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Research Article

Kuz and TACE can activate Notch independent of ligand

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Abstract. A central mechanism in activation of the Notch signaling pathway is cleavage of the Notch receptor by ADAM metalloproteases. ADAMs also cleave Delta, the ligand for Notch, thereby down-regulating Notch signals. Two ADAMs, Kuzbanian (Kuz) and TNF-α converting enzyme (TACE), are known to process both Delta and Notch, yet the role of these cleavages in signal propagation has remained controversial. Using an *in vitro* model, we show that Kuz regulates Notch signaling primarily by activating the receptor and has little overall effect on signaling *via* disabling Delta. We confirm that Kuz-dependent activation of Notch requires stimulation of Notch by Delta. However, over-expression of Kuz gives ligand-independent Notch activation. In contrast, TACE, which is elevated in expression in the developing *Drosophila* nervous system, can efficiently activate Notch in a ligand-independent manner. Altogether, these data demonstrate the potential for Kuz and TACE to participate in context- and mechanism-specific modes of Notch activation.

Keywords. Notch, Delta, ADAM protease, Kuzbanian, TACE, proteolysis.

Introduction

Notch signaling is a conserved mechanism of cell-cell signaling that is critical for specifying cell fates during development (reviewed in [1, 2]). The fundamental role of Notch signaling has directed considerable attention to mechanisms that activate the Notch receptor. Core components that initiate Notch signaling are the Delta ligand and the Notch receptor, both being single-pass transmembrane proteins. Additional components include an ADAM (a disintegrin and metalloprotease) protease and a γ-secretase complex. The current model of activation holds that engagement of the Notch receptor by Delta ligand binding provokes two sequential cleavages of Notch [3–5]. The first rate-limiting cleavage, which requires an ADAM protease, is followed by a constitutive intramembranous γ-secretase cleavage involving Presenilin [6, 7]. The resulting intracellular Notch product (N^{ICD}) translocates to the nucleus and activates transcription of genes of the *Enhancer of Split* locus [E(Spl)] [8]. This locus encodes seven basic helix-loop-helix (bHLH) proteins (Mδ, Mβ, Mγ, M3, M5, M7, and M8) that in turn act as transcriptional repressors for a number of genes that regulate cell fate decisions [8, 9]. Several aspects of this model remain unresolved. For instance, there is contradictory evidence for the role of specific ADAM proteases in propagating Notch signals. Genetic analyses in *Drosophila*, mice and *Caenorhabditis elegans* strongly implicate Kuzbanian (Kuz, a mammalian ADAM10 homolog and one of five *Drosophila* ADAMs) as the ADAM required for Notch activation [10–13]. In *Drosophila* S2 cells, Kuz and Notch can physically associate and Kuz is able to cleave a Notch construct that is lacking parts of the

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extracellular and intracellular domains [14]. Yet, the relationship between Notch cleavage and Kuz activity with respect to activation of E(Spl) targets remains unresolved. Delta also undergoes Kuz-dependent cleavage [15–17] that inactivates its ligand activity and leads to down-regulation of Notch signaling [18]. However, the relative contribution of Kuz to Notch activation *versus* Delta inactivation remains unclear. Experimental data are contradictory on the role of TNF-α converting enzyme (TACE, a mammalian ADAM17 homolog) in Notch activation. Biochemical studies show that in mammalian cells TACE and not Kuz is required for cleavage of various Notch1 constructs that are missing extracellular domain [7, 19]. In *Drosophila* S2 cells, overexpressed TACE can noticeably compensate for the loss of Kuz-dependent cleavage of Notch [14]. These facts are in contradiction with the phenotypic data from TACE null mice that do not exhibit neurogenic phenotypes akin with Notch mutations [20] whereas Kuz null mice do [21]. Thus, it is still not clear whether TACE-dependent cleavage of Notch leads to its activation. Furthermore, the potential for ADAMs to act in a ligand-independent manner has not been thoroughly characterized.

To investigate the explicit activity of ADAMs in signal-sending *versus* signal-receiving cells, we developed an *in vitro* model using neuronally derived *Drosophila* ML-DmBG2-c6 cells that utilize endogenous Notch and Kuz expressed at relevant physiological levels. We demonstrate that Kuz acts primarily in signal-receiving cells and is required for Notch-dependent activation of E(Spl) targets. Kuz activity toward regulating Delta function is small by comparison. Overexpression of Kuz can activate Notch independent of Delta stimulation. In contrast to Kuz, we find that TACE at near-physiological expression levels is highly effective at activating Notch in a ligand-independent manner. We find an example of differential levels of TACE expression in developing *Drosophila* tissues, pointing to specific contexts where these ADAMs may act. Altogether, these data clarify a more explicit role for ADAMs in regulating Notch signals predominantly through the signal receiving cells and demonstrate the potential for Kuz and TACE to act on Notch in a ligand-independent manner.

Materials and methods

Cell culture. All cells are from *Drosophila melanogaster* and available at the *Drosophila* Genome Resource Center (Bloomington, IN). Cells were routinely maintained in Schneider's *Drosophila* medium (Lonza, Walkersville, MD, cat. no. Q4–351Q) supplemented with 13 % fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA; cat. no. S11550), 0.1 % human insulin (Sigma; cat. no. I9278) and penicillin/streptomycin solution at 50 µg/ml (Mediatech, Herndon, VA; cat. no. 30-001-CI). The S2 cell line was originally derived from Oregon R embryos on the verge of hatching [22]. The S2-Mt-Dl (DlS2) cell line was made from S2 cells stably transfected to express wild-type Delta from a copper-inducible metallothionein promoter [23]. ML-DmBG2-c6 (C6) cell line was made from brain and ventral ganglion of late 3rd instar larvae [24]. These cells were originally characterized as being immunoreactive to HRP (a neuronal marker in insects), and having detectable levels of acetylcholine, L-DOPA, substance P, proctolin, and somatostatin, and no detectable GABA [24–26]. We observe that knockdown of Kuz or Notch expression does not effect proliferation rate or cell morphology of C6 cells. $C6^{Kuz}$ and $C6^{TACE}$ cell lines were made by stable transfection of C6 cells with pIZ Kuz and pIZ TACE plasmids, respectively, according to the manufacturer's protocol for making a polyclonal cell line (Invitrogen, document. no. 25–0283). pIZ Kuz with V5 tag at the 3' end was made by cloning *Drosophila* Kuz cDNA (gift from Spyros Artavanis-Tsakonas, Harvard Medical School [15]) into pIZ/V5-His vector (Invitrogen, cat. no. V8010–01). The pIZ TACE construct was made by cloning *Drosophila* TACE cDNA with an HA tag at the 3'-end from the pUAST-TACE plasmid (gift from Ben Shilo, [16]) into pIZ/V5-His vector (Invitrogen, cat. no. V8010–01) using *Eco*RI and *Xba*I restriction enzymes and verified by direct DNA sequencing. The expression of Kuz-V5 in $C6^{Kuz}$ cells and TACE-HA in $C6^{TACE}$ cells were validated by immunostaining and Western blotting. For both TACE and Kuz, we observe bands on Western blots indicative of approximately 50 % of the total protein in the latent pro-enzyme form, with the remainder in the active form.

For transient transfections, cells were plated in a 12-well plate and allowed to adhere until 60–70 % confluent. Media was then aspirated and replaced with a transfection mix consisting of 1 µg plasmid DNA and 5 µl Cellfectin (Invitrogen, cat. no. 10362–010) in 150 µl serum free Schneider's *Drosophila* insect media. Transfection mix was applied for 4 h under rocking motion followed by recovery in complete medium for 16–24 h prior to assays.

RNA interference (RNAi). Double-stranded interference RNA treatment of *Drosophila* cells was done essentially as described [27]. PCR products (~ 500 bp) from the 5′ coding region of target genes were amplified with following primers that incorporated 5′ flanking T7 sequence (5′-GAA TTA ATA CGA CTC ACT ATA GGG AGA-3′): *Kuz* (5′-ATG TCA TCA

AAA TGT GCT TTC AAC-3′, 5′-GTG ACT GTT GTT GCT GAG GAT G-3′), *TACE* (5′-GGA CGA TGT GGT GCA CAG G-3′, 5′-GTG CAG CTC ATT GTC CAG AG-3′), *Delta* (5′-CAC AGT CAT CGT GCA GGT TC-3′, 5′-CAG CGA CGT GTA CTG CGA TTC -3′), *Notch* (5’-ATG CAA TCG CAG CGC AGC C-3′, 5’-GCT GAC AGG TGC CTC CAT TG-3′). These products were used as a template for *in vitro* RNA synthesis using the MEGAscript T7 kit (Ambion, cat. no. 1334). Double-stranded RNA was added to cells at concentrations of 40 μg/100-mm culture dish and incubated for 3 days. RNAi-treated cells were then used in subsequent assays.

***In vitro* assay of Notch activation.** Signal sending cells (DlS2 and control S2 cells) were plated into 6-well plates and allowed to adhere until 100 % confluent. Medium was aspirated and cells were washed once in Robbs PBS [28]. Cells were then fixed in 5 % formalin (made in Robbs PBS) for 10 min at room temperature to cross-link polynucleotides and proteins. After two additional washes in Robbs PBS, 15×10^7 signal-receiving cells (C6) were added per well for 1.5 h to induce Notch signaling. Following this induction, cells were processed for RNA extraction and cDNA synthesis as described below. All assays except for the dose-response experiment relied on the basal expression of Delta in DlS2 cells, and did not require use of $CuSO_4$ induction of Delta expression. For the dose-response assay, protein expression in DlS2 cells was induced with 50 μM and 350 μM $CuSO_4$ addition to the medium for 16 h prior to assay. No $CuSO_4$ was added to DlS2 cells that were treated with Delta RNAi. All experiments were done in a minimum of triplicates.

***In vivo* analysis of gene expression.** Brains (abdominal ganglia, thoracic ganglia and optic lobes only) and wing disks (with haltere disks) of wandering 3rd instar larvae from wild-type *D. melanogaster* (Canton S strain) were dissected in a drop of Robbs PBS on silicone plates (Sylgard® 184 silicone elastomere kit, Dow Corning, Midland, MI) under Nikon SMZ1000 dissecting microscope and transferred directly into 1.5-ml microfuge tubes filled with 100 μl TRIzol (Invitrogen, cat. no. 15596–026). Ten brains or wing disks were collected per experiment. Collected tissue was homogenized (Kontes Pellet Pestle® cordless homogenizer) and processed for RNA extraction and cDNA synthesis as described below.

RNA extraction and cDNA synthesis. Cells and larval tissues were lysed in 1 ml TRIzol. RNA extraction was performed according to the manufacturer’s protocol. Briefly, RNA was separated into aqueous phase by addition of 200 μl chloroform followed by vigorous mixing and centrifugation at +4°C in a microfuge at 16 100×*g* for 15 min. Aqueous phase was transferred to a new 1.5-ml Eppendorf tube and RNA was precipitated by addition of 500 μl 100 % isopropanol followed by 10-min incubation at room temperature and centrifugation at + 4°C at 16 100 g for 15 min. The resulting RNA pellet was washed in 75 % ethanol and dissolved in DEPC-treated water at +55°C for 10 min. Concentration of RNA was measured using Nanodrop® ND-100 spectrophotometer (NanoDrop, Wilmington, DE). Samples of RNA from cell lysates (10 μg) were treated with Turbo DNA-free Kit (Ambion, cat. no. AM1907) to remove any contaminating DNA. DNase treatment was omitted for lysates from *Drosophila* tissues (due to low RNA yield). cDNA was generated using 800 ng RNA with oligo(dT) in the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, cat. no. 11904, 18080). Samples that were not treated with DNase were processed in duplicates that included no RT control. In all cases, the contribution of genomic DNA to the PCR signal was less than 1 %.

Quantitative analysis of gene expression. Levels of gene expression were determined by real-time, quantitative PCR (qPCR) analysis using SYBR® Green dye (Sigma, cat. no. S5193) and an ABI PRISM 7500 Fast Sequence Detection System. Expression of target genes was normalized to the expression of RP49 ribosomal gene as a housekeeping gene. We observed that levels of RP49 expression were stable and comparable across various cell lines and *Drosophila* tissues. Expression values were determined using either the comparative $\Delta\Delta C_T$ method [29] or straight ΔC_T values. Analysis was done in ABI 7500 Fast System SDS software (version 1.3.1) and Microsoft Excel (Office 2003). Statistical significance of the difference in expression was determined by running a Student’s *t*-test in Microsoft Excel (Office 2003). PCR primers were manually designed to amplify 200–300-bp fragments at the 3′ end of the coding region: *Kuz* (5′-GAA TTT GTT GCT CAA CCG GAA G-3′, 5′-CTC CGC CGC GTG AAT AAT G-3′), *TACE* (5′-CAT CAC AGG ATT CTG CAA CAA G-3′, 5′-GAA TCA CTC GAC GCC TCT C-3′), *Delta* (5′-CAT ATG CGG AGT GCC GCA G-3′, 5′-GGC GAG GGT TCC TAC TGT AG-3′), *Notch* (5′-GAA TCT GCC CAG TCC GTAC-3′, 5′-CCA TTC ATC CCG AGT CCT-3′), Mβ (5′-CTA CGT TCA TGC TGC CAA TG-3′, 5′-ATT CAG AGG GTG GTG GAG TG-3′), Mγ (5′-GTC AAT GAG GTC TCC CGT TC-3′, 5′-GGT CAA CAG GGA ATG ACT GG-3′), *rp49* (5′-AGT ATC TGA TGC CCA ACA TCG-3′, 5′-TTC CGA CCA GGT TAC AAG AAC-3′). Primers were validated by template dilution standard curve analysis,

identification of single PCR products on agarose gel and direct DNA sequencing. The contribution of genomic DNA to the PCR signal was determined by analyzing samples omitting the RT step.

Western blot analysis. Cells were lysed for 10 min on ice with 50 mM Tris, 1 % IGEPAL CA-630 (Sigma, St. Louis, MO), and 150 mM NaCl, containing the protease inhibitors 5 mM EDTA, 1 mM PMSF, and 5 μg/mL each of aprotinin, leupeptin, and pepstatin. SDS-PAGE sample buffer containing β-mercapto-ethanol was added to cell lysates and samples were boiled for 5 min. Proteins were separated on SDS-PAGE and transferred to PVDF membranes (Millipore Corporation, Danvers, MA) for quantitative Western blot analysis using an Odyssey infrared imager (Li-Cor, Lincoln, NE). The BCA assay (Pierce, Rockford Il.) was used to determine protein content in samples and to ensure equal loading of protein on a gel. Primary antibodies were specific for *Drosophila* proteins and included: rat monoclonal 10D5 anti-Delta intracellular domain [30] and mouse monoclonal C17.9C6 anti-Notch intracellular domain (gift from Spyros Artavanis-Tsakonas, Harvard Medical School). Secondary antibodies included goat anti-rat IRDye700 (Rockland, Gilbertsville, PA) and goat anti-mouse Alexa680 (Molecular Probes, cat. no. A-21057). All primary antibodies were used at 1:5000 dilution. Protein bands were quantified using Odyssey software (version 1.2) and Microsoft Excel (Office 2003). Images were processed using Adobe Photoshop software (Adobe).

Immunostaining. Cell surface staining of Delta was done in non-permeabilized cells. Briefly, DlS2 cells were fixed in 2 % paraformaldehyde in Robb's PBS [28] for 10 min, washed twice in Robbs PBS, and then blocked with 1 % normal goat serum (NGS) in Robbs PBS for 1 h. C594.9B (9B) antibody (DSHB, University of Iowa), specific for extracellular domain of *Drosophila* Delta, was prepared in 1 % NGS Robbs PBS at 1:5000 dilution and samples were incubated at room temperature for 1 h. After two washes in Robbs PBS, samples were incubated with anti-mouse Alexa488 secondary antibody (Molecular Probes, cat. no. A-11029) for 1 h at room temperature. After additional wash in Robbs PBS, cells were immersed in Citifluor mounting medium (University of London) and mounted on a coverslip. Images were captured using a Nikon C1 confocal microscope system and processed using Adobe Photoshop software (Adobe).

Results

Development of a robust *in vitro* assay of Notch activation. To investigate the contribution of Kuz and TACE to Notch activation and Delta inactivation, we developed an *in vitro* assay where signal-sending cells that selectively express Delta are co-cultured with signal-receiving cells that selectively express Notch (Fig. 1A). In designing this assay we sought to use endogenous Notch that is expressed at relevant physiological levels. Since one of the long-term goals in our lab is to understand the role and regulation of Notch signaling during development of *Drosophila* larval nervous system, we sought to carry out *in vitro* experiments in cells that mimic this context. ML-DmBG2-c6 (C6) cells derived from larval nervous system [24] are particularly well suited for the role of signal-receiving cells due to the fact these cells express Notch and no detectable Delta as determined by Western blotting (Fig. 1B and [31]). Comparatively, Notch expression is somewhat lower than the average level seen in the larval brain (approximately 25 %, Fig. 1D), yet, the expression level of Kuz is similar to that found in the larval brain (Fig. 1D). In contrast, the expression level of TACE in C6 cells is less than 5 % of that seen in larval brains (Fig. 1D). We found this expression profile an optimal attribute for selective analysis of Kuz in the absence of TACE in initial experiments (see below).

For signal-sending cells we were unable to identify a neuronally derived cell line that selectively expresses Delta, and instead used the well-characterized Delta-S2 (DlS2) stable transformant line [23]. Of technical importance, we found that DlS2 and its parent cell line, S2 cells, express significant levels of Mβ and Mγ (Fig. 1E), which is a spurious observation since these cell lines fail to express Notch [32]. To overcome the contribution of Mβ and Mγ mRNA from these cells to the signal-receiving C6 cells, we introduced a brief formalin-fixation step to DlS2 and S2 cells prior to presentation to C6 cells. This fixation procedure is a modified version of the one reported previously [18]. We found that formalin fixation effectively abolishes the ability to detect Mβ and Mγ mRNA extracted from these cells by our qPCR methods (Fig. 1E), presumably due to cross-linking of protein-RNA complexes that is subsequently removed during phenol-chloroform extraction. Formalin treatment does not grossly alter the level of full-length mature Delta in DlS2 cells (Fig. 1F). However, a small fraction of high molecular species that is immunoreactive with Delta antibody is present (marked by asterisk in Fig. 1F) indicative of cross-linking of Delta either to itself or to other proteins. In addition, with formalin treatment we see less C-terminal derived cleavage product (Dl^{CTF},

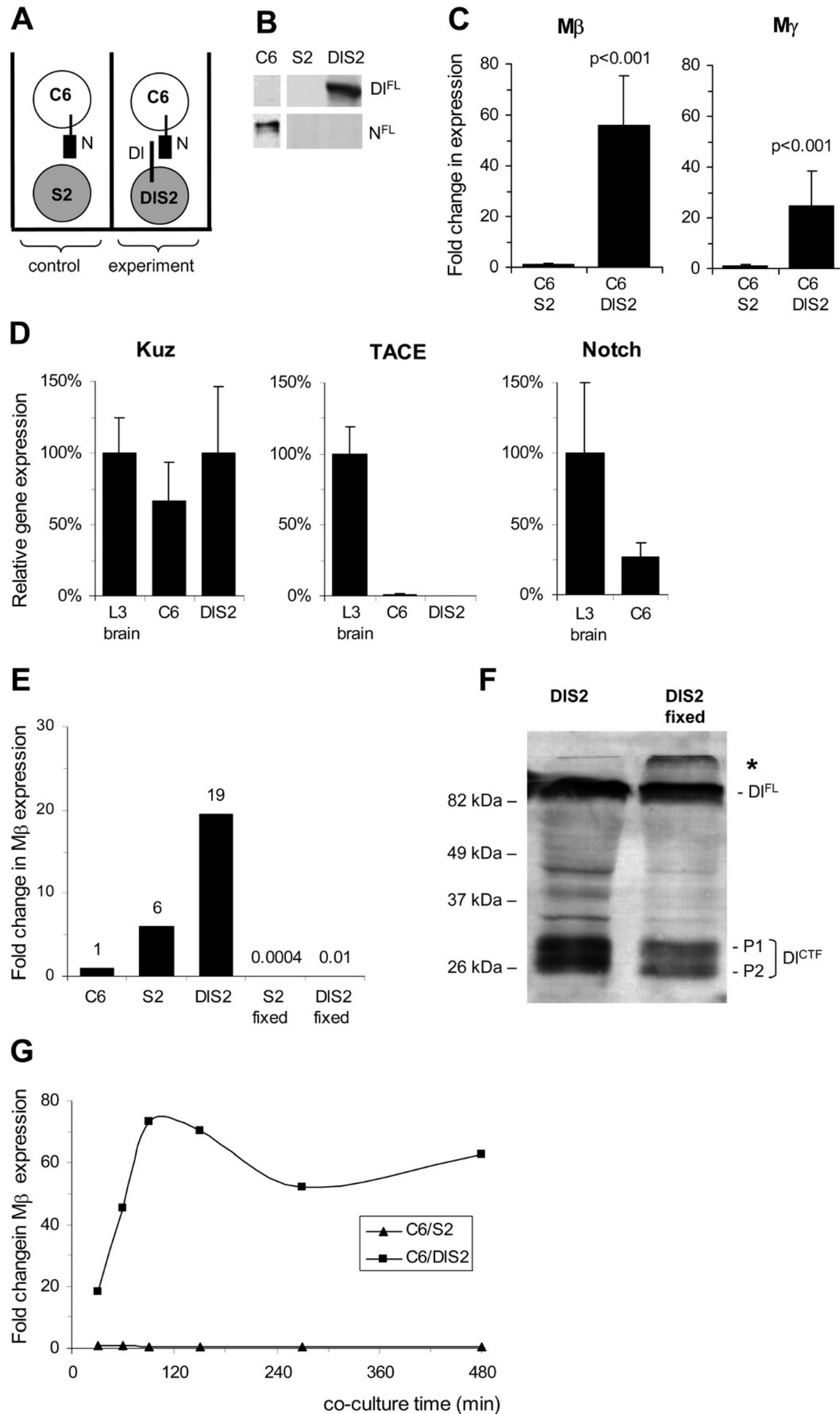

Figure 1. *In vitro* assay of Notch activation. (*A*) Schematic of co-culture assay showing control and experimental wells with signal-sending (gray) and signal-receiving (white) cells. (*B*) Western blot showing protein expression of full-length Notch (N^{FL}) and full-length Delta (Dl^{FL}) in signal-sending (DlS2) and signal-receiving (C6) cells. S2 cells that do not express detectable Notch and Delta are used as a control for non-specific stimulation. (*C*) Fold difference in gene expression of two E(Spl) genes, Mβ and Mγ as measured by qPCR (see Methods), in C6 cells following co-culture with S2 *versus* DlS2 cells. Error bars indicate one standard deviation from the mean. All subsequent experiments that show difference in expression are done using qPCR unless otherwise noted. (*D*) Gene expression levels in *Drosophila* larval brain (L3) and cell lines showing fold difference in expression levels of Kuz, TACE and Notch. (*E*) Mβ gene expression levels in signal sending cells showing the levels before and after fixation in 5 % formalin. (*F*) Western blot of cell lysates from non-fixed and formalin-fixed DlS2 cells stained with Delta-specific antibody [30], showing full length Delta (Dl^{FL}) and the C-terminal fragments of cleaved Delta (Dl^{CTF}), the Kuz-dependent P1 fragment and the Kuz-independent P2 fragment [17]. Asterisk denotes high molecular weight immunoreactive material, indicating possible Delta species resulting from cross-linking Delta either to itself or to other proteins. (*G*) Time course of Delta-induced Notch activation in C6 cells (filled squares). Y-axis shows fold change in Mβ gene expression relative to the basal level of Mβ expression in S2 cell control-stimulated C6 cells (filled triangles) at 30-min time point.

Fig. 1F), which likely reflects a decreased immunoreactivity of these fixed fragments to anti-Delta antibody.

Stimulation of Notch in C6 cells with Delta on DlS2 cells leads to a dramatic increase in Notch activation as measured by the expression level of E(Spl) mRNA (Fig. 1C). Time-course analysis of Delta-induced Notch activation revealed that E(Spl) expression increases within 30 min after Notch-expressing cells make contact with Delta-expressing cells and reaches

maximum level at 90 min (Fig. 1G). We therefore used 90-min stimulation for all subsequent assays. While two representative E(Spl) genes, Mβ and Mγ, were used in this study, both of these Notch targets showed similar responses in all experiments, therefore only Mβ results are shown. The wide dynamic range of Delta-induced Notch activation presented optimal conditions for the analysis of the contribution of Kuz (and other elements of the Notch pathway) to the regulation of Notch signaling.

To confirm that activation of E(Spl) genes in this assay is Notch specific, we down-regulated expression of Notch using RNAi [27] (Fig. 2A, C, D). Knockdown of Notch expression (Fig. 2C) led to complete unresponsiveness to Delta stimulation as seen by a lack of induction of Mβ expression (Fig. 2B). We noted that RNAi-mediated knockdown of Notch expression led to a 70 % reduction in mRNA (Fig. 2C), a 90 % reduction in protein (Fig. 2D) and 100 % reduction in activity (Fig. 2B), confirming that RNAi is very effective at removing the activity of target proteins in this cell line. Based on this robust reduction of Notch gene products with RNAi, in subsequent experiments where we could not test the level of protein due to unavailable immunoreagents, we assumed that removal of >70 % of target mRNA corresponds with an equal or greater reduction in the associated protein. To confirm that Notch activation was specific to Delta stimulation, we titrated Delta expression in signal-sending cells (Fig. 2E, F). Notch activation showed linear response to the dose of Delta ligand (Fig. 2G).

Kuz is required for Delta-induced activation of Notch. To investigate the requirement for Kuz in signal-receiving cells, we down-regulated its expression in C6 cells with RNAi and analyzed the level of Delta-induced Notch activation (Fig. 3A). First, we demonstrated that RNAi-mediated knockdown of Kuz expression in C6 cells leads to more than 80 % reduction in Kuz mRNA (Fig. 3C) and loss of Kuz-dependent cleavage of Delta (Fig. 3D and [15, 17, 30]), showing that Kuz RNAi is effective at down-regulating both Kuz expression and Kuz activity. This knockdown of Kuz expression leads to 90 % reduction in Notch activation (Fig. 3B), indicating that Notch activation is highly sensitive to the presence of Kuz in the same cell.

In the course of monitoring Kuz expression levels subsequent to Delta stimulation, we found that Kuz is moderately up-regulated in C6 cells after 90 min of stimulation by Delta (Fig. 3H), suggesting a potential positive feedback loop that can amplify Delta/Kuz-mediated Notch signaling. For comparison, Notch activation does not affect the expression of itself or another ADAM, TACE (Fig. 3H). To further study the effect of Kuz expression levels on Notch activation, we created a stably transformed C6 cell line $C6^{KUZ}$ (see Methods). In this context where an 8-fold increase in Kuz expression is achieved (Fig. 3E), a 14-fold increase in Notch activation is seen even in the absence of ligand stimulation (Fig. 3F). Notch in these cells still responds to Delta stimulation, and the final level of Notch activation is similar to that observed with Delta stimulation in the parent C6 cell line (Fig. 3F). Furthermore, the ligand-independent activation of Notch in $C6^{KUZ}$ cells is due to increased Kuz expression since partial knockdown of the overexpressed Kuz in these cells leads to a decrease of Mβ expression (Fig. 3G).

Altogether, these results demonstrate that Notch activation is highly responsive to Kuz activity on the signal-receiving cell. Whereas ligand engagement is required to invoke Notch activity with endogenous levels of Kuz, elevated levels of Kuz are sufficient to invoke ligand-independent Notch activation.

Kuz cleavage of Delta has little effect on Notch signaling. Several studies point to a role for Kuz cleavage of Delta in regulating Notch signaling [10, 15, 18, 33, 34]. However, this role has remained somewhat unresolved, in part due to attempts to interpret outcomes in the complex context of developing tissues [10,13,15]. We therefore investigated the contribution of Kuz to regulating Delta ligand activity in our assay system where ligand activity can be assayed more directly. First, we found that Notch activation responds in a linear manner to the level of Delta protein expression in the signal-sending cell (Fig. 2G). We next down-regulated the Kuz expression in signal sending cells (DlS2) and measured the ability of these cells to activate Notch (Fig. 4A, D). In DlS2 cells, we find approximately 30 % of Delta is cleaved at a basal steady state. Removal of Kuz in DlS2 cells with RNAi essentially abolishes generation of the P1 cleavage product (Fig. 4B, E also see [17]). Despite this inhibition of Delta cleavage, Kuz RNAi results in a negligible increase in Delta ligand activity (Fig. 4C). However, this level of Delta expression results in strong stimulation of Notch. At lower levels of Delta expression, achieved with Delta RNAi, Kuz RNAi similarly inhibits Delta cleavage (Fig. 4E) and gives a mild (approximately 2-fold) increase in Notch signaling (Fig. 4F). Overall, these results reinforce that Delta is sensitive to Kuz cleavage; however, Kuz has comparatively little overall effect on Notch activation *via* modulation of Delta ligand activity.

TACE can activate Notch in ligand-independent manner. The TACE ADAM protease has been

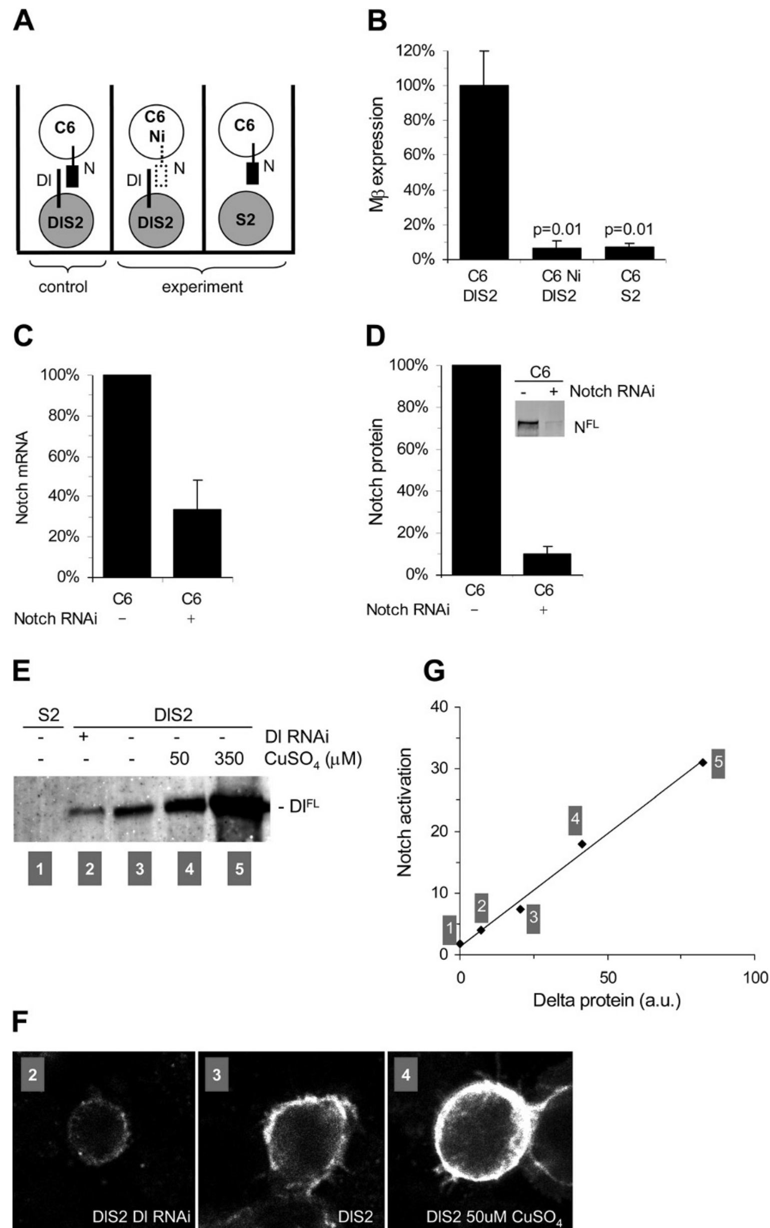

Figure 2. Notch activation in C6 cells is Notch and Delta dependent. (*A–D*) Induction of Mβ expression in C6 cells is Notch dependent. (*A*) Schematic of the experiment showing signal sending cells (gray) and signal receiving (white) cells with and without Notch RNAi (Ni) treatments. (*B*) Delta-induced Mβ expression in C6 cells is greatly reduced after the treatment with Ni. Basal level of Mβ expression (C6/S2) is shown for comparison. *p* values are with reference to C6/DlS2. (*C*) Average reduction in Notch gene expression following RNAi treatment against Notch in C6 cells. (*D*) Average reduction in Notch protein expression as measured by quantifying band densities on the Western blot. Insert shows a sample of the Western blot of cell lysates from C6 cells ± RNAi against Notch stained with 9C6 antibody against intracellular domain of Notch. Full-length Notch protein (N^{FL}). (*E–G*) Notch activation in C6 cells is Delta specific. (*E*) Western blot showing Delta expression in response to $CuSO_4$ induction. S2 cells do not express Delta and serve as a control for nonspecific stimulation. Delta expression in DlS2 cells is under the control of Cu^{2+}-inducible metallothionein promoter [23]. Since DlS2 cells express detectable level of Delta in the absence of $CuSO_4$ (lane 3), further titration can be achieved by reducing Delta expression in these cells with RNAi against Delta (lane 2). Lanes 4 and 5 show increased Delta expression using 50 μM and 350 μM $CuSO_4$. Full-length Delta (Dl^{FL}). (*F*) Immunostaining of Delta in DlS2 cells corresponding to lanes 2–4 in (*E*) showing increased amount of Delta expression on the cell surface. Non-permeabilized DlS2 cells were stained with anti-Delta antibody specific for extracellular domain (see Methods). (*G*) Effect of Delta expression levels in signal-sending cells (DlS2) on Notch activation in signal-receiving cells (C6). Y-axis indicates fold change in Delta-induced Notch activation in C6 cells relative to basal level of Notch activation in C6 cells as measured by Mβ gene expression. X-axis indicates Delta protein expression in signal-sending cells [in arbitrary units (a.u.)] as determined by quantification of both full-length and cleaved Delta from the Western blot in (*E*) (see Methods). Numbers represent signal-sending cells as indicated in (*E*): 1, S2; 2, DlS2 with Delta RNAi; 3, DlS2; 4, DlS2 stimulated with 50 μM $CuSO_4$; 5, DlS2 stimulated with 350 μM $CuSO_4$.

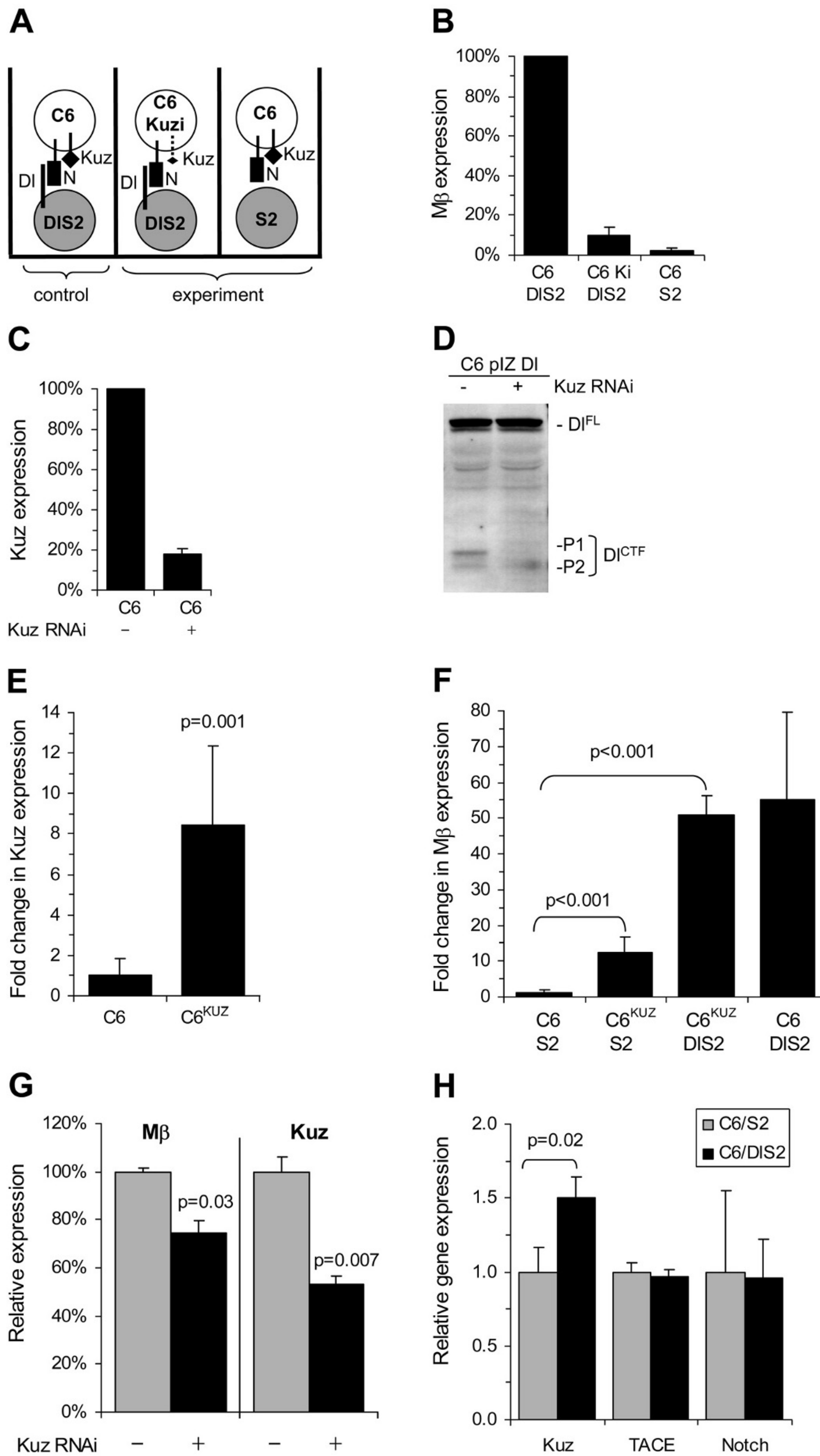

Figure 3. Notch activation in C6 cells is Kuz dependent. (*A*) Schematic of the experiment showing signal-sending cells (gray) and signal-receiving cells (white) and Kuz RNAi (Kuzi) treatments. (*B*) Average reduction in Delta-induced Notch activation in C6 cells that were treated with RNAi against Kuz as measured by Mβ expression. Basal level of Mβ expression is shown for comparison (C6/S2). (*C*) Average reduction in Kuz expression in C6 cells following treatment with RNAi against Kuz. (*D*) Validation of the effectiveness of Kuz RNAi in C6 cells. Kuz RNAi is effective in preventing the Kuz-dependent P1 cleavage of Delta. Western blot of C6 cells transiently transfected with wild-type *Drosophila* Delta (pIZ Dl) and treated with RNAi against Kuz. (*E*) Relative levels of Kuz expression in C6 and $C6^{KUZ}$ cell lines. (*F*) Effect of increase in Kuz expression on ligand-dependent and -independent Notch activation as measured by Mβ expression. (*G*) Ligand-independent activation of Notch in $C6^{KUZ}$ cells is due to increased levels of Kuz expression. Mβ expression in $C6^{KUZ}$ cells is reduced following knockdown of Kuz expression. (H) Effect of Notch activation on the expression levels of Kuz, TACE and Notch.

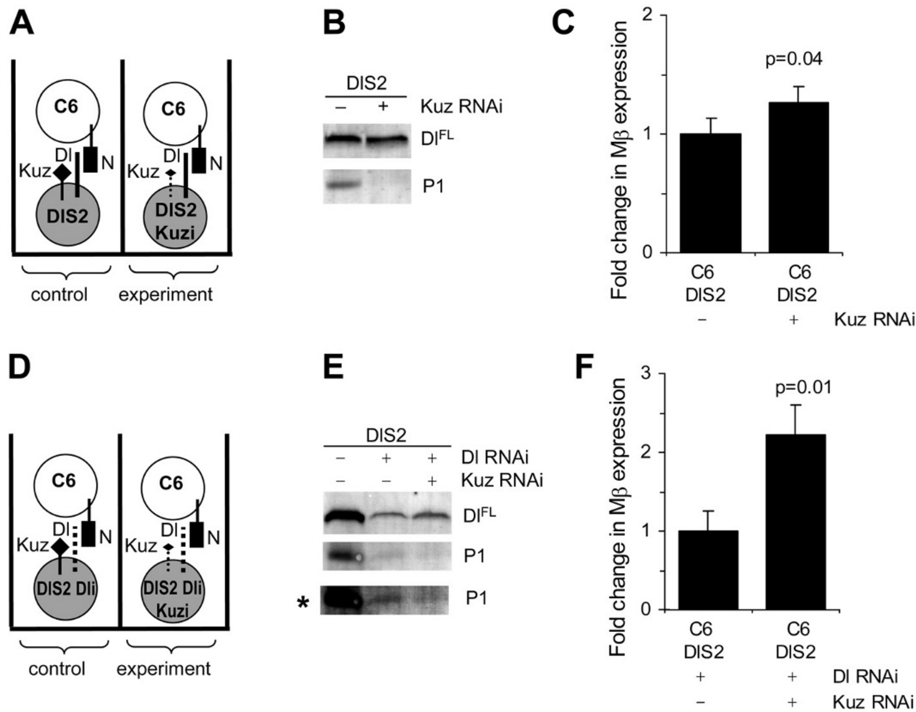

Figure 4. Kuz has minimal effect on regulating signal sending capacity of Delta. (*A*, *D*) Schematics of the experiments showing signal-sending (gray) and signal-receiving (white) cells and RNAi treatments. (*B*, *E*) Western blots showing the effect of Kuz RNAi on Delta cleavage. Asterisk in (*E*) represents overexposed version of the P1 band. (*C*, *F*) Effect of decreasing Kuz expression in signal-sending cells (DlS2) on Notch activation in signal-receiving cells (C6).

implicated in the cleavage of Notch [7, 14, 19] while also showing activity in Delta cleavage [16]. However, the contribution of TACE to Notch activation remains unclear. We first noted that TACE mRNA expression is significantly enriched in the brain of 3rd instar larvae as compared to the levels in the whole larvae and wing disks (Fig. 5A), suggesting that TACE has a specific role in nervous system development. We therefore asked whether TACE functions in regulation of Notch signaling in our *Drosophila* neuronal-derived cell culture assay. In contrast to the larval brain, C6 cells show very little TACE expression (Fig. 1D). We therefore established a stably expressing $C6^{TACE}$ cell line (see Methods), which resulted in constitutive TACE expression at levels 25-fold higher than those observed in larval brain (Fig. 5B). At this level of TACE expression, we see robust Notch cleavage (Fig. 5E) and very strong ligand-independent activation of Notch (Fig. 5C). Treatment with Notch RNAi confirms that TACE-induced Mβ activation is Notch dependent (Fig. 5D). Furthermore, removal of Kuz with Kuz RNAi from $C6^{TACE}$ cells does not affect constitutive Notch activation by TACE (data not shown), indicating that TACE does not act indirectly through Kuz, but is likely to act on Notch directly. We next attempted to obtain TACE expression levels that approximate a relevant endogenous level. Using RNAi we were able to reduce steady-state TACE expression in these cells [$C6^{TACE}$(i)] to levels approximating those found in the larval brain (Fig. 5B). We observe a 17-fold activation of Notch in $C6^{TACE}$(i) cells relative to control C6 cells, again indicating a constitutive activation of Notch by TACE (Fig. 5C). Overall, these data point to a potential role for TACE in ligand-independent activation of Notch at expression levels that approximate those seen in the developing larval CNS.

Discussion

Our *in vitro* assays of Notch activation show that in signal-receiving cells where Kuz is the predominant ADAM expressed, Notch activation is very sensitive to the level of Kuz expression. This study therefore provides molecular evidence to corroborate earlier genetic and biochemical studies that suggest that Kuz acts directly on the Notch receptor to activate it [10, 11, 14].

We also demonstrate that at basal levels of Kuz expression in C6 cells, which approach those observed in the larval brain, Notch responds robustly to Delta ligand activity in a Kuz-dependent manner. As the levels of Kuz decrease, Notch loses responsiveness to Delta stimulation. On the other hand, as the Kuz levels exceed relevant endogenous levels, Notch becomes activated without stimulation by Delta. Unlike Kuz, when TACE is expressed at levels approximating those observed in the brain, it efficiently activates Notch in a ligand-independent manner. As TACE expression exceeds endogenous levels, its ability to activate Notch becomes even more

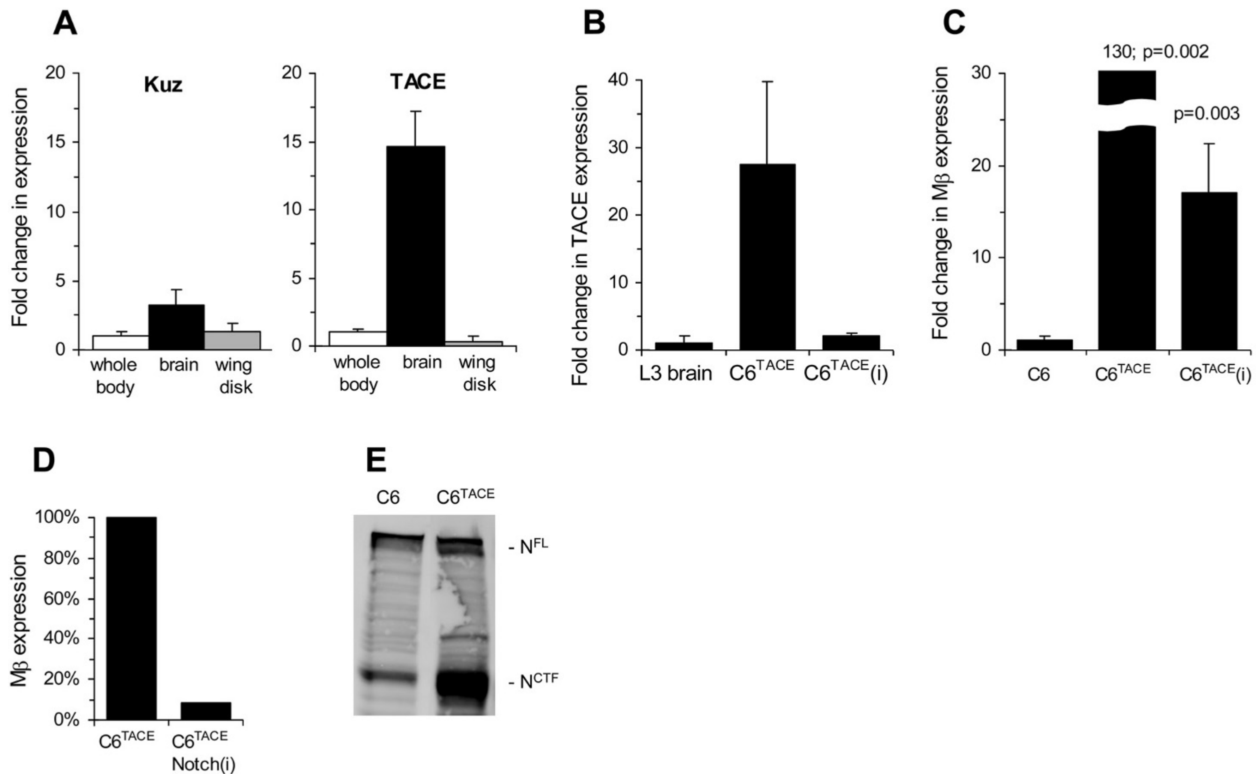

Figure 5. TACE effectively activates Notch in a ligand-independent manner. (*A*) Expression of Kuz and TACE in *Drosophila* 3rd instar (L3) larval brains and wing disks relative to whole larvae. (*B*) TACE expression in $C6^{TACE}$ cells and $C6^{TACE}$ cells treated with TACE RNAi [$C6^{TACE}$(i)] relative to larval brain. (*C*) Delta-independent Notch activation in $C6^{TACE}$ and $C6^{TACE}$(i) cells relative to basal level of Notch activation in C6 cells. *p* values are with reference to C6 cells. (*D*) TACE-induced activation of Mβ expression is Notch specific. Removal of Notch expression with RNAi against Notch in $C6^{TACE}$ cells leads to a dramatic drop in the expression of Mβ. (E) TACE promotes Notch cleavage. Western blot of cell lysates from C6 and $C6^{TACE}$ cells stained with 9C6 antibody against intracellular domain of Notch. Full-length Notch (N^{FL}) and C-terminal fragment(s) of cleaved Notch (N^{CTF}).

potent. These results suggest that in the developing larval brain, TACE has the capacity to contribute to ligand-independent activation of Notch. Altogether, the data support a mechanism whereby Notch activation and the responsiveness of Notch to Delta are intimately linked to the expression levels of Kuz and TACE. As Notch signaling is a ubiquitous pathway used for morphogenesis of a number of tissues, this mechanism is likely utilized in specific contexts during development. Two prominent examples from *Drosophila* are border cell migration in follicle development [35] and cardiogenesis [36] where Kuz plays a dominant role. Ligand-independent activation of Notch in these and other contexts remains to be investigated. Nonetheless, our data provide rationale to investigate ADAM-initiated ligand-independent Notch activation in development and in pathological states. In support of this notion elevated expression of TACE and Kuz are detected in multiple human pathologies [37–39], suggesting that Notch signaling can potentially be uncoupled from ligand in these contexts. Indeed, in pancreatic cancer, aberrant expression of a related protease, matrix metalloproteinase-7, was recently reported to instigate Notch signaling possibly by cleaving Notch in a ligand-independent manner [40].

In contrast to signal-receiving cells where Notch activation is very sensitive to the presence of Kuz, removal of Kuz activity from Delta expressing cells does not greatly change their signaling capacity. This is in contrast to the previously suggested role for Kuz to affect Delta ligand activity [15, 18]. Our conclusion is with the caveat that Delta-expressing cells in our assay system are necessarily fixed prior to presentation to Notch expressing cells. Nonetheless, we have previously reported that Kuz-dependent cleavage of Delta happens prior to Delta endocytosis [30] either on the way to the cell surface or on the cell surface itself. The simplest interpretation of our data follows from the hypothesis that full-length un-cleaved Delta at the cell surface is the signaling competent form of the ligand [18]. In this regard, since only ~30% of Delta is cleaved (this study and see [17]), removal of Kuz would yield only an approximately 1.5-fold increase in full-length ligand. Since we demonstrate a linear response of Notch signaling to Delta concentration,

it follows that a meager increase in signaling is anticipated, and indeed observed, with removal of Kuz from the signal-sending cell. One prediction from this hypothesis is that as the basal level of Delta processing increases, modulation of Kuz activity (and activity of other ADAMs that can cleave Delta) can exert greater regulatory function on the ligand signal-sending capacity. In this regard, mouse and human homologs of Delta show a greater basal level of cleavage in cultured mammalian cells (A.D. and M.D.R., unpublished observation and [41]), which may be the underlying reason that ADAM10 shows activity in regulating the Delta ligand in a mouse model [34].

Additional insight into Notch signaling comes from our observation that formalin-fixed DlS2 cells elicit Notch activation. This observation corroborates previous studies where Notch activation was achieved by the extracellular domain of Delta immobilized on beads [42] or clustered with an antibody [43, 44]. Fixation will terminate normal cell metabolism, including endocytic trafficking of cell surface proteins. The fact that "fixed" Delta elicits Notch activity is inconsistent with the current paradigm of endocytosis-dependent Delta ligand activity. Several lines of evidence indicate endocytic trafficking is required for Delta ligand activity (reviewed in [45]). One model maintains that the endocytic uptake of Delta provides the "force" necessary to pull on and dissociate the Notch heterodimer, leading to receptor cleavage and activation [46]. While our data refute this model, it remains a possibility that endocytosis, or general motility of the signal-receiving cell may provide the "mechanical" force necessary to induce Notch activation when bound to immobilized ligand. A second model points to a role for endocytic recycling of the ligand as a necessary pathway for ligand to become active [47, 48]. However, with the formalin-fixation protocol, our results are unable to lend additional insight into this latter model.

In summary, we demonstrate with a defined *in vitro* experimental model of Delta-Notch signaling that ADAM proteases predominantly act at the level of the receptor. Whereas Kuz is necessary for ligand-induced activation of Notch, it is sufficient for ligand-independent receptor activation when overexpressed. By contrast, the related ADAM, TACE, is sufficient to induce ligand-independent Notch activation when expressed at levels approaching those seen in developing brain tissue. These data demonstrate the potential for Kuz and TACE to participate in unique modes of Notch activation during development.

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