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RESEARCH ARTICLE

Requirements for Mediator Complex Subunits Distinguish Three Classes of Notch Target Genes at the *Drosophila* Wing Margin

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Spatial and temporal gene regulation relies on a combinatorial code of sequence-specific transcription factors that must be integrated by the general transcriptional machinery. A key link between the two is the mediator complex, which consists of a core complex that reversibly associates with the accessory kinase module. We show here that genes activated by Notch signaling at the dorsal–ventral boundary of the *Drosophila* wing disc fall into three classes that are affected differently by the loss of kinase module subunits. One class requires all four kinase module subunits for activation, while the others require only Med12 and Med13, either for activation or for repression. These distinctions do not result from different requirements for the Notch coactivator Mastermind or the corepressors Hairless and Groucho. We propose that interactions with the kinase module through distinct cofactors allow the DNA-binding protein Suppressor of Hairless to carry out both its activator and repressor functions. *Developmental Dynamics 240:2051–2059, 2011.* © 2011 Wiley-Liss, Inc.

Key words: Med12; Med13; Cdk8; CycC; Skuld; Kohtalo; Notch; Suppressor of Hairless; Mastermind; mediator complex

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INTRODUCTION

Intercellular signaling pathways drive many processes during development. Their activation results in changes in transcription factor activity that lead to the activation or repression of specific target genes. An important goal is to understand the transcriptional regulatory codes that allow the combinations of proteins bound to enhancer elements to direct precise patterns of gene expression. One well-characterized developmental paradigm is the specification of the *Drosophila* wing margin by Notch signaling. The Notch receptor is specifically activated at the dorsal–ventral boundary of the larval wing imaginal disc, due to the restricted expression of its ligands Delta and Serrate and of the glycosyltransferase Fringe (Panin et al., 1997; Bruckner et al., 2000; Moloney et al., 2000). Notch activation results in expression of the target genes *Enhancer of split m8* (*E(spl)m8*), *cut*, *wingless* (*wg*), and *vestigial* (*vg*) (Lecourtois and Schweisguth, 1995; Rulifson and Blair, 1995; de Celis et al., 1996; Kim et al., 1996; Micchelli et al., 1997), the last through a specific enhancer element known as the boundary enhancer (*vgBE*) (Williams et al., 1994; Kim et al., 1996). Wg signaling then leads to the differentiation of characteristic sensory bristles adjacent to the margin of the adult wing (Rulifson and Blair, 1995).

Upon ligand binding, Notch is cleaved by the γ-secretase complex, and its intracellular domain (N_{intra}) enters the nucleus, where it interacts with the DNA-binding protein Suppressor of Hairless (Su(H)) (Bray, 2006). In the absence of Notch activation, Su(H) represses target gene expression through interactions with the corepressor Hairless (H), which binds to Groucho (Gro) and C-terminal binding protein (CtBP). N_{intra}

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displaces these corepressors from Su(H) and recruits coactivators such as Mastermind (Mam) (Bray, 2006). It has been proposed that only a subset of Notch target genes require Su(H) to recruit coactivators, while others require Notch signaling only to relieve Su(H)-mediated repression, allowing transcription to be activated by other factors (Bray and Furriols, 2001). However, the mechanisms by which Su(H) directs both activation and repression are not fully understood.

The mediator complex is thought to promote transcriptional activation by recruiting RNA polymerase II (Pol II), the general transcriptional machinery, and the histone acetyltransferase p300 to promoters, and by stimulating transcriptional elongation by Pol II molecules paused downstream of the promoter (Malik and Roeder, 2010; Taatjes, 2010). The "head" and "middle" modules of the core complex bind to Pol II and general transcription factors, while the "tail" module consists largely of adaptor subunits that bind to sequence-specific transcription factors (Chadick and Asturias, 2005). This core complex reversibly associates with a fourth "kinase" module that consists of the four subunits Med12, Med13, Cdk8, and Cyclin C (CycC) (Borggrefe et al., 2002). Several studies have implicated the kinase module in transcriptional repression, which can be mediated by phosphorylation of Pol II and other factors by Cdk8, by histone methyltransferase recruitment, and by occlusion of the Pol II binding site (Hengartner et al., 1998; Song and Carlson, 1998; Akoulitchev et al., 2000; Ding et al., 2008; Knuesel et al., 2009). However, this module also appears to function in activation in some contexts (Larschan and Winston, 2005; Meyer et al., 2008; Belakavadi and Fondell, 2010); for example, it promotes Wnt target gene expression during *Drosophila* and mouse development, in mammalian cells, and in colon cancer (Kim et al., 2006; Carrera et al., 2008; Firestein et al., 2008; Rocha et al., 2010). Although all four subunits have very similar mutant phenotypes in yeast (Song and Carlson, 1998; Samuelsen et al., 2003; van de Peppel et al., 2005), loss of Med12 or Med13 has more severe effects on *Drosophila* development than loss of Cdk8 or CycC (Loncle et al., 2007; Carrera et al., 2008; Gobert et al., 2010), suggesting that Med12 and Med13 have evolved additional functions in higher eukaryotes.

Here, we show that Notch target genes at the wing margin can be divided into three classes based on their requirements for kinase module subunits. An *E(spl)m8* reporter requires all four subunits for its activation, *cut* requires only Med12 and Med13 (known as Kohtalo [Kto] and Skuld [Skd], respectively, in *Drosophila*) (Treisman, 2001; Janody et al., 2003) for its activation, and *wg* and the *vgBE* enhancer require Med12 and Med13 for their repression in cells close to the wing margin. Because Med12 and Med13 coimmunoprecipitate with Su(H), regulate an artificial reporter driven by Su(H) binding sites, and can be replaced by a VP16 activation domain or a WRPW repression signal fused to Su(H), we propose that the kinase module directly regulates Notch target genes. All four Notch target genes fail to be expressed in the absence of Mam and are similarly affected by the loss of Hairless or Gro, suggesting that other more specific cofactors might recruit kinase module subunits to these genes.

RESULTS

Med12 and Med13 Differentially Affect Notch Target Genes

We and others have previously shown that clones of cells mutant for *kto* or *skd*, the *Drosophila* homologues of Med12 and Med13, have identical effects on gene expression (Treisman, 2001; Janody et al., 2003; Lim et al., 2007; Carrera et al., 2008). Clones mutant for either gene inappropriately cross the dorsal–ventral boundary in the wing disc (Janody et al., 2003), a phenotype characteristic of mutations that impair Notch signaling (Micchelli and Blair, 1999; Rauskolb et al., 1999). Because the Notch-regulated genes that control boundary crossing are currently unknown, we instead examined whether Med12 and Med13 were required for the expression of Notch target genes that control cell fate specification at the wing margin. We found that four genes that are expressed in a stripe at the dorsal–ventral boundary in a Notch-dependent manner showed two distinct responses to the loss of Med12 or Med13. Expression of *E(spl)m8-lacZ* and *cut* was lost in clones homozygous for null alleles of either *skd* or *kto* (Fig. 1A–H). In contrast, *wg* and *vgBE-lacZ* were still expressed in *skd* or *kto* mutant cells at the wing margin, and their expression domains were expanded into cells close to the margin in *skd* or *kto* mutant regions (Fig. 1I–P). These observations show that Med12 and Med13 are required for the activation of *E(spl)m8* and *cut* at the wing margin, but for the repression of *wg* and *vgBE* in neighboring cells.

Med12 and Med13 Are Required for the Function of the Notch Transcriptional Complex

To test whether Med12 and Med13 act downstream of Notch activation to promote the transcription of target genes, we expressed a constitutively active form of Notch that lacks the extracellular domain (N_{intra}) (Doherty et al., 1996) in *skd* or *kto* mutant cells using the MARCM system (Lee and Luo, 1999). When expressed in clones of wild-type cells, N_{intra} strongly activated *cut* expression (Fig. 2A,B). However, N_{intra} failed to restore *cut* expression to *skd* or *kto* mutant cells (Fig. 2C,D), indicating that Med12 and Med13 are required downstream of Notch cleavage for transcriptional activation of *cut*. Med12 and Med13 could directly enable transcriptional activation by N_{intra} bound to Su(H); alternatively, they might mediate the function of other transcription factors also required for *cut* expression, such as Vg and Scalloped (Guss et al., 2001). To distinguish these two possibilities, we used a fusion of the Su(H) DNA-binding domain to the transcriptional activation domain of VP16 (Kidd et al., 1998). This domain interacts with the mediator complex through a different subunit, Med17 (Ito et al., 1999). The chimeric Su(H)-VP16 protein ectopically activated *cut* when expressed in wild-type cells (Fig. 2E,F). Unlike N_{intra}, Su(H)-VP16

could also activate *cut* when expressed in *skd* or *kto* mutant cells (Fig. 2G,H). Linking a different activation domain to the Su(H) complex thus alleviates the requirement for Med12 and Med13, suggesting that these subunits specifically promote the functions of N_{intra} or its cofactors.

We wondered whether repression of Notch target genes by Skd and Kto also reflected effects on the Notch transcriptional complex. To test this, we used a fusion of Su(H) to the sequence WRPW, which recruits the corepressor Gro and represses Notch target genes (Nagel et al., 2005). We found that expression of Su(H)WRPW in wild-type cells repressed endogenous *wg* expression at the wing margin (Fig. 2I,J). Similarly, expressing Su(H)WRPW in *skd* or *kto* mutant clones repressed endogenous *wg* expression and prevented *wg* misexpression in cells near the wing margin (Fig. 2K,L). This rescue by Su(H)WRPW supports a function for Med12 and Med13 in repression by the Notch complex rather than by

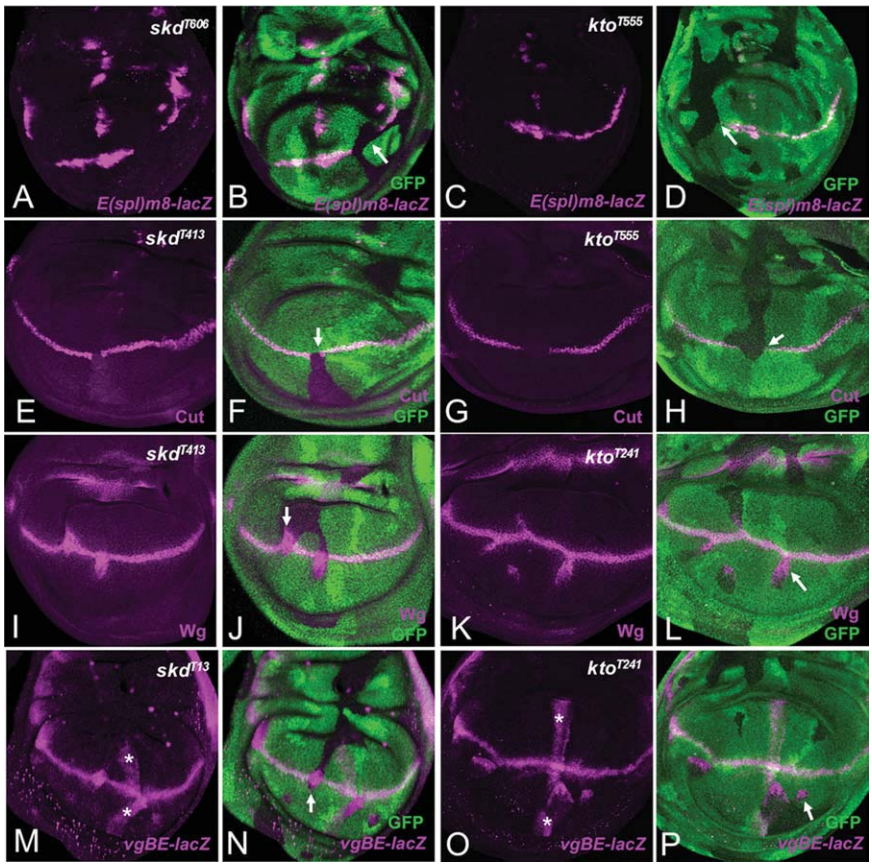

Fig. 1.

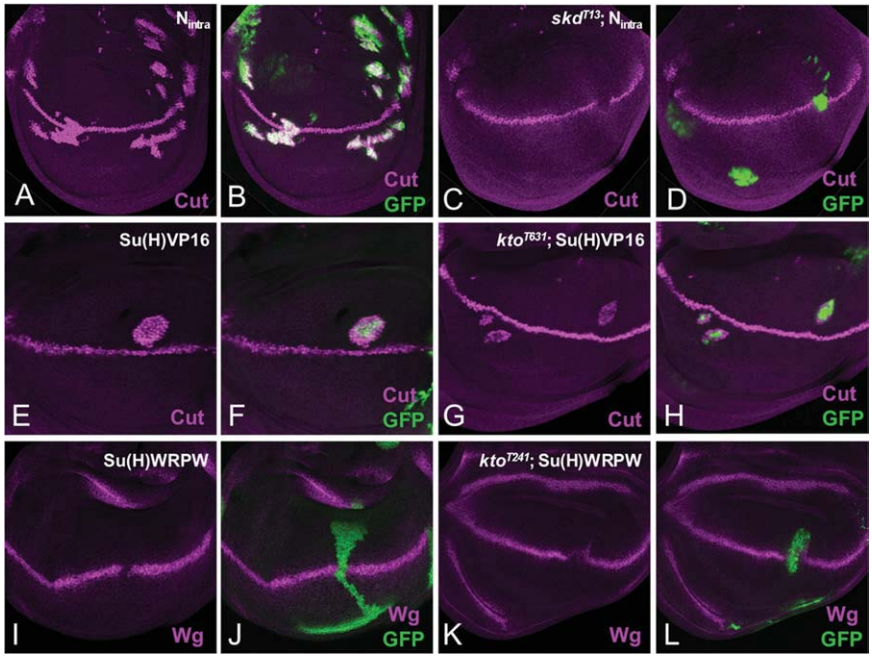

Fig. 2.

Fig. 1. Med12 and Med13 have distinct effects on Notch target genes at the wing margin. All panels show third-instar wing imaginal discs with dorsal up and posterior to the right. **A–P**: Clones of cells homozygous for *skd*^{T606} (A,B), *skd*^{T413} (E,F,I,J), *skd*^{T13} (M,N), *kto*^{T555} (C,D,G,H), or *kto*^{T241} (K,L,O,P) are marked by the absence of green fluorescent protein (GFP; green in B,D,F,H,J,L,N,P). Discs are stained with anti–β-galactosidase to reveal *E(spl)m8-lacZ* expression (magenta in A–D); anti-Cut (magenta in E–H); anti-Wg (magenta in I–L); or anti–β-galactosidase to reveal *vgBE-lacZ* expression (magenta in M–P). B,D,F,H,J,L,N,P: Arrows indicate representative clones. Asterisks in (M,O) mark endogenous expression of *vgBE* at the anterior–posterior boundary. *E(spl)m8-lacZ* and Cut are lost from *skd* or *kto* mutant clones, while Wg and *vgBE-lacZ* are expanded into regions adjacent to the wing margin in *skd* or *kto* mutant clones. Because *skd* and *kto* have identical effects on gene expression, in subsequent figures only one mutant is shown for each experiment.

Fig. 2. Activator or repressor forms of Su(H) can substitute for Med12 and Med13. **A–L**: All panels show wing imaginal discs in which Cut is stained in magenta (A–H) or Wg is stained in magenta (I–L). Clones of cells misexpressing N_{intra} (A,B), homozygous for *skd*^{T13} and misexpressing N_{intra} (C,D), misexpressing Su(H)VP16 (E,F), homozygous for *kto*^{T631} and misexpressing Su(H)VP16 (G,H), misexpressing Su(H)WRPW (I,J), or homozygous for *kto*^{T241} and misexpressing Su(H)WRPW (K,L), are positively marked by green fluorescent protein (GFP) expression (green in B,D,F,H,J,L). Med12 and Med13 are required downstream of N_{intra}, but their activator function can be replaced by Su(H)VP16 and their repressor function by Su(H)WRPW.

other factors. We obtained additional evidence for this conclusion using an artificial reporter consisting only of binding sites for Su(H) and for Grainyhead, a ubiquitous activator. This reporter (*Gbe+Su(H)-lacZ*) is repressed by the Su(H) complex in cells in which Notch is inactive (Furriols and Bray, 2001). *Gbe+Su(H)-lacZ* was misexpressed in *skd* or *kto* mutant clones (Fig. 3A,B), while a version of this reporter in which the Su(H) binding sites are mutated (Furriols and Bray, 2001) did not show increased expression in *skd* or *kto* mutant cells (Fig. 3C,D), suggesting that Med12 and Med13 are required for repression by Su(H).

If Med12 and Med13 directly regulate the activity of the Su(H) complex, we might expect them to physically associate with this complex. Indeed, we were able to coimmunoprecipitate a Myc-tagged form of Su(H) (Kidd et al., 1998) with both endogenous and overexpressed Skd and Kto (Fig. 3E). Taken together, these results argue that Med12 and Med13 interact with the Su(H) transcriptional complex to modulate gene expression.

Cdk8 and Cyclin C Regulate a Subset of Notch Target Genes

We next tested whether the other two subunits of the kinase module of the mediator complex were required for Notch-mediated transcriptional regulation. Deletion alleles of *cdk8* and *CycC* have the same phenotypes in all contexts examined so far (Loncle et al., 2007; Carrera et al., 2008; Gobert et al., 2010), and also showed indistinguishable effects on Notch target genes. Both subunits were required for the expression of *E(spl)m8-lacZ* (Fig. 4A,B), but unlike *skd* and *kto*, *cdk8* and *CycC* mutant cells maintained expression of *cut* (Fig. 4C,D). The *cut* and *E(spl)m8* genes must therefore be activated by different mechanisms. The two genes repressed by Med12 and Med13, *wg* and *vgBE*, showed only a slight expansion in *cdk8* or *CycC* mutant clones (Fig. 4E–H). These differences show that Cdk8 and CycC are not required for all the functions of the kinase module; in their absence, Med12 and Med13 are still able to activate *cut* and to repress *wg* and *vgBE*.

Mastermind Is a Coactivator for All Four Notch Target Genes

N_{intra} binding to Su(H) has been proposed to induce target gene expression by two different mechanisms. Genes for which N_{intra} acts instructively, such as *E(spl)m8*, cannot be expressed in its absence, presumably because coactivators bound to N_{intra} play an essential role. In contrast, N_{intra} appears to have only a permissive function in relieving the repression of genes such as *vgBE* (Bray and Furriols, 2001). N_{intra} displaces the corepressors Hairless, Gro, and CtBP from Su(H) (Morel et al., 2001; Barolo et al., 2002), allowing *vgBE* to be activated by other positively acting transcription factors. Such permissively regulated genes can be activated by removing Hairless or by overexpressing Su(H), which sequesters the corepressors (Furriols and Bray, 2000; Klein et al., 2000; Nagel et al., 2005). This model suggests the possibility that activation by Med12 and Med13 is limited to the instructive Notch target genes, and that this specificity might be due to interactions with coactivators recruited by N_{intra}. The primary coactivator characterized so far is Mastermind (Mam), which has been shown to bind to Cdk8 (Fryer et al., 2004; Wu and Griffin, 2004). We therefore tested whether Mam function could distinguish instructive from permissive Notch target genes. Surprisingly, we found that cells homozygous for a null allele of *mam* failed to activate not only *E(spl)m8* and *cut*, the genes that are positively regulated by Med12 and Med13, but also *wg* and *vgBE*, the genes that are negatively regulated by Med12 and Med13 (Fig. 5E–H). Because Mam is required for both modes of Notch target gene induction, it is unlikely to be the factor that specifically recruits Med12 and Med13 to instructive target genes.

Hairless, but Not Groucho, Is Required for Notch Target Gene Repression at the Wing Margin

The misexpression of *wg* and *vgBE* in *skd* and *kto* mutant cells suggested that Med12 and Med13 play a role in the repression of Notch target genes in the absence of Notch signaling and might be recruited by Hairless, Gro, or CtBP (Morel et al., 2001; Barolo et al., 2002; Nagel et al., 2005). Like *skd* and *kto* mutant clones, clones homozygous for the null *Hairless* allele H^{E31} showed misexpression of *wg* and *vgBE* in cells close to the wing margin, and low levels of *vgBE* could be detected at some distance from the margin (Fig. 6E–H). Hairless does not mediate the positive effects of Med12 and Med13 on *E(spl)m8* and *cut*; these genes were still expressed in *Hairless* mutant clones, and *cut* expression was expanded, suggesting that it is also sensitive to repression by *Hairless* (Fig. 6A–D).

We found that Gro is unlikely to be the protein that recruits Med12 and Med13 to repressed genes, because all four target genes at the wing margin in fact showed reduced or absent expression in clones homozygous for an amorphic *gro* allele. *E(spl)m8-lacZ* expression was lost in *gro* mutant clones at the wing margin (Fig. 6I,J), although its expression in proneural clusters in the notum was expanded to include sensory organ precursors (arrows, Fig. 6J), confirming that the mutant cells lacked the repressor activity of Gro (Castro et al., 2005). Expression of *cut* was also reduced in *gro* mutant cells (Fig. 6K,L) and no expansion of *wg* or *vgBE* was seen (Fig. 6M–P). Gro is thus not required to repress *wg* and *vgBE* in cells adjacent to the wing margin, and appears to play a positive role in the expression of *E(spl)m8* and *cut* at the wing margin, although it is possible that its effect on these genes is indirect. Hairless itself, CtBP or other currently unknown corepressors may recruit Med12 and Med13 to repress *wg* or *vgBE*, or these mediator complex subunits may interact directly with Su(H).

DISCUSSION

Activation and Repression by the Four Kinase Module Subunits

The kinase module of the mediator complex is conserved throughout eukaryotes, yet its functions in

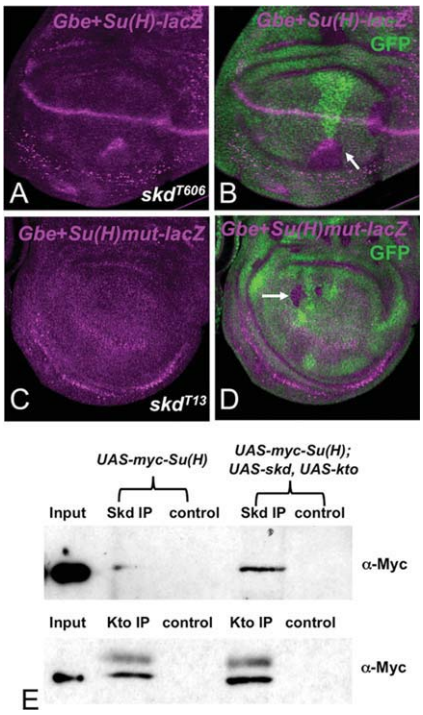

Fig. 3.

Fig. 3. Med12 and Med13 act on a Su(H) reporter. **A–D**: Show wing discs in which clones of cells homozygous for *skd*^{T606} (A,B) or *skd*^{T13} (C,D) are marked by the absence of GFP (green in B,D), and stained with anti–β-galactosidase to reveal the expression of *Gbe+Su(H)-lacZ* (magenta in A,B) or of *Gbe+Su(H)mut-lacZ* (magenta in C,D). B,D: Arrows indicate representative clones. The artificial Su(H)-dependent reporter is misexpressed in *skd* mutant cells, but the reporter with mutated Su(H) sites is not. **E**: Shows anti-Myc Western blots of extracts from embryos in which ubiquitously expressed *da*-GAL4 drives UAS-myc-Su(H), with or without UAS-*skd* and UAS-*kto* as indicated, immunoprecipitated with anti-Skd, anti-Kto, or preimmune serum (control). Myc-tagged Su(H) is pulled down by both endogenous and overexpressed Skd or Kto, indicating that Su(H) interacts directly or indirectly with these kinase module subunits.

transcription remain poorly understood (Bourbon, 2008; Malik and Roeder, 2010). In yeast, loss of any of the four subunits has a very similar effect (Song and Carlson, 1998; Samuelsen et al., 2003; van de Peppel et al., 2005). In *Drosophila*, however, loss of Med12 or Med13 has more dramatic effects than loss of Cdk8 or CycC (Loncle et al., 2007; Carrera et al., 2008; Gobert et al., 2010). The kinase module was originally thought to be primarily important for transcriptional repression, mediated by the kinase activity of Cdk8 (Hengartner et al., 1998; Akoulitchev et al., 2000). However, Med12 and Med13 appear to directly activate genes regulated by Wnt signaling in *Drosophila* and mammalian systems (Kim et al., 2006; Carrera et al., 2008; Rocha et al., 2010), and also play a positive role in gene activation by the Gli3 and Nanog transcription factors (Zhou et al., 2006; Tutter et al., 2009). The data we present here confirm that Med12 and Med13 have functions distinct from Cdk8 and CycC. In addition, we provide evidence that all four kinase module subunits contribute to the activation of *E(spl)m8*.

The human Mastermind homologue MAM has been shown to recruit Cdk8 and CycC to promoters of Notch target genes, where Cdk8 phosphorylates the intracellular domain of Notch, leading to its ubiquitination by

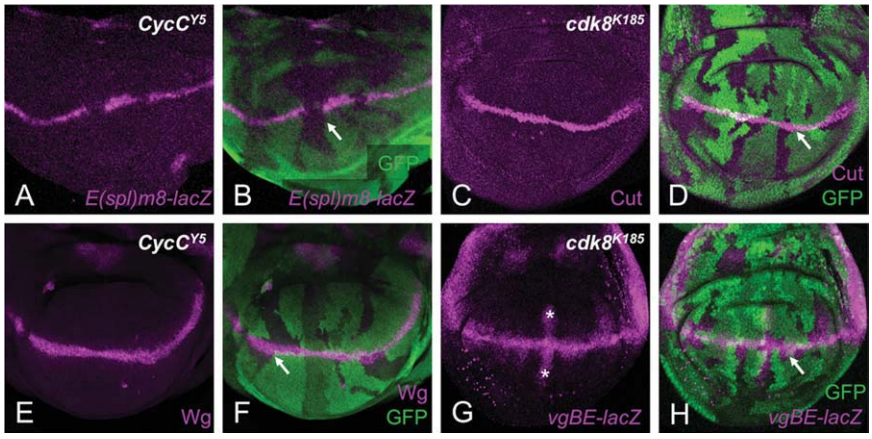

Fig. 4.

Fig. 4. Cdk8 and CycC are required for the expression of *E(spl)m8-lacZ*, but not for Cut, Wg, or *vgBE-lacZ*. **A–H**: All panels show wing imaginal discs in which clones of cells homozygous for *CycC*^{Y5} (A,B,E,F) or *cdk8*^{K185} (C,D,G,H) are marked by the absence of green fluorescent protein (GFP; green in B,D,F,H). Discs are stained with anti–β-galactosidase to reveal *E(spl)m8-lacZ* expression (magenta in A,B), anti-Cut (magenta in C,D), anti-Wg (magenta in E,F), or anti–β-galactosidase to reveal *vgBE-lacZ* expression (G,H). B,D,F,H: Arrows indicate representative clones. G: Asterisks mark endogenous expression of *vgBE* at the anterior–posterior boundary. *E(spl)m8* expression is lost in *cdk8* or *CycC* mutant clones, but Cut expression is unaffected and Wg and *vgBE-lacZ* are only slightly expanded.

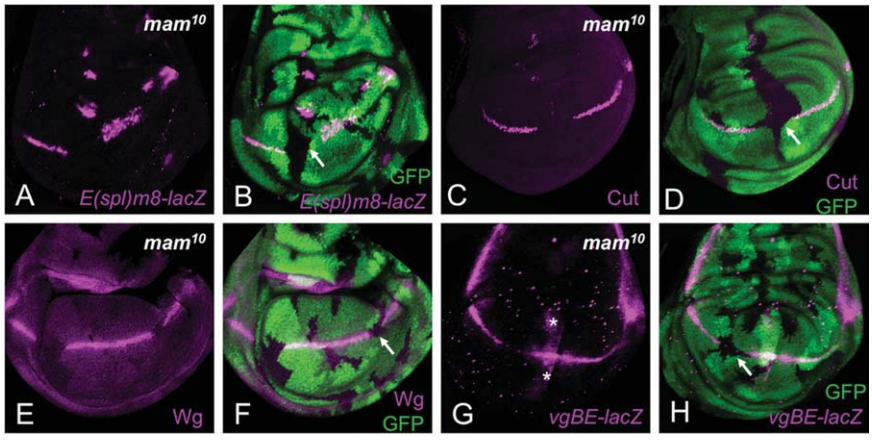

Fig. 5.

Fig. 5. Mam is required for the expression of all four Notch target genes. **A–H**: All panels show wing imaginal discs in which clones of cells homozygous for *mam*^{10} are marked by the absence of green fluorescent protein (GFP; green in B,D,F,H). Discs are stained with anti–β-galactosidase to reveal *E(spl)m8-lacZ* expression (magenta in A,B); anti-Cut (magenta in C,D); anti-Wg (magenta in E,F); or anti–β-galactosidase to reveal *vgBE-lacZ* expression (G,H). B,D,F,H: Arrows indicate representative clones. G: Asterisks mark endogenous expression of *vgBE* at the anterior–posterior boundary. Expression of all four genes is lost in *mam* mutant clones.

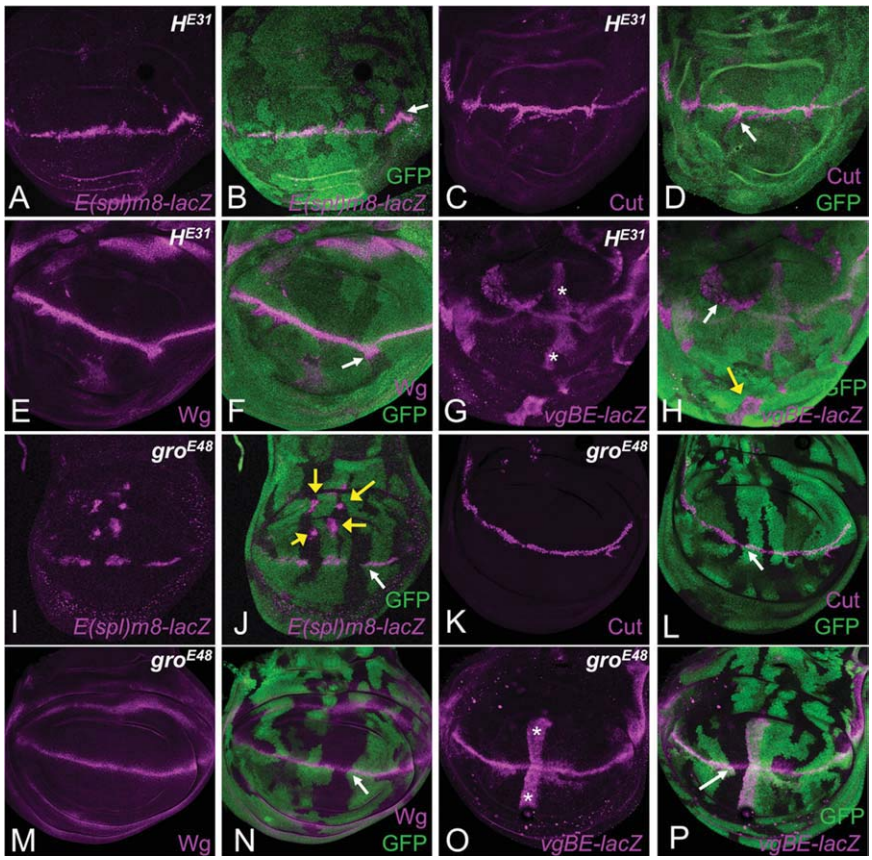

Fig. 6. Hairless is required to repress Notch target genes, but Gro is not. **A–P**: All panels show wing imaginal discs in which clones of cells homozygous for H^{E31} (A–H) or gro^{E48} (I–P) are marked by the absence of green fluorescent protein (GFP; green in B,D,F,H,J,L,N,P). Discs are stained with anti–β-galactosidase to reveal *E(spl)m8-lacZ* expression (magenta in A,B,I,J); anti-Cut (magenta in C,D,K,L); anti-Wg (magenta in E,F,M,N); or anti–β-galactosidase to reveal *vgBE-lacZ* expression (G,H,O,P). B,D,F,H,J,L,N,P: White arrows indicate representative clones. G,O: Asterisks mark endogenous expression of *vgBE* at the anterior–posterior boundary. *E(spl)m8* is still expressed in *H* mutant clones, but its expression is lost in *gro* mutant clones at the wing margin, although expression is expanded into sensory organ precursors in the notum (yellow arrows in J). Cut, Wg, and *vgBE-lacZ* all show expanded expression in *Hairless* mutant clones, but slightly reduced expression in *gro* mutant clones. In *Hairless* clones, *vgBE* is ectopically expressed even in cells distant from the wing margin (yellow arrow in H).

the Fbw7 ligase and degradation (Fryer et al., 2004). This mechanism would be expected to reduce Notch target gene expression, consistent with the increase in *E(spl)mβ* expression seen in clones lacking the *Drosophila* Fbw7 homologue Archipelago (Nicholson et al., 2011); thus it cannot explain the positive effects of Cdk8 and CycC on *E(spl)m8*. A function for Cdk8 and CycC in Notch-mediated activation would be analogous to recent findings showing that Cdk8 phosphorylation of Smad transcription factors and of histone H3 promotes activation (Meyer et al., 2008; Alarcon et al., 2009). Cdk8 phosphorylation of RNA polymerase II (Pol II) is also important for transcriptional elongation (Liu et al., 2004; Donner et al., 2010).

Of interest, our data also suggest that Med12 and Med13 are involved in the repression of *wg* and the *vgBE* enhancer in the absence of Notch signaling. The kinase module has been proposed to inhibit transcription through steric hindrance of Pol II binding, independently of Cdk8 kinase activity (Elmlund et al., 2006). Removal of this module on the *C/EBP* promoter is thought to convert the mediator complex to its active form (Mo et al., 2004). In contrast, we find that *wg* and *vgBE* require Med12 and Med13 for their repression but not their activation (Fig. 7A,B), while *cut* and *E(spl)m8* require Med12 and Med13 only for their activation (Fig. 7C,D), arguing that the two functions occur on different promoters. We

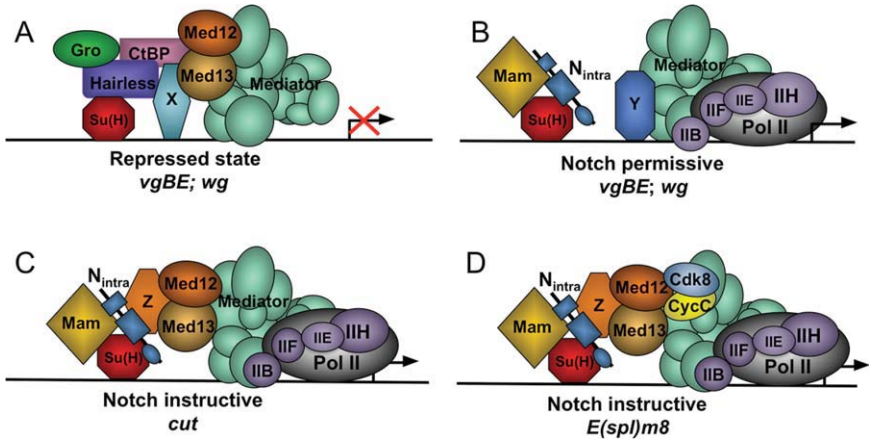

Fig. 7. Model for kinase module regulation of Notch target genes. **A:** Diagram of the promoter of a permissive Notch target gene such as *wg* or *vgBE* in its repressed state. Su(H) is bound to Hairless, which recruits Gro and CtBP. CtBP, Hairless, or another cofactor (X) recruits Med12 and Med13, leading to transcriptional repression. **B:** Same promoter in its activated state. N_{intra} and Mam displace Hairless, Gro, and CtBP, allowing Notch-independent transcriptional activators (Y) to drive transcription independently of the kinase module. **C:** *cut* promoter in its activated state. A N_{intra} cofactor other than Mam (Z) recruits Med12 and Med13, leading to transcriptional activation. **D:** *E(spl)m8* promoter in its activated state. In addition to Med12 and Med13, Cdk8 and CycC are required for activation of this promoter.

Fig. 7.

cannot rule out the possibility that Med12 and Med13 have only indirect effects on some of the genes we examined; however, their physical association with Su(H) and the requirement for Su(H) binding sites for misexpression of an artificial reporter in *skd* and *kto* mutant clones are consistent with a direct effect of Med12 and Med13 on the Su(H) complex.

Med12 and Med13 are found associated with both active and inactive promoters in genome-wide chromatin immunoprecipitation studies (Andrau et al., 2006; Zhu et al., 2006), suggesting that they can have different effects on transcription when bound to distinct interaction partners (X and Z in Fig. 7). Although both are very large proteins, they contain no domains predicted to have enzymatic activity, and may instead act as scaffolds for the assembly of transcriptional complexes.

Distinct Mechanisms of Notch Target Gene Activation

Bray and Furriols (2001) proposed that Notch target genes could be categorized into two classes: permissive genes, for which the primary function of Notch is to relieve repression by the Su(H) complex, and instructive genes, for which Notch plays an essential role in activation by recruiting specific coactivators. These differences presumably depend on the combinatorial code of transcription factors that regulate each promoter. We show here that *vgBE*, an enhancer previously placed in the permissive category (Furriols and Bray, 2000), as well as *wg*, require Med12 and Med13 for their repression but not their activation (Fig. 7A,B). During eye development, the proneural gene *atonal* is likewise regulated permissively by Notch (Li and Baker, 2001), and ectopically expressed in *skd* or *kto* mutant clones (Treisman, 2001; Lim et al., 2007). Unexpectedly, we found that Gro, previously thought to be a cofactor through which Hairless mediates repression (Barolo et al., 2002; Nagel et al., 2005), is not required for the repression of *vgBE* or *wg*. Hairless may repress target genes at the wing margin through CtBP, its other binding partner (Morel et al., 2001; Barolo et al., 2002; Nagel et al., 2005). Alternatively, Gro may affect the expression of other upstream regulators of wing margin fate, masking its repressive effect on the genes we examined.

We also show here that instructive Notch target genes can be further subdivided into two classes based on their requirement for kinase module subunits; *E(spl)m8* requires all four subunits, while *cut* requires Med12 and Med13, but not Cdk8 and CycC (Fig. 7C,D). Cdk8 and CycC may simply increase the ability of the mediator complex to recruit Pol II or promote transcriptional initiation; this model would suggest that *E(spl)m8* has a higher activation threshold than *cut*. Alternatively, Cdk8 and CycC might enhance the function of a transcription factor that is specifically required for the expression of *E(spl)m8* but not *cut*. Good candidates for such factors would be the proneural proteins Achaete or Scute or their partner Daughterless (Cooper et al., 2000).

The mechanism by which the kinase module is recruited to promote the activation of instructive target genes is not yet clear. Although Mam proteins are well-characterized coactivators for N_{intra} (Wu and Griffin, 2004), we find that Mam is necessary for the activation of both instructive and permissive genes. It may thus have a general function in transcriptional activation, such as recruiting histone acetyltransferases or stabilizing the Notch-Su(H) complex (Fryer et al., 2002; Wallberg et al., 2002; Kovall, 2007). A coactivator that recruits Med12 and Med13 specifically to instructive target genes to promote activation (Z in Fig. 7) may remain to be identified. Our results, like recent reports demonstrating that the arrangement of Su(H) binding sites can affect the interactions between Notch and its coactivators (Cave and Caudy, 2008; Arnett et al., 2010; Cave et al., 2011), highlight the complexity in the mechanisms through which promoter elements respond to Notch signaling.

EXPERIMENTAL PROCEDURES

Fly Strains and Genetics

Fly strains used were skd^{T606}, skd^{T413}, skd^{T13}, kto^{T241}, kto^{T555}, kto^{T631} (Treisman, 2001), $cdk8^{K185}$, $CycC^{Y5}$ (Loncle et al., 2007), gro^{E48} (Jennings et al., 2006), mam^{10} (Xu et al., 1990), H^{E31} (Furriols and Bray, 2000), *E(spl)m8-lacZ* (Lecourtois and Schweisguth, 1995), *vgBE-lacZ* (Kim et al., 1996), *Gbe+Su(H)-lacZ*, *Gbe+Su(H)mut-lacZ* (Furriols and Bray, 2001), UAS-N_{intra} (Doherty et al., 1996), UAS-Su(H)VP16, UAS-myc-Su(H) (Kidd et al., 1998), UAS-Su(H)WRPW (Nagel et al., 2005), *daughterless* (*da*)-GAL4 (Wodarz et al., 1995), UAS-*skd*, and UAS-*kto* (Janody et al., 2003). Clones on 3L were generated by crossing (*E(spl)m8-lacZ* or *vgBE-lacZ*); FRT80, *skd* (or *kto* or *cdk8*)/SM6-TM6B to *hs*FLP; FRT80, Ubi-GFP or to *teashirt*-GAL4, UAS-FLP; FRT80, Ubi-GFP/SM6-TM6B. Clones on 3R were generated by crossing (*E(spl)m8-lacZ* or *vgBE-lacZ*); FRT82, *CycC* (or *gro* or *H*)/SM6-TM6B to *hs*FLP; FRT82, Ubi-GFP or to *teashirt*-GAL4, UAS-FLP; FRT82, Ubi-GFP/SM6-TM6B. Clones expressing N_{intra}, Su(H)VP16, or Su(H)WRPW were generated by crossing UAS-N_{intra} (or UAS-Su(H)-VP16 or UAS-Su(H)WRPW); FRT80 (or FRT80, *skd* or FRT80, *kto*)/SM6-TM6B to UAS-GFP, *hs*FLP; *tub*-GAL4; FRT80, *tub*-GAL80. When *hs*FLP was used to generate the clones, larvae were heat shocked for 1 hr at 38.5°C in both first and second instar.

Immunohistochemistry

Third-instar wing discs were fixed in 4% formaldehyde in PEM (0.1 M PIPES pH 7.0/2 mM $MgSO_4$/1 mM EGTA) for 25–35 min on ice, washed in 0.1 M sodium phosphate pH 7.2/0.2% Triton X-100, and incubated in primary antibody in 0.1 M sodium phosphate pH 7.2/0.2% Triton X-100/10% normal donkey serum (Jackson Immunoresearch) overnight at 4°C. Discs were washed 3 times in 0.1 M sodium phosphate pH 7.2/0.2% Triton X-100, incubated in secondary antibody for 2–4 hr at 4°C, washed 3 times as above and mounted in 80% glycerol in 0.1 M sodium phosphate pH 7.2. Antibodies used were rabbit anti–β-galactosidase (1:5,000; Cappel), mouse anti-Cut (1:10, Developmental Studies Hybridoma Bank [DSHB]), mouse anti-Wg (1:10, DSHB), and chicken anti- green fluorescent protein

(GFP; 1:500; Aves Labs). Secondary donkey anti-mouse and anti-rabbit antibodies conjugated to TRITC or Cy5 were purchased from Jackson Immunoresearch and used at 1:200, and donkey anti-chicken antibodies conjugated to Alexa 488 were purchased from Invitrogen and used at 1:1,000. Images were obtained using Zeiss LSM510 and Leica SP5 confocal microscopes.

Immunoprecipitation

Extracts from embryos of the genotype *da*-GAL4/UAS-*myc-Su(H)-VP16* or UAS-*skd*, UAS-*kto*/+; *da*-GAL4/UAS-*myc-Su(H)-VP16* were immunoprecipitated as described (Janody et al., 2003) with rabbit anti-Skd (1:100), guinea pig anti-Kto (1:100), or preimmune serum (control lanes). Western blotting was performed as described (Carrera et al., 2008) using mouse anti-Myc (Santa Cruz Biotechnology) at 1:8,000. Input lanes contain 5% of the amount included in the IP.

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