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Bre1 Is Required for Notch Signaling and Histone Modification

Short Article

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Summary

Notch signaling controls numerous cell fate decisions during animal development. These typically involve a Notch-mediated switch in transcription of target genes, although the details of this molecular mechanism are poorly understood. Here, we identify dBre1 as a nuclear component required cell autonomously for the expression of Notch target genes in *Drosophila* development. dBre1 affects the levels of Su(H) in imaginal disc cells, and it stimulates the Su(H)-mediated transcription of a Notch-specific reporter in transfected *Drosophila* cells. Strikingly, *dBre1* mutant clones show much reduced levels of methylated lysine 4 on histone 3 (H3K4m), a chromatin mark that has been implicated in transcriptional activation. Thus, dBre1 is the functional homolog of yeast Bre1p, an E3 ubiquitin ligase required for the monoubiquitination of histone H2B and, indirectly, for H3K4 methylation. Our results indicate that histone modification is critical for the transcription of Notch target genes.

Introduction

The Notch signaling pathway is highly conserved in animal cells and controls numerous cell fate decisions during development (Artavanis-Tsakonas et al., 1999). Most notably, this pathway operates in the selection of individual cells from cellular equivalence groups, a process called "lateral inhibition" (Lewis, 1998; Simpson, 1997), as well as implementing cell fate decisions in stem cell lineages (e.g., Brittan and Wright, 2002; Gaiano and Fishell, 2002; Krause, 2002), and contributing to the oscillator that regulates vertebrate segmentation (Pourquie, 2003). The Notch pathway has also been implicated in various human diseases including cancer (Gridley, 2003; Joutel and Tournier-Lasserve, 1998; Maillard and Pear, 2003).

During classical Notch signaling, the Notch transmembrane receptor is stimulated by its ligands Delta or Serrate. This results in proteolytic cleavage of Notch and release of an intracellular Notch fragment (N^{ICD}) that translocates to the nucleus (Lecourtois and Schweisguth, 1998; Schroeter et al., 1998; Struhl and Adachi, 1998). Here, N^{ICD} binds to transcription factors of the CSL family and functions as their coactivator. CSL factors include Suppressor of Hairless (Su(H)) in *Drosophila* (Fortini and Artavanis-Tsakonas, 1994), LAG-1 in *C. elegans* (Christensen et al., 1996), and CBF1 in vertebrates (Hsieh et al., 1996; Jarriault et al., 1995). CSL/N^{ICD} then activates Notch target genes, which include the Enhancer of split/HES genes (Artavanis-Tsakonas et al., 1999). Thus, there are three components at the core of the Notch pathway—ligand, Notch, and CSL—and the relay of the signal from the membrane to the nucleus depends upon N^{ICD}. An implication of this may be that, in order to regulate the pathway, the activities of these core components must be modulated by interactions with other proteins and/or by posttranslational modifications.

This is indeed the case: genetic studies in *Drosophila* and *C. elegans* have identified a large number of genes that control Notch signaling (e.g., Justice and Jan, 2002; Panin and Irvine, 1998). Among these are at least five different E3 ubiquitin ligases, each of which targets a core component of the Notch pathway (Itoh et al., 2003; Lai, 2002). Here, we identify a RING finger protein, predominantly nuclear, that is necessary for the transcription of Notch target genes. This RING protein has two close counterparts in mammals, and has further relatives in other organisms including *C. elegans*, *Arabidopsis*, and yeast. Its relative Bre1p in the yeast *S. cerevisiae*—the only Bre1 protein whose function has been studied so far—is required for the monoubiquitination of histone H2B and, indirectly, for the methylation of histone 3 on lysine 4 (H3K4) and 79. Yeast can survive without Bre1p function but they are slow growing and the transcription of inducible genes is compromised (Hwang et al., 2003; Osley, 2004; Wood et al., 2003). Interestingly, *Drosophila Bre1* (*dBre1*) mutant clones also show reduced methylation of H3K4 (H3K4m), indicating that dBre1 is the functional homolog of yeast Bre1p. The relatively specific phenotypes and transcriptional defects argue that the Su(H)-dependent transcription of Notch target genes is particularly reliant on Bre1-dependent histone modification.

Results and Discussion

The lethal allele E132 was fortuitously identified among a collection of mutants that modify the wing notching phenotype caused by Armadillo depletion (Sanson et al., 1996). Genetic mapping of the lethality associated with E132 placed this at 64E8, and it was found to be allelic to an existing mutation, *l(3)01640*, caused by the P element insertion P1541. Using plasmid rescue of the P element, we localized the site of insertion to the first intron of the open reading frame CG10542, which encodes a predicted protein of 1044 amino acids (Figure 1A). The insertion site is 48 nucleotides upstream of the translation initiation codon. Precise excision of P1541

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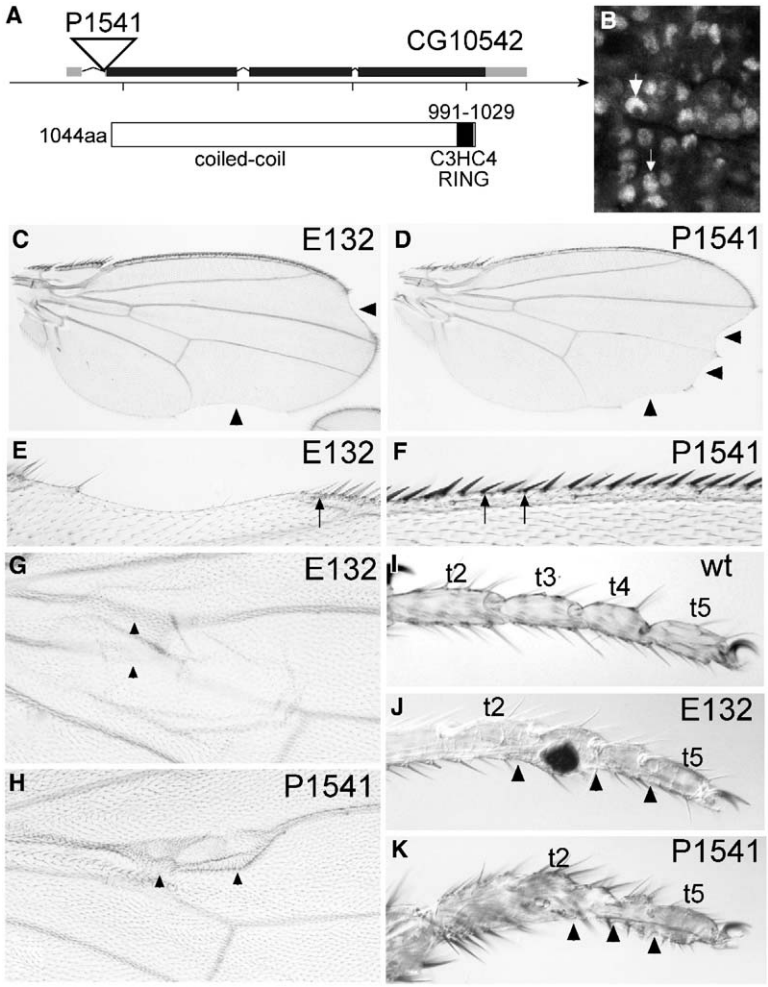

Figure 1. Map of the *dBre1* Locus, and Adult Phenotypes of *dBre1* Mutant Cells

(A) Top, primary structure of the CG10542 open reading frame (protein coding exons black, noncoding exons gray), with the position of the P1541 insertion marked in the first intron. Underneath, layout of dBre1 (residues refer to C1 and C7 of RING domain, flanked at both ends by ~15 conserved residues [Hwang et al., 2003]). (B) Ubiquitous expression of GFP-dBre1 in the embryonic epithelium with arm.GAL4 (arrows indicate nuclei); GFP-ΔRING shows the same subcellular distribution (not shown). (C–E) Clones of (C and E) E132 and (D) P1541 mutant cells (arrowheads indicate nicks, arrows occasional mutant bristles adjacent to nicks). (F) Small clones of P1541 can contribute to margin tissue with aberrantly spaced bristles (arrows). (G) E132 and (H) P1541 mutant clones associated with blisters and widened veins (arrowheads). (I) Wild-type tarsal leg segments (t2–t5, tarsomeres). (J) E132 and (K) P1541 mutant clones associated with reduced growth, and fusions or truncations of tarsal segments (arrowheads mark missing joints).

restores viability, confirming that the P element insertion and, by inference, E132 are lethal alleles of CG10542. In support of this, ubiquitous overexpression of the full-length protein encoded by CG10542 rescues the lethality of E132 or P1541 mutant embryos and sustains development to give essentially normal adult flies (with a few minor defects including slightly reduced bristles). CG10542 encodes a conserved protein with close relatives in mammals, *C. elegans*, plants, and fungi (Hwang et al., 2003). We shall name the *Drosophila* protein dBre1, after its relative Bre1p in the yeast *S. cerevisiae*.

dBre1 Is a Nuclear RING Domain Protein

The hallmarks of the Bre1 proteins are a C-terminal RING finger domain linked to an extensive N-terminal coiled-coil region (Figure 1A). The 39 amino acid C3HC4 RING domain is flanked on both sides by ~15 conserved amino acids, suggesting that the fly and mammalian proteins are true orthologs of yeast Bre1p (Hwang et al., 2003). RING domains are typically found in E3 ubiquitin ligases (Freemont, 2000) and frequently mediate the interaction with the E2 ubiquitin-activating enzyme while the other parts of the protein are involved in substrate recognition. The RING domains are therefore critical to catalyze the transfer of ubiquitin from the E2 to the substrate (Weissman, 2001). To confirm the functional importance of the RING domain in dBre1, we tested whether an N-terminal fragment of dBre1 that lacks the RING domain (ΔRING) could rescue *dBre1* mutants. No rescue was observed with any of the 4 transgenic lines (from a total of 814 flies scored), confirming that the RING domain is essential for the function of dBre1 as it is for yeast Bre1p (Hwang et al., 2003; Wood et al., 2003).

To examine the subcellular location of full-length dBre1 and the derivative that lacks the RING domain, we tagged both forms of the protein with GFP at the N terminus. Both GFP-dBre1 and GFP-ΔRING are predominantly nuclear in embryonic and imaginal disc cells (Figure 1B and data not shown), although a low level of protein is also detectable in the cytoplasm. This nuclear-cytoplasmic distribution is similar to that of a ΔRING derivative of human Bre1-B when it is overexpressed in mammalian cells (Wen and Ao, 2000). Thus dBre1 appears to be a nuclear protein, like its mammalian counterpart, and deletion of the RING domain does not alter its subcellular distribution even though it abolishes its ability to rescue the mutants.

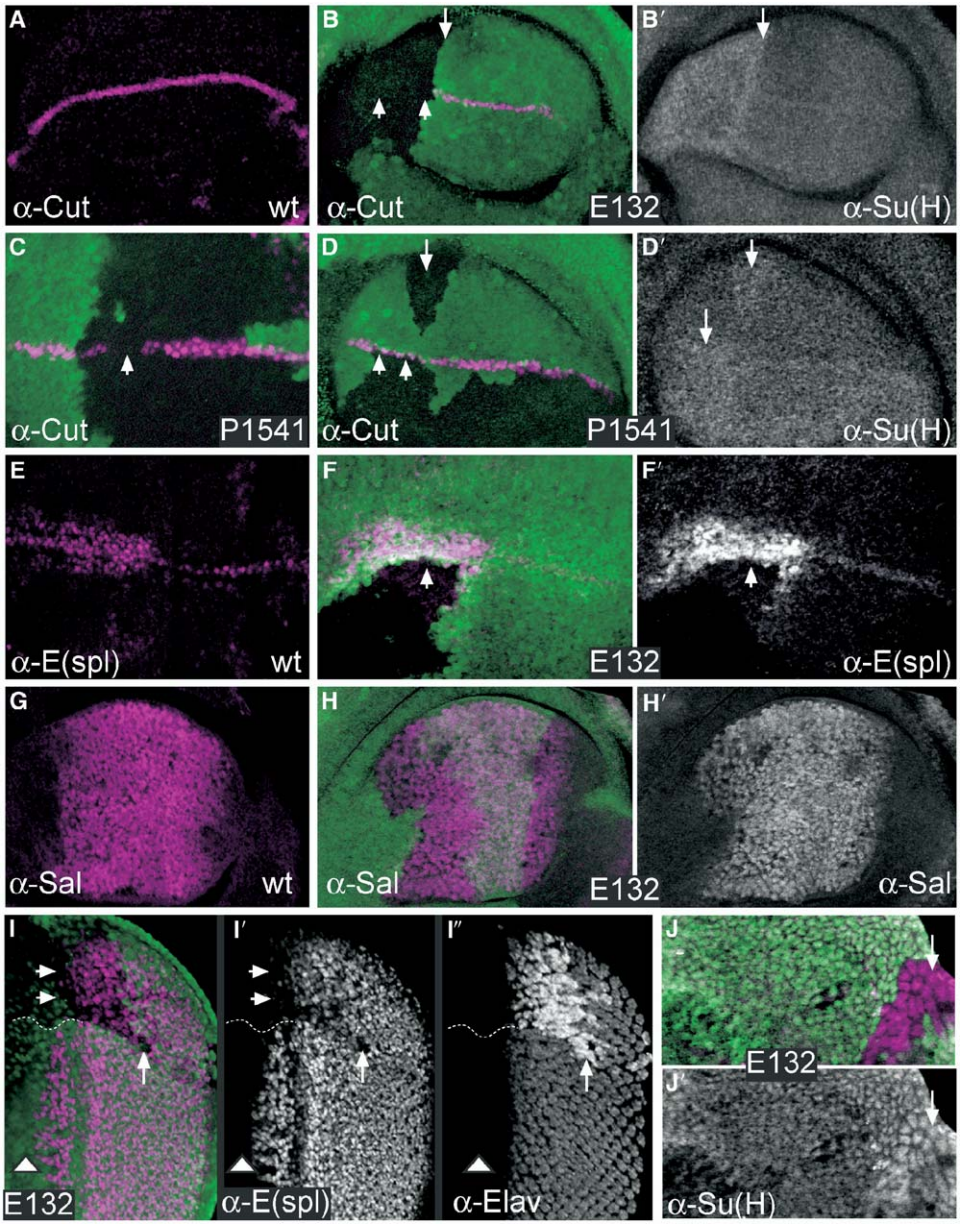

Figure 2. *dBre1* Mutant Cells Show Loss of Notch Target Gene Expression and Elevated Levels of Su(H)

(A–H) Wing discs, with (B) E132 or (C and D) P1541 mutant clones marked by absence of GFP (green); magenta, expression of (A–D) Cut, (E and F) E(spl), or (G and H) Spalt; double and single channels from the same optical section are shown. Arrowheads indicate loss of (B and D) Cut or (F) E(spl) expression; arrows point to (B) edge of clone or (D) elevated levels of Su(H). (I) Eye discs, with E132 mutant clone (edge of the clone marked by dotted line; green nuclei above the line are from the overlaying peripodial membrane). Reduced E(spl) expression near the morphogenetic furrow (marked by large arrowhead) is indicated by arrowheads, derepression of the neuronal marker Elav by arrows. (J) Wing disc from early third instar larva, with E132 mutant clones generated without a growth advantage; arrows point to elevated levels of Su(H) and large cells.

dBre1 Mutant Clones in Imaginal Discs Show Notch Signaling Defects

To investigate the role of dBre1 in the fly, we generated homozygous mutant clones in the imaginal disc precursors of the adult structures. Surprisingly, we found that the majority of defects were similar to those caused by defects in Notch signaling. Thus, adult flies bearing E132 or P1541 mutant clones show notches in the wing margin (Figures 1C–1E) and aberrant spacing of wing margin bristles (Figure 1F), wing blistering and vein defects (Figures 1G and 1H), fusions of leg segments (Figures 1I–1K), and loss of notal bristles and rough eyes (not shown). Most of these phenotypes are characteristic of reduced Notch signaling (e.g., Cagan and Ready, 1989; de Celis and Garcia-Bellido, 1994; Shellenbarger and Mohler, 1975) and are distinct from those produced by loss-of-function of other signaling pathways, such as Wingless, Dpp, or Hedgehog signaling that also op-

erate during imaginal disc development (Strigini and Cohen, 1999). The phenotypic data suggest therefore that dBre1 has a role in promoting Notch signaling.

To confirm this, we examined the expression of Notch target genes in *dBre1* mutant clones (Figure 2). Since *dBre1* mutant clones are considerably smaller than their matched wild-type twin clones (see below), we used the *Minute* technique to compensate for the growth defect of the mutant clones. In wing imaginal discs, *cut* and *Enhancer of split* (*E(spl)*) are expressed along the prospective wing margin, and their expression depends directly on Notch signaling (Bailey and Posakony, 1995; de Celis et al., 1996b; Lecourtois and Schweisguth, 1995; Micchelli et al., 1997; Neumann and Cohen, 1996). We found that Cut expression is absent in large E132 mutant clones (9/9; Figures 2A and 2B), and is lost (3/11) or reduced (6/11) in most P1541 mutant clones (Figures 2C and 2D). Likewise, E(spl) expression is lost cell autonomously from all E132 mutant clones in the wing (Figures 2E, 2F, and 2F′). Conversely, expression of *spalt*, a target of Dpp signaling in the wing, is not reduced in P1541 and E132 mutant cells (Figures 2G, 2H, and 2H′, and data not shown), indicating that the effects of *dBre1* mutation are relatively specific. Similar results are obtained in the eye, where E(spl) expression is also disrupted in E132 clones (Figures 2I and 2I′). Expression in the neurogenic region at the furrow is lost, and elsewhere it is absent or severely reduced, except in the basal layer of undifferentiated cells where expression is independent of *Notch* (Baker et al., 1996). In addition, we observe a derepression of the neuronal cell marker *Elav* in eye disc clones (Figure 2L′′). The latter indicates excessive neuronal recruitment due to diminished Notch-mediated lateral inhibition (Baker et al., 1996) (note, however, that the phenotypes are not identical to those produced by complete absence of *Notch*, which in the eye results in loss of neuronal markers because Notch is needed to promote neural development by alleviating Su(H)-mediated repression [Li and Baker, 2001]). Our results demonstrate that dBre1 functions in multiple developmental contexts and, specifically, that it is required for the subset of Notch functions that involve Su(H)-dependent activation of Notch target genes.

Functional Interactions between dBre1 and Notch Signaling

To further confirm the importance of dBre1 during Notch signaling, we asked whether we could detect any genetic interactions between overexpressed dBre1 or ΔRING and mutations in *Notch* (*N*) or its ligand *Delta* (*Dl*). Indeed, overexpression of either protein in the wing disc results in adult phenotypes (Figures 3A–3F). In each of 5 ΔRING-expressing lines, we observed mild if consistent mutant phenotypes in both males and females, namely upward-curved wings (due to stronger expression in the dorsal wing compartment), tiny vein deltas, and a significant decrease in wing size (Figure 3B, compare to Figure 3A). These defects are more severe after overexpression of ΔRING in *dBre1* heterozygotes (Figure 3D; data not shown), indicating that ΔRING acts as a weak dominant-negative. Consistent with this, excess ΔRING significantly enhances the phenotypes of *N*/+ and *Dl*/+ heterozygotes, resulting in increased vein thickening and additional vein material and, in the case of *N*/+, also in more frequent wing notching (Figures 3F and 3H; data not shown). These genetic interactions support the link between dBre1 and Notch signaling.

Excess full-length dBre1 in wing discs causes vein defects whose strength, however, varies considerably between different dBre1-expressing lines, and between males and females (probably because the ms1096. GAL4 driver produces higher expression levels in males). In most lines (4/6), we observe vein thickening and additional vein material only in males, while female wings appear normal. These vein defects in male wings are suppressed to almost normal in *dBre1* heterozygotes (not shown), suggesting that they are due to increased levels of functional dBre1 protein. The remaining 2 lines produce similar vein defects also in females (Figure 3C; the corresponding males have small and severely crumpled wings; not shown). Unexpectedly, these defects are enhanced in *N*/+ and *Dl*/+ heterozygotes (Figure 3E, compare to [G]; not shown), suggesting that the overexpressed dBre1 interferes with Notch signaling, rather than enhancing it as we might have expected. This anomalous result could be explained if dBre1 is part of a multiprotein complex, in which case its overexpression might interfere with the function of this complex by titrating one of its components. Nevertheless, the genetic interactions between overexpressed dBre1 and *Notch* and *Delta* further underscore the link between dBre1 and Notch signaling

To test whether dBre1 directly influences Notch-dependent transcription, *Drosophila* S2 cells were transfected with Flag-tagged or untagged dBre1, and the activity of a Notch-specific reporter containing 4 Su(H) binding sites (NRE, a luciferase derivative of $Gbe+Su(H)_{m8}$ [Furriols and Bray, 2001]) was measured in the presence or absence of low levels of N^{ICD}. As a control, we used a reporter with mutant Su(H) binding sites (NME, or $Gbe+Su(H)_{mut}$ [Furriols and Bray, 2001]). These experiments revealed a significant stimulation of the NRE reporter by dBre1, especially in the presence of N^{ICD} (Figure 3I). The degree of stimulation is similar to that observed when the ubiquitin ligase Hdm2 is added to transcription assays of Tat activity (Bres et al., 2003). dBre1 also elicits a slight stimulation of NME; however, it has no effect on transcription of the internal renilla control nor of a Wingless-specific reporter (Figure 3I; data not shown). The fact that overexpressed dBre1 has stimulatory effects on Notch in the transfection assays but not in imaginal discs presumably reflects differences either in the levels of dBre1 or in the amounts of other limiting factors in the two cell contexts. Nevertheless, the transfection assays reveal an intrinsic potential of dBre1 in stimulating the transcription mediated by Su(H) and its coactivator N^{ICD}.

Elevated Levels of Su(H) in *dBre1* Mutant Clones

All our results point to a role of dBre1 in promoting Notch signaling. As other ubiquitin ligases have been shown to influence the levels of specific protein components of the Notch pathway (Itoh et al., 2003; Lai, 2002), we investigated whether there were any alter-

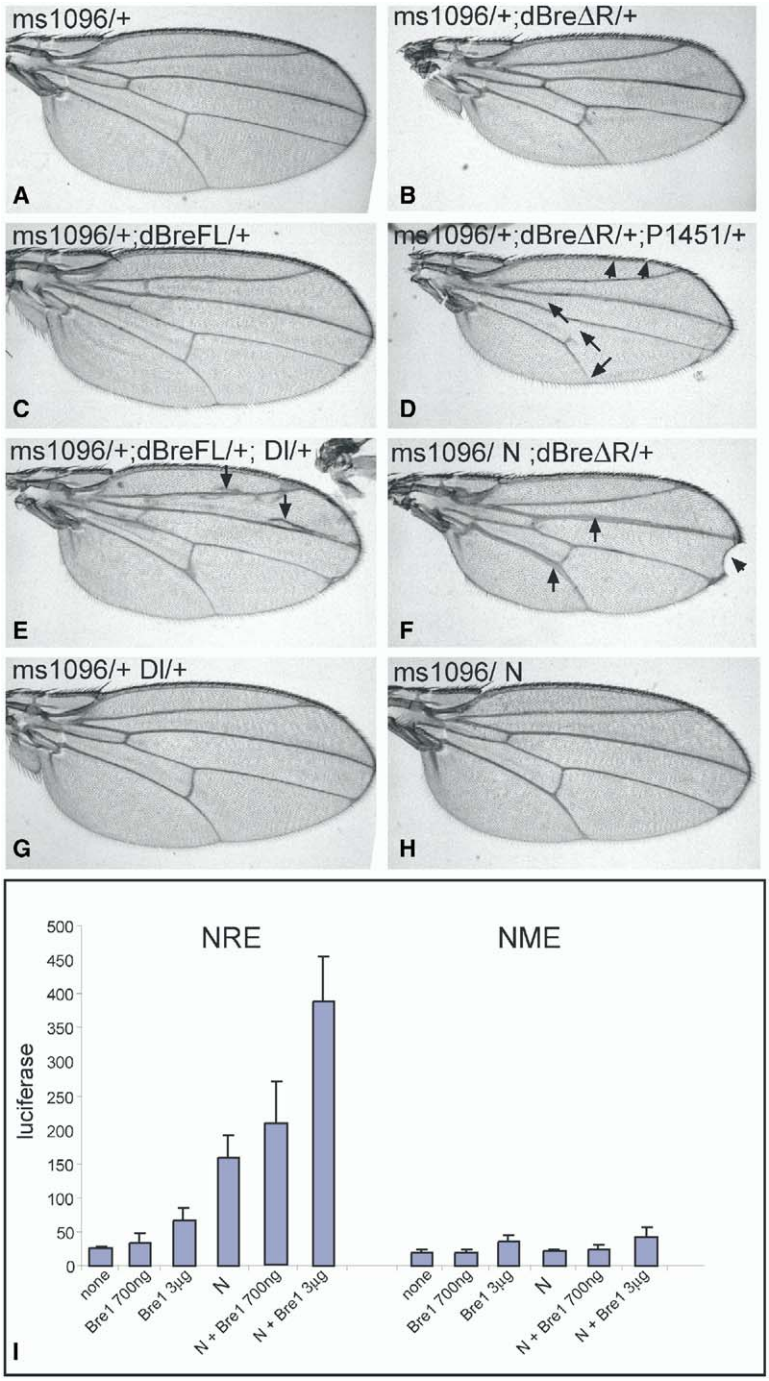

Figure 3. Functional Interactions between Excess dBre1 and *Notch* Signaling in Wings and S2 Cells

(A–F) Mutant phenotypes in female wings due to overexpressed ΔRING (ΔR [B, D, and F]) or full-length dBre1 (dBreFL [C and E]); genotypes are indicated at the top of each panel (N, N^{55e11}; Dl, Dl^{RF}). (G and H) Phenotypes of N/+ (G) or Dl/+ (H). (D) Loss of vein material is indicated by arrows, margin defects by arrowheads (both seen in ~80% of wings). (E and F) Arrows point to increased vein thickening and/or additional vein material (seen in 100% of wings), arrowhead to wing notch (seen in 57% of *N*/+ wings with ΔRING, versus 18% without ΔRING). (I) dBre1 stimulates Notch-dependent transcriptional activation in *Drosophila* S2 cells. The activity of luciferase reporters containing wild-type (NRE) or mutant (NME) Su(H) binding sites were assayed in extracts from S2 cells cotransfected with combinations of plasmids (as indicated) expressing dBre1 and N^{ICD}, and with an internal renilla control. Values indicated on the left represent mean ratios of firefly luciferase to renilla control from at least 3 independent experiments (standard deviations indicated by error bars).

ations to Notch, Delta, or Su(H) levels in *dBre1* mutant clones. While there are no detectable changes in Notch or Delta staining in *dBre1* mutant cells (not shown), we found that the levels of Su(H) staining are enhanced slightly but consistently, and cell autonomously, in mutant clones of both *dBre1* alleles, regardless of the location of these clones within the disc (Figures 2B′ and 2D′). This is also obvious in clones induced early in larval development in a non-*Minute* background in which the mutant *dBre1* clones remain small (Figures 2J and 2J′). As an aside, these clones reveal that individual *dBre1* mutant cells are enlarged (Figures 2J and J′), reminiscent of the yeast *bre1p* mutant which also shows a "large cell" phenotype (Hwang et al., 2003). This phenotype has not been observed in cells lacking Notch signaling, so this aspect of dBre1 function appears distinct from its role in the Notch pathway, and suggests that there are additional molecular targets. Nevertheless, the elevated levels of Su(H) in the *dBre1* mutant clones identify Su(H) as one molecular target of dBre1 and suggest that, in the wild-type, dBre1 may expose Su(H) to ubiquitin-mediated degradation. The

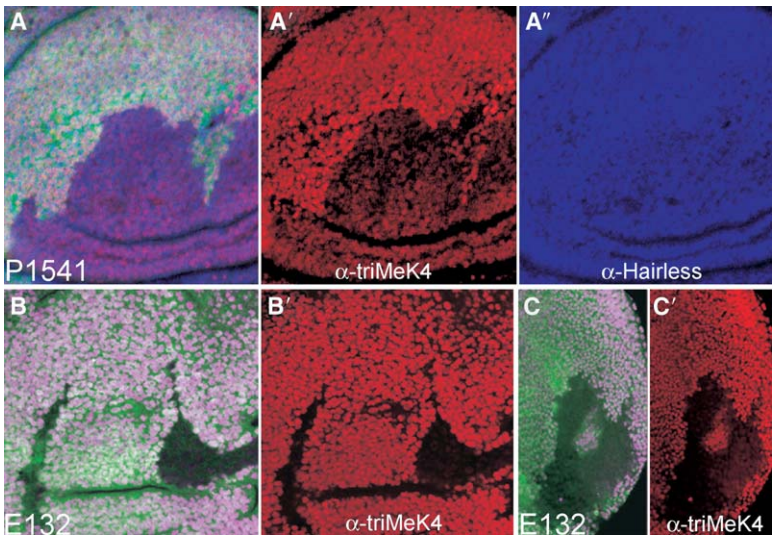

Figure 4. Loss of H3K4 Trimethylation in *dBre1* Mutant Clones

Wing (A and B) and eye (C) imaginal discs bearing (A) P1541or (B and C) E132 mutant clones (marked by absence of GFP, green), stained with antibodies against H3K4m (red) and Hairless (blue), as internal control for nuclear staining.

effects on Su(H) are consistent with the cell-autonomous action of dBre1 on Notch target gene expression, but the fact that removal of dBre1 has a stabilizing effect on Su(H) appears to contradict its stimulating effect on Notch-dependent transcription. As Su(H) functions as both a repressor and an activator, this may be explained if loss of dBre1 specifically stabilizes the repressor complex. Alternatively, the effect of *dBre1* mutations on Su(H) may reflect an indirect bystander activity of dBre1 (see below).

dBre1 Is Required for Histone Modification

Finally, we asked whether dBre1 has a similar molecular function as its relative yeast Bre1p. The latter is required for the monoubiquitination of histone H2B, which is a prerequisite for the subsequent methylation of histone H3 at K4 by SET1-containing complexes. H3K4 methylation appears to be a chromatin mark for transcriptionally active genes (Lachner and Jenuwein, 2002), and yeast *bre1p* mutants show defects in the transcription of inducible genes that have been ascribed to the lack of H2B ubiquitination and H3K4 methylation at the promoters of these genes (Kao et al., 2004; Wood et al., 2003). As there are no in vitro assays for H2B ubiquitination and no antibodies that detect this modified form of H2B, we investigated effects of *dBre1* mutations on the linked H3K4 methylation. Wing discs bearing *dBre1* mutant clones were stained with an antibody specific for trimethylated H3K4 (H3K4m). This revealed a significant reduction of H3K4m in *P1541* mutant clones (Figure 4A). More strikingly, in clones of the stronger *E132* allele, H3K4m is barely detectable (Figures 4B and 4C). In contrast, staining of these clones with an antibody against H3K9m does not show any changes in the mutant territory (not shown), indicating that the effect in *dBre1* mutant clones on the methylation of H3K4 is relatively specific. We note that, in wild-type wing discs, there is slight modulation of trimethylated H3K4, with higher levels at the dorsoventral boundary where Notch is activated. However, *Notch* mutant cells retain robust H3K4m staining, although occasionally show slightly lowered levels compared to adjacent wild-type cells (not shown). Thus, the reduced H3K4m staining in *dBre1* mutant cells is primarily due to an activity loss of dBre1 rather than due to loss of Notch signaling. Based on its effects on trimethylated H3K4, we conclude that dBre1 is indeed the functional homolog of yeast Bre1p. Furthermore, it appears that the activity of dBre1 is essential for the bulk of trimethylated H3K4 in imaginal disc cells.

In yeast, H2B ubiquitination and H3K4 methylation are associated with sites of active transcription, but the only identified natural target gene is GAL1 (Kao et al., 2004). In *Drosophila*, the target genes of dBre1evidently include genes regulated by Notch, given the requirement of *dBre1* for their transcription. It is therefore conceivable that Su(H) may have a role in targeting dBre1 to their promoters (although we have not been able to detect direct binding or coimmunoprecipitation between dBre1 and Su(H) [S.B., unpublished results]). It is puzzling that dBre1 has a slight destabilizing effect on Su(H), despite being an activating component of Notch signaling. We believe that this could be a bystander effect of dBre1: evidence suggests that the Bre1p-mediated monoubiquitination of H2B leads to a transient recruitment of proteasome subunits to chromatin, and that the subsequent methylation of H3K4 depends on the activity of these proteasome subunits (Ezhkova and Tansey, 2004). Their transient presence at specific target genes may have a destabilizing effect on nearby DNA binding proteins, and the mildly increased levels of Su(H) in *dBre1* mutant cells could therefore reflect a failure of proteasome recruitment due to loss of H2B monoubiquitination.

Perhaps the most interesting implication of our results is that the dBre1-mediated monoubiquitination of H2B and methylation of H3K4 may be critical steps in the transcription of Notch target genes. Indeed, it appears that the Notch target genes belong to a group of genes whose transcription is particularly susceptible to the much reduced levels of H3K4m in *dBre1* mutant cells. Based on the *dBre1* mutant phenotypes, there

are likely to be other genes in this group, including for example genes controlling cell survival and cell size. Nevertheless, it would appear that the transcription of Notch target genes is particularly reliant on the activity of dBre1. Other examples are emerging where the transcriptional activity of a subset of signal responsive genes is particularly sensitive to the function of a particular chromatin modifying and/or remodelling factor (e.g., Kadam and Emerson, 2003). This sensitivity presumably reflects the molecular mechanisms used by signaling pathways to activate transcription at their responsive enhancers. Understanding why Notch-induced transcription is particularly susceptible to loss of dBre1 function will require knowledge of these underlying molecular mechanisms.

Experimental Procedures

Fly Strains, Genetics, and Plasmids

E132 was isolated in an ethylmethanesulfonate screen (Thompson et al., 2002) described by H.M. (PhD thesis, Cambridge University, 2002), and found to be allelic to *l(3)01640* (formerly called P1541) described in FlyBase. Viable *ry* lines were isolated by standard P excision, and precise excision of the P element was confirmed by PCR analysis. Four lethal *ry* lines were also obtained, all of which still retain residual P sequences.

Standard FRT chromosomes (described in FlyBase) and marked strains were used for clonal analysis of E132 or P1541 alleles (see also Supplemental Data). Somatic clones were induced by 1 hr heat shock at 38°C at 48–72 hr of development. Clones induced at later stages rarely produced phenotypes, probably due to perdurance of the wild-type dBre1 protein in the mutant cells.

Full-length dBre1 was tagged with GFP or FLAG at its N terminus, and subcloned into pUAST (Brand and Perrimon, 1993) for GAL4-mediated expression in transgenic flies, and into pMT-B (Invitrogen) for cotransfection assays in *Drosophila* S2 cells. Western analysis confirmed that these tagged Bre1 proteins are expressed as full-length proteins in *Drosophila* embryos and in S2 cells (not shown). GFP-tagged and untagged versions of C-terminal truncations of dBre1 (ΔRING; amino acids 1–983) were also generated, and transgenic fly strains were established using standard procedures.

For rescue assays, stocks were established bearing *dBre1* alleles and a second-chromosomal ubiquitous driver (arm.GAL4) or pUAST construct (GFP-dBre1 or ΔRING), and eclosion of homozygous *dBre1* flies (lacking TM balancers) was scored. Of six individual UAS.GFP-dBre1 lines, three produced homozygous *dBre1* flies at the expected frequency of ~1/4.

GAL4 driver lines used for expression of GFP-Bre1, and mutant alleles of *N* and *Dl* are described in Flybase. Flies expressing GFP-dBre1 were identified by their phenotypes, or by their green fluorescence if necessary. The overexpression phenotypes due to GFP-tagged ΔRING were essentially the same as those produced by untagged ΔRING (Figure 3).

Immunofluorescence

Discs were dissected from wandering third instar larvae and indirect immunofluorescence was carried out as previously described (de Celis et al., 1996a). Primary antibodies were rabbit α-GFP (1:1000; Molecular Probes); rabbit α-Su(H) (1:500; kindly provided by F. Schweisguth); mouse α-E(spl)323 (1:3) (Jennings et al., 1994); rat α-Spalt (1:250) (de Celis et al., 1996a); rat α-Elav (1:50) (O'Neill et al., 1994) and mouse α-Cut (1: 20) (Blochlinger et al., 1990), both obtained from Developmental Studies Hybridoma Bank, University of Iowa, Department of Biological Sciences; and rabbit α-H3K4m and α-H3K9m (1:1000; Abcam). FITC, Cy3- or Cy5-conjugated secondary antibodies were obtained from Jackson Immunological.

To examine expression of GFP-dBre1 and GFP-ΔRING in embryos or imaginal discs, green fluorescence was recorded with an MRC1024 confocal microscope.

Cell Culture Assays

The transcription-factor binding-site and promoter regions were excised from Gbe+Su(H)m8 and from Gbe+Su(H)mut (Furriols and Bray, 2001) and inserted upstream of luciferase in pGL3 (Promega). Transfections were carried out using lipofectin (Invitrogen) according to the manufacturers recommendations. For each assay, 1 well of a 24-well plate was transfected with 1 μg of luciferase reporter, 200 ng of control renilla reporter (pRL-TK; Promega) along with 200 ng pMT-N^{icd} (Eastman et al., 1997) and 700 ng or 3 μg of pMT-FLAG-dBre1 where indicated. The empty vector pMT-A was used to bring the total DNA concentration to 4.5 μg. Expression from pMT-N^{icd} and pMT-FLAG-dBre1 was induced after 24 hr using 600 μM $CuS0_4$, and cells were harvested 18 hr later. Expression of the luciferase reporter was assayed using the Promega Dual luciferase reporter assay kit.

Supplemental Data

Supplemental Data associated with this article can be found in the online version at http://www.developmentalcell.com/cgi/content/8/2/279/DC1/. The supplemental data set contains Experimental Procedures.

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