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A positive role of Sin3A in regulating Notch signaling during *Drosophila* wing development

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ABSTRACT

Notch is a transmembrane receptor that mediates intercellular signaling through a conserved signaling cascade in all animal species. Transcriptional and posttranscriptional regulation of Notch receptor are important for maintaining Notch signaling activity. Here, we show that depletion of *Drosophila* Sin3A leads to loss of the adult wing margin and downregulation of Notch target gene expression in the developing wing disc. Sin3A regulates the Notch pathway downstream of Delta and upstream of Notch activation. The role of Sin3A in the Notch pathway is partly mediated by its ability to modulate Notch receptor transcription. Furthermore, the transcriptional activation of Notch receptor is autoregulated by Notch itself. We also provide evidence that Sin3A is required for Notch activation mediated Notch transcription. Together, our data demonstrate that Sin3A activates Notch signaling by promoting Notch transcription and reveal a previously unknown autoregulatory mechanism for Notch signaling activation during *Drosophila* wing development.

1. Introduction

The Notch signaling pathway is widely used to control cell fate and cell behavior in various developmental processes across different species [1–3]. The core components of the Notch pathway are the receptors Notch, Delta/Serrate/LAG-2 (DSL) ligands and nuclear factors [1–3]. Ligand induced Notch activation leads to the release of the intracellular domain of the Notch receptor, which subsequently translocates to the nucleus and interacts with transcription factors and coactivators to activate transcription [1–3]. Extensive studies in *Drosophila* development have identified numerous factors required for the activity of the Notch pathway in different tissues and cell types [1–3]. A number of mechanisms for Notch signaling regulation have been described, such as Notch protein modification, trafficking, recycling and degradation [1–3]. Recently, several studies reveal that the regulation of Notch receptor transcription is also essential for Notch pathway activation [4–7]. However, the underlying molecular mechanism and significance of Notch receptor transcriptional regulation remains less understood.

Sin3 is a large scaffold protein and functions to modify chromatin structure and regulate gene expression through recruiting the histone deacetylase and other transcriptional factors [8,9]. Although initially identified as a repressor, Sin3 has been shown to function in both positive and negative transcriptional regulation [8,9]. In mammals, two Sin3 homologues, Sin3A and Sin3B, are known, whereas in *Drosophila* there is only one well characterized Sin3 protein, Sin3A [10–12]. Previous studies have shown that Sin3 is implicated in the regulation of multiple biological processes, including cell proliferation, differentiation, apoptosis and cell cycle progression [8,9]. *Drosophila* Sin3A associates with HDAC1/Rpd3 and forms a transcriptional repressor complex to control target gene expression [9,13]. Genetic and biochemical analysis has demonstrated that Sin3A is associated with multiple signaling pathways, including steroid hormone, Hippo and JNK pathways [9,14,15]. It has been reported that 3% of *Drosophila* genes is regulated by Sin3A, indicating that Sin3A has a profound effect on gene expression [16]. Impaired function of Sin3A also promotes tumorigenesis in both a *Drosophila* tumor model and human tumors [17]. Thus, further studies will be necessary to understand diverse roles of Sin3A in both development and disease.

In the present study, we performed tissue specific knockdown analysis in the *Drosophila* developing wing, an effective system to study Notch signaling. Our results reveal that Sin3A positively regulates Notch signaling by promoting Notch transcription. Furthermore, we

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show that Notch is able to autoregulate its own expression, which is also dependent on Sin3A. Together, these findings demonstrate a novel role of Sin3A in Notch regulation and uncover Notch autoregulation as one important regulatory mechanism in *Drosophila* wing development.

2. Materials and methods

Drosophila stocks-Drosophila melanogaster stocks were maintained and crosses were performed at 25 °C. The fly stocks we used were as follows: *UAS-sin3A$^{RNAi(I)}$* (v10808, Vienna *Drosophila* Resource Center). *UAS-sin3A$^{RNAi(II)}$* (BL32368, Bloomington *Drosophila* Stock Center), *UAS-Delta* (BL5614), *UAS-NICD* (BL52008), *UAS-Notch^{RNAi}* (BL28981), *UAS-Su(H)^{RNAi}* (BL28900), *hs-Flp1.22; act > FRT y + FRT > GAL4 UAS-GFP / CyO, Notch-lacZ; en-Gal4 UAS-GFP/Cyo, E(spl)m8-lacZ/Cyo; hh-Gal4 UAS-GFP/TM6B* and *Su(H)-lacZ; en-Gal4 UAS-GFP/Cyo. Vg-Gal4, Daughterless-Gal4*.

2.1. Immunostaining

Third instar larval wing imaginal discs were dissected in cold PBS before fixing them in 4% formaldehyde in PBS at room temperature for 2 h. Discs were washed in 0.1% PBT (0.1% Triton X-100 in PBS), blocked with 3% BSA in PBT for 1 h and then incubated with specific primary antibody diluted in 3% BSA in PBT at 4 overnight. After washing several times in PBT, discs were incubated with secondary antibody at room temperature for 2 h. After incubation with DAPI for 20 min, discs were mounted in VECTASHIELD (H-1200; Vector laboratories). Primary antibodies used were as follows: chicken anti-GFP (1:2000; ab13970; Abcam), mouse anti-Cut (1:50; 2B10s; DSHB), mouse anti-Wg (1:100; 4D4; DSHB), mouse anti-NICD (1:100; C17.9C6; DSHB), mouse anti-β-gal (1:2000; Z378A; Promega). Fluorescent secondary antibodies used were Alexa 488 (as well as 555 and 633)-conjugated anti-rabbit, anti-mouse and anti-chicken IgG antibodies (Molecular Probes). Images were taken using an Olympus FV1000 confocal microscope and processed using Adobe Photoshop.

qPCR-Total RNA was prepared from 50 third instar larval wing imaginal discs using TRIzol reagent (Thermo Fisher Scientific). cDNA was synthesized from 1 μg of total RNA using oligo dT primers and SuperScript III reverse transcriptase (Thermo Fisher Scientific). qPCR was run in triplicate for each sample on an ABI 7900HT Fast Real-time PCR system using the Power SYBR Green PCR Master Mix (Applied Biosystems). Rp49 expression level was used for normalization. The primers used were as follows:

Sin3A: 5′-GGGAGATGTCGTATCAGCGG-3′ and 5′-GGCTTATCGACT TCCTCGGG-3’.

Wingless: 5′-CCAAGTCGAGGGCAAACAGA-3′ and 5′-CCCAGTACA CCGGGATTGTC-3′.

Cut: 5′-CCGTCATGCTGCTCAAATCG-3′ and GCAATCCGATCCTCG GACAT-3′.

E(spl)m8: 5′-CCTGAAAACACTTGTCGCCG-3′ and 5′-GTCTTTTGCT GGCGCATGAA-3′.

Notch: 5′-CGACACCAAGTACAAGCCCT-3′ and 5′-CGAAACCTTTGG GGCACTTG-3′.

Rp49: 5′-GCTAAGCTGTCGCACAAA-3′ and 5′-TCCGGTGGGCAGCA TGTG-3′.

Drosophila wings-Adult flies were soaked in 75% ethanol at least 2 h and then put in 80% glycerol overnight. Wings were removed and mounted on the glass slide. Images were taken using a Nikon Eclipse 80i microscope and processed using Adobe Photoshop.

3. Results

3.1. Reduction of Sin3A causes wing notching

Sin3A is essential for *Drosophila* development as loss-of-function *Sin3A* alleles cause late embryonic or first instar larval lethality [11,12].

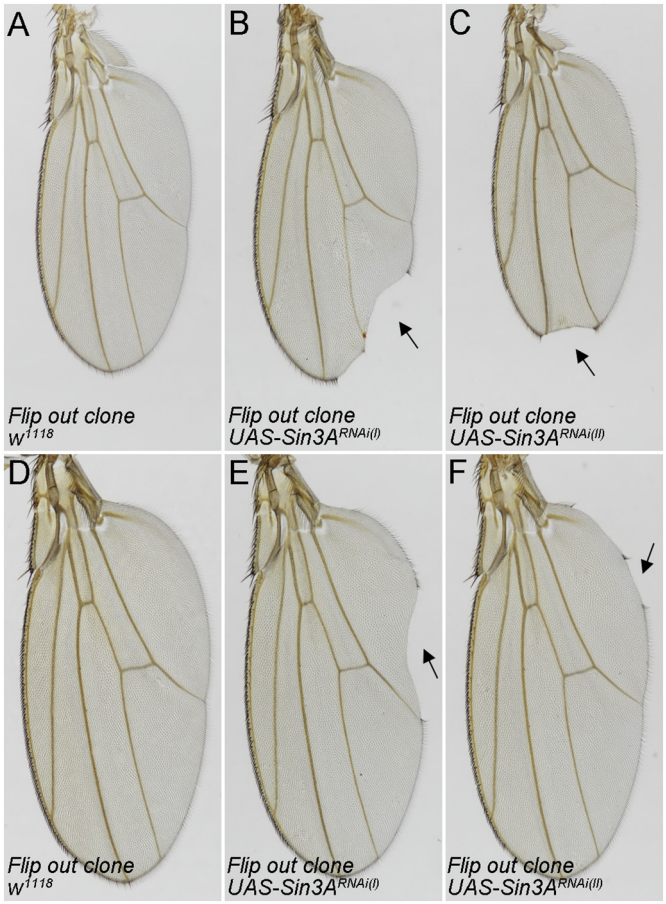

Fig. 1. Knockdown of Sin3A causes wing notching in *Drosophila*.
A-C, Adult wings from male flies harboring control or Sin3A RNAi flip out clones. *D-F,* Adult wings from female flies harboring control or Sin3A RNAi flip out clones. Note the presence of notched wing margin when Sin3A was knocked down. Arrows indicate wing notches.

A recent RNA-seq study shows that Notch is downregulated upon *Sin3A* knockdown in *Drosophila* S2 cells [18]. Therefore, we speculated that Sin3A might play a positive role in regulating the Notch pathway during development. To test this idea, we knocked down Sin3A activity in the developing wing by expressing a *UAS-Sin3A RNAi* construct under the control of the actin flip-out *Gal4* driver. *Sin3A* knockdown wing disc clones were induced at first instar larvae, and adult wings were analyzed. The adult wings of male and female flies harboring *Sin3A RNAi* clones exhibited notches at the wing margin, a phenotypic hallmark of reduced Notch signaling (Fig. 1A, B, D and E). To avoid an off target *RNAi* effect, we also generated the flip-out clones using an additional RNAi line, which targets a different region of *Sin3A* mRNA. Similarly, wing notches were observed in wings of both male and female flies expressing this RNAi line, although the phenotype was much weaker in female flies (Fig. 1C and F). In addition, RNAi of *Sin3A* by a *vg-Gal4* driver, which is specifically expressed at the dorsal-ventral (DV) boundary of the wing disc, caused wing notches in the adult flies (Fig. S1A–F). These results suggest that Sin3A might positively regulate Notch signaling during *Drosophila* wing development.

3.2. Sin3A is required for Notch target gene activation

To further confirm a specific role of Sin3A in the Notch pathway, we examined its ability to modulate Notch target gene expression. In the developing wing imaginal disc, Notch is strongly activated in a small stripe of cells along the DV boundary, which will later become the adult wing margin. The activation of the Notch pathway results in the

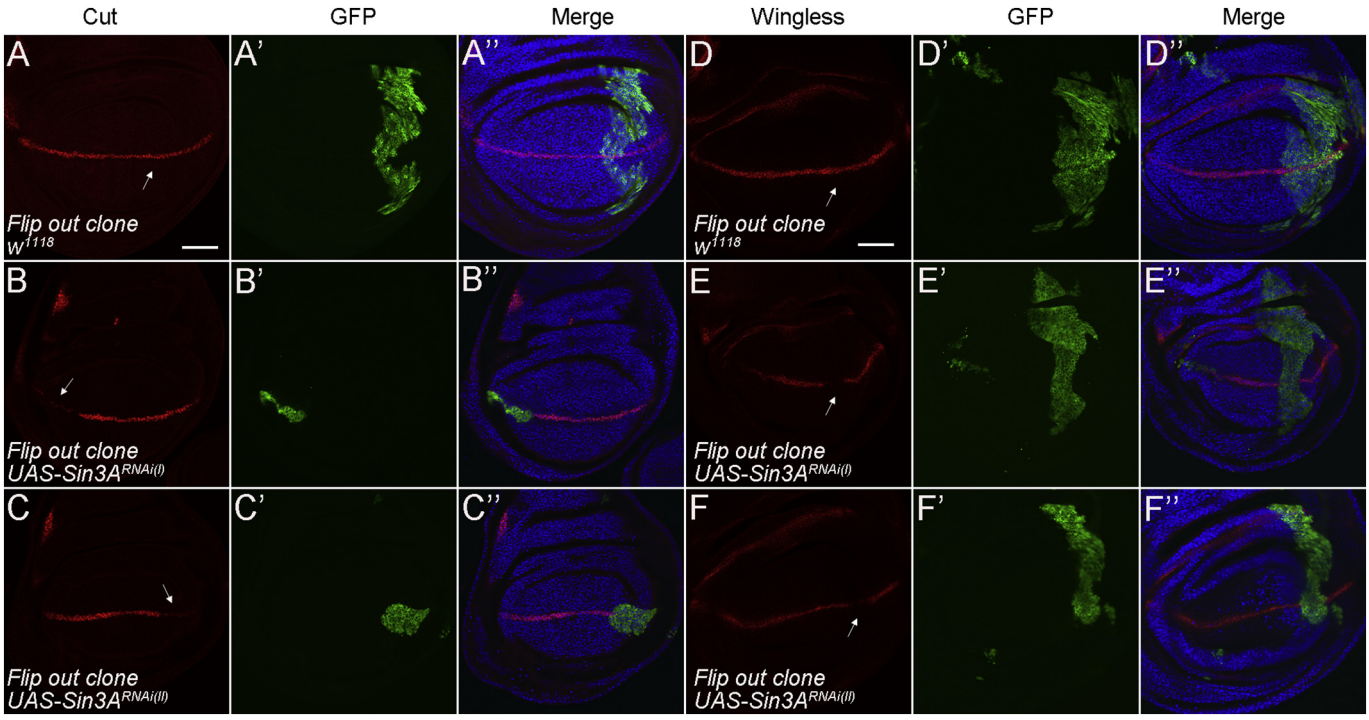

Fig. 2. Sin3A is required for Notch target gene expression.
A-C″, RNAi knockdown of Sin3A in the wing disc reduces Cut protein levels. Wing discs with flip-out clones expressing the following transgenes: *(A-A″)* control, *(B-B″) UAS-Sin3A*$^{RNAi(I)}$, *(C-C″) UAS-Sin3A*$^{RNAi(II)}$, stained with anti-Cut and anti-GFP. *D-F″,* RNAi knockdown of Sin3A in the wing disc reduces Wingless protein levels. Wing discs with flip-out clones expressing the following transgenes: *(D-D″)* control, *(E-E") UAS-Sin3A*$^{RNAi(I)}$, *(F-F″) UAS-Sin3A*$^{RNAi(II)}$, stained with anti-Wingless and anti-GFP. DNA was stained with DAPI. Clones were labelled by the presence of GFP. Scale bars: 50 μm.

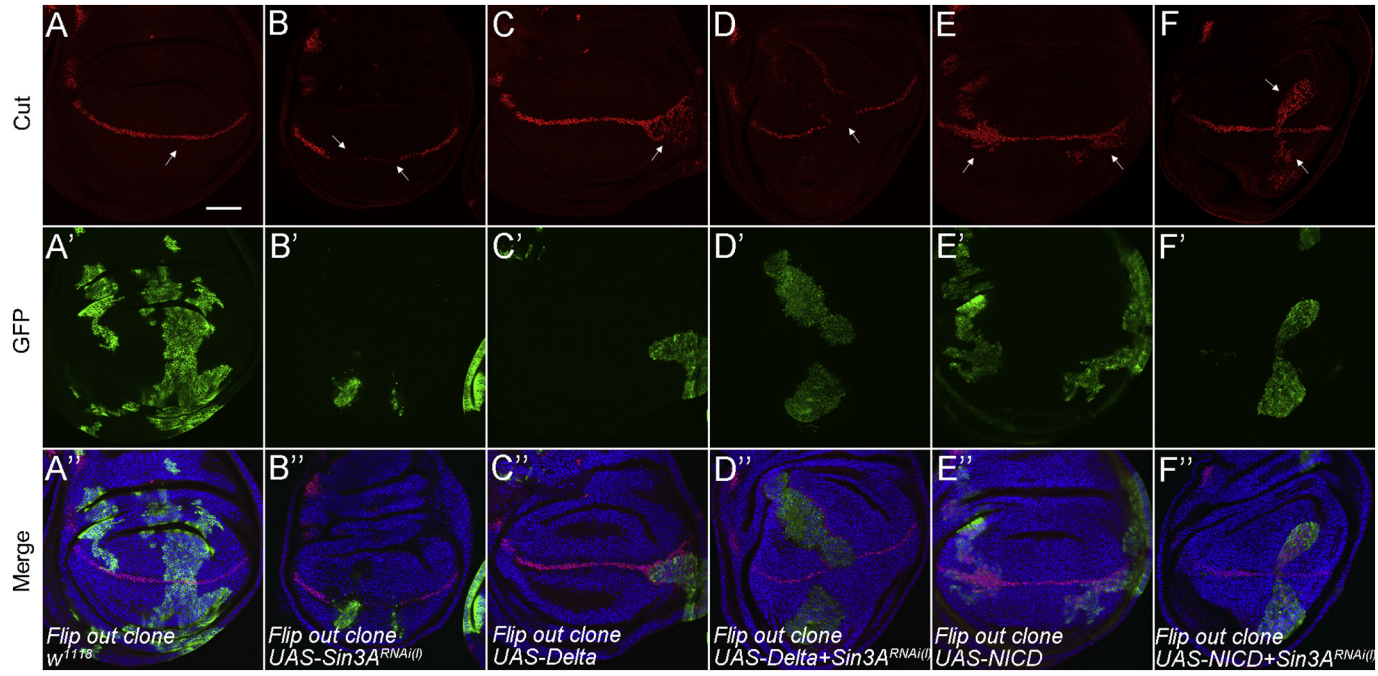

Fig. 3. Sin3A acts downstream of Delta expression and upstream of Notch activation.
A-D", Co-expression of *Sin3A RNAi* and *Delta* suppresses the *Delta*-induced ectopic Cut expression. Wing discs with flip-out clones expressing the following transgenes: *(A-A″)* control, *(B-B″) UAS-sin3A*$^{RNAi(I)}$, *(C-C″) UAS-Delta*, *(D-D″) UAS-Delta* and *UAS-sin3A*$^{RNAi(I)}$, stained with anti-cut and anti-GFP. *E-F″,* Co-expression of *Sin3A RNAi* and the active form of Notch (NICD) does not suppress NICD-induced ectopic Cut expression. Wing discs with flip-out clones expressing the following transgenes: *(E-E″)* UAS-NICD *(F-F″)* UAS-NICD and UAS-sin3A$^{RNAi(I)}$, stained with anti-Cut and anti-GFP. DNA was stained with DAPI. Clones were labelled by the presence of GFP. Scale bars: 50 μm.

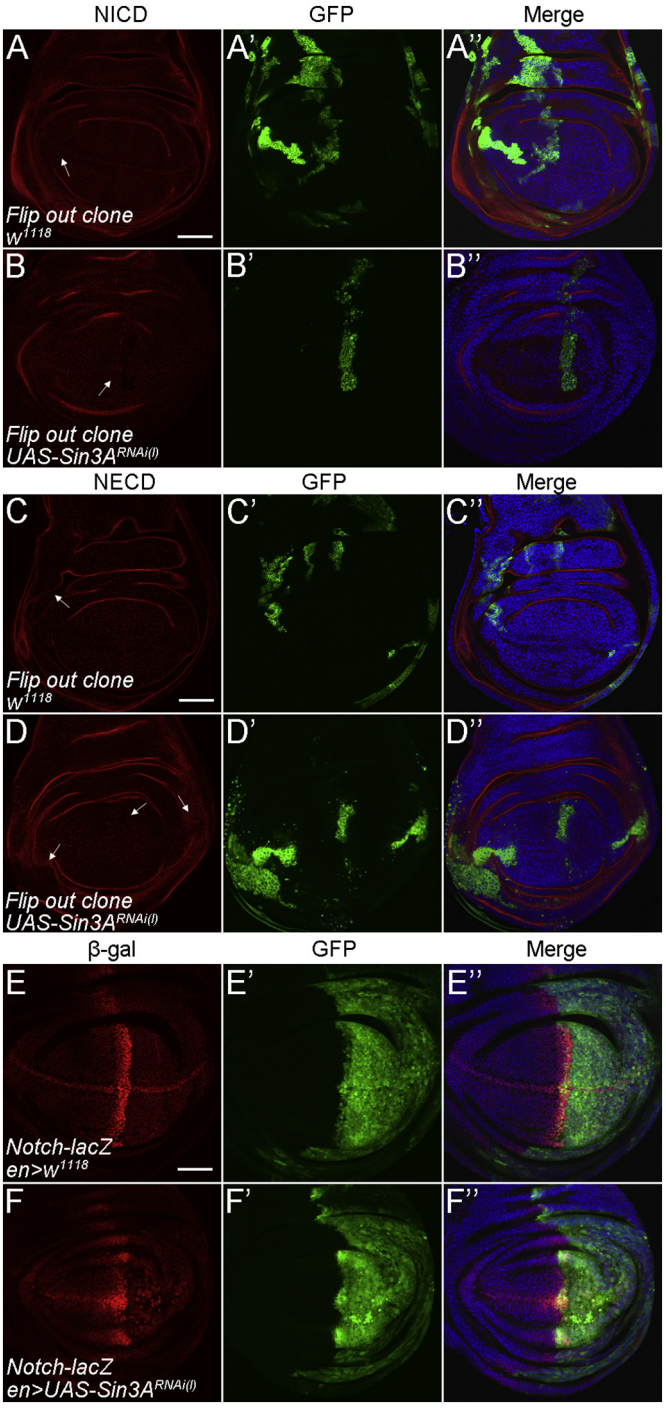

Fig. 4. Sin3A is required for Notch receptor transcription.
A-D″, Knockdown of Sin3A in the wing disc reduces NICD and NECD protein levels. Wing discs with flip-out clones expressing the following transgenes: *(A-A″* and *C-C″)* control, *(B-B″ and D-D")* *UAS-Sin3A*$^{RNAi(l)}$, stained with anti-NICD (or anti-NECD) and anti-GFP. DNA was stained with DAPI. Clones were marked by the presence of GFP. Arrows indicates clone area. *E-E″,* Expression pattern of the *Notch-lacZ* reporter gene in a control wing disc. *F-F″,* RNAi knockdown of Sin3A by *en-Gal4* reduces *Notch-lacZ* expression in the posterior compartment of the wing disc. Scale bars: 50 μm.

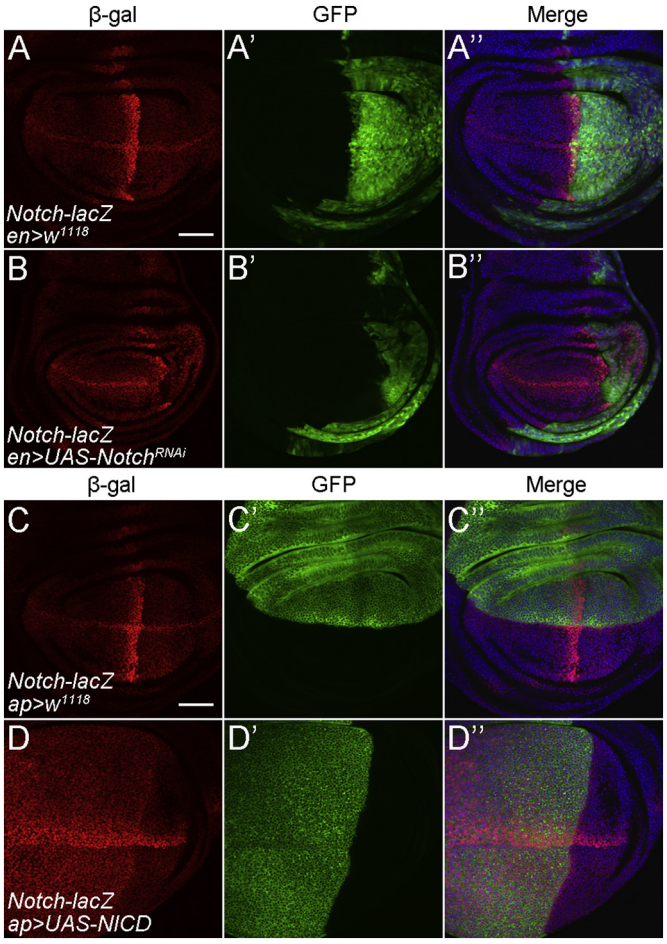

Fig. 5. Notch autoregulates its own transcription.
A-A″, Expression pattern of the *Notch-lacZ* reporter gene in a control wing disc. *B-B″,* RNAi Knockdown of Notch by *en-Gal4* reduces *Notch-lacZ* expression in the posterior compartment of the wing disc. *C-C″,* Expression pattern of the *Notch-lacZ* reporter gene in a control wing disc. *D-D",* Overexpression of NICD by *ap-Gal4* increases *Notch-lacZ* expression in the dorsal compartment of the wing disc. Posterior and dorsal cells were marked by the presence of GFP. Scale bars: 50 μm.

expression of several Notch target genes, including Cut and Wingless, which together are essential for the development of the wing. The expression of both *Sin3A RNAi* lines in wing imaginal disc clones along the DV boundary using the flip-out Gal4, resulted in a strong reduction of Cut and Wingless protein levels, while the control clones exhibited no such defects (Fig. 2A–F″). In addition, RNAi of *Sin3A* by a posterior expressed *en-Gal4* also led to the reduced expression of two other Notch target gene reporters, *E(spl)m8-lacZ* and *Su(H)-lacZ*, at the DV boundary of the posterior wing disc (Fig. S2A–F″). To further verify these findings, we performed quantitative PCR analysis to determine the mRNA levels of the endogenous Notch target genes, including *Cut*, *Wingless* and *E(spl)m8*. For this analysis, the ubiquitously expressed *Daughterless-Gal4* (*Da-Gal4*) was used to knockdown Sin3A activity. RNAi of *Sin3A* by *Da-Gal4* reduced *Sin3A* mRNA levels, confirming the knockdown was efficient and specific (Fig. S3). Consistent with the above results, we observed a reduction of *Cut*, *Wingless* and *E(spl)m8* mRNA levels in *Sin3A* knockdown wing imaginal discs (Fig. S3). Together, these data support that Sin3A has a role in promoting Notch target gene expression.

3.3. Sin3A acts downstream of Delta expression and upstream of Notch activation

In the canonical Notch signaling pathway, the interaction between Delta ligand and Notch receptor triggers a series of proteolytic cleavages of Notch, leading to the generation of the Notch intracellular domain (NICD) [1–3]. NICD travels into the nuclear and activates specific target gene expression [1–3]. To determine at which point

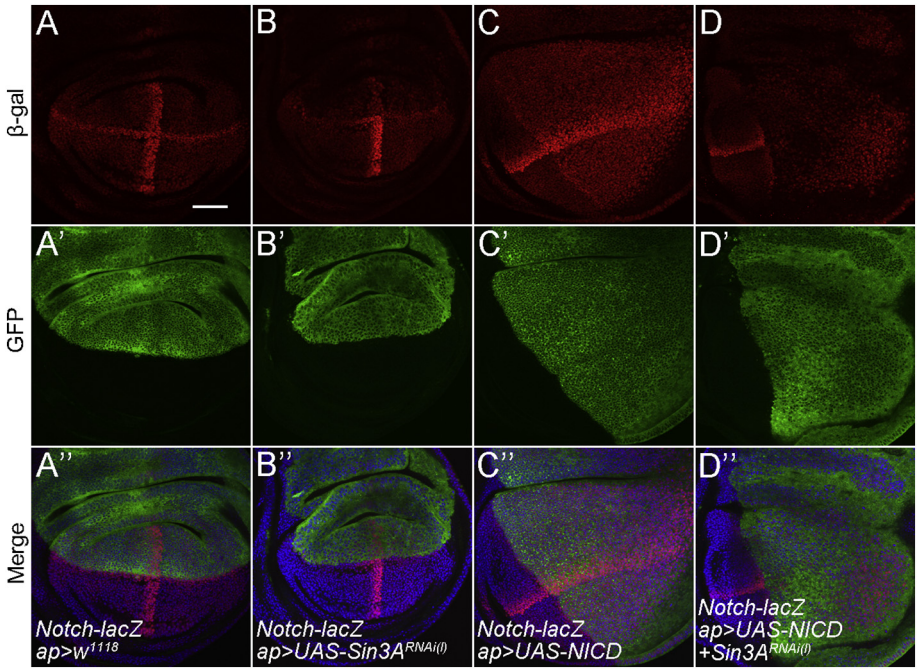

Fig. 6. Notch transcription induced by Notch activation requires Sin3A.
A-A″, Expression pattern of the *Notch-lacZ* reporter gene in a control wing disc. *B-B″,* RNAi Knockdown of Sin3A by *ap-Gal4* reduces *Notch-lacZ* expression in the dorsal compartment of the wing disc. *C-C″,* Overexpression of NICD by *ap-Gal4* increases *Notch-lacZ* expression in the dorsal compartment of the wing disc. *D-D",* Co-expression of Sin3A RNAi by *ap-Gal4* suppresses NICD-induced increase of *Notch-lacZ* expression in the dorsal compartment of the wing disc. Dorsal cells were marked by the presence of GFP. Scale bars: 50 μm.

Sin3A acts on the Notch pathway, we again used the flip-out technique to express Delta (*UAS-Delta*) or an active form of Notch (*UAS-NICD*) in *Sin3A RNAi* clones. If Sin3A functions upstream of Delta expression or Notch activation, overexpression of Delta or NICD would restore the Cut expression defects caused by Sin3A knockdown. The opposing result would indicate that Sin3A acts downstream of Delta expression or Notch activation. As described above, RNAi of *Sin3A* reduced Cut expression along the DV boundary of the wing disc as compared to the control (Fig. 3A-B″). Ectopic expression of *Delta* resulted in the upregulation of Cut expression within the clones as well as the adjacent cells (Fig. 3C-C″). However, co-expression of *Delta* had no effects on the reduced Cut expression induced by *Sin3A* knockdown (Fig. 3D–D″). On the other hand, overexpression of *NICD* autonomously induced Cut expression, and the increase of Cut expression was not altered upon *Sin3A* knockdown (Fig. 3E–F″). These results demonstrates that Sin3A functions downstream of Delta expression, but upstream of Notch activation.

3.4. Sin3A activates Notch transcription

Activation of Notch target genes by Sin3A could result from its direct regulation of Notch receptor levels. To test this, we first examined the effect of *Sin3A* knockdown on Notch protein levels using two antibodies against either intracellular or extracellular domains of Notch. Upon depletion of *Sin3A*, Notch protein levels were strongly reduced in the wing disc clones (Fig. 4A-D″). The reduction of Notch protein levels might reflect a reduced transcription of Notch receptor. We then used a previously reported *Notch-lacZ* insertion line to determine Notch transcription [19,20]. Consistent with our speculation, knockdown of *Sin3A* by *en-Gal4* caused an obvious reduction of *Notch-lacZ* expression in the posterior wing compartment as compared to the control (Fig. 4E–F″). Moreover, our qPCR analysis also revealed that knockdown of *Sin3A* by *Da-Gal4* led to the downregulation of *Notch* mRNA levels (Fig. S4). Thus, we conclude that Sin3A promotes Notch signaling by activating Notch transcription.

3.5. Notch autoregulates its own transcription

Transcriptional activation of Notch receptor requires several chromatin factors and transcriptional co-repressors during *Drosophila* wing development [4–7]. It has also been shown that the interaction between Notch and these factors regulates Notch target gene expression [1–3]. However, it remains unknown whether Notch can autoregulate its own transcription. To examine this possibility, we obtained a *Notch* RNAi line and knocked down Notch activity in the posterior wing disc using *en-Gal4*. Our results revealed that *Notch-lacZ* expression was reduced in the wing posterior compartment where *Notch* was knocked down (Fig. 5A-B″). We also confirmed the specificity of the *Notch* RNAi line by performing anti-NICD antibody staining, as RNAi of *Notch* by *en-Gal4* reduced NICD protein levels (Fig. S5A–B″). These data strongly argue that Notch promoter activity is suppressed upon loss of *Notch*.

To further confirm the role of Notch in regulating its own expression, we next investigated the effect of NICD overexpression on *Notch* transcription. Ectopic expression of *NICD* under the control of *ap-Gal4* led to a strong upregulation of *Notch-lacZ* expression in the dorsal compartment of the wing disc (Fig. 5C-D″). Taken together, these data support the notion that Notch can autoregulate its own transcription.

3.6. Sin3A is required for Notch activation mediated Notch transcription

Have shown that Notch has an autoregulatory role for its own transcription, we then want to determine whether Sin3A is involved in this regulation of *Notch* transcription upon Notch activation. For this purpose, we overexpressed *Sin3A RNAi* and *NICD* in the dorsal cells of the wing disc using *ap-Gal4* and assayed the expression of *Notch-lacZ*. As shown above, *Sin3A* knockdown reduced *Notch-lacZ* expression, and *NICD* overexpression increased *Notch-lacZ* expression in the wing dorsal compartment (Fig. 6A–C″). Interestingly, the elevation of *Notch-lacZ* expression upon Notch activation was offset by simultaneously expressing *Sin3A RNAi* (Fig. 6D–D″). These results indicate that Sin3A is required for Notch activation mediated *Notch* transcription.

4. Discussion

The precise control of gene expression is extremely important in animal development. The data presented here demonstrate that the scaffold protein Sin3A plays a critical role in promoting Notch receptor transcription and Notch signaling activity during *Drosophila* wing development. Our recent analysis reveals that *Drosophila* HDAC1/Rpd3 has a similar role with Sin3A in regulating Notch receptor transcription

in the developing wing [7]. These findings together support the notion that the Sin3A/HDAC1 co-repressor complex might be involved in activating Notch gene expression. Transcriptional fine-tuning is an important function of many co-repressor complexes [21]. It appears that the Sin3A/HDAC1 co-repressor complex can activate transcription directly. Previous studies report that HDAC1 is associated with the repressor complex at Notch target gene loci and functions to suppress target gene expression [2,22]. It remains unclear that how the Sin3A/HDAC1 co-repressor complex can exert opposing effects on transcription at different loci. The involvement of Sin3A/HDAC1 in controlling Notch transcription suggests that the chromatin of the Notch promoter region appears to be in a dynamic state. Notch receptor transcriptional regulation provides an additional layer regulation of Notch signaling. Consistent with this, a number of chromatin modifiers have been implicated in the positive regulation of Notch receptor transcription [4–7].

Moreover, we show that the transcription of Notch receptor is autoregulated by its own product. This positive autoregulation by stimulating its own transcription might provide a feedback mechanism to prolong Notch signaling during wing development. Such regulation may be important in maintaining cell fate. It is possible that Notch expression is reinforced in some cells, and not others, leading to difference in cell fate. Sin3A is required for Notch activation mediated Notch transcription, raising the possibility that Notch and Sin3A/HDAC1 complex can act at the same site in the Notch promoter region.

5. Conclusion

Our identification of Sin3A as a positive regulator of Notch signaling and discovery of an autoregulation mechanism for Notch signaling during *Drosophila* wing development potentially open a new avenue for the analysis of Notch pathway in animal development.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2018.10.008.

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Conflict of interest

The authors declare no conflict of interest.

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