E(y)1/TAF9 mediates the transcriptional output of Notch signaling in *Drosophila*

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ABSTRACT

Transcriptional activation of Notch signaling targets requires the formation of a ternary complex that involves the intracellular domain of the Notch receptor (NICD), DNA-binding protein Suppressor of Hairless [Su(H), RPBJ in mammals], and coactivator Mastermind (Mam). Here we report that E(y)1/TAF9, a component of the transcription factor TFIID complex, interacts specifically with the NICD/Su(H)/Mam complex to facilitate the transcriptional output of Notch signaling. We identified E(y)1/TAF9 in a large-scale in vivo RNAi screen for genes involved in a Notch-dependent mitotic-to-endocycle transition in Drosophila follicle cells. Knockdown of e(y)1/TAF9 displayed Notch-like phenotypes and defects in target gene and activity reporter expression in both the follicle cells and wing imaginal discs. Epistatic analyses in these two tissues indicate that E(y)1/TAF9 functions downstream of the Notch cleavage. Biochemical studies in S2 cells demonstrated that E(y)1/TAF9 physically interacts with the transcriptional effectors of Notch signaling, Su(H) and NICD. Together, our data suggest that the association of the NICD/Su(H)/Mastermind complex with E(y)1/TAF9 in response to Notch activation recruits the transcription initiation complex to induce Notch target genes, coupling Notch signaling with the transcriptional machinery.

KEY WORDS:
E(y)1; TAF9; Notch pathway; Drosophila; TFIID complex; transcriptional regulation
INTRODUCTION

The eukaryotic transcription factor IID (TFIID) complex, composed of the TATA box-binding protein (TBP) and 13 or 14 TBP-associated factors (TAF), plays an essential role in the transcriptional regulation of gene expression, and all components of this complex were generally thought to be required for RNA polymerase II-initiated transcription in all eukaryotic cells (Goodrich and Tjian, 2010; Thomas and Chiang, 2006). However, increasing studies suggest that some TAFs found in different species are vital in specific events like apoptosis, spermatogenesis and adipogenesis (Goodrich and Tjian, 2010). For example, mouse TAF7L is specifically required for male germ-cell differentiation (Cheng et al., 2007; Pointud et al., 2003); in both zebrafish and mouse embryonic stem cells, interaction between TRF3 and TAF3 is essential for hematopoiesis (Bartfai et al., 2004; Hart et al., 2007; Hart et al., 2009).

Kalogeropoulou et al. demonstrated that TAF4b is specifically associated with c-Jun and other AP-1 family members to regulate the expression of Integrin α6 in the context of cancer progression (Kalogeropoulou et al., 2010). Although a recent report has shown that TAF4 interacts with Pygopus, a transcriptional activator of Wg signaling, to induce transcription of naked cuticle in Drosophila (Wright and Tjian, 2009), how other signaling pathways, such as Notch, are regulated by specific TAFs is poorly understood.

Notch signaling is an evolutionally conserved pathway across species that plays a pivotal role in many different developmental events, including cell fate determination, control of cell proliferation and apoptosis, and the maintenance of stem cells (Artavanis-Tsakonas and Muskavitch, 2010; Guruharsha et al., 2012; Tien et al., 2009). Its dysregulation is implicated in a number of diseases including cancer (Louvi and Artavanis-Tsakonas, 2012; Ntziachristos et al., 2014). Notch signaling activation is mediated by a direct interaction between the Notch receptor in one cell and its ligand on the neighboring cell (Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996). Such an interaction induces two consecutive proteolytic processes that result in the release of the Notch intracellular domain (NICD) (Struhl, 1998), which is then translocated to the nucleus and activates transcription of its target genes by interacting with the DNA-binding protein Suppressor of Hairless [Su(H)] and the coactivator Mastermind (Mam) to form a functional Su(H)/NICD/Mam ternary complex (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). Although numerous Notch target genes have been identified in different
tissues or organs during different developmental stages, how the Su(H)/NICD/Mam ternary complex regulates their expression is largely unclear. Recent findings in different systems have suggested that chromatin-associated epigenetically-regulatory mechanisms are very important for proper Notch target gene expression (Bray et al., 2005; Domanitskaya and Schupbach, 2012; Endo et al., 2012; Kugler and Nagel, 2007; Mulligan et al., 2011; Yu et al., 2013; Zeng et al., 2013). In flies, histone chaperones Asf1 and Nap1 are differentially associated with LAF (LID-associated factor) and RLAF (RPD3-LID-associated factor) silencing complexes to mediate epigenetic silencing at the Notch target Enhancer of Split [E(spl)] cluster (Moshkin et al., 2009). A SIRT1-LSD1 co-repressor complex regulates Notch target gene expression in both mammalian and Drosophila cultured cells (Mulligan et al., 2011). Our very recent study has demonstrated that the histone chaperone CAF-1 complex epigenetically and positively regulates Notch target gene expression in Drosophila (Yu et al., 2013). But the most basic questions of how the Su(H)/NICD/Mam complex activates Notch target gene expression and what cofactor(s) brings it to the general transcriptional machinery are not answered.

The Drosophila egg chamber is an ideal system to study the regulation of Notch signaling and its developmental consequences (Bastock and St Johnston, 2008; Klusza and Deng, 2011). During mid-oogenesis (stages 7-10A), Notch signaling is activated in entire follicular epithelia by the germline-expressed ligand, Delta (Dl) (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). This activation induces the follicle cells from the mitotic cycle to endoreplication, which is mediated by the suppression of Cut and activation of Hindsight (Hnt) by Notch signaling (Sun and Deng, 2007; Sun and Deng, 2005). As in other processes, this Notch-dependent cell-cycle transition also requires Notch protein cleavage that is achieved by γ-secretase components Presenilin and Nicastrin. In addition, the nuclear effector of Notch signaling, Su(H), is needed for the switch. The precise timing of Notch signaling during mid-oogenesis is regulated by multiple mechanisms (Domanitskaya and Schupbach, 2012; Heck et al., 2012; Poulton et al., 2011). Our recent finding demonstrated that the microRNA pathway regulates the temporal pattern of Notch signaling by repressing Dl-mediated inhibition of Notch in Drosophila follicle cells (Poulton et al., 2011). Interestingly, transcriptional cofactors are also involved in precise control of Notch signaling (Domanitskaya and Schupbach, 2012; Heck et al., 2012): the transcriptional corepressor SMRTER inhibits Notch activity in a temporally-restricted manner in
follicular epithelium (Heck et al., 2012); whereas the transcriptional cofactor Corepressor for
element-1-silencing transcription factor (CoREST) promotes Notch signaling in a spatially-
restricted manner by affecting H3K27 tri-methylation and H4K16 acetylation in the follicle cells
(Domanitskaya and Schupbach, 2012). In a genetic RNAi screen for regulators of Notch
signaling in follicle cells, we identified e(y)1 as required for proper Notch signaling in mediating
the mitotic-to-endoreplicating cycle transition of follicle cells. Further studies extended e(y)1’s
requirement for the transcriptional regulation of Notch target genes during wing development
and in *Drosophila* cultured S2 cells. Epistatic analyses in both follicle cells and adult wings
suggested that E(y)1 functions downstream of the Notch cleavage. Biochemical studies in S2
cells demonstrated that E(y)1 physically interacts with both transcriptional effectors of Notch
signaling, Su(H), and NICD. Together, our data revealed that the association of the
NICD/Su(H)/Mastermind complex with E(y)1 in response to Notch activation recruits the
transcription initiation complex to induce Notch target genes, coupling Notch signaling with the
transcriptional machinery.
RESULTS

e(y)1 is required for Notch signaling-mediated follicle cell transition from mitotic cycle to endocycle during Drosophila oogenesis

To identify genes that modulate Notch signaling, we carried out a large-scale in vivo RNAi screen for defects in Notch target gene expression in Drosophila follicle cells. The follicle cell epithelium shows a temporal Notch activation pattern during stages 7-10A of oogenesis, with both negative and positive targets identified (Sun and Deng, 2007; Sun and Deng, 2005). We tested around two thousand TRiP (Transgenic RNAi Project) RNAi lines (Ni et al., 2009; Ni et al., 2008; Ni et al., 2011), which contain either long double-strand hairpin (Ni et al., 2009; Ni et al., 2008) or short hairpin RNAs (Ni et al., 2011) (both referred to as RNAi) under the control of the upstream activating site (UAS). These lines were crossed to the flip-out Gal4 driver (Ito et al., 1997; Pignoni and Zipursky, 1997) to generate random follicle-cell RNAi clones that were marked by the expression of green (GFP) or red (RFP) fluorescent proteins. The follicle-cell RNAi clones were then screened for defects in the expression pattern of negative Notch target Cut, a homeodomain-containing transcription factor (Sun and Deng, 2005). Cut is normally expressed in early oogenesis (stages 1-6) and then downregulated at stage 7 upon Notch activation in follicle cells (Sun and Deng, 2005). Failure to downregulate Cut at stage 7 suggests a defect in Notch signaling. From this screen, we found that knockdown of the gene CG6474 [enhancer of yellow 1 (e(y)1)], which encodes a homolog of TAF9, a core component of the TFIID transcription initiation complex (Soldatov et al., 1999; Thomas and Chiang, 2006), resulted in prolonged Cut expression in 74% (n = 72) of follicle cell RNAi clones at stages 7-8 (Fig. 1A), suggesting a Notch defect in e(y)1-depleted follicle cells.

To confirm that E(y)1 is required for Notch signaling in follicle cells, we examined the expression of zinc finger protein Hindsight (Hnt), a positive follicle-cell-specific Notch target, which is normally expressed in the entire follicular epithelium from stage 7 to 10A (Sun and Deng, 2007). Downregulation of Hnt in mid-oogenesis (stages 7-10A) is an indication of disrupted Notch signaling in follicle cells (Sun and Deng, 2007). Indeed, Hnt downregulation was detected in 68% (n = 51) of e(y)1-knockdown follicle cells (Fig. 1B). Both Cut upregulation and Hnt downregulation were detected in an independent e(y)1-RNAi line (#6474R-1) from the...
National Institute of Genetics (NIG) that was used to knock down \( e(y)^1 \) expression in mid-staged egg chambers (supplementary material Fig. S1A and data not shown).

To further exclude the potential off-target effect on Notch signaling caused by \( e(y)^1 \)-RNAi, we set out to analyze the phenotypes of \( e(y)^1 \) mutants. An allele named \( e(y)^1^{190} \) was generated through imprecise excision of a P-element inserted in the 5’-UTR of the \( e(y)^1 \) locus (Fig. 2A and Materials and Methods for details). This allele was both hemizygous and homozygous lethal during early larval stages and could be rescued to adulthood by a duplication [\( Dp(1;3)DC335 \) or \( Dp(1;3)DC336 \]) harboring the \( e(y)^1 \) genomic sequence (data not shown). Flippase-flippase recognition target (FLP-FRT) induced follicle cell mitotic clones of \( e(y)^1^{190} \) showed upregulated \( \text{Cut} \) and downregulated \( \text{Hnt} \) during stages 7-8, when compared with neighboring wild-type cells (Fig. 2B-2C’’), supporting our findings in \( e(y)^1 \) knockdown cells.

Notch-induced transcriptional activation can be assessed in a more direct manner with transgenic reporters. Both \( E(spl)^{m7-}\beta^\text{-CD2} \) (de Celis et al., 1998; Furriols and Bray, 2001) and \( E(spl)^{m7-}\text{lacZ} \) (Assa-Kunik et al., 2007; Pines et al., 2010), which contain the Su(H) binding sites, are expressed in follicle cells during mid-oogenesis upon Notch activation. Indeed, the expression of these two reporters was significantly reduced in \( e(y)^1 \)-RNAi follicle cells (Fig. 1C,D). In addition, the expression of another follicle-cell specific Notch reporter newly identified by our laboratory, \( \text{broad-early-enhancer-lacZ (brE-lacZ)} \) (Jia et al., 2014), was also decreased in \( e(y)^1 \)-depleted follicle cells (supplementary material Fig. S1B). These results suggest that \( e(y)^1 \) is required for proper transcriptional regulation of the Notch target genes in follicle cells.

A developmental consequence of follicle-cell Notch activation during stage 7 is the induction of a switch from the mitotic divisions of early oogenesis to endoreplication cycles (endocycles) (the M/E transition) during mid-oogenesis (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). To determine whether \( e(y)^1 \) is required for this Notch-dependent event, we examined nuclear size and expression of mitotic markers in mosaic egg chambers containing \( e(y)^1 \) RNAi-expressing follicle-cell clones. Knockdown of \( e(y)^1 \) resulted in smaller and more densely distributed nuclei than those of the neighboring wild-type follicle cells, suggesting a failure in the M/E switch (supplementary material Fig. S2A’). To determine whether \( e(y)^1 \)-knockdown
follicle cells remained in the mitotic cycle, we monitored two mitotic markers, phosphorylated histone H3 (PH3) and Cyclin B (CycB), by staining the mosaic egg chambers. In wild-type egg chambers, PH3 and CycB oscillate in early mitotic cycles up to stage 6 and are absent during endoreplication stages (stages 7-10A) (Deng et al., 2001; Shcherbata et al., 2004), whereas in e(y)I-knockdown follicle cells both markers showed random expression after stage 6 (supplementary material Fig. S2B,C). Together, these data indicate that e(y)I is potentially required for the Notch-dependent M/E switch in Drosophila follicle cells.

Disruption of e(y)I affects the expression of Notch target genes in the wing imaginal disc and cultured S2 cells

To assess whether e(y)I also modulates Notch signaling in other tissues, we examined the expression of two well-documented Notch target genes, wingless (wg) and cut, in the wing imaginal disc. During wing disc development, the Notch signaling pathway is activated at the dorsal-ventral boundary by an interaction between the Notch receptor on one cell and its ligand (Dl or Serrate) on the neighboring cell (Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996). Since e(y)I mutant cells in wing disc generated by FLP-FRT mosaic analysis displayed very poor viability (Fig. S3A-B’‘), we employed the RNAi line to study e(y)I’s role in Notch signaling during wing development. To circumvent early-larval lethality of engrailed-Gal4 (en-Gal4)-induced e(y)I-RNAi (data not shown), we used the temporally-controlled Gal4/Gal80ts system (McGuire et al., 2003) to express UAS-e(y)IRNAi in the posterior compartment of a wing disc marked by GFP expression. The control discs did not show detectable changes of Wg (Fig. 3I) or Cut expression (Fig. 3J) (Compare the GFP-positive posterior region with the GFP-negative anterior region). However, in en-Gal4, UAS-GFP; tub-Gal80ts>e(y)IRi discs, Wg or Cut in the GFP-expressing posterior compartment was decreased as compared with those in the anterior compartment (Fig. 3K,L). These results demonstrate that e(y)I is involved in the expression of Notch target genes in the wing disc.

To determine whether E(y)I is necessary for Notch target gene transcription when Notch signaling is artificially induced, we employed a well-documented transient Notch activation system in cultured S2 cells by transfecting a Notch-expressing plasmid, pMT-Notch (Krejci and Bray, 2007; Yu et al., 2013). In this assay, the overexpressed full-length Notch can be cleaved to
produce a constitutively active form of NICD upon EDTA treatment. NICD translocates into the nucleus and physically interacts with Su(H), which activates the transcription of Notch target genes. In this transient Notch activation system, assuming that E(y)1 is required for regulating Notch target gene expression, the induced expression of Notch target genes by pMT-Notch should be compromised by a perturbation of e(y)1. As expected, the introduction of interfering e(y)1 dsRNAs [dse(y)1], but not dsGFP, into the Notch-activating S2 cells led to a significant reduction in expression of the primary endogenous Notch target genes, E(spl)m7 and E(spl)m8 (Fig. 5D,E). These results suggest that E(y)1 is also required for artificially-induced Notch signaling in cultured S2 cells.

*e(y)1 genetically interacts with components of the Notch pathway*

To determine whether e(y)1 is functionally involved in Notch signaling in the wing imaginal disc, we knocked down e(y)1 by use of a tissue-specific Gal4 driver, C96-Gal4, which is expressed in the cells of the dorsal/ventral (D/V) boundary of the wing disc, the precursor of the adult wing margin (Gustafson and Bouliaune, 1996; Saj et al., 2010). Defective Notch signaling in the D/V boundary leads to loss of wing margins (de Celis et al., 1996). Depletion of e(y)1 under the C96-Gal4 driver also led to partial wing margin loss in 96.9% (n = 64) of wings (Fig. 3B), similar to the Notch loss-of-function phenotype. This notched wing margin phenotype was also observed in mosaic adult flies in which e(y)1190 random clones were induced at larval stages by heat-shock (hs) induced Flp (Fig. 2D). Notch heterozygous animals (N264-39/+0) showed a mild wing margin notching in the distal region in 48.5% (n = 101) of wings (Fig. 3C). Combination of C96-G4>e(y)1Ri and N264-39/+ resulted in a synergistically enhanced notched wing phenotype in terms of both penetrance and expressivity (100%, n = 32; Fig. 3D). Furthermore, while e(y)1 heterozygous flies [(y)1190] did not show any visible wing defects (data not shown), incorporation of a copy of e(y)1190 mutation into Notch heterozygotes greatly enhanced the wing margin notching phenotype in both expressivity (Fig. 3F, compared with Fig. 3E) and penetrance [increasing from 48.5% (n = 132; Fig. 3E) to 90% (n = 60; Fig. 3F)]. Similarly, e(y)1190 also displayed a synergistic interaction with another Notch allele, N (Fig. 3G,H). In addition to D/V boundary formation in the wing disc, Notch signaling also plays an important role in cell fate determination through lateral inhibition (Artavanis-Tsakonas and Muskavitch, 2010). Indeed, we observed lateral specification defects in bristle and wing vein development when e(y)1 was
depleted (Fig. S3C-H). These genetic experiments suggest that e(y)1 functionally interacts with the Notch pathway during Drosophila wing development.

**E(y)1 mediates the transcriptional output of Notch signaling through association with NICD and Su(H)**

The Notch signaling pathway is delicately regulated at multiple levels, including ligand and receptor posttranslational modification, endocytosis and vesicle trafficking, and epigenetic regulation of target gene expression (Fischer et al., 2006; Fortini and Bilder, 2009; Tien et al., 2009; Yu et al., 2013; Zeng et al., 2013). Interaction between Notch ligand and receptor induces two consecutive proteolytic processes of the Notch receptor, S2 and S3 cleavages, which results in the release of NICD (Guruharsha et al., 2012). Translocation of the cleaved NICD to the nucleus activates transcription of the target genes through formation of a ternary complex with the DNA-binding protein Su(H) and the coactivator Mam (Guruharsha et al., 2012). e(y)1 putatively encodes a core component of the TFIIID transcription initiation complex, which is required for polymerase II-mediated gene expression (Dynlacht et al., 1991). Thus, two possibilities for its role in Notch signaling exist: first, E(y)1 is required for the transcriptional expression of a core component or a regulator of the Notch pathway; second, E(y)1 is directly engaged in the transcriptional control of Notch target genes that are mediated by the core NICD/Su(H)/Mam complex. To distinguish between these two possibilities, we conducted epistatic analyses in follicle cells by expressing transgenes that encode components acting in different steps of the Notch pathway. These transgenic constructs include a membrane-tethered active form of Notch, N^EXT, a constitutively activated Notch, N^ICD (Rebay et al., 1993); and a fusion protein Su(H)-VP16 consisting of the DNA-binding domain of Su(H) and the strong activation domain of viral transcription-factor protein VP16 (Kidd et al., 1998; Shyu et al., 2009). N^EXT is produced after S2 cleavage and N^ICD after S3 cleavage. Since the majority of Notch regulators and core components function upstream of either S2 cleavage or S3 cleavage, if E(y)1 is required for the expression of one of the players, expression of N^EXT and/or N^ICD would rescue the defects caused by e(y)1 depletion. However, if E(y)1 is directly involved in the expression of Notch target genes, N^EXT and/or N^ICD would have no effect, but Su(H)-VP16 may activate Notch target gene expression in the absence of e(y)1, because Su(H)-VP16 is a more potent transcription activator than Su(H) and can activate a Notch target independent of Notch.
Since, in addition, the VP16 activation domain can directly interact with multiple components of different general transcription factor complexes (Hall and Struhl, 2002), we speculate that expression of certain Notch targets by Su(H)-VP16 may bypass E(y)1. Expression of these constructs can therefore distinguish whether the involvement of E(y)1 is at the level of target gene expression or component expression. Cut upregulation in e(y)1-knockdown follicle cells was employed as a readout in this epistatic analysis. During stages 7-8 of oogenesis, 76% (n = 66) of e(y)1-RNAi follicle cell clones showed Cut upregulation (Fig. 4A). Similar percentages of Cut upregulation were detected when N^EXT (79%, n = 46; Fig. 4B), or N^ICD (70%, n = 82; Fig. 4C) was co-expressed with e(y)1-RNAi. In contrast, Cut upregulation resulting from e(y)1-RNAi was dramatically reduced by co-expression of Su(H)-VP16 (24%, n = 57; Fig. 4D). These results suggest that the involvement of E(y)1 in Notch signaling is probably not related to the expression of Notch regulators or core components upstream of the release of NICD. We employed similar epistatic analysis for the function of E(y)1 in the adult wing. As shown in Fig. 3B, C96-Gal4>e(y)1^Ri adults had notched wing margins (Fig. 4F). While co-expression of N^ICD did not alleviate this defect (Fig. 4G), C96-Gal4>e(y)1^Ri animals incorporated with Su(H)-VP16 showed greatly alleviated wing margin defects (Fig. 4H). C96-Gal4>Su(H)-VP16 alone displayed typical Notch gain-of-function phenotypes during Drosophila wing development (Fig. S4). Taken together, these epistatic results in both follicle cells and developing wings suggest that E(y)1 acts downstream of NICD and is engaged in transcriptional activation of Notch target genes.

Previous studies have shown that specific transcription factors can directly interact with specific TAFs to recruit the transcriptional machinery to their target genes (Kalogeropoulou et al., 2010; Wright and Tjian, 2009). Su(H) is the executive core transcription factor of Notch signaling. Genetic analyses encouraged us to hypothesize that Su(H) physically interacts with E(y)1 to recruit the transcriptional machinery to initiate Notch target gene expression. To test this hypothesis, we turned to the Drosophila S2 cell system again. We co-transfected two constructs to express Flag-tagged E(y)1 and Myc-tagged Su(H), respectively, both under the control of the actin5C promoter in S2 cells. As expected, Flag-tagged E(y)1 was able to immunoprecipitate Myc-tagged Su(H) (Fig. 5A). The NICD/Su(H)/Mam ternary complex has been reported to regulate Notch target gene activation in many organisms, and we therefore asked whether E(y)1
is also physically associated with NICD to promote Notch activation. Indeed, the association
between E(y)1 and NICD was detected in the co-IP assay (Fig. 5B). Thus, E(y)1 mediates the
transcriptional output of Notch signaling by interacting with NICD and Su(H) biochemically.

Requirement of other TAFs in Notch signaling

The results described thus far indicate that E(y)1 is required for the NICD/Su(H)/Mam complex-
mediated transcriptional program of Notch signaling. E(y)1, the *Drosophila* homolog of hTAF9,
was initially identified as one of the core components of the TFIID complex (Dynlacht et al.,
1991; Goodrich et al., 1993). To determine whether E(y)1 is the specific factor of the TFIID
complex for Notch signaling and/or whether all components of the TFIID complex are required
for Notch target gene activation, we examined Notch activity during *Drosophila* wing
development after depleting other components of the TFIID complex by RNAi from the
Transgenic RNAi Project. Knockdown of TAF12 by the en-Gal4 driver did not obviously affect
Notch activity, as revealed by the expression of Wg (Fig. 6A), and consistently, the adult wings
of C96-G4>TAF12*Ri animals had normal wing margins (Fig. 6D). However, ablation of TAF1
under the en-Gal4 driver visibly decreased the Wg level in the posterior compartment of the
wing disc (Fig. 6B), and expectedly, overexpression of TAF1*Ri induced by C96-Gal4 caused
notched wing margins in adult flies (Fig. 6E). Furthermore, we tested several RNAi lines
corresponding to specific components of the TFIID complex (TAF2, TAF5 and TAF6) available
at the Vienna *Drosophila* RNAi Center under the same C96-Gal4 driver, and all of them showed
notched wing margin defects with different expressivity (data not shown). These results suggest
that all components of the TFIID complex we tested, except TAF12, are required for Notch
signaling, at least during *Drosophila* wing development. The e(y)1, e(y)2 and e(y)3 genes were
genetically identified as the respective mutations enhance the phenotype of the y2 mutation
(Georgiev and Gerasimova, 1989; Georgiev et al., 1990), but only E(y)1 is believed to be a
component of the TFIID complex. To investigate whether e(y)2 or e(y)3 plays a similar role in
Notch signaling as that of e(y)1, we compromised e(y)2 function by expression of e(y)2-RNAi
and found that it was not required for Notch activity during wing development (Fig. 6C,F),
implying that unlike e(y)2, e(y)1 is specifically required for Notch signaling.

**DISCUSSION**
The Notch signaling pathway has been well documented in a variety of tissues and in species ranging from *Drosophila* to humans (Artavanis-Tsakonas and Muskavitch, 2010; Fortini and Bilder, 2009; Tien et al., 2009). The regulation of this pathway occurs at different levels, and is especially well-characterized prior to nuclear entry of NICD (Andersson et al., 2011; Guruharsha et al., 2012; Tien et al., 2009). Our and others’ recent findings have shown that Notch signaling can be regulated epigenetically by the CAF1 chromatin chaperone complex or by the SWI/SNF chromatin remodeling complex in different tissues (Yu et al., 2013; Zeng et al., 2013). Our studies address how the Su(H)/NICD/Mam transcriptional complex of the Notch pathway interacts with the general transcription factor complexes to initiate Notch target gene expression.

The results suggest that E(y)1/TAF9, a component of the TAFIID transcription initiation complex, directly interacts with Su(H)/NICD/Mam complex and recruits the TFIID complex to mediate the transcriptional activation of Notch signaling in *Drosophila*.

*Drosophila e(y)1* was initially phenotypically characterized by enhancing the phenotype of the y^2^ allele when mutated (Georgiev et al., 1990). Following study demonstrated that e(y)1 is highly expressed in follicle cells and oocytes during *Drosophila* oogenesis and that decreased e(y)1 transcription causes dramatic underdevelopment of the ovaries and sterility of female flies (Soldatov et al., 1999). Consistent with this, our studies indicate that the effect of e(y)1 loss in follicle cells is stronger than that in the wing disc, suggesting that intensity of e(y)1 involvement in Notch signaling is probably context dependent. A protein-protein interaction assay revealed direct binding between E(y)1/TAF9 and the activation domains of VP16 and p53 (Goodrich et al., 1993; Thut et al., 1995). A human homologue of E(y)1, hTAFII31, was also identified as a critical protein required for p53- and VP16-dependent activation of transcription (Uesugi et al., 1997). A recent report has shown that TAF4 interacts with Pygopus, a transcriptional activator of Wg signaling, to induce transcription of *naked cuticle* (Wright and Tjian, 2009). Our study suggests that TAF9 mediates the transcriptional output of Notch signaling. It is very likely that for a given signaling pathway, specific TAF of the TFIID complex is required for transcriptional output of this signaling, through its interaction with transcription effector.

Genome-wide transcriptome studies in different cell types from a variety of species have revealed a considerable diversity in the expression of Notch-induced target genes (Aoyagi-Ikeda...
et al., 2011; Chadwick et al., 2009; Krejci et al., 2009; Meier-Stiegen et al., 2010; Weerkamp et al., 2006), which might explain the function of Notch signaling in so many different cellular contexts. However, the basis for this transcriptome diversity is only partially understood. The conventional view holds that Su(H) is statically associated with its target enhancers and that it represses transcription when Notch is not activated (Andersson et al., 2011). Upon Notch activation, NICD, together with Mam, then displaces co-repressors and brings co-activators to the Su(H)/NICD/Mam complex, which leads to transcriptional activation of target genes, but a systemic analysis of Su(H) binding at 11 E(spl) Notch target genes has revealed that in Drosophila, Su(H) occupancy at target loci is largely different before and after Notch activation (Krejci and Bray, 2007). Prevailing models imply that the general TFIID complex binds core promoters even in the absence of specific transcription activator (Goodrich and Tjian, 2010). Our biochemical study shows that E(y)1/TAF9 can interact with both NICD and Su(H). We propose that after Notch activation-induced cleavage, the TFIID complex mediates the change of Su(H) occupancy on Notch target gene enhancers through the association between E(y)1/TAF9 and the Su(H)/NICD/Mam ternary complex, leading to transcriptional activation of Notch target genes.

Many Notch target genes have been found in different tissues and their activation by Notch signaling is controlled in a spatiotemporal manner (Borggrefe and Oswald, 2009). In our studies, although many TAFs of the TFIID complex we tested were required for Notch signaling during Drosophila wing development, TAF12 seemed to be dispensable for Notch signaling in wing margin formation, consistent with studies showing that some TAFs are not required for activation of certain genes in specific tissues (Goodrich and Tjian, 2010). In addition, the effect of e(y)1 loss in the wing disc appears to be weaker than that in follicle cells, suggesting that intensity of e(y)1 involvement in Notch signaling is probably context dependent. Whether E(y)1 is required for Notch target gene expression in all tissues remains unclear. It is plausible that certain TAFs are involved in regulating the output and intensity of Notch target gene expression in a spatial- and temporal-specific manner. Our study suggests that, upon Notch signaling activation, Su(H) is released from the Su(H)/Hairless complex to form a transcriptional activator complex with NICD and Mam and this Su(H)/NICD/Mam complex is then recruited by
E(y)1/TAF9 to the TFIID transcription initiation complex to activate Notch target gene expression.
MATERIALS AND METHODS

Generation and characterization of the e(y)I^{190} allele

Fly P-element, w^{1118} P[GT1]e(y)I^{BG00948}, was inserted 78 base pairs (bps) upstream of the start codon of the e(y)I gene. Isogenized P-element flies that are both homozygous and hemizygous viable and fertile were used for generation of the e(y)I^{190} allele by a standard P-element-mediated imprecise jump-out strategy. Excisions of P[GT1]e(y)I^{BG00948} that were hemizygous lethal were sequenced by the use of a pair of primers (5’-CATAAGCTCACCGATTTC and 5’-CCTCCATCTTGAGATCTC) flanking the genomic region of the P[GT1]e(y)I^{BG00948} insertion site. The e(y)I^{190} mutation is a deletion of 339 bps including part of 5’-UTR, exon 1, intron 1, and part of exon 2 of the e(y)I locus (See Fig. 2A).

Fly stocks and genetics

For clonal analyses, the mutation of e(y)I^{190} was recombined with hsFLP^{122} and FRT19A on the X chromosome. Two independent UAS-e(y)I^{RNAi} lines were used to knock down e(y)I level in this paper: e(y)I^{TRiP#HMS00336} from the Bloomington Drosophila Stock Center and e(y)I^{6474R-1} from the National Institute of Genetics, Japan. To induce random clones in follicle cells, the flip-out Gal4 driver [hsFLP; act>CD2 (or y+)>Gal4] was crossed to flies carrying an RNAi or an overexpression construct under the control of the UAS promoter or combination of interest. Their adult progeny were heat-shocked twice at 37°C for 30 minutes, and then cultured in a wet-yeast-pasted vial at 29°C for 2-3 days prior to dissection. w ubi-GFP M(1)osp FRT19A was a gift from Dr. Richard S. Mann (Estella and Mann, 2010). Other fly stocks and genotypes related to figures listed are listed in supplementary material.

Immunocytochemistry

Antibody staining was performed as previously described (Deng et al., 2001). Antibodies from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA) were Cut (1:20), CycB (1:10), Wg (1:20) and Hnt (1:15). Other antibodies used were PH3 (rabbit, 1:400; Upstate), CD2 (mouse, 1:50; Serotec) and β-Galactosidase (rabbit, 1:2000; Sigma). Nuclei were co-stained with DAPI (1:1000, Invitrogen) for 15 minutes at room temperature. Images were captured on a Zeiss LSM-510 confocal microscope in the Biological Science Imaging Resource Facility at FSU. Figures were processed and arranged in Adobe Illustrator.
Drosophila S2 cells were cultured at room temperature in Hyclone serum-free insect cell culture media (Roche). Transfection of S2 cells was performed using FuGENE HD transfection kit reagents (Roche) following the manufacturer’s instructions. Constructs used for transfections are available upon request. The S2 cells were normally harvested 48 hours after transfection for further experiments. Full-length Notch expression was induced by 500 µM CuSO₄ for 24 hours after pMT-Notch transfection (Fehon et al., 1990). Double-stranded (ds) RNA was prepared with the Ribomax large-scale RNA production system-T7 kit (Promega) as previously described (Huang et al., 2011). Primers used were:

**e(y)1**
- Forward: 5’-tttaatacgactcactataggggagATCATGTCCATCCTGAAGGAG-
- Reverse: 5’-tttaatacgactcactataggggagaCGCTAGTTGGTCACAAACTC;

control GFP
- Forward: 5’-tttaatacgactcactataggggagaATGGTGAGCAAGGGCGAGGAGCTG-
- Reverse: 5’-tttaatacgactcactataggggagaCTTGTACAGCTCGTCCATGCCGAGAG (lowercase letters indicate T7 promoter sequences). For a 6-well plate, cells in 2 ml medium in each well were treated with 15 µg dsRNA for 4 days prior to plasmid transfection or CuSO₄ induction.

**Co-immunoprecipitation (co-IP) assay**

Total protein extracts from S2 cells were prepared in IP lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA pH 7.4, 1% Triton X-100, 0.1% SDS) in the presence of protease inhibitors [1 mM PMSF; protease inhibitor cocktail (Calbiochem)]. For co-IP assays, extracts were incubated with specific antibodies and protein A/G agarose beads (Abmart) at 4°C overnight before washes and elution. Immunoprecipitates were boiled in 2× SDS loading buffer for elution from the beads. Rabbit anti-Flag (1:100, Sigma) and rabbit anti-Myc (1:200, Sigma) antibodies were used for co-IP experiments; mouse anti-E(y)1 (1:1000, Sigma), rabbit anti-Flag (1:1000, Sigma), rabbit anti-Myc (1:2000, Sigma) and mouse anti-Tubulin (1:2000, Sigma) antibodies were used for Western blots.
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Competing interests

The authors declare no competing financial interests.

Author contributions

W.-M.D., G.X. and R.J. conceived and designed the experiments. G.X., Z.Y. and D.J. performed the experiments. G.X., Z.Y., R.J. and W.-M.D. analyzed the data. G.X., W.-M.D. and R.J. prepared and wrote the paper with inputs from Z.Y. and D.J.

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References


Figure Legends

Fig. 1. E(y)1 is required for the Notch signaling pathway in Drosophila follicle cells

(A and A’) Cut is upregulated in the e(y)1-RNAi follicle cells (expressing RFP) of a stage-7 egg chamber.
(B and B’) Expression of Hnt is suppressed in the e(y)1-knockdown follicle cells (expressing RFP) of an egg chamber at stage 7.
(C-D’) The expression of two direct reporters of Notch signaling, E(spl)m6-CD2 and E(spl)m7-lacZ, is markedly decreased in e(y)1-depleted follicle cells in mid-stage egg chambers, labeled by the expression of lacZ in red (C) and of GFP in red (D), respectively.

Fig. 2. Generation of an e(y)1 null mutant allele, e(y)1190 and characterization of its Notch defects in follicle cells and adult wings

(A) Genomic organization of the e(y)1 locus, with the p{GT}-BG00948 P-element insertion site (white triangle) and the deletion in e(y)1190 (black double arrows). The single black arrow displays the transcription start site of e(y)1 and its direction. Black bars indicate the coding regions of e(y)1; white bars indicate 5’- and 3’-UTRs. Scale bar: 200 bps.
(B-C’’) Stage 7 egg chambers bearing e(y)1190 homozygous mutant follicle-cell clones labeled by the absence of RFP (B and C) outlined by white lines. Egg chambers were stained for Cut in (B’) and Hnt in (C’), both shown in green. Yellow arrows (B’, B’’, C and C’’) point to polar cells, in which Cut is normally expressed while Hnt is suppressed. Cell nuclei were stained with DAPI in blue (B’’ and C’’). B’’ and C’’ are the merged images.
(D and E) An e(y)1190 mosaic wing induced at larval stages having defective wing edge (D), which can be fully restored to normal wing margin by a duplication line carrying the e(y)1 genomic region (E).

Fig. 3. Genetic interaction between e(y)1 and Notch in wing development

(A) A wild-type adult wing with a normal wing margin.
(B) Knockdown of e(y)1 under the C96-Gal4 driver caused partial wing margin loss and Notch heterozygous flies (N264-39/+ ) showed very mild wing margin notching in the distal region (C).
Combination of [C96-G4 > e(y)1[RI] + N264-39/+] , however, resulted in nearly complete wing margin defects (D). An e(y)1 mutant allele [e(y)1190] was generated; see Materials and Methods
for details. Transheterozygotes of $N^{264/39}/e(y)^{1BG00948}$ (E) showed a similar phenotype to Notch heterozygotes (C), with the same penetrance [64/132 in (E) versus 49/101 in (C)]. $e(y)^{190}$ heterozygous flies showed no visible wing defects (data not shown), while introducing one copy of $e(y)^{190}$ into $N^{264/39/+}$ flies significantly enhanced the wing margin defects of $N^{264/39/+}$ (F). (G and H) Synergistic enhancement of notching wing margin between $N^{+/+}$ and $e(y)^{190/+}$. $N^I$ is a hypomorphic Notch allele, with 12/81 wings showing mild notching when heterozygous (G, compared to 49/101 of $N^{264/39}$). When a copy of $e(y)^{190}$ is incorporated, the notched wing margin phenotype was visibly enhanced and penetrance was remarkably increased to 50/82 (H). (I-L) Knockdown of $e(y)I$ in wing discs compromised the expression of Notch target genes. Anterior and posterior compartments of wing discs are separated by white lines; anterior is to the left and posterior to the right. (I and J) Wing discs from wild-type control flies showed normal expression patterns of Wg in red (I) and of Cut in red (J) in the third instar larvae. (K and L) Wg level was obviously reduced in the $e(y)I$-depleted posterior compartment marked by the expression of GFP in green (K, compared with internal control level in the anterior compartment). Posterior Cut level was also dramatically decreased in posterior $e(y)I$-RNAi area (L).

**Fig. 4. Epistatic analyses of E(y)1 in the Notch pathway in endocycling follicle cells and adult wings**

(A-D’) E(y)1 regulates Notch signaling downstream of the Notch proteolytic cleavage in mid-stage follicle cells. Cell nuclei were stained with DAPI in blue (A, B, C and D). Flip-out clones were marked by the expression of RFP in red (A, B, C and D) and outlined by white lines. Mid-stage egg chambers were stained for Cut in green (A-D’). Since a stau:GFP transgene was recombined in the hsFLP-carrying X chromosome, punctated green signals were detected within the germ cells. (A, B, C and D) Merged pictures of three channels; (A’, B’, C’ and D’) Single channel of Cut staining. (A and A’) Follicle cell clones of $e(y)I$-RNAi in a mid-stage egg chamber showed Cut upregulation. (B-C’) Expression of $N^{EXT}$ (B and B’) or $N^{ICD}$ (C and C’) failed to stop Cut upregulation caused by $e(y)I$ depletion.
(D-D’) Induction of strong Su(H)-VP16 activator prevented Cut expression in mid-stage e(y)1-RNAi follicle cells.

(E-H) Ectopic expression of Su(H)-VP16 suppressed notched wing margin defect caused by C96-Gal4-driven e(y)1 RNAi.

(E) C96-Gal4 control adult flies had normal wing margin.

(F) A typical wing-margin loss in C96-Gal4>e(y)1-RNAi flies.

(G) Coexpression of NICD under the same C96-Gal4 driver did not suppress wing margin defects caused by e(y)1 RNAi.

(H) Coexpression of Su(H)-VP16 significantly suppressed the wing margin loss resulting from C96-Gal4>e(y)1-RNAi. All wings were from male flies.

Fig. 5. E(y)1 is physically associated with NICD and Su(H) and required for Notch target gene expression in S2 cells

(A) Flag-tagged E(y)1 interacts with Myc-tagged Su(H) in Drosophila S2 cells.

(B) Myc-tagged NICD is associated with Flag-tagged E(y)1 in S2 cells.

Flag-tagged E(y)1 and Myc-tagged Su(H) in (A), and Myc-tagged NICD and Flag-tagged E(y)1 in (B) were cotransfected into cultured S2 cells, respectively. Total cellular extracts were prepared for the co-IP assay with IgG (control) or the indicated antibodies. Antibodies used for IP in (A and B) are indicated at the top and proteins detected by Western blot after IP are indicated to the left. Input lanes represent 5% of the S2 cell extracts that were used for IP.

(C) E(y)1 protein levels are greatly reduced by dse(y)1 but not dsGFP treatment in S2 cells, while NICD and Tubulin protein levels are not changed by dse(y)1 treatment.

(D and E) Expression of two Notch target genes, E(spl)m7 (D) and E(spl)m8 (E), strongly induced by transfected Myc-tagged NICD in S2 cells, was significantly suppressed by e(y)1 knockdown. The basal expression of E(spl)m7 and E(spl)m8 is very low, indicated by transfection of the pAc5.1 empty vector, shown in the left side of each chart, and e(y)1-RNAi manipulation had little effect on their expression. E(spl)m7 and E(spl)m8 levels were increased 39 folds (D) and five folds (E), respectively, when Notch signaling is induced by transfection of pAc5.1-Myc-NICD in normal S2 cells. Knockdown of e(y)1 significantly suppressed this activation induced by the expression of NICD. ***p < 0.001.
Fig. 6. Effects of depleting e(y)2 or other TAF-encoding genes on Notch signaling

(A-C) Confocal images of wandering third-instar wing discs with en-Gal4 driving GFP plus the indicated constructs. Wing discs were stained for Wg displayed in red. GFP in green is to mark the posterior compartment of wing discs, in which en-Gal4 is expressed.

(D-F) Pictures of adult wings from flies expressing the indicated constructs induced by the C96-Gal4 driver.
Fig. 1

A, A' Cut RFP
B, B' Hnt RFP
C, C' E(spl)mß-CD2 lacZ
D, D' E(spl)m7-lacZ GFP
Fig. 2

A

3'-UTR of CG6398

GT-BG00948

e(y)1

deleted region 200 bp

B

B'

B''

B'''

C

C'

C''

RFP Cut Cut RFP DAPI

RFP Hnt Hnt RFP DAPI

Fig. 2
en-G4, UAS-GFP/++; tub-G80ts/+; en-G4, UAS-GFP/++; tub-G80ts/e(y)1Ri

Fig. 3
Fig. 4
Fig. 5
en-G4, UAS-GFP/+; tub-G80ts/+  

C96-G4 >

Fig. 6