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Genome Engineering-Based Analysis of *Bearded* Family Genes Reveals Both Functional Redundancy and a Nonessential Function in Lateral Inhibition in Drosophila

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ABSTRACT

Lateral inhibition mediated by Notch receptor signaling regulates the determination of sensory organ precursor cells (SOPs) in Drosophila. The selection of SOPs from proneural cluster cells appears to rely on a negative feedback loop linking activation of the Notch receptor to downregulation of its ligand Delta within each cell. The molecular basis of this regulatory feedback mechanism is not known. Here, we have tested the role of the *Bearded* (*Brd*) family genes in this process. The Drosophila genome encodes eight Brd family members that interact with the E3 ubiquitin ligase Neuralized (Neur) and act as inhibitors of Neur-mediated Delta signaling. Genome engineering technologies were used to create specific deletions of all eight *Brd* family genes. We find that the *Brd* family genes *mα*, *m4*, and *m6* encoded by the *Enhancer of split Complex* (*E(spl)-C*) are dispensable for Drosophila development and that deletion of the five *Brd* family genes encoded by the *Brd Complex* only reduces viability. However, deletion of all *Brd* family genes results in embryonic lethality. Additionally, the *mα*, *m4*, and *m6* genes act redundantly with the other five *Brd* family genes to spatially restrict Notch activation in stage 5 embryos. These data reveal that the *Brd* family genes have an essential but redundant activity. While the activity of all eight *Brd* genes appears to be dispensable for SOP determination, clone border studies indicate that both the relative activity levels of Neur and Brd family members influence competition for the SOP fate during lateral inhibition. We propose that inhibition of Neur–Delta interaction by Brd family members is part of the feedback loop that underlies lateral inhibition in Drosophila.

SIGNALING through the Notch receptor is widely used in animal development to control cell fate choices and regulate pattern formation. One of the best-known functions of Notch is to mediate lateral inhibition, a patterning process that regulates the formation of differentiated structures at regular spatial and/or temporal intervals. In Drosophila, inhibitory cell–cell interactions mediated by the Notch receptor are responsible for the regular spacing of sensory bristles at the surface of the dorsal thorax (Simpson 1990). Sensory organ precursor cells (SOPs) are singled out from within groups of proneural cluster (PNC) cells that can differentiate as either epidermal cells or as SOPs (Ghysen and Dambly-Chaudiere 1989). Selection of SOPs among PNC cells is classically viewed as the result of a competition for adoption of the SOP fate (Heitzler and Simpson 1991). All PNC cells are thought to initially express similar levels of the receptor Notch and its ligand Delta (Dl). As a consequence, all cells in the cluster may initially inhibit one another. However, activation of Notch in a given cell is thought to decrease that cell's potential to become a SOP and to also reduce its ability to send back the Dl signal that activates Notch at the surface of neighboring cells. This negative feedback loop therefore ensures that a single cell emerges as a winner of this competition. This model whereby PNC cells compete for the adoption of the SOP fate is supported by clonal studies demonstrating that the level of Notch activity within a cell can influence the fate of its neighbors (Heitzler and Simpson 1991).

Downregulation of Dl in response to Notch receptor activation has been proposed to be under transcriptional control, as originally shown for the feedback loop operating between the Lin-12 receptor and its ligand Lag-2 (Wilkinson *et al.* 1994). In this model, transcriptional activation of the bHLH genes of the *Enhancer of split Complex* (*E(spl)-C*) by activated Notch results in the downregulation of proneural gene activity, thereby leading to a downregulation of *Dl* gene transcription (Heitzler *et al.* 1996). This molecular scenario is, however, not supported by the analysis of *Dl* transcription: *Dl* transcription levels in emergent and newly specified SOPs were found to be similar to those observed in neighboring PNC cells (Parks *et al.* 1997). This observation therefore argues against negative

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regulation of Dl transcription as a central mechanism for competition. Thus, the molecular basis underlying the negative feedback linking Notch activation to Dl inhibition remains unknown.

The activity of Dl is positively regulated at the post-transcriptional level by the E3 ubiquitin ligase Neuralized (Neur) and Neur-dependent Dl signaling is essential for proper SOP specification (Boulianne *et al.* 1991; Lai *et al.* 2001; Pavlopoulos *et al.* 2001; Le Borgne and Schweisguth 2003; Le Borgne *et al.* 2005). Recent studies have shown that regulation of Dl activity by Neur is antagonized by proteins of the Bearded (Brd) family. Molecular data indicate that Brd family proteins physically interact with Neur and antagonize its interaction with Dl (Leviten *et al.* 1997; Lai *et al.* 2000a,b; Bardin and Schweisguth 2006; De Renzis *et al.* 2006). Additionally, most Brd family genes are expressed in PNC cells (Wurmbach *et al.* 1999; Lai *et al.* 2000a,b). While transcriptional regulation studies are suggestive of a role of these genes in lateral inhibition (Singson *et al.* 1994; Castro *et al.* 2005), whether inhibition of Neur by Brd family proteins regulates lateral inhibition has not been tested using loss-of-function analysis.

The Brd family consists of the proteins BobA, BobB, Tom, Brd, and Ocho that are encoded by the *Bearded Complex* (*Brd-C*) and of the proteins mα, m4, and m6 that are encoded by the *E(spl)-C*. The structurally related protein m2 is not considered here as a Brd family member as it does not include the Neur binding motif and does not antagonize Notch (Lai *et al.* 2000b; Bardin and Schweisguth 2006). To investigate the function of the *Brd* family genes, we have used here genomic engineering approaches to delete each of these genes. Our analysis demonstrates that *Brd* family genes act redundantly in the embryo and suggests that inhibition of Neur by Brd family members participates in the negative feedback loop linking Notch activation to down-regulation of Dl within a cell during lateral inhibition.

MATERIALS AND METHODS

Drosophila stocks: The *P*-elements RBe00084 and XPd08311 from the Exelixis collection (https://drosophila.med.harvard.edu/) were used to generate the *Df(3)E(spl)δ-6* deficiency using Flp-mediated recombination as described in (Thibault *et al.* 2004). The *Dp(3;2)E(spl)δ-8*, *Dp(3;2)E(spl)δ-8Δα46*, *Dp(3;2)Brd-C*, and *Dp(3;2)CG13466* were generated using phiC31-mediated site-specific integration (Venken *et al.* 2006; Bischof *et al.* 2007). The *DpE(spl)δ-8* and *DpE(spl)δ-8Δα46* were micro-injected in vas-phiC31-zh-2A; ZH-attP-51D embryos (Bischof *et al.* 2007). Micro-injection of *DpBrd-C* and *DpCG13466* into vas-phiC31-zh-2A; ZH-attP-58A embryos was performed by BestGene (http://www.thebestgene.com). Other stocks used in this study include: *Df(3)Brd-C1* (Bardin and Schweisguth 2006), UAS-mα (Apidianakis *et al.* 1999), *neur*^{IF65} (FlyBase), and tub>GFP, y+>Gal4 (Pinal *et al.* 2006).

Clones of *mα* overexpression were generated in hs-flp; UAS-mα; tub>GFP, y+>Gal4 pupae. Mitotic clones were induced by a 45-min heat-shock at 36.5° in first and second instar larvae of the following genotypes (numbering as in Figure 4):

(1) Control wild-type clones for 3L: hs-flp tub-Gal4 UAS-GFP;; FRT2A/tub-Gal80 FRT2A.
(2) *Df(3)Brd-C1* loss-of-function clones: hs-flp tub-Gal4 UAS-GFP;; FRT2A *Df(3)Brd-C1*/FRT2A tub-Gal80.
(3) *Df(3)Brd-C1* mutant clones in *mα m4 m6* triple mutant flies: hs-flp tub-Gal4 UAS-GFP; *Dp(3;2)E(spl)δ-8Δα46*; *Df(3)Brd-C1* FRT2A *Df(3)E(spl)δ-6*/tub-Gal80 FRT2A *Df(3)E(spl)δ-6*.
(4) Control wild-type clones for 2R: hs-flp tub-Gal4 UAS-GFP; FRT42B/FRT42B tub-Gal80.
(5) *mα*, *m4*, *m6* loss-of-function clones: hs-flp tub-Gal4 UAS-GFP; FRT42B *Dp(3;2)E(spl)δ-8Δα46*/FRT42B *Dp(3;2)E(spl)δ-8* tub-Gal80; *Df(3) E(spl)δ-6*.
(6) Control wild-type clones for 3R: hs-flp tub-Gal4 UAS-GFP;; FRT82B ubi-nls-GFP/FRT82B tub-Gal80.
(7) *neur* mutant clones: hs-flp tub-Gal4 UAS-GFP;; FRT82B ubi-nls-GFP *neur*^{IF65}/FRT82B tub-Gal80.

Molecular biology: Duplications were generated from BACs RPCI-98-13F13 and RPCI-98-01H12 (http://bacpac.chori.org) cloned into the attB-P[acman]-Ap vector using recombineering mediated gap repair as described in Venken *et al.* (2006). The PCR-amplified 5′ and 3′ homology arms were cloned into attB-P[acman]-Ap using *Not*1 and *Eco*R1. Resulting plasmids were linearized using *Bam*HI, digested with ExoSAP (USB) and used for recombineering with RPCI-98-13F13 and RPCI-98-01H12 in *Escherichia coli* SW102 as described at http://recombineering.ncifcrf.gov/Protocol.asp.

The following primers were used to PCR amplify the 5′ and 3′ homology arms subsequently used to generate duplications:

*DpE(spl)*_UF: CCCGGCCGTTAAACCAAGTTCACCTCTC
*DpE(spl)*_UR: CAATAAAGGGATCCCTTGTTTAATGCGGATAACG
*DpE(spl)*_DF: AACAAGGGATCCCTTTATTGGGATGTTGGGAG
*DpE(spl)*_DR: CGAATTCAGATCTGTACATGTTCTTCAGG
*DpBrd*_UF: CCGCGGCCGCTGGGGTTTCTTGCAACCAAC
*DpBrd*_UR: CATTTTGTGGATCCCTTATTAGTGGTAAGGGCAG
*DpBrd*_DF: CATATAAGGGATCCACAAAATGTTGGGGTAACAGC
*DpBrd*_DR: CCGAATTCCTATTGTTACCCTCTTCTAGG
*DpCG13466*_UF: CCCGGCCGTAGTGTGCCGGTTGTGTGTG
*DpCG13466*_UR: AACCCCAGGATCCGACTCCCACAACAGCGAGAG
*DpCG13466*_DF: GAGTCGGATCCTGGGGTTTCTTGCAACCAAC
*DpCG13466*_DR: CCGAATTCCTTATTAGTGGTAAGGGCAG.

The *mα*, *m4*, and *m6* genes were deleted from the RPCI-98-13F13 using recombineering with a positive/negative selection galK cassette (Warming *et al.* 2005).

The following primers were used to PCR amplify the 5′ and 3′ homology arms used to delete the *mα*, *m4*, and *m6* genes:

mα-UF: GAATGCCCATTAGGAATAC
mα-UR: TGTGTGTAACCGCTCGAGCGACAGAGAGG
mα-DF: CGCTCGAGCGGGTTACACACAACAAGTAG
mα-DR: CAATGACCCAGTTAGATAG
m4-UF: CATCACCTCAAATGTATGC
m4-UR: GTTTCTCCACTCGTATGAAATGGGCCTTCTC
m4-DF: TTTCATACGAGTGGAGAAACCCGAAGCCGAG
m4-DR: GGCGCCTCCGTTCGAGCGTTG
m6-UF: GCAAGTGCAATATGTGTC
m6-UR: ACAGTGACGAGTGCCACATGTGGCGGAC

m6-DF: ACATGTGGCATCGTCACTGTTTGTTACGTGG
m6-DR: TCAGGCCTGAAGCTTGAGAATC.

Immunostainings and RNA *in situ* hybridization: The following antibodies were used: guinea pig anti-Sens (1:3000; H. Bellen), rabbit anti-GFP (Molecular Probes; 1:1000). RNA *in situ* hybridization of stage 5 embryos was performed using standard procedures with DIG-labeled RNA probes. Embryos were genotyped using the TM3 hb-lacZ balancer.

RESULTS

***Brd* family genes of the *E(spl)-C* are not essential:** To genetically study the function of the *m*α, *m4*, and *m6* genes, we have used combinations of molecularly defined deletions and duplications. We first generated a 41-kb deletion, *Df(3)E(spl)*δ*-6*, that removes all four *Brd* genes and five of the seven bHLH repressors (Figure 1A). This deletion is associated with a strong neurogenic phenotype in embryos (not shown). This phenotype was fully rescued by a 46-kb duplication covering the entire *E(spl)-C* locus, *DpE(spl)*δ*-8*, which was designed and generated from a BAC carrying the *E(spl)-C* genomic DNA by gap repair in *E. coli* and integrated within the fly genome using phiC31-mediated transgenesis (Venken *et al.* 2006; Bischof *et al.* 2007) (Figure 1A). Indeed, *DpE(spl)*δ*-8 Df(3)E(spl)*δ*-6* embryos are viable (only 10% of these embryos do not hatch, $n = 115$, as compared with 2%, $n = 273$, for wild-type control embryos). We further engineered this duplication using recombineering in *E. coli* to precisely delete the *m*α, *m4*, and *m6* genes, from the TATA box to the end of the 3′-UTR included, resulting in *DpE(spl)*δ*-8*Δα*46* (Figure 1A). This mutant duplication rescued the neurogenic mutant phenotype associated with the *E(spl)-C* deletion, demonstrating that this duplication is functional and that the neurogenic phenotype results from the loss of the bHLH genes (not shown). Additionally, only 6% ($n = 299$) of the embryos triply mutant for the *m*α, *m4*, and *m6* genes fail to hatch, and flies homozygous for the *E(spl)-C* deletion and carrying one copy of the mutant duplication are viable and fertile with no detectable morphological phenotype. We therefore conclude that the *m*α, *m4*, and *m6* genes are not essential.

Functional redundancy between *Brd* family genes: To test whether the *m*α, *m4*, and *m6* genes are dispensable due to genetic redundancy with other genes of the *Brd* family, we have produced a genetic background deleted of all *Brd* family genes. In a first step, we have analyzed the phenotype associated with the loss of the *Brd-C* genes *BobA*, *BobB*, *Tom*, *Brd*, and *Ocho*. The *Brd-C1* deletion that removes the *Brd-C* and truncates *CG13466* is largely lethal: 60% ($n = 121$) of *Brd-C1* individuals die as embryos and only 6% reach adulthood. This lethality is partially rescued by a genomic duplication of the *Brd-C* but not by the *CG13466* duplication (Figure 1B): 20% ($n = 133$) of the *DpBrd-C Brd-C1* embryos gave adult flies that are fertile and can be kept as a stock whereas *DpCG13466 Brd-C1* embryos gave no escapers. We conclude that the reduced viability associated with the *Brd-C1* deletion is due to the loss of the *Brd-C* genes.

We then generated embryos mutant for all eight *Brd* family genes, *i.e.*, homozygous for the *Brd-C1* and *Df(3)E(spl)*δ*-6* deletions with one copy of the mutant *DpE(spl)*δ*-8*Δα*46* duplication and found that this genotype is 100% embryonic lethal ($n > 120$). The wild-type *DpE(spl)*δ*-8* duplication was used as a positive control: 39% ($n = 120$) of embryos homozygous for the *Brd-C1* and *Df(3)E(spl)*δ*-6* deletions with one copy of the wild-type *DpE(spl)*δ*-8* duplication fail to hatch and only 2% reach pupal stages. The reduced viability associated with this genotype is likely to result from the loss of the *Brd-C* genes since reduced viability was also observed with the *Brd-C1* deficiency. Together, these data clearly demonstrate that *Brd* family genes have an essential and redundant function in the embryo.

***Brd* family genes act redundantly to restrict the domain of Notch activity along the dorsoventral axis in early embryos:** Previous studies have shown that genes of the *Brd-C* are collectively required to restrict Dl signaling to mesodermal cells in stage 5 embryos (Bardin and Schweisguth 2006; De Renzis *et al.* 2006). At this stage, *neur*-dependent Dl endocytosis in mesodermal cells results in the activation of Notch in a single row of cells on either side of the mesoderm. These cells express the Notch target gene *single-minded* gene (*sim*) and form the mesectoderm (Figure 2, A and F) (Martin-Bermudo *et al.* 1995; Morel and Schweisguth 2000; Cowden and Levine 2002; Morel *et al.* 2003; Bardin and Schweisguth 2006; De Renzis *et al.* 2006) (Figure 2, A, B, and G). *Brd* family genes encoded by both complexes are expressed in nonmesodermal cells at this stage (Nagel *et al.* 2000; Zaffran and Frasch 2000). We first confirmed that *Brd-C* genes contribute to restrict the expression of *sim* to the mesectoderm (Figure 2, C and G) (Bardin and Schweisguth 2006; De Renzis *et al.* 2006). We next investigated the role of the *m*α, *m4*, and *m6* genes in this process. We found that loss of *m*α, *m4*, and *m6* gene activities had no detectable effect on *sim* expression (Figure 2D). However, deletion of these genes strongly enhanced the *Brd-C* phenotype: *sim* transcripts were detected in 3–5 rows of cells dorsal to the mesoderm in embryos mutant for all eight *Brd* family members (Figure 2E). This ectopic expression of *sim* was strongly suppressed by the loss of zygotic *neur* activity, indicating that *Brd* family genes inhibit the activity of *neur* (Figure 2F). Of note, the *neur* loss of *sim* expression phenotype was suppressed by the zygotic loss of *Brd* family genes (compare Figure 2, B and F). We interpret this suppression to suggest that maternally provided Neur is sufficient to activate Notch only in the complete absence of all Brd family antagonists. Together, these data demonstrate that *Brd* family genes of the *E(spl)-C* and *Brd-C* act redundantly

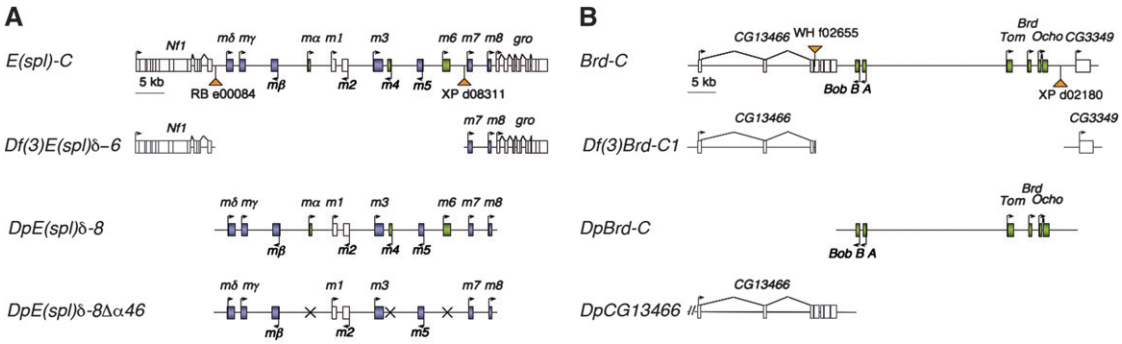

FIGURE 1.—Genome engineering of the *E(spl)-C* and *Brd-C*. (A) Structure of the *E(spl)-C* locus, *Df(3)E(spl)*δ*-6* deficiency, and BAC-based *DpE(spl)*δ*-8* and *DpE(spl)*δ*-8*Δα*46* duplications. The *E(spl)-C* encodes seven bHLH repressors (blue) and four *Brd* family genes (green). The *P*-elements used to generate *Df(3)E(spl)*δ*-6* are shown (orange triangles). The *m*α, *m4*, and *m6* genes are deleted in *DpE(spl)*δ*-8*Δα*46*. (B) Structure of the *Brd-C*, *Df(3)Brd-C1* deficiency, *DpBrd-C* and *DpCG13466* duplications. The *P*-elements used to generate *Df(3)Brd-C1* are shown (orange triangles).

to restrict the spatial domain of Neur-dependent Dl signaling, hence Notch receptor activation, in the early embryo.

***Brd* family genes are not required for SOP determination:** Previous studies have shown that *Brd* family genes of the *E(spl)-C* and *Brd-C* are expressed in PNC cells and are positively regulated by both proneural factors and Notch signaling (SINGSON *et al.* 1994; CASTRO *et al.* 2005). These observations have suggested that Notch activation within a cell may result in Neur inhibition via the transcriptional regulation of *Brd* family genes (BARDIN and SCHWEISGUTH 2006). To test whether the loss of *Brd* gene activity actually results in increased Dl activity accompanied by a SOP loss phenotype, we have studied the bristle phenotype of flies lacking some or all of the *Brd* family genes. No SOP and/or bristle loss was detected in *Brd-C* mutant clones, *Brd-C* homozygous mutant escaper flies, triple *m*α *m4 m6* mutant flies or in clones of cells mutant for all eight *Brd* family genes (Figure 3). These observations indicate that *Brd* family genes are not strictly required for SOP determination. We speculate that other mechanisms, including transcriptional repression of Notch target genes by Hairless and Su(H), may act to buffer Notch signaling activity in SOPs (BANG *et al.* 1995; CASTRO *et al.* 2005).

***Brd* family genes act during SOP selection:** To further test the possible role of the *Brd* family genes in lateral inhibition, we have used a fate competition assay (HEITZLER and SIMPSON 1991). This assay compares the relative ability of cells of different genotypes to compete for the adoption of the SOP fate along mosaic clone borders. For instance, cells with a twofold higher level of *Dl* activity were shown to be more likely to inhibit their neighbors than to be inhibited by them and most often emerge as winners of this competition. Here, we have examined the genotypes of SOPs along clone borders separating cells with varying copy numbers of *Brd* family genes in mosaic pupae at 16–18 hr after puparium formation (APF). We found that cells homozygous for the *Brd-C1* deletion are significantly more likely to become SOPs than cells with one or two copies of the

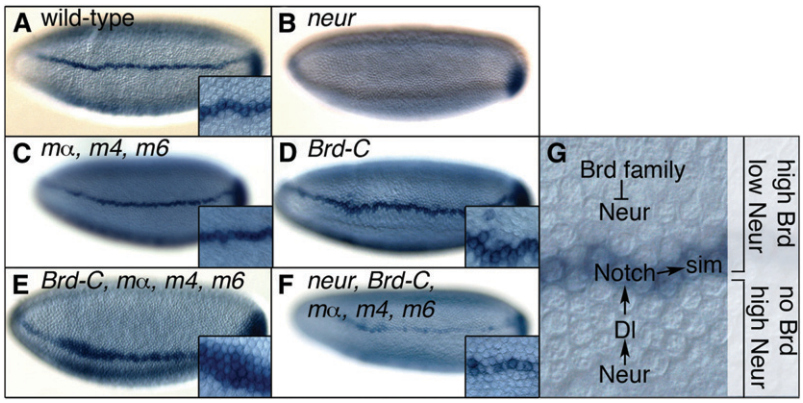

FIGURE 2.—*Brd* family genes act redundantly. (A) Wild-type embryos express *sim* in a single row of cells at stage 5. (B) *sim* transcripts are not detected in *neur*^{IF65} mutant embryos. (C) Deletion of the *m*α, *m4*, and *m6* genes in *Dp(3;2)E(spl)*δ*-8*Δα*46*; *Df(3)E(spl)*δ*-6* embryos does not affect *sim* expression. (D) Deletion of the *Brd-C* in *Dp(3;2)E(spl)*δ*-8*; *Df(3)Brd-C1 Df(3)E(spl)*δ*-6* embryos leads to the ectopic expression of the *sim* gene in a few cells dorsal to the mesectoderm. (E) Deletion of all eight *Brd* family members in *Dp(3;2)E(spl)*δ*-8*Δα*46*; *Df(3)Brd-C1 Df(3) E(spl)*δ*-6* embryos leads to strong ectopic expression of the *sim* gene in 3–5 cell rows. (F) The *sim* gene is weakly expressed in *Dp(3;2)E(spl)*δ*-8*Δα*46*; *Df(3)Brd-C1 neur*^{IF65} *Df(3)E(spl)*δ*-6* embryos, indicating that the phenotype seen upon loss of the *Brd-C*, *m*α, *m4*, and *m6* genes is largely rescued by loss of zygotic *neur* activity. (G) Spatial regulation of Dl signaling by Brd family proteins. Mesodermal cells express *neur* but not *Brd* family genes. Brd family proteins inhibit Neur in nonmesodermal cells. As a result, Neur-dependent Dl signaling is restricted to the mesoderm. Notch is activated in cells in direct contact with the mesoderm, as shown by expression of the *sim* gene.

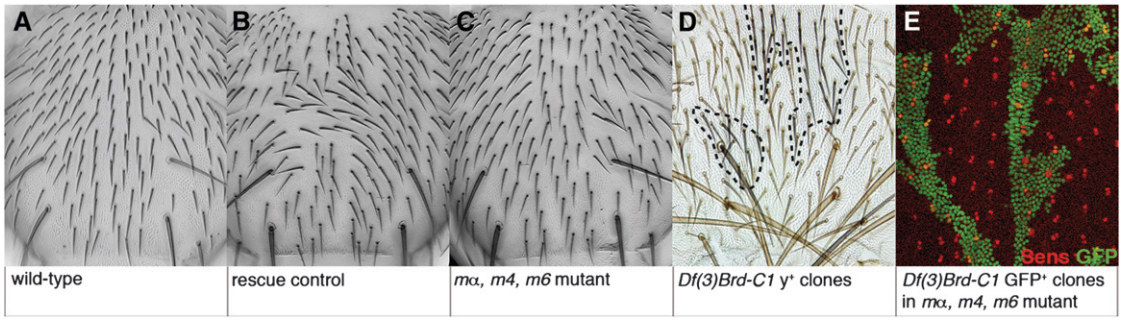

FIGURE 3.—*Brd* family genes are not required for SOP determination. Sensory organ formation was analyzed in adult cuticle preparations (A–D) and dissected pupal nota (E). Controls include wild type (A) and *Df(3)E(spl)*δ*-6 Dp(3;2)E(spl)*δ*-8* flies (B). Deletion of the *mα, m4*, and *m6* genes in *Dp(3;2)E(spl)*δ*-8*Δα*46*; *Df(3)E(spl)*δ*-6* flies did not affect bristle formation (C). Likewise, loss of *Brd-C* genes in *Df(3)Brd-C1* clones marked by *yellow* (*y*+) did not perturb bristle development (D). Loss of *Brd-C* genes in *Df(3)Brd-C1* clones marked by GFP (green) in *Dp(3;2)E(spl)*δ*-8*Δα*46*; *Df(3)E(spl)*δ*-6* pupae did not detectably affect SOP specification (sensory cells marked by Sens in red; E).

Brd-C (65%, $n = 145$; Figure 4, A and D). Similarly, cells that are triply mutant for the *mα*, *m4*, and *m6* genes are more likely to become SOPs than cells with one or two copies of each of these genes (60%, $n = 194$; Figure 4, B and D). For technical reasons, we could not study clone borders separating cells mutant for all *Brd* family genes and cells with one or two copies of each *Brd* family gene. These data indicate that the level of *Brd* family gene activity influences the choice of cell fate along mosaic border. We therefore conclude that *Brd* family genes of the *E(spl)-C* and *Brd-C* act in the lateral inhibition process prior to stable commitment to the SOP fate.

The level of Neur within a cell influences the fate of neighboring cells: Since Brd family members likely act during SOP selection by inhibiting Neur in PNC cells, we predict that the relative levels of *neur* activity should also influence the outcome of this competition, albeit in a manner opposite to the one seen for the *Brd* family

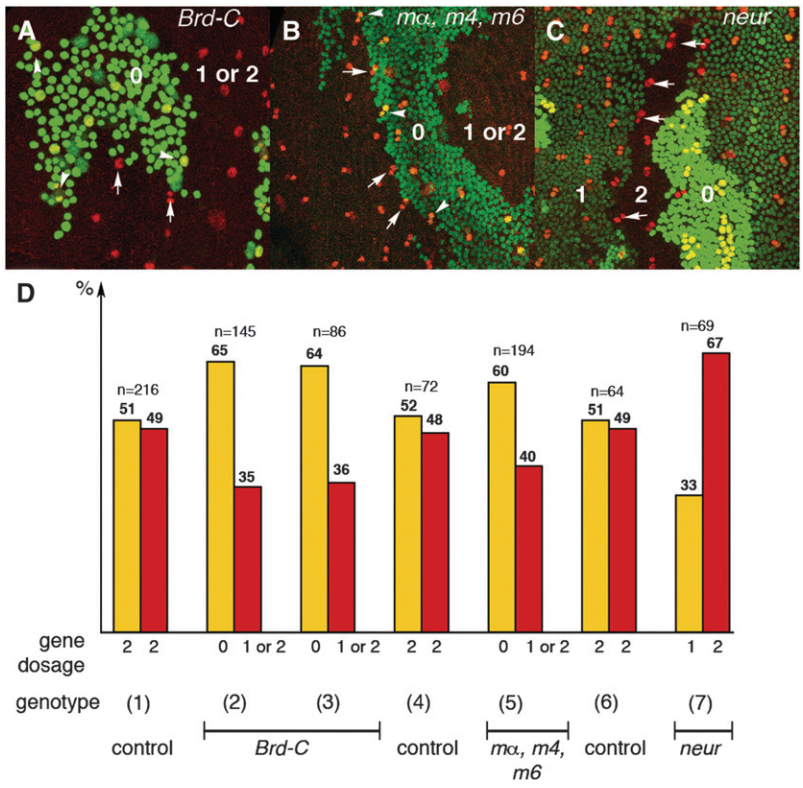

FIGURE 4.—The relative levels of *neur* and *Brd* family genes influence fate decisions. Competition for the adoption of the SOP fate was studied by scoring the genotype of SOPs located along the clone border separating cells that differ in the copy number of *Brd* family (A and B) and *neur* genes (C) (as numbered in A–C). *Df(3)Brd-C1* mutant clones (A) and *mα m4 m6* triple mutant clones (B) were marked by GFP whereas cells with one or two copies of either the *Brd-C* or the *mα*, *m4*, and *m6* genes do not express GFP. In panel C, the three different genoptypes were identified: *neur* mutant cells were marked by strong GFP expression, cells with one copy of the wild-type *neur* gene were marked by weak GFP expression, and cells with two copies of the wild-type *neur* gene were GFP-negative. SOPs were identified using Sens (red). SOPs located along clone borders are indicated by arrows (GFP-negative SOPs) and arrowheads (GFP-positive SOPs). (D) Plots showing the percentage of GFP-negative SOPs (in red) and GFP-positive SOPs (in yellow) along clone borders (% values are indicated above each bar; *n* is the number of SOPs scored along clone borders; "gene dosage" indicates the number of wild-type copies). Genotypes 1–7 are described in the MATERIALS AND METHODS section. Competition along *Brd-C* mutant clones was monitored in a wild-type background (2) as well as in a *mα m4 m6* triple mutant background (3). Similar results were obtained. Control wild-type clones were studied for each corresponding chromosomal arm (1, 4, and 6). Significantly more GFP-positive SOPs were observed in 2 and 3 compared to 1, and in 5 compared to 4. In contrast, more GFP-negative SOPs were observed in 7 compared to 6 (χ^2 test, $P < 0.01$).

genes. While previous clonal studies have indicated that *neur* is required for lateral inhibition (Yeh *et al.* 2000; Le Borgne and Schweisguth 2003), the influence of *neur* gene dosage on sensory cell fate has not been studied. A role for Neur during competition is not necessarily expected since *neur* transcripts and Neur proteins have been detected in SOPs but not in PNC cells (Boulianne *et al.* 1991; Le Borgne and Schweisguth 2003). Indeed, this expression pattern suggests that *neur* may only act in singled out SOPs to reinforce lateral signaling and that it does not act when PNC cells still compete for the SOP fate. Alternatively, it is conceivable that *neur* is expressed at low levels when PNCs compete for the SOP fate and is eventually upregulated in singled-out SOPs. Consistent with this view, expression of a *neur* enhancer-trap line is detected, albeit infrequently, in epidermal cells located next to SOPs (Huang *et al.* 1991). To functionally test whether *neur* acts during the competition phase prior to the stable commitment to the SOP fate, we have used the fate competition assay described above and have examined the genotypes of SOPs along clone borders separating cells with one or two wild-type copies of the *neur* gene. Cells with two copies of the *neur* gene were found to be more likely to become SOPs than cells with one copy (67%, $n = 69$; Figure 4, C and D). Thus, cells with a twofold reduction in *neur* activity appear to send a weaker inhibitory signal and are less likely to win the competition. We conclude that the level of *neur* activity influences the choice of cell fate along mosaic border and that *neur* acts to regulate the singling out of SOPs. Together, our results suggest that inhibition of Neur by Brd family members participates in SOP selection by modulating the activity of Neur in response to Notch activation within each PNC cell.

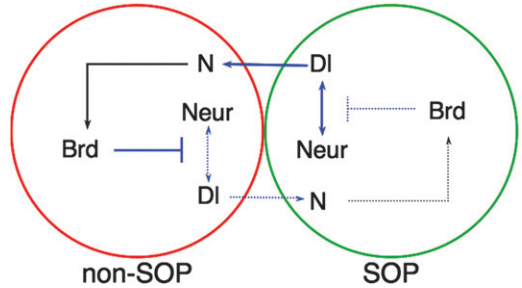

Figure 5.—Model A negative feedback loop linking activation of Notch at the cell surface to downregulation of Dl within the same cell operates during lateral inhibition. We propose that inhibition of Neur–Dl interaction by Brd family members is part of this feedback loop, so that the transcriptional upregulation of *Brd* family genes by activated Notch results in the downregulation of Dl activity in non-SOP cells. Transcriptional regulation of *Brd* genes by Notch is indicated in black while post-transcriptional regulatory steps are in blue.

DISCUSSION

Recent advances in genome engineering now permit the genetic analysis of complex gene families in Drosophila (Venken *et al.* 2006; Bischof *et al.* 2007; Venken and Bellen 2007). In this study, we have addressed the function of the eight *Brd* family proteins that interact with Neur and inhibit Neur-dependent Dl signaling. We have used FLP/FRT deletion, BAC recombineering, and phiC31-mediated integration technologies to delete specific sets of *Brd* family members. Our analysis reveals for the first time that *Brd* family genes have an essential function in the embryo and that they act in a redundant manner. Indeed, deletion of the *mα*, *m4*, and *m6* genes has no major effect on viability and fertility while the combined loss of the *BobA*, *BobB*, *Tom*, *Brd*, and *Ocho* genes reduces viability and has a weak effect on Notch target gene expression in early embryos. However, deletion-based inactivation of all eight *Brd* family genes known to encode direct Neur interactors results in fully penetrant embryonic lethality that is associated with ectopic Neur-dependent Notch signaling in early embryos. This study therefore establishes that *Brd* family genes act in a partly redundant manner to antagonize the activity of Neur. Functional redundancy is not, however, strict and the strength of the phenotypes associated with the progressive loss of *Brd* family genes may be, at least in part, dosage dependent. For instance, loss of *Brd-C* genes has milder effect on both viability and *sim* ectopic expression than the complete loss of all eight *Brd* family genes.

Our genetic analysis revealed that cells with lower levels of *Brd* family gene activity relative to their neighbors are more likely to win the competition and to adopt the SOP fate in the pupal notum. Conversely, cells with lower levels of *neur* activity relative to their neighbors are more likely to loose competition and to differentiate as epidermal cells. These data indicate that Neur-dependent Dl signaling regulates the singling out of SOPs and that inhibition of Neur by Brd family members operates during the process of SOP selection. Since expression of *Brd* family genes is regulated by Notch (Wurmbach *et al.* 1999; Lai *et al.* 2000b; Castro *et al.* 2005), we propose that inhibition of Neur by Brd family members is one of the mechanisms whereby activation of Notch in a given cell results in the downregulation of Dl. Thus, our study provides experimental support for a novel molecular mechanism underlying the negative feedback loop mechanism operating during lateral inhibition in Drosophila (Figure 5). While our clone border analysis indicates that *Brd* family genes influence SOP selection, it is likely that the molecular mechanism proposed here based on the activity and regulation of the *Brd* family genes acts in parallel with other feedback mechanisms since SOPs are properly selected in the absence of all *Brd* family genes.

Several features of our model deserve mention. First, this model involves a single transcriptional step followed by one post-transcriptional regulatory step. Second, the

Notch transcriptional target genes are small, typically less than 1 kb, and encode small cytoplasmic proteins, indicating that the transcription/translation time delay between Notch receptor activation and Delta inhibition is minimal. Third, Notch activates several functionally redundant target genes at once. This may therefore serve to amplify the signal of Notch activation. We speculate that these features may be important for rapid and efficient feedback regulation.

A single *Brd* family gene has been identified in other insect genomes. So, how could we explain the increase in gene copy number in Drosophila? First, functional diversification may be associated with this increase. For instance, unlike other *Brd* family members, *m6* is expressed predominantly in muscles, suggesting that it may have acquired a specific function in this tissue. Similarly, the Brd and Bob proteins lack motifs 3 and 4, suggesting that their molecular activity and/or localization may slightly differ (Lai *et al.* 2000b). Second, an increase in the number of Notch target genes acting in parallel may have been selected as a means to selectively amplify one specific response of the genome to Notch activation. Finally, this increase may have been evolutionarily selected as a response to constraints exerted internally on the gene regulatory network by miRNAs acting to inhibit gene expression (Lai *et al.* 2005). This interpretation is consistent with the notion that miRNAs provide a mechanism for internal selection favoring the emergence of a stable complex gene network.

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