant causes gaps in the wing margin and reduces blade size (Fig. 4). This phenotype was quantified by measuring the anterior-posterior width of the wing midway between the two crossveins (Fig. 4). A narrower wing indicates lower Notch activity. The $N^{Ax\text{-}E2}$ hypermorphic mutant has shortened L4 and L5 veins (Fig. 4). This was quantified by measuring the length of the L4 vein from the posterior crossvein to the tip of the wing, and a longer vein indicates reduced Notch signaling (Fig. 4). The heterozygous *mam*^{g2} mutation, which was induced by γ rays and behaves genetically as a null allele (42), causes rare nicks (gaps in the bristle

TABLE 1. *Nipped-A* mutations in *Tra1/TRRAP*

Allele	Mutation(s)[a]	Type	Encoded protein[b]	Reference
NC96	T73046C	3′ UTR	1–3790 (wild-type)	This work
NC105	A62691T	Splice acceptor	1–2048 + 5	This work
NC106	T33610A, G33613C	Splice acceptor	1–263 + 2	This work
NC186	T52327A	V1191D	1–3790 (missense)	38
NC194	A54590T	K1501STOP	1–1500	This work

[a] The numbering is based on a hybrid sequence with the first nucleotide of the most 5′ RACE product from *cn bw* defined as nucleotide 1. The hybrid sequence was generated by substituting the *cn bw* exon sequences determined here into the wild-type genomic sequence generated by the *Drosophila* genome project (8). There are multiple polymorphic differences in both exon and intron sequences between the *cn bw* and *Drosophila* genome sequences, but the hybrid sequence is used because we did not fully sequence all *cn bw* introns.

[b] The numbers refer to the residues encoded by the major *Nipped-A*/*Tra1/TRRAP* transcript determined in this work.

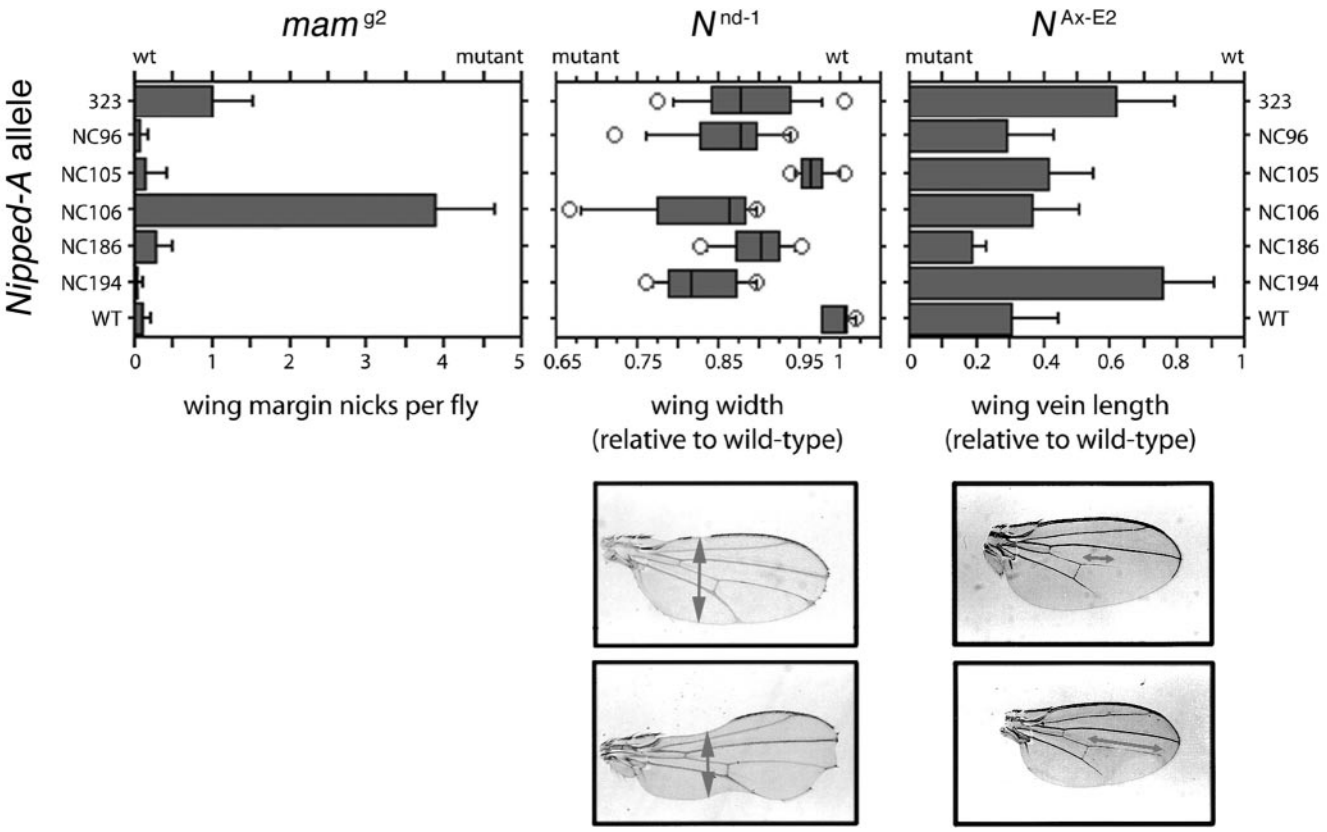

FIG. 4. Effects of heterozygous *Nipped-A* mutations on *Notch* and *mastermind* mutant phenotypes. The left panel shows that the heterozygous *mam*^{g2} null *mastermind* mutation (45) causes rare wing margin nicks and some *Nipped-A* mutations increase the frequency of these nicks, indicating reduced mastermind function. The wild-type *Nipped-A* allele is the parental allele in the *cn bw* stock used to generate the EMS-induced *Nipped-A* mutations (NC). The error bars indicate the 95% confidence intervals. The middle panel shows box plots of the distribution of wing widths of N^{nd-1} hypomorphic *Notch* mutant flies relative to the wild type (wt) in the presence of the indicated *Nipped-A* alleles. Wing widths were measured from the anterior to the posterior margin between the anterior and posterior crossveins, as shown at the bottom. Some *Nipped-A* mutations decrease the width, indicating reduced Notch function. The median width is indicated by the black line within each box, the ends of each box indicate the 25th and 75th percentiles, and the ends of the "whiskers" indicate the 10th and 90th percentiles. Circles show the lowest and highest values. The right panel shows the relative lengths of the L4 vein in N^{Ax-E2} hypermorphic *Notch* mutant flies in the presence of the *Nipped-A* alleles. As shown below, the veins were measured from the posterior crossvein toward the tip of the wing. Some *Nipped-A* mutations increase the length of the vein, indicating reduced Notch activity. The error bars show the 95% confidence intervals.

rows) in the wing margin. This phenotype was quantified by counting the nicks, with more nicks indicating reduced mastermind activity.

As observed previously (45), the *Nipped-A*^{323} null allele had significant effects in all three genetic tests, increasing the severity of the N^{md-1} mutant phenotype, decreasing the severity of the N^{Ax-E2} gain-of-function phenotype, and increasing the number of margin nicks caused by *mam*^{g2} (Fig. 4). All these effects indicate reduced Notch activity.

The *Nipped-A*^{NC106} mutation, predicted to truncate Tra1/TRRAP near the N terminus, had substantial effects on N^{md-1} and *mam*^{g2}, similar to the null allele, but little or no effect on N^{Ax-E2} (Fig. 4). We cannot quantitatively compare the effects of *Nipped-A*^{NC106} to those of *Nipped-A*^{323} because they were generated in different genetic backgrounds. Because other *Nipped-A* mutations generated in the same isogenic chromosome as *Nipped-A*^{NC106} do affect N^{Ax-E2}, however, we postulate that *Nipped-A*^{NC106} is not a null mutation and retains some activity. Because *Nipped-A*^{NC106} is a splice site mutation, it is possible that an alternatively spliced product provides some Tra1/TRRAP activity. In support of this idea, the splice site affected by *Nipped-A*^{NC106} is not used in the originally reported transcript (Fig. 2) (29), demonstrating that at least one known splicing pattern can produce a full-length Tra1/TRRAP protein in this mutant. The recessive lethality of *Nipped-A*^{NC106} and its effects on N^{nd-1} and *mam*^{g2}, however, also demonstrate that the alternative transcript is not sufficient to fulfill some critical Tra1/TRRAP functions.

Nipped-A^{NC96}, which has a point mutation in the 3′ UTR just upstream of the polyadenylation site, behaves as a hypomorph. *Nipped-A*^{NC96} increased the severity of the N^{nd-1} wing phenotype but had no significant effect on N^{Ax-E2} or *mam*^{g2}. *Nipped-A* null mutants die at the second- to third-instar molt (45), but flies hemizygous for *Nipped-A*^{NC96} survive at a significant frequency to the pupal stage (Table 2). Third-instar larvae hemizygous for *Nipped-A*^{NC96} have smaller or missing imaginal disks, and acridine orange staining suggests that there is apoptosis in the mutant disks (not shown). The *Nipped-A*^{NC186} mutant, which encodes a Tra1/TRRAP protein with a missense mutation (38), also survives as a hemizygote past pupariation, suggesting that it is also a hypomorph.

Intriguing results were obtained with *Nipped-A*^{NC105} and *Nipped-A*^{NC194}. Although *Nipped-A*^{NC105} produces reduced

TABLE 2. Survival of *Nipped-A*^{NC96} and *Nipped-A*^{NC186} mutants to pupae

Genotype[a]	Temp (°C)	No. of pupae	% Mutant pupae[b]
Nipped-A^{NC96}/Df(2R)*Nipped-E*^{43}	18	230	36.5
Nipped-A^{NC96}/*Nipped-A*^{NC106}	18	261	14.9
Nipped-A^{NC96}/*Nipped-A*^{NC194}	18	203	17.7
Nipped-A^{NC186}/Df(2R)*Nipped-E*^{43}	18	577	14.9
Nipped-A^{NC186}/*Nipped-A*^{NC106}	18	408	3.7
Nipped-A^{NC186}/*Nipped-A*^{NC194}	18	389	4.6
Nipped-A^{NC96}/Df(2R)*Nipped-E*^{43}	25	255	8.6
Nipped-A^{NC186}/Df(2R)*Nipped-E*^{43}	25	221	2.3

[a] *Nipped-E*^{43} is a deficiency that deletes *Nipped-A* and the surrounding genes (45). *Nipped-A* mutations heterozygous with *Nipped-E*^{43} are hemizygous.
[b] Mutant pupae were detected by the lack of green fluorescent protein produced by the balancer chromosome (see Materials and Methods).

levels of a short transcript lacking exon 17 (Fig. 1; Fig. 2; Table 1) and encodes a protein lacking most of the C-terminal half of Tra1/TRRAP (Fig. 3), it had little dominant effect on Notch signaling in any of the three genetic assays (Fig. 4). None of the known or theoretical splicing patterns could generate a full-length protein, and thus one possible interpretation is that the C-terminal half of Tra1/TRRAP is not required for Notch and mastermind activity in the developing wing margin and veins. This is surprising, because the available evidence indicates that the missing region, which contains the ATM-PI3 kinase motif (Fig. 3), is required for association of Gcn5 and Tip60 with mammalian TRRAP (41) and is also involved in interactions with acidic activator proteins (6). An alternative is that the mutant allele somehow increases the activity of the remaining wild-type *Nipped-A* gene or Tra1/TRRAP protein.

Nipped-A^{NC194}, which has a nonsense mutation in exon 14 (Fig. 2; Table 1) and encodes a shorter protein than *Nipped-A*^{NC105} (Fig. 3), had strong effects on both the hypomorphic wing margin and hypermorphic wing vein *Notch* mutant phenotypes but no significant effect on the *mam*^{g2} wing-nicking phenotype (Fig. 4). Thus, heterozygous *Nipped-A*^{NC194} reduces Notch, but not mastermind, activity. *Nipped-A*^{NC194}, which supports fewer functions than *Nipped-A*^{NC105}, produces more mRNA (Fig. 1, bottom panel). We cannot exclude the possibility, however, that the *Nipped-A*^{NC194}-encoded proteins are less stable than the *Nipped-A*^{NC105}-encoded proteins, and thus the difference between the two alleles may be caused by a difference in the levels of the two mutant proteins. As discussed below, other evidence suggests that Nipped-A-containing complexes have multiple roles in Notch signaling, and thus it is also possible that *Nipped-A*^{NC194} is a separation-of-function allele that does not disrupt the ability of Nipped-A to support mastermind activity.

We think it unlikely that the ability of the *Nipped-A*^{NC105} allele to support mastermind and Notch functions in wing development or the ability of the *Nipped-A*^{NC194} allele to support mastermind function is caused by linked second-site mutations that counteract or mask the effects of these *Nipped-A* mutations on the *Notch* and *mastermind* mutant phenotypes. The *Nipped-A*^{NC105} and *Nipped-A*^{NC194} chromosomes were backcrossed to the parental *cn bw* chromosome during balancing to reduce the number of unlinked mutations. Of the many reported genetic effects on the wing margin phenotype of N^{nd-1} (http://flybase.bio.indiana.edu/.bin/fbidq.html?FBal0012890&content=geneinter), most enhance the phenotype, and only mutations in *Nipped-B* and *Notchless* (*Nle*) reduce its severity (45, 46). *Nipped-B* is tightly linked to *Nipped-A*, but complementation tests show that the *Nipped-A*^{NC105} and *Nipped-A*^{NC194} chromosomes do not contain *Nipped-B* mutations. *Nle* is located near the tip of 2L (21C) and thus is essentially unlinked from *Nipped-A*, which is on 2R. Similarly, most mutations that affect $N^{Ax\text{-}E2}$ suppress the vein-shortening phenotype, as do *Nipped-A* mutations (http://flybase.bio.indiana.edu/.bin/fbidq.html?FBal0012858&content=geneinter). The chromosome 2 mutations with opposite effects are (paradoxically) particular *Su*(*H*) mutations and *Suppressor of deltex* [*Su*(*dx*)] mutations. *Su*(*dx*) is also located near the tip of 2L (22C) and thus is essentially unlinked to *Nipped-A*. Many other *Su*(*H*) mutations suppress the $N^{Ax\text{-}E2}$ phenotype, and a chromosome with both *Su*(*H*) and *Nipped-A* mutations, *Nipped*$^{226.1}$, displays a dominant wing-nicking phenotype (45). The *Nipped-A*^{NC105} and *Nipped-A*^{NC194} chromosomes do not show such a phenotype. Thus, it is also unlikely that the lack of a significant effect of *Nipped-A*^{NC105} on the $N^{Ax\text{-}E2}$ phenotype is caused by a second-site mutation.

Finally, screens of deficiencies covering much of the genome and known Notch and wingless signaling pathway mutants for modifiers of a phenotypes caused by expression of a dominant-negative mastermind protein identified many enhancers of the mutant phenotypes and few suppressors (22, 53). None of the suppressors map to chromosome 2. Thus, it is also improbable that both the *Nipped-A*^{NC105} and *Nipped-A*^{NC194} chromosomes contain linked second-site mutations that suppress the *mastermind* mutant phenotype. In the case of *Nipped-A*^{NC105}, it would likely require multiple linked second-site mutations to mask effects on all three phenotypes.

Effects of *Nipped-A* mutations on Tra1/TRRAP proteins. We developed antisera against Nipped-A to evaluate the effects of the *Nipped-A* mutations on Tra/TRRAP protein. Antisera were generated against polypeptides encoded primarily by exon 12 (NipA12) and exon 14 (NipA14). As expected, both antisera recognize a major band larger than 400 kDa in Western blot assays of various developmental stages and cultured cell extracts. The highest levels of Nipped-A protein are found in embryonic nuclear extract, most of which is provided maternally. We examined the levels of Nipped-A protein in nuclear extracts of embryos produced by wild-type mothers and mothers heterozygous for *Nipped-A*^{NC105} and *Nipped-A*^{NC194}. As expected, the mutant mothers both produced embryos with roughly half the amount of full-length Nipped-A protein compared to the wild type (Fig. 5). This confirms that the major band recognized by the antibodies is Nipped-A (Tra1/TRRAP). It also indicates that there is not substantial overexpression of the wild-type *Nipped-A* allele in these mutants, which could potentially mask the effects of these mutations on *Notch* and *mastermind* mutant phenotypes.

Based on the abilities of the *Nipped-A*^{NC105} and *Nipped-A*^{NC194} genes to support or partially support Notch and mastermind activities in wing development, we thought it possible that truncated mutant proteins could be detected in mutant embryo extracts. Although both mutant proteins should contain the polypeptide used to generate the NipA12 serum, we did not detect truncated proteins of the expected sizes (234 and 171 kDa for *Nipped-A*^{NC105} and *Nipped-A*^{NC194}, respectively)

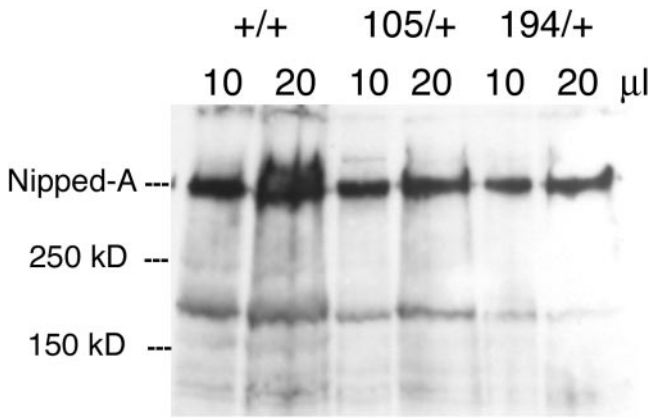

FIG. 5. Western blot assay of *Nipped-A* mutants. Each lane contained 10 or 20 μl of extract of 0- to 12-h-old embryos from mothers of the indicated genotypes: +/+, wild type; 105/+, *Nipped-A*^{NC105}/+; 194/+, *Nipped-A*^{NC194}/+. The amounts loaded are the equivalent of 115 and 230 whole embryos. The blot was probed with affinity-purified NipA12 antibody. Densitometry indicates that the mutants contain 35 to 53% of the full-length Nipped-A protein present in the wild type.

(Fig. 5). We considered the possibility that the proteins encoded by *Nipped-A*^{NC105} and *Nipped-A*^{NC194} might be less stable in embryos than at other developmental stages. We were also unable, however, to detect the expected truncated proteins in Western blots of heterozygous mutant larvae (not shown). It should be noted, however, that the levels of full-length protein are much lower in larvae than in embryos and near the limit of detection by Western blot assay with either antibody. It is also feasible that proteins encoded by the mutant alleles are modified after synthesis and lack the critical epitopes.

We also examined protein expression by immunostaining, which can be more sensitive than Western blot assays. The *Nipped-A*^{NC105}, *Nipped-A*^{NC194}, and *Nipped-A*^{NC106} mutants all die at the second- to third-instar molt as hemizygotes, so we immunostained several second-instar larval tissues of these mutants for Nipped-A and compared them to hemizygous wild-type *Nipped-A* (Fig. 6). Wild-type larvae show cytoplasmic and brighter nuclear staining in multiple tissues, as exemplified by the polytene cells of the second-instar proventriculus shown in Fig. 6. Staining above the background was also observed in imaginal disks and several other tissues, including the gut and salivary glands, and the same staining pattern was seen with both the NipA12 and NipA14 antibodies (not shown). No staining above the background was seen with hemizygous *Nipped-A*^{NC106}, demonstrating that the staining is specific for the Nipped-A protein (Fig. 6).

The NipA12 antibody is predicted to detect the *Nipped-A*^{NC105} and *Nipped-A*^{NC194} truncated proteins, and the NipA14 antiserum should detect the *Nipped-A*^{NC105} protein, but we did not see staining above the background with either antiserum in either mutant (Fig. 6). Thus, although the genetic analysis indicates that these mutant alleles retain the ability to support some Notch and mastermind functions in wing development, we were unable to detect protein products. It is possible that mutant proteins are not recognized by the antisera or that they are present at levels below the detection limit.

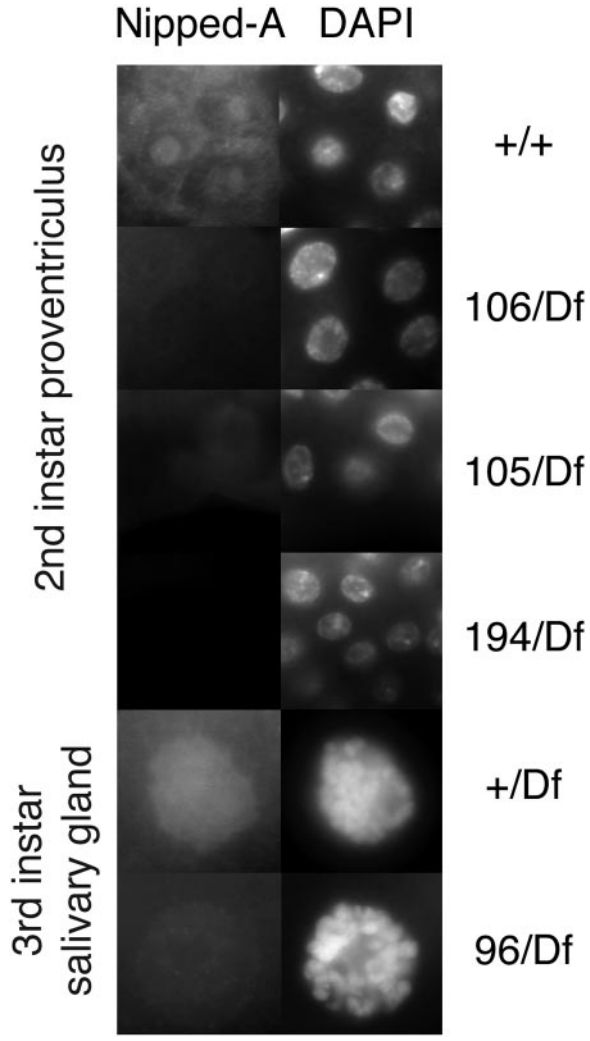

FIG. 6. Fluorescent immunostaining of larval tissues for Nipped-A. The examples shown were performed with affinity-purified NipA14 antibody, and essentially identical results were obtained with NipA12 antibody. The genotypes are indicated on the right: +/+, wild type; 106/Df, *Nipped-A*^{NC106}/*Nipped-E*^{43}; 105/Df, *Nipped-A*^{NC105}/*Nipped-E*^{43}; 194/Df, *Nipped-A*^{NC194}/*Nipped-E*^{43}; +/Df, +/*Nipped-E*^{43}; 96/Df, *Nipped-A*^{NC96}/*Nipped-E*^{43}. The micrographs were taken with a 60× objective. All second-instar micrographs were taken with identical exposure times, and both third-instar micrographs were identical exposures.

The genetic analysis indicates that *Nipped-A*^{NC96} is a hypomorph, even though it produces more mRNA than the wild type. We immunostained larval tissue hemizygous for *Nipped-A*^{NC96} and wild-type *Nipped-A* and observed that staining is reduced in the mutant compared to the wild type (Fig. 6). The wild type and mutant were stained simultaneously, and the photographic exposure times were identical. *Nipped-A*^{NC96} does not contain mutations in the open reading frame but has a point mutation in the 3′ UTR. Because the immunostaining indicates that this mutant allele produces less protein, we speculate that the point mutation reduces translation. Because *Nipped-A*^{NC96} is a hypomorph and, unlike a null allele, affects only the N^{nd-1} phenotype in the functional genetics tests, we conclude that the N^{nd-1} phenotype is more sensitive to

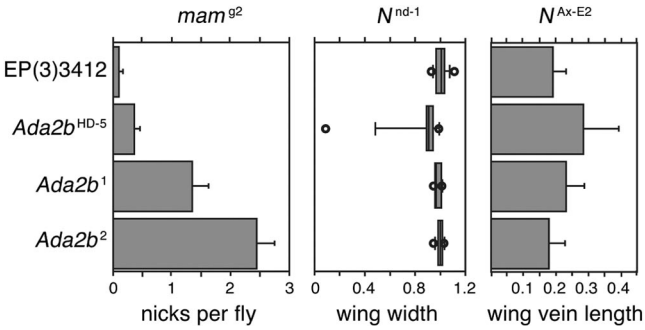

FIG. 7. Effects of *Ada2b* mutations on *Notch* and *mastermind* mutant phenotypes. The effects of the indicated *Ada2b* mutations and the parental P insertion [EP(3)3412] used to generate them on the mam^{g2} wing nicking phenotype, the N^{nd-1} wing width phenotype, and the N^{Ax-E2} vein-shortening phenotype were determined and diagrammed as described in the legend to Fig. 1. Homozygous $Ada2b^{HD-5}$ mutations occasionally survive to eclosion, indicating that they are hypomorphic, while the $Ada2b^1$ and $Ada2b^2$ alleles both delete significant portions of the transcribed regions and die during pupal development (44).

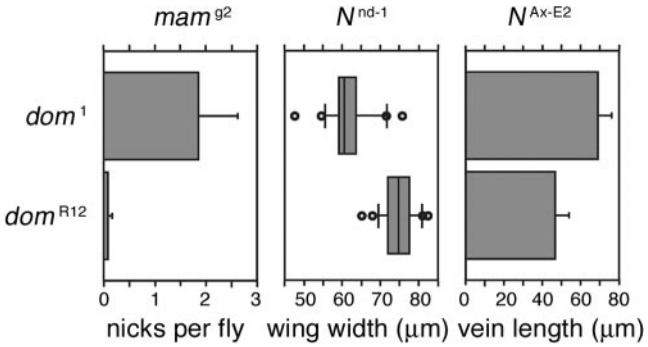

FIG. 8. Effects of *domino* mutations on *Notch* and *mastermind* mutant phenotypes. Dominant effects of the recessive lethal dom^1 P insertion allele of *domino* (44) and a viable revertant (dom^{R12}) generated for this work on the mam^{g2}, N^{nd-1}, and N^{Ax-E2} phenotypes were measured as described in the legend to Fig. 1, except that the wing widths and vein lengths are presented in micrometers instead of relative values.

Nipped-A levels than are the N^{Ax-E2} and mastermind phenotypes.

The Ada2b subunit of SAGA is required for mastermind activity. Tra1/TRRAP (Nipped-A) is a component of the SAGA complex that contains the Gcn5 HAT protein. To determine if SAGA is required for Notch and mastermind activity, we tested the effects of mutations affecting the Ada2b subunit of SAGA on the *Notch* and *mastermind* mutant phenotypes. Biochemical analysis indicates that Ada2b is found primarily in SAGA (29). All three *Ada2b* mutations tested increased the number of wing margin nicks displayed by heterozygous mam^{g2} but did not have any significant effects on the N^{nd-1} and N^{Ax-E2} mutant phenotypes (Fig. 7). All three mutant alleles were generated by P element excision and were compared to the original viable P element-bearing chromosome [EP(3)3412] used to generate them (44; T Kusch, personal communication). The $Ada2b^1$ and $Ada2b^2$ alleles have large deletions (1.1 and 2.8 kbp) that remove the transcription start site and much of the gene, and homozygotes die during pupariation (44). The $Ada2b^{HD-5}$ allele, an unpublished allele provided by Thomas Kusch and Jerry Workman (Stowers Institute), appears to be hypomorphic because a small percentage of homozygotes eclose but die shortly thereafter. Consistent with this idea, the effect of $Ada2b^{HD-5}$ on mam^{g2} was not as strong as the effects of the other alleles (Fig. 7).

Compared to the $Nipped\text{-}A^{NC194}$ mutation, the *Ada2b* mutations have the opposite specificity in their effects on Notch signaling: $Nipped\text{-}A^{NC194}$ affected the *Notch* mutants but had no detectable effect on mam^{g2} (Fig. 4), while the *Ada2b* mutations affected mam^{g2} but not the *Notch* mutants (Fig. 7). We conclude, therefore, that only a mastermind-dependent function of Nipped-A requires the Ada2b subunit of SAGA. This also indicates that *Nipped-A* has functions in Notch signaling that are not Ada2b dependent.

The domino subunit of the Tip60 complex is required for Notch and mastermind activities. Domino is a Tip60 (NuA4) component in drosophila and mammals (15, 28), but it is unknown if this is the only complex that contains domino in vivo. The *domino* gene encodes two proteins, domino-A and domino-B, which have significant sequence similarity to the mammalian p400 and SRCAP proteins (47). Both are putative ATPase nucleosome-remodeling enzymes. Domino-A is more restricted in expression than domino-B, and domino-B is likely the major *domino* product expressed in wing disks (47). Previous studies indicated that a *domino* mutation increased the severity of the N^{nd-1} mutant phenotype, suggesting that domino-B participates in Notch signaling at the wing margin, and cultured cell transfection experiments with mammalian SRCAP suggest that it supports transcriptional activation by Notch (16).

Figure 8 shows that, similar to the previously reported effect of the dom^7 mutation, the dom^1 mutation increased the severity of the N^{nd-1} mutant phenotype. The dom^1 mutation had only a slight effect on the length of the N^{Ax-E2} wing veins but strongly increased the number of wing margin nicks displayed by heterozygous mam^{g2}. The dom^1 mutation is a recessive lethal P element insertion allele, and these effects were measured relative to a viable revertant, dom^{R12}, generated by excision of the P insertion. We conclude, therefore, that domino supports Notch and mastermind activities in the developing wing margin and has little effect in the wing veins. In this respect, dom^1 behaves similarly to the $Nipped\text{-}A^{NC106}$ mutation (Fig. 4). Because the effects of the dom^1 mutation are similar to those observed with a specific *Nipped-A* mutant allele, we postulate that domino functions as a component of the Tip60 complex in Notch signaling, although we cannot rule out the possibility that it can also act independently of Tip60.

Nipped-A is required for binding of mastermind to chromosomes. As a test of the possibility that the effect of Nipped-A on mastermind activity is direct, we looked to see if they bind to the same sites on salivary gland polytene chromosomes. The NipA12 antisera generated in a guinea pig and the NipA14 antisera generated in a rabbit show virtually identical staining patterns, confirming that the staining is specific (Fig. 9). The secondary antibodies do not show species cross-reactivity.

Both Nipped-A antisera show considerable overlap with mastermind staining (Fig. 9). Virtually all mastermind binding sites also bind Nipped-A, but there are many minor Nipped-A sites that do not show significant staining for mastermind. The

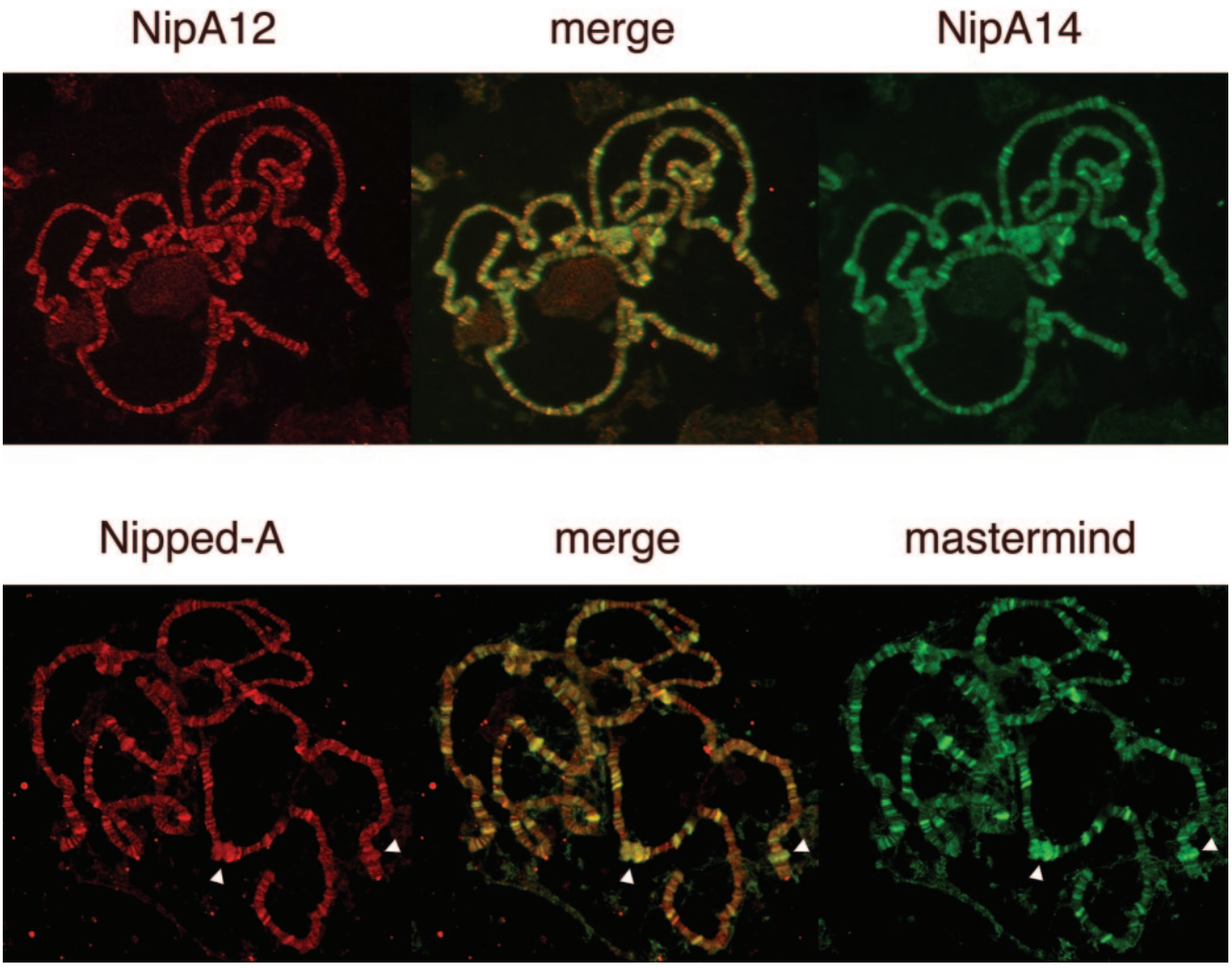

FIG. 9. Nipped-A and mastermind bind to the same sites on salivary gland chromosomes. The top panels show double immunostaining with the two Nipped-A antibodies, NipA12 and NipA14. The staining patterns are virtually identical, providing evidence additional to the Western blot data (Fig. 5) and the tissue immunostaining (Fig. 6) that the antibodies are specific for Nipped-A. The bottom panels show double immunostaining for Nipped-A and mastermind. As seen in the merge, virtually all sites that stain for mastermind also stain for Nipped-A, and there are several minor Nipped-A staining sites that that do not stain for mastermind. Arrowheads indicate puffs that stain for both Nipped-A and mastermind.

secondary antibodies do not show cross-species reactivity. Although the functional target genes of Nipped-A and the mastermind target genes in salivary glands are unknown, both proteins associate with some chromosomal puffs, which are highly transcribed (Fig. 9, arrows).

The survival of flies hemizygous for the *Nipped-A*^{NC96} or *Nipped-A*^{NC186} allele to the third-instar larval stage of development provided an opportunity to examine the effects of Nipped-A on the binding of SAGA and mastermind to polytene chromosomes. Relative to wild-type control chromosomes, hemizygous *Nipped-A*^{NC96} mutant polytene chromosomes displayed significantly reduced immunostaining for the Gcn5 HAT component of SAGA and dramatically reduced staining for mastermind (Fig. 10). These effects were observed in multiple nuclei in multiple salivary glands and indicate that Nipped-A is required for efficient binding of mastermind to chromosomes. The reduced association of Gcn5 confirms that Nipped-A, as expected, also affects binding of the SAGA complex. We obtained similar results with Gcn5 and mastermind immunostaining with flies hemizygous for *Nipped-A*^{NC186} (not shown). We conclude that Nipped-A (Tra1/TRRAP) regulates the association of mastermind with chromosomes. These effects are unlikely to be general effects on chromosome structure. The polytene chromosomes of the *Nipped-A* mutants are normal in structure and contain puffs indicating that they are transcriptionally active. Moreover, a significant fraction of the *Nipped-A*^{NC96} hemizygous mutants live beyond the third-instar stage and pupariate (Table 2), indicating that there is unlikely to be a generalized defect in chromosome structure and function.

DISCUSSION

The evidence provided here, combined with the previous report that two *Nipped-A* mutants have point mutations in the Tra1/TRRAP gene (38), demonstrates conclusively that *Nipped-A* encodes Tra1/TRRAP. All EMS-induced *Nipped-A*

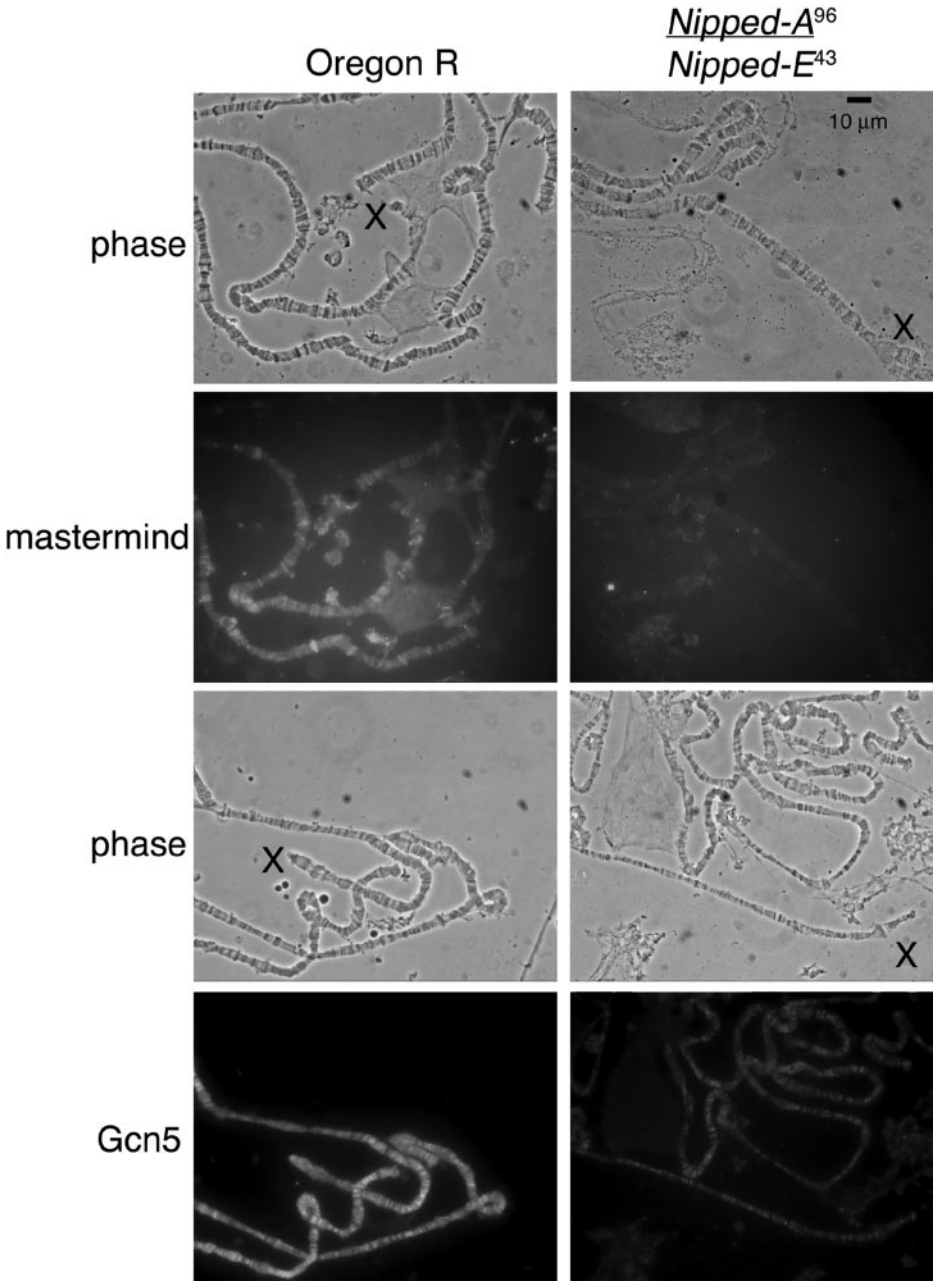

FIG. 10. The *Nipped-A*^{NC96} hypomorphic mutation reduces binding of mastermind and Gcn5 to chromosomes. The panels show representative wild-type (Oregon R) and hemizygous *Nipped-A*^{NC96} mutant (*Nipped-A*^{NC96}/*Nipped-E*^{43}) third-instar salivary gland polytene chromosome squashes immunostained for either the mastermind or the Gcn5 protein. For each antibody, the wild-type and mutant glands were immunostained under identical conditions. The digital micrographs were taken with the same illumination and exposure times. Examination of several nuclei in multiple squashes revealed that Gcn5 staining intensity is reproducibly reduced but not abolished, while mastermind staining is severely reduced. Similar results were obtained with hemizygous *Nipped-A*^{NC186} mutant polytene chromosomes (not shown).

alleles sequenced to date have point mutations in the *Tra1/TRRAP* gene that affect the protein coding sequence or, in one case, the 3′ UTR. A seventh allele generated by γ rays, *Nipped-A*^{323}, does not produce Tra1/TRRAP mRNA. We have also sequenced additional *Nipped-A* mutant alleles, and all contain point mutations that alter the protein coding sequence (M.G. and D.D., unpublished data).

Our results show that the major *Nipped-A* transcript differs from the previously reported splicing pattern, which appears to be a rare variant (29). Antibodies against a polypeptide encoded largely by the rare exons detect a weak Tra1/TRRAP signal in Western blot assays of concentrated nuclear extracts or purified complexes, confirming that the variant produces Tra1/TRRAP protein in vivo (29; M.G. and D.D., unpublished). The rare transcript does not, however, support at least one essential function of Nipped-A and Notch signaling in the wing margin because, as reported here, mutation of a splice site in *Nipped-A*^{NC106} for an exon that is not included in the

rare variant is lethal and causes defects in Notch signaling. *Nipped-A*^{NC106}, however, had little effect on the $N^{Ax\text{-}E2}$ wing vein phenotype, raising the possibility that the alternatively spliced product can support Notch function in developing wing veins.

Effects of *Nipped-A* mutants encoding truncated proteins on Notch and mastermind function. An unexpected finding is that the *Nipped-A*^{NC105} allele, which encodes the N-terminal 2,048 residues of Tra1/TRRAP, suffices to replace one wild-type copy of *Nipped-A* to support Notch and mastermind function in vivo. This was unexpected because the protein encoded by *Nipped-A*^{NC105} lacks the ATM-PI3 kinase motif which, in mammalian cell culture experiments, is required for Tra1/TRRAP to associate with Gcn5 and Tip60 (41). One possible explanation is that the C terminus of the Nipped-A protein is not required for Notch and mastermind function and that the truncated protein can replace the full-length protein. Because we could only assay the effects of the *Nipped-A* mutations on Notch functions in wing development in the presence of a wild-type allele, it is also possible that a truncated protein somehow increases the activity of the remaining full-length Nipped-A protein. We were unable to detect the truncated protein in Western blot assays of extracts or by immunostaining, suggesting that if this is the case, only a small amount of the mutant protein is sufficient. We consider it improbable that linked second-site mutations are masking effects of *Nipped-A*^{NC105} on both *Notch* mutant phenotypes and the *mastermind* phenotype. Many mutations have effects similar to Nipped-A, and few have opposing effects, and it would likely require multiple mutations to counteract the effects of *Nipped-A*^{NC105} on all three phenotypes. It is also unlikely that there is a linked second-site mutation that counteracts the effects of *Nipped-A*^{NC105} by increasing the expression of wild-type *Nipped-A*, because mutant embryos and larvae show the expected decrease in full-length Nipped-A protein.

The *Nipped-A*^{NC194} allele, which encodes residues 1 to 1500, had a significant effect on both of the Notch mutant phenotypes but did not increase the severity of the wing-nicking phenotype displayed by *mam*^{g2}. Again, this differs from null alleles of *Nipped-A*, which affect all three phenotypes, suggesting that *Nipped-A*^{NC194} retains sufficient activity to replace one copy of the wild type in support of mastermind activity. Again, one possible explanation is that Nipped-A residues 1 to 1500 are sufficient to support mastermind function, although it is conceivable that the truncated protein somehow increases the activity of the remaining wild-type Nipped-A protein. We were also unable to detect this truncated protein, suggesting that if a truncated protein is responsible, only low levels are required. Despite extensive screens with a deficiency collection and candidate genes, no mutations that suppress *mastermind* mutant phenotypes have been mapped to chromosome 2 (22, 53). Thus, it is unlikely that a linked second-site mutation masks an effect of *Nipped-A*^{NC194} on the *mastermind* phenotype. Similar to *Nipped-A*^{NC105}, heterozygous *Nipped-A*^{NC194} mutants display the expected reduced levels of full-length protein, although we cannot exclude the possibility of a subtle increase in the expression of the wild-type *Nipped-A* allele that is sufficient to rescue the *mastermind* phenotype but not the *Notch* mutant phenotypes.

Isolation and analysis of additional *Nipped-A* truncation alleles and development of more sensitive biochemical assays will lead to a fuller understanding of how *Nipped-A* alleles encoding truncated proteins support Notch signaling.

The roles of Nipped-A (Tra1/TRRAP) in Notch signaling involve both the SAGA and Tip60 complexes. The experiments presented here indicate that the roles of Nipped-A in supporting mastermind function likely involve both the SAGA and Tip60 complexes. The Ada2b protein is specific to SAGA (29), and *Ada2b* mutations affect the *mastermind* phenotype but not the two *Notch* mutant phenotypes. We think it unlikely that the effect of the *Ada2b* mutations is more specific than *Nipped-A* mutations because the *mastermind* phenotype is more sensitive. As shown by the *Nipped-A*^{NC96} hypomorph, the $N^{nd\text{-}1}$ phenotype is more sensitive to the Nipped-A dosage than is *mastermind*. Moreover, the *Nipped-A*^{NC194} allele has the opposite specificity to the *Ada2b* mutations and affects the *Notch* mutant phenotypes but not the *mastermind* phenotype. Combined, the contrasts in the effects of *Ada2b* and various *Nipped-A* mutations show that Nipped-A and its complexes play multiple roles in Notch signaling. They suggest that the SAGA complex, or at least the Ada2b subunit, is more specific for mastermind function and that Nipped-A has additional functions.

Another possibility raised by the specificity of the effects of *Ada2b* mutations for effects on mastermind activity in wing margin development is that mastermind may have functions in margin development independent of Notch. For example, mastermind could conceivably function as a coactivator for other activator proteins in addition to Notch. This possibility is consistent with the binding of mastermind to several sites in polytene chromosomes, including the ecdysone-dependent puffs.

The domino protein, a putative ATPase remodeling enzyme, is a subunit of the Tip60 complex (28). The $N^{nd\text{-}1}$ and $N^{Ax\text{-}E2}$ phenotypes and the mastermind phenotype are modified by *domino* mutations, although the effect on $N^{Ax\text{-}E2}$ is modest. These effects are similar to those of the *Nipped-A*^{NC106} allele and thus suggest that that the Tip60 complex also supports mastermind function and Notch signaling during wing development. It is possible, however, that domino functions independently of Tip60 and Nipped-A because the human domino homologue SRCAP interacts directly with the CBP HAT enzyme (24) that interacts with mastermind (18). Nevertheless, the likely involvement of the Tip60 complex raises the possibility that histone exchange could facilitate transcriptional activation by Notch because, in addition to acetylating histone H4, Tip60 exchanges histone H2 variants during DNA repair (28).

Is the effect of Nipped-A on mastermind function direct? As revealed by immunostaining of salivary gland polytene chromosomes, at least one function of Nipped-A is to regulate the binding of mastermind to chromosomes. The reduction in binding of mastermind to polytene chromosomes caused by the hypomorphic *Nipped-A*^{NC96} and *Nipped-A*^{NC186} alleles is dramatic. Supporting the idea that Nipped-A directly regulates mastermind binding, virtually all sites on polytene chromosomes that bind mastermind also bind Nipped-A. We envision a few possible explanations for these results. The SAGA and Tip60 complexes that contain Nipped-A could acetylate mastermind, proteins in the Notch activator complex, and/or possibly histones to facilitate binding of the Notch activator complex to chromatin. These modifications could be made by Gcn5

and/or Tip60, which acetylate histones H3 and H4, respectively. Alternatively, Nipped-A or its complexes could bind to chromosomes cooperatively with mastermind. This would be consistent with the published observation that the ankyrin repeats of the NICD fragment of Notch, which help recruit mastermind to the Notch activator complex, are also required to recruit Gcn5/PCAF SAGA subunit in transfected mouse cells (27). Both the Ada2b component of SAGA and the domino subunit of Tip60 affect mastermind function, so it is likely that Nipped-A supports mastermind function in more than one way.

Because the evidence suggests that Nipped-A supports mastermind function through both the SAGA and Tip60 chromatin-modifying complexes, we theorize that, in addition to controlling the binding of mastermind to chromosomes, Nipped-A could also cooperate with mastermind to recruit these complexes to facilitate transcriptional activation through chromatin modification.

Other possible functions for Nipped-A in Notch signaling. Our data indicate that the SAGA complex, or at least its Ada2b subunit, is not required for some functions of Nipped-A in Notch signaling. Unlike *Nipped-A* and *domino* mutations, *Ada2b* mutations did not affect *Notch* mutant phenotypes, while they did enhance the phenotype caused by a *mastermind* mutation. We postulate, therefore, that the Tip60 complex is also required for functions of Nipped-A beyond controlling the binding of mastermind to chromosomes. The Tip60 complex could affect the expression of Notch activator complex components, or it could modify proteins in the Notch activator complex. It is also possible that Tip60 modifies chromatin to either aid binding of the Su(H) protein to the Notch target genes or, as mentioned above, to aid transcriptional activation by the Notch activator complex. In any case, the evidence indicates that two subunits of Tip60, Nipped-A and domino, play more than one role in Notch signaling during wing development.

ACKNOWLEDGMENTS

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M.G. conducted the genetic analysis and sequencing of the *Nipped-A* and *Ada2b* mutants, generation of Nipped-A antibodies, Western blot assays of *Nipped-A* mutants, and immunostaining of larval tissues and participated in immunostaining of polytene chromosomes and preparation of the manuscript. J.C.E. conducted the genetic analysis of the *domino* mutations and immunostaining of polytene chromosomes and participated in preparation of the manuscript. A.M. participated in the Northern analysis of the *Nipped-A* mutants. M.D. designed and tested the RT-PCR sequencing strategy and helped construct the *Nipped-A* RNA probe vector. Z.M. assisted in the preparation of the *Nipped-A* probe vector and conducted the 5′ and 3′ RACE analysis. D.D. participated in the design and interpretation of all experiments, conducted the Northern analysis of the *Nipped-A* mutants, and participated in preparation of the manuscript.

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