An *in vivo* screen for proteolytic switch domains that can mediate Notch activation by force

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Abstract

Notch proteins are single pass transmembrane receptors that are activated by proteolytic cleavage, allowing their cytosolic domains to function as transcription factors in the nucleus. Upon binding, Delta/Serrate/LAG-2 (DSL) ligands activate Notch by exerting a "pulling" force across the intercellular ligand/receptor bridge. This pulling force is generated by Epsin-mediated endocytosis of ligand into the signal-sending cells, and results in cleavage of the force-sensing Negative Regulatory Region (NRR) of the receptor by an ADAM10 protease [Kuzbanian (Kuz) in *Drosophila*]. Here, we have used chimeric Notch and DSL proteins to screen for other domains that can substitute for the NRR in the developing *Drosophila* wing. While many of the tested domains are either refractory to cleavage or constitutively cleaved, we identify several that mediate Notch activation in response to ligand. These NRR analogues derive from widely divergent source proteins and have strikingly different predicted structures. Yet, almost all depend on force exerted by Epsin-mediated ligand endocytosis and cleavage catalyzed by Kuz. We posit that the sequence space of protein domains that can serve as force-sensing proteolytic switches in Notch activation is unexpectedly large, a conclusion that has implications for the mechanism of target recognition by Kuz/ADAM10 proteases and is consistent with a more general role for force dependent

ADAM10 proteolysis in other cell contact-dependent signaling mechanisms. Our results also validate the screen for increasing the repertoire of proteolytic switches available for synthetic Notch (synNotch) therapies and tissue engineering.

Introduction

Many processes in biology depend on contact-dependent, juxtacrine signaling in which transmembrane ligands and receptors form intercellular bridges between "sending" and "receiving" cells. Such bridges are likely exposed to pulling forces as they undergo Clathrin-mediated endocytosis and the resulting mechanical tension across the bridge can lead to ectodomain cleavages that regulate signaling. A prototypical example is the receptor Notch, a highly conserved single pass transmembrane protein that has profound and pervasive roles in development, physiology and disease (Kovall et al. 2017; Gazave et al. 2009; Greenwald 1998). Notch is activated by single pass transmembrane ligands of the conserved Delta/Serrate/LAG-2 (DSL) family. Upon binding, DSL ligands induce cleavage and shedding of the Notch ectodomain by an ADAM10 protease tethered to the surface of the receiving cell (Lieber 2002; Pan and Rubin 1997a; Brou et al. 2000). Ectodomain shedding renders the rest of the receptor a substrate for intramembrane cleavage by γ-secretase, which allows the cytosolic domain—a transcription factor—to enter the nucleus (Kidd, Lieber, and Young 1998; Struhl and Adachi 1998; Struhl and Greenwald 1999; De Strooper et al. 1999; Lecourtois and Schweisguth 1998).

For Notch, activation depends on mechanical tension exerted across the intercellular ligand/receptor bridge by endocytosis of the ligand into the sending cell. Specifically, upon binding to Notch, DSL ligands are recruited to the Clathrin endocytic pathway by the adapter protein Epsin, and the process of ligand recruitment and/or internalization generates a pulling force across the ligand/receptor bridge that renders it subject to ADAM10 proteolysis (Gordon et al. 2015; Langridge and Struhl 2017; Wang and Struhl 2004, 2005; Overstreet, Fitch, and Fischer 2004). Both *in vivo* and biophysical studies

indicate that cleavage depends on the exposure of an otherwise buried site in a conserved, juxtamembrane “Negative Regulatory Region” (NRR) that behaves as a force sensitive proteolytic switch (Sloas et al. 2023; Gordon et al. 2015). Indeed, the key role of the NRR can be recapitulated using a well-defined, heterologous proteolytic switch, the A2 domain from Von Willebrand Factor, in place of the NRR, provided it is tuned to the appropriate force level (Langridge and Struhl 2017).

The ectodomains of many other cell surface proteins are also cleaved by ADAM10 proteases (Tosetti et al. 2021; Hayashida et al. 2010), raising the possibility that force-dependent, ADAM10 proteolysis plays a more general role in juxtacrine signaling mechanisms. In support, although many ADAM10 substrate proteins are cleaved constitutively [e.g. (Weskamp et al. 2006)], proteolysis of at least some depends on ligand [(Pan and Rubin 1997b; Janes et al. 2005a)]. However, the sheer number of ADAM10 substrates identified to date [at least 100 (Lichtenthaler, Lemberg, and Fluhrer 2018)], as well as the lack of a defined consensus cleavage site (Caescu, Jeschke, and Turk 2009), make it difficult to assess the prevalence of ADAM10-dependent force-sensitive switches in juxtacrine signaling. Moreover, the possibility that any given ADAM10-sensitive domain functions as a proteolytic switch needs to be validated by experiment. Notably, such tests have been performed in a cell culture screen of Sea urchin Enterokinase Agrin-like (SEA) domains, which have structural similarity with Notch NRRs and are found in many transmembrane receptors and other cell surface proteins (Hayward, Aird, and Gordon 2019). The capacity of SEA domains to function as NRR-like proteolytic switches in cell culture is consistent with a more general role for such domains in force-sensitive signaling events. However, it is unclear how well cell culture conditions capture the native signaling processes *in vivo*, where the relevant interactions occur between cells in polarized epithelia, and for which ligand and/or receptor endocytosis may be necessary to generate the requisite, activating force.

Here we present the results of a screen designed to identify protein domains that can function as proteolytic switches in place of the Notch NRR *in vivo*. The screen is predicated on the use of chimeric forms of *Drosophila* Notch and Delta in which the native ligand and receptor ectodomains are replaced by heterologous ligand and receptor binding domains, and productive signaling monitored *in vivo* by the induction of Notch target genes in *Drosophila* wing imaginal discs (Langridge and Struhl 2017). Of 43 chimeric Notch receptors that contain different candidate switch domains in place of the native Notch NRR, we have identified 11 that respond to ligand in an Epsin and Kuz dependent manner. Strikingly, these putative proteolytic switches derive from a wide range of protein domains and appear remarkably diverse in structure, indicating (i) that the repertoire of possible switch domains is surprisingly large, and (ii) that many different cell surface proteins have the potential to be force-dependent targets for Kuz/ADAM10 proteases. Our results also validate the screen as a means to identify new switch domains that can be used to diversify the collection of available synthetic Notch (synNotch) receptors for biomedical applications.

Results

In vivo screen for putative force sensors able to substitute for the Notch Negative Regulatory Region (NRR)

To screen protein sequences for their capacity to function as NRR-like switch domains, we generated transgenes that encode chimeric Notch receptors in which (i) the ligand-binding, EGF-repeat domain of the native receptor is replaced by the ligand-binding domain of the Follicle Stimulating Hormone Receptor (FSHR), and (ii) the juxtamembrane Negative Regulatory Region (NRR) is replaced by candidate force sensors from other proteins. All such FSHR-Notch receptors retain the transmembrane and downstream intracellular domains of the native Notch receptor (Fig. 1A). Hence, when activated, they drive well-characterized transcriptional and morphological outputs of the Notch signaling pathway. We then asked whether each of these receptors can

be activated *in vivo* by a chimeric form of Delta (Dl) in which the native ectodomain is replaced by Follicle Stimulating Hormone (FSH-Dl). Importantly, FSH is a heterodimer of distinct α and β subunits. As in our prior studies (Langridge and Struhl 2017; Langridge et al. 2022), we generate the functional FSH-Dl ligand by co-expressing the otherwise inactive FSHβ-Dl ligand together with secreted FSHα. Because virtually all of our experiments are performed in the presence of co-expressed FSHα, we refer to the FSHβ-Dl coding sequence and encoded protein—for convenience—as FSH-Dl, except when we perform negative controls in which it is expressed in the absence of FSHα.

To execute this approach at scale, we incorporated three strategies to optimize the protocols for generating chimeric FSHR-Notch receptors and testing their capacity to respond to FSH-Dl.

First, the coding sequences for a collection of FSHR-Notch chimeric receptors, each having a different protein domain in place of the native NRR, were introduced into a series of transformation vectors bar-coded by using different transgenic markers (e.g., variously deleted forms of a genomic *yellow*$^{+}$ rescuing fragment that generate different patterns of body and bristle pigmentation). This allowed us to micro-inject mixtures of several transgenes at a time, and then distinguish transformants of each transgene by phenotype in the G_1 progeny of the injected G_0 embryos, thus saving time, materials and cost (Fig 1B).

Second, we used transformation vectors designed to be compatible with our Mosaic Analysis by Promoter Swap (MAPS) technique (Langridge and Struhl 2017). This allows us to generate potential signaling interfaces between clones of FSH-Dl expressing cells and surrounding FSHR-Notch expressing tissue. Specifically, the coding sequences of the chimeric FSHR-Notch receptors were placed downstream of an *Upstream Activating Sequence* (*UAS*)-dependent promoter controlled by the yeast Gal4 transcription factor (Brand and Perrimon 1993), but separated from the promoter by a target for mitotic

recombination catalyzed by the yeast Flp recombinase [a Flp Recombination Target, FRT, symbolized by ">" (Golic 1991)]. All of the resulting *UAS>FSHR-Notch* transgenes were then introduced by PhiC31 integrase mediated transformation at the same attP genomic docking site [at cytological position 86Fb (Bischof et al. 2007)], and in the same orientation, as a *Ø>FSH-Dl* transgene that lacks a functional promoter ("*Ø*") but contains the coding sequence of FSH-Dl positioned downstream of the FRT (Langridge and Struhl 2017). Consequently, transheterozygous *UAS>FSHR-Notch/Ø>FSH-Dl* cells express only FSHR-Notch in response to Gal4. However, when subjected to heat-shock induced Flp recombinase, these cells can undergo mitotic recombination across the FRTs to generate single descendent cells in which the *UAS* promoter has, in effect, been moved from the receptor transgene to the ligand transgene, placing FSH-Dl rather than FSHR-Notch expression under Gal4 control [Fig 1C; described in detail (Langridge and Struhl 2017)]. In all experiments except negative controls, FSHα was provided by co-expression from a separate *UAS.FSHα* transgene not subject to recombination. During subsequent development, the descendants of *UAS>FSH-Dl* founding cells form clones in a surround of *UAS>FSHR-Notch* tissue.

Third, we used the *nubbin.Gal4* (*nub.Gal4*) transgene to drive the expression of the *UAS>FSHR-Notch*, *UAS>FSH-Dl* and *UAS.FSHα* transgenes in the developing wing imaginal disc. Normally, peak DSL/Notch signaling in the prospective wing is restricted to cells flanking the dorso-ventral (D/V) boundary, where it (i) induces expression of the transcription factor Cut, (ii) specifies formation of wing margin bristles, and (iii) directs local production of Wingless (Wg), a morphogen that controls wing growth. Accordingly, early heat shock induction of *UAS>FSH-Dl* clones in *UAS>FSHR-Notch/Ø>FSH-Dl* wing discs that are also *nub.Gal4* generates clonal interfaces between dedicated FSH-Dl and FSHR-Notch expressing cells. If a given FSHR-Notch chimera can be activated by FSH-Dl, these interfaces will induce ectopic Cut expression, the formation of ectopic wing margin bristles, and ectopic wing growth, all outcomes that alter the normal morphology of the adult wing (Fig 1D). Because activity of *nub.Gal4* is

limited predominantly to the developing wing, flies in which such ectopic signaling occurs are usually viable, fertile and phenotypically wild type.

To perform the screen (Fig 1D), G_0 flies derived from embryos micro-injected with each mixture of *UAS>FSHR-Notch* transgenes were outcrossed to screen their G_1 progeny for successful transgene insertions identified by the marker barcode. Females carrying each transgene were then outcrossed to "tester" mates carrying the *nub.Gal4*, *Ø>FSH-Dl*, *UAS.FSHα* and *hsp70.flp* transgenes, heat-shocked early in larval life, and the resulting transheterozygous *UAS>FSHR-Notch/Ø>FSH-Dl* adults scored for the abnormal wing phenotypes indicating FSHR-Notch activation.

In principle, *UAS>FSHR-Notch* transgenes that score positively in this assay could encode FSHR-Notch receptors that are either ligand-dependent or constitutively active. These outcomes can be readily distinguished because ligand-dependent receptors require co-expression of FSHα, as well as heat-shock induction of *UAS>FSH-Dl* expressing clones, whereas constitutively active receptors do not.

In contrast, interpretation of *UAS>FSHR-Notch* transgenes that score negatively is not as straight forward. Some may contain candidate NRR domains that simply do not function as proteolytic switches. However, others may be *bona fide* force-dependent switches but not activated in FSH/FSHR signaling assay, either because they are tuned to a sufficiently high force threshold that they cannot be activated by FSH-Dl [*e.g.*, as is the case for the native A2 domain from VWF (Langridge and Struhl 2017)] or because they are cleaved sufficiently far amino-terminal to the transmembrane domain that they are no longer substrates for transmembrane cleavage by γ-secretase (Struhl and Adachi 2000). Alternatively, they may be inadvertently truncated, or alternatively, perturbed by neighboring protein sequences so they can no longer function as proteolytic switches. As a consequence, we draw no conclusions about the chimeric receptors that test negatively, but instead, have focused our analysis exclusively on *UAS>FSHR-Notch* transgenes that yielded positive results.

We present, below, the results of a screen of 43 *UAS>FSHR-Notch* transgenes, each encoding a different candidate proteolytic switch domain.

Protein domains tested for NRR-like proteolytic switch properties

We chose to test domains from a broad class of proteins to assess the efficacy of the screen as well as the range of structures that can mediate Notch cleavage in response to force exerted across the intercellular ligand/receptor bridge by ligand endocytosis (Table 1). Kuzbanian/ADAM10 proteolysis typically targets the extracellular juxtamembrane regions of surface proteins, and the amino acid sequence cleaved varies among substrates. For this reason, we mostly tested extracellular domains and amino-acid sequences immediately N-terminal to the transmembrane domain of cell surface proteins involved in juxtamembrane signaling and did not restrict the choice to domains with particular structural features. We also included a small number of miscellaneous peptide sequences and protein domains. Domains differed in length and structural composition, ranging from juxtamembrane domains from surface receptors (e.g. from Dscam) to proteolytic switches previously identified in cell culture [SEA domains (Hayward, Aird, and Gordon 2019)], to unstructured domains (e.g., flagelliform, a spider silk protein that is a concatemer of glycine-proline-alanine repeats), to protein domains suggested by prior serendipitous findings (e.g., Venus fluorescent protein). Finally, we included a few truncated versions of the native Notch NRR and, as benchmarks, the *Drosophila* NRR together with the NRR from the *C.elegans* Notch protein, LIN-12.

Classification of candidate proteolytic switch domains based on Notch target gene expression in the wing imaginal disc

Of the 43 FSHR-Notch chimeric receptors assayed in this study, 20 scored negatively, as indicated by the failure to detect any departure from normal wing morphology in the MAPS outcross assay, whereas the remaining 23 scored positively (Table 1). Of these 23, 12 behaved as constitutively active, consistent with many ADAM10 substrate

proteins being subject to constitutive cleavage (Lichtenthaler, Lemberg, and Fluhrer 2018). However, the remaining 11 receptors fit the criteria for being ligand-dependent. Specifically, they generated severe abnormal wing morphologies when FSHβ-Dl expressing clones were generated in a background of receptor expressing cells, provided the FSHα subunit was available to form a fully functional FSH-Dl ligand (Fig 1D'). Without FSHα, the same receptors led to wildtype or near wildtype wing morphologies (Fig 1D'').

To classify *UAS>FSHR-Notch* transgenes according to their activation and to corroborate classifications based on adult wing morphology, we repeated the MAPS experiments, this time using the wing imaginal disc and examining the expression of the wing Notch target gene, *cut*, that normally responds to peak Notch signaling, as monitored by staining for Cut protein (Fig 2A). By design, both the canonical FSH-Dl and FSHR-Notch proteins have epitope tags: FSH-Dl contains a juxtamembrane Horse Radish Peroxidase (HRP) tag between the FSH domain and the transmembrane domain and FSHR-Notch contains a mCherry fluorescent protein tag between the FSHR and NRR domains. Hence, both *UAS>FSH-Dl* clones and the surrounding *UAS>FSHR-Notch* tissue can be identified with single cell resolution by monitoring HRP and/or mCherry expression, allowing signaling between the two sub-populations to be assessed by assaying Cut expression.

The 43 transgenes fell into one of three categories that could be clearly distinguished in the wing imaginal disc: *UAS>FSHR-Notch* transgenes that had (i) no ectopic Cut expression scored as refractory to cleavage, (ii) Cut expression in FSHR-Notch expressing cells not close to FSH-Dl scored as constitutive, and (iii) ectopic Cut expression largely restricted to FSHR-Notch expressing cells that abut FSH-Dl expressing cells scored as ligand-dependent (Fig. 2B).

<u>Reliance of ligand-dependent FSHR-Notch receptors on Epsin-mediated ligand endocytosis</u>

Given our prior evidence that cleavage of the native NRR depends on force exerted by Epsin-mediated ligand endocytosis (Langridge and Struhl 2017), we next asked if

ligand-dependent FSHR-Notch receptors are likewise dependent on Epsin. FSH-Dl normally depends on ubiquitination of cytosolic Lysine residues for recruitment to the Clathrin endocytic pathway by Epsin (Langridge and Struhl 2017). Hence, FSH-Dl ligands in which all the Lysines in the cytosolic domain are replaced by Arginine (FSH-Dl-KtoR) or that lack a cytosolic domain entirely (FSH-Dl-ΔIC) are unable to enter the Clathrin endocytic pathway or to activate the canonical FSHR-Notch receptor. Accordingly, to test whether the 11 ligand-dependent chimeric receptors require an Epsin endocytic "pull" to be activated by ligand, we compared their responses to the wild type, KtoR, and ΔIC forms of FSH-Dl.

To perform this comparison, we have taken advantage of our previous findings that the distance from the D/V boundary that FSH-Dl/FSHR-Notch signaling can induce ectopic Cut expression provides an indication of the strength of signaling. To normalize for variations in wing disc size, this measurement is expressed as the ratio of the distance of ectopic Cut expression from the D/V boundary relative to the width of the D/V border [Fig 3A, (Langridge and Struhl 2017; Langridge et al. 2022)]. Using this approach, we found that the 11 candidate force sensing domains fell into three classes.

The first class (5/11) behaved similarly to the native Notch NRR and contained protein domains from the receptors Deleted in Colorectal Cancer (DCC), Ephrin-type receptor B2 (EphB2) and *Drosophila* Eph (DEph), the human protocadherin FAT1, and the *Drosophila* DSL ligand Serrate (Ser). Specifically, they show a readily detectable response to FSH-Dl, but little or no response to FSH-Dl-KtoR or FSH-Dl-ΔIC, indicating a strong, if not absolute requirement for Epsin-dependent ligand endocytosis (Fig 3B). This is similar to what we have observed previously for both the native Notch NRR as well as disease-associated mutant forms of the A2 domain from Von Willebrand Factor (Langridge and Struhl 2017), consistent with these 5 domains being cleaved in response to similar force thresholds.

The second class (3/11) differs from the first in that the domains show a detectable response to FSH-Dl-KtoR and FSH-Dl-ΔIC, but one that is significantly weaker than their

response to FSH-Dl (Fig 3C). These domains came from Venus fluorescent protein (Venus), Down Syndrome Cell Adhesion Molecule (DSCAM) and Dystroglycan (DAG). Hence, they appear to be force-sensitive, but tuned to a sufficiently low force threshold that tonic mechanical tension across the intercellular ligand/receptor bridge may suffice to induce an activating cleavage. In this regard, they appear intermediate in their force tuning between the native *Drosophila* Notch NRR, which is strictly Epsin-dependent, and the *C. elegans* Notch NRR LIN-12, which is largely Epsin-independent [Fig 3A, (Langridge et al. 2022)].

The third class (3/11) of domains appears to be activated in a manner intermediate to ligand-dependent and constitutive receptors. These came from Flagelliform, ephrinB and a mutation of the fly Notch NRR (ΔLNR) that removes the Lin-12 Notch Repeats (LNR) and a portion of the heterodimerization domain (HD), but retains the native Kuz cleavage site (Fig. 4). In these cases, we find that Cut is present at peak levels in FSHR-Notch expressing cells near FSH-Dl or FSH-Dl KtoR expressing cells but at significantly lower levels further away. In the absence of ligand in the wing, ectopic Cut expression can be detected, but it is restricted to the D/V boundary, where we infer it boosts the native response above the threshold sufficient to achieve detectable Cut expression. Hence, we interpret these receptors as being hypersensitive, having low constitutive activity as well as a readily detectable response to ligand and this forms a sub-category of the ligand-dependent receptors (Table 1, chartreus).

We note that FSHR-Notch receptors carrying the different ligand-dependent domains show a wide range of responses to FSH-Dl, as monitored by the distance over which Cut can be induced away from the D/V boundary (Fig. 3). However, given (i) that transmembrane domain cleavage by γ-secretase depends on the size of the residual ectodomain stub left behind after ectodomain shedding (Struhl and Adachi 2000), and (ii) that we don't know where the different candidate domains are cleaved, we do not draw any inferences about differences in force tuning based on the spatial responses of the different FSHR-Notch receptors to FSH-Dl. Instead, we draw such inferences only

from comparing the responses of the same FSHR-Notch receptor to FSH-Dl versus FSH-Dl-KtoR or FSH-Dl-ΔIC, which we interpret as indicating the relative reliance on Epsin-dependent endocytic force.

Candidate force sensitive domains require the ADAM protease Kuzbanian for cleavage

The 11 force sensitive protein domains have little common sequence similarity and remarkable structural diversity based on Alpha Fold predicted structures (Fig S1). This opens the possibility that proteases other than ADAM10/Kuz may be involved in their activation. Hence, we tested the requirement for Kuz for activation of each of our ligand-dependent FSHR-Notch receptors. We used a more complex version of our MAPS technology to generate *UAS>FSH-Dl* clones that abut *UAS>FSHR-Notch* clones in which Kuz activity is knocked down by expression of a *UAS.Kuz-RNAi* transgene.

Briefly, expression of *UAS.Kuz-RNAi* under *nub.Gal4* control in otherwise wildtype wing discs renders Cut undetectable at the DV boundary. However, this knock-down can be rescued cell-autonomously by co-expression of a *UAS.KuzHA* transgene, which encodes an HA tagged form of the Kuz protein (Coleman et al. 2010). Hence, *UAS.KuzHA/UAS.Kuz-RNAi* transheterozygous cells are wildtype for Kuz function, whereas homozygous *UAS.Kuz-RNAi/UAS.Kuz-RNAi* clones generated by mitotic recombination have severely reduced Kuz activity. Accordingly, we generated animals that are *UAS>FSHR-Notch, UAS.Kuz-RNAi/Ø>FSH-Dl, UAS.KuzHA* in the presence of the *nub.Gal4*, *UAS.FSHα*, and *hsp70.flp* transgenes. As diagrammed in Figure 4, MAPS recombination across the FRTs in this background can generate clones of FSH-Dl expressing cells that abut clones of FSHR-Notch expressing cells that are severely deficient for Kuz, as well as FSHR-Notch expressing cells that retain Kuz activity (neighboring transheterozygous *UAS>FSHR-Notch/Ø>FSH-Dl* cells). This allows us to compare the capacity of the FSH-Dl expressing cells to induce Cut expression in abutting FSHR-Notch expressing cells depending on whether they do, or do not, retain Kuz activity (see Fig. 4 legend for details).

We applied this assay to the 8 strictly ligand-dependent FSHR-Notch receptors identified in the screen as well as the canonical and LIN-12 forms of receptor [containing the native NRR or the LIN-12 NRR; (Langridge et al. 2022); (Fig 5B)]. We have previously demonstrated that the canonical receptor requires Kuz for activation by analyzing FSHR-Notch clones homozygous for a Kuz-null allele (Langridge and Struhl 2017). For all 8 FSHR-Notch receptors, as well as the FSHR-Notch receptor containing the LIN-12 NRR, we found that FSH-Dl could induce Cut in FSHR-Notch cells that retain Kuz, but not in FSHR-Notch cells in which Kuz activity is knocked down [(Fig 5, the arrowhead indicates FSHR-Notch/Kuz knock-down cells in viable signal receiving positions and with control FSHR-Notch cells expressing Cut, arrow)].

We also examined the Kuz requirement for activation of a selection of 6 constitutively cleaved or hypersensitive FSHR-Notch receptors. In this case, we used an alternative, "twin-spot" MAPS strategy in *nub.Gal4 UAS.FSHα hsp70.flp* animals in which all cells are initially *UAS>FSHR-Notch/UAS.FSH-Dl* and either carry (experimental), or do not carry (control), the *UAS.Kuz-RNAi* transgene in cis with the *UAS>FSHR-Notch* transgene (see Fig 6A & B for genetic details). When MAPS twin spots are induced in the control (no Kuz-RNAi) version of this experiment for each of three benchmark, ligand-dependent FSHR-Notch receptors (FSHR-Notch receptors carrying the native NRR, the A2 E1638K domain, or the LIN-12 NRR; Fig. 6C, left), ectopic Cut expression was detected in *UAS>FSHR-Notch* expressing cells that abut *UAS>FSH-Dl* expressing cells. However, in the experimental (Kuz-RNAi) version of this experiment no ectopic Cut expression was detected, except in the case of clones expressing the FSHR-Notch receptor carrying the LIN-12 NRR (Fig 6C, inset). We interpret this result as due to cleavage of the unusually sensitive LIN-12 NRR by residual Kuz activity.

For the constitutive/hypersensitive receptors, the cells of interest are the *UAS>FSHR-Notch/UAS>FSH-Dl* transheterozygous cells (magenta cells, inset Fig 6C right and entire wing blade Fg 6D). In the control (no Kuz-RNAi) *UAS>FSHR-Notch/UAS>FSH-Dl* cells ectopic Cut was detected. In contrast, Cut was undetectable in the experimental,

when Kuz was knocked-down (Fig 6C and 6D), with the exception of three constitutive FSHR-Notch receptors with the E-cad, Delta or *Drosophila* ephrin (Dephrin) cleavage domains. For the E-cad domain receptor, the great majority of activation was eliminated by KuzRNAi co-expression, and the faint Cut at the D/V border may reflect residual Kuz activity. This may also be true of the Delta and Dephrin cleavage domains, although the residual activity was extensive, raising the possibility that it results from cleavage by another protease.

Hence, except for two possible exceptions, it appears that activation of all of the chimeric FSHR-Notch receptor tested, whether ligand-dependent, hypersensitive or constitutive, depends on cleavage by the Kuz protease.

Discussion/Conclusions

We present a readily scalable, *Drosophila* screen to identify protein domains that can substitute for the Negative Regulatory Region (NRR) domain of Notch to activate the receptor in response to force exerted by Epsin-mediated ligand endocytosis. In an initial proof-of-principle test of 43 candidate domains, around one quarter (11/43) have this capacity and comprise a surprisingly diverse repertoire of seemingly unrelated structural motifs. Further, in virtually every case, activation requires Kuzbanian (Kuz), the sole *Drosophila* ADAM10 protease responsible for cleaving the NRR to activate the native receptor. These results (i) validate the screen, (ii) provide insight into the structural basis and cleavage of force-sensitive proteolytic switches by ADAM10 proteases, and (iii) raise the possibility that force-sensitive signaling mechanisms may be more prevalent than currently appreciated.

A scalable screen to identify protein domains that are cleaved in response to force

In our initial screen of 43 candidate proteolytic switches, we were able to quickly and unequivocally identify around half (23/43) as capable of mediating Notch activation when introduced in place of the native, force-dependent NRR domain. Of these, 12/23 conferred constitutive activation, while the remaining 11/23 exhibited ligand-

dependence, whether stringent (8/11) or more relaxed (3/11). Importantly, the initial designations made on the basis of alterations in adult wing morphology following simple genetic crosses were validated by subsequent investigations of target gene expression in developing wing imaginal discs. Further, we successfully utilized additional genetic paradigms to quickly and unequivocally determine the requirements for Epsin-dependent ligand endocytosis and Kuz proteolysis for each of the 11 "positive" proteolytic switches. Finally, we optimized the methods used to introduce transgenes encoding different chimeric receptors, providing the potential to scale up the screen to assess hundreds rather than tens of different candidate proteolytic switches.

As noted in the Results, there are several technical limitations in the screen. These include (i) a requirement for the candidate switches to be tuned to respond to the force level exerted by Epsin-dependent ligand endocytosis, (ii) the position of the cleavage site within the switch domain (which governs the efficacy of the subsequent γ-secretase cleavage of the transmembrane domain of the receptor required for signal transduction), and (iii) the structural integrity of the candidate domain when inserted in place of the native Notch NRR. In addition, some of the candidate switch domains were chosen based on their potential to function as proteolytic switches in their normal molecular contexts, and hence do not represent a random sample. Nevertheless, we find it striking that around a quarter of the candidate domains behave as force-sensitive proteolytic switches. In our view, these results, as well as both the ease and efficiency of the methods employed, validate the screen as a means to explore the structural requirements and basic mechanisms of force-sensitive signaling mechanisms.

<u>An unexpectedly large and diverse collection of sequences can behave as force-sensitive proteolytic switches</u>

Our initial choice of candidate proteolytic switches was biased, in part, by prior evidence that other contact-dependent signaling systems aside from DSL/Notch might likewise depend on force-dependent cleavage mechanisms. These include Eph/Ephrin, Frazzled/DCC and the DSCAM family of proteins, all of which have roles in neuronal

guidance (Zang, Chaudhari, and Bashaw 2021). Some of these proteins share phenomenology with the Notch receptor, including γ-secretase cleavage following ADAM10 cleavage (Janes et al. 2005b), transendocytosis of parts of the ligand/receptor bridge from one cell to another (Greene et al. 2014; Marston, Dickinson, and Nobes 2003), and intracellular domains that may act as transcription factors (Neuhaus-Follini and Bashaw 2015; Coleman et al. 2010). Other protein domains we tested were chosen based on their structural or functional properties [(SEA domains (Hayward, Aird, and Gordon 2019)]; Flagelliform), or serendipitous observations (e.g., Venus fluorescent protein). However, regardless of the justifications for choosing the various candidate domains, those domains that tested positively for ligand, Epsin and Kuz dependent activation exhibit remarkable structural diversity as assessed by alpha-fold predictions. That we observe such diversity amongst the candidates tested in a screen suggests that the sequence space for Kuz-dependent proteolytic switches is unexpectedly large.

Two aspects of our findings merit special note: First, we assayed 6 domains that are identical to those assessed in the synNotch based SNAPS cell culture assay (Hayward, Aird, and Gordon 2019). 3/6 of these domains were not cleaved significantly in the SNAPS assay (EpCAM, KIAA0319, muc13), and in agreement, we found that these were also not active in our screen. Conversely, the remaining 3/6 SEA-domains did exhibit switch activity in the SNAPS assay, and 2/3 of these domains (from Dystroglycan (DAG) and FAT) were ligand, Epsin and Kuz dependent in our screen, whereas the remaining domain (from CDHR2) was cleaved constitutively. Hence, our findings are, for the most part, consonant with the results of the SNAPS assay, suggesting conservation of the switch properties of these domains, and posing the question of whether the DAG and FAT domains act as Kuz-dependent switches in their native signaling contexts.

Second, the 11 candidate domains that tested positively for ligand, Epsin and Kuz dependence appear to fall on a spectrum reflecting requirements for different levels of

Epsin-mediated, endocytic force. Specifically, 5/11 are strictly dependent on Epsin-mediated ligand endocytosis, as determined by the failure of modified forms of the chimeric ligand that cannot enter the Epsin pathway to activate the receptor. In contrast, 3/6 of the remaining domains are weakly activated by such defective ligands and the final 3/6 behave similarly except that they also show evidence of modest constitutive activity. Accordingly, we infer that these three classes are tuned to respond to high, moderate and low levels of ligand-dependent force, with moderately tuned receptors being able to respond, albeit weakly, to tonic mechanical tension across the ligand/receptor bridge, and weakly tuned receptors being subject to detectable cleavage even in the absence of ligand. Interestingly, this last "weak" class includes a partially deleted form of the Notch NRR that retains little else except the defined Kuz cleavage site, suggesting that the peptide sequence containing the cleavage site retains some residual capacity to function as a force-sensitive proteolytic switch. Significantly, these results contrast with our previous analysis of the native NRR from the *C. elegans* Notch protein LIN-12 (Langridge et al. 2022). In this case, the receptor can be strongly activated by ligand, whether or not it has access to the Epsin pathway, but is devoid of activity in the absence of ligand. We suggest that the LIN-12 NRR comprises a special class of proteolytic switches that has been evolutionarily selected to respond non-linearly, such that it can be activated even in absence of Epsin-dependent endocytic force while remaining strictly ligand dependent.

Insight into the broad promiscuity of the ADAM10/Kuz protease

We have determined that all of the candidate domains that depend on ligand, as well as almost all that are constitutively active, require Kuz to mediate activation of the receptor. This is despite the diverse source of the candidate domains (some of which, like Venus fluorescent protein, are not known substrates of Kuz) and their striking sequence and structural differences. The rules governing the vulnerability of a substrate to ADAM10 cleavage are unknown, but our study shows that in the context

of chimeric Notch they are unexpectedly lenient and the substrate sequence space large.

Possible force dependent cleavage events in signaling pathways besides Notch

A motivation for screening for new NRR-like proteolytic switches that depend on force and Kuz is the conjecture that force-dependent Kuz cleavage may apply to many different contact-dependent signaling interactions between cells, aside from the few examples like DSL/Notch signaling that we currently know. The success of this initial screen, limited as it is, in identifying a diverse repertoire of such switches is consistent with this possibility. Indeed, one clear implication is that it will be difficult, if not impossible, to anticipate the presence of such a switch in any given signaling protein by sequence or structural properties. Instead, identification will depend on functional tests, ideally *in vivo*.

We note that force-sensitive proteolytic switches may not be restricted to receptors, as one of the force sensitive switch domains we identified in the screen derives from the juxtamembrane domain of the Notch ligand Serrate. We have previously shown that in certain circumstances, productive signaling between chimeric forms of Delta and Notch can be aborted by receptor-mediated, trans-endocytosis of the entire ligand from the signal-sending cell into the signal-receiving cell. Force-dependent cleavage of Serrate bound to Notch could provide another means to abort signaling across an intercellular Serrate/Notch bridge.

Increasing the repertoire of proteolytic switches for synthetic Notch biomedical applications.

Synthetic Notch (synNotch) technologies employing chimeric ligand and receptors to generate new, heterologous signaling systems offer significant potential for disease therapies and tissue engineering (Morsut et al. 2016; Roybal et al. 2016). For future purposes, it will be desirable to have synNotch receptors with distinct characteristics that combat common problems, such as receptor leakiness or lack of potency, or that

are optimized for certain environments and applications, e.g. receptor activation within epithelia vs lymphocytes. The cleavage domains identified in this study may well impart useful characteristics on synNotch activation, such as a distinct force tuning or signal to noise ratios, as has recently been found using truncations of the NRR and heterologous cleavage domains in the context of the SNIPR synNotch receptor (Zhu et al. 2022). As the screen we have devised is both effective and scalable, it should make it possible to identify new proteolytic switches that can be used to optimize and diversify the current repertoire of synNotch signaling systems. Finally, as there is a gap between the development of synthetic tools and their clinical application (Brooks and Alper 2021), we believe our use of a model organism to test synthetic tools represents an effective general approach to accelerating progress in the synthetic biology field.

Acknowledgements

Thanks to Dominic Huntley, Olivia Mosley, Simran Bhikot, Abby Bryant, and Corey Andrews for technical assistance. Research reported in this publication was supported by the National Institutes of Health through the Institute of General Medicine of the under award number R35 GM127141 (to G.S) and the Institute of Child Health and Human Development under award number R21 HD107414 (to P.L.), in addition to support from the Roy and Diana Vagelos Precision Medicine Award (G.S., P.L.) and the Research Scholarship & Creativity Activity Program at Augusta University (to P.L.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Methods

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Paul Langridge (plangridge@augusta.edu).

Drosophila

All flies were maintained at 25°C on standard corn food. Fly stocks were maintained at

18°C, whilst experiments were performed at 25°C.

Drosophila Transgenes

All ligand and receptor coding sequences, with the exception of FSHα, were inserted into a modified form of *pUAST-attB* (www.flyc31.org) that contains a single Flp Recombinase Target (FRT, '>') positioned between the UAS promoter and the coding sequence, and the resulting *UAS>ligand* and *UAS>receptor* transgenes were introduced at a single genomic docking site, *attP-86Fb* located on the right arm of the third chromosome, oriented so that the promoter is centromere proximal to the coding sequence. A "no promoter" (Ø) element encoding the transcriptional terminating 3'UTR of the *hsp70* gene is used in place of the UAS promoter in *Ø>ligand* transgenes (generated by Flp/FRT mitotic recombination in the male germline (Langridge and Struhl 2017)). A single *UAS.FSHα,* transgene inserted by conventional P-element mediated transformation onto the X chromosome was used in all experiments.

The WT, KtoR, and ΔIC chimeric forms of Dl (Fig. 1) are described in detail in (Langridge and Struhl 2017). Briefly, the native extracellular domain of Dl was replaced in its entirety by the β subunit of human Follicle Stimulation Hormone (FSHβ) (Fan and Hendrickson 2005) and an HRP tag was inserted immediately downstream of the C-terminal YCSFGEMKE sequence of FSHβ and immediately upstream of the Dl transmembrane domain. In all experiments, FSHα was co-expressed with the FSHβ-Dl protein to reconstitute FSH, which is an FSHα/FSHβ heterodimer (for simplicity, we refer to all ligands as FSH-Dl chimeras and do not include the HRP tag in their designations).

The chimeric forms of Notch (Fig 1) are based on the FSHR-Notch receptor described in detail (Langridge and Struhl 2017). Briefly the amino-terminal Epidermal Growth Factor (EGF) Repeat containing portion of the native extracellular domain of N was replaced by the ectodomain of FSHR. The extracellular domain was tagged by the insertion of mCherry just upstream of the juxtamembrane NRR domain.

For the screen, the native NRR was replaced with the coding sequence of the domain of interest. In each case, the same restriction enzyme sites and junction sequences was used. The sequence of the NRR insertion position is between the upstream mCherry epitope tag and the downstream Notch TM domain are as follows: delyk. . .rppanvkyvit. The sequence of each of the ligand dependent and constitutive force sensors is given in Supplementary Table 1. The *UAS KuzHA* transgene and the *UAS KuzRNAi* originate from (Coleman et al. 2010). Complete DNA sequences for both the ligands and receptors are available on request.

Analysis of signaling between dedicated ligand and receptor expressing cells.

Signaling between dedicated ligand and receptor cells was analyzed using Mosaic Analysis by Promoter Swap (MAPS; see (Langridge and Struhl 2017) for detailed description). In essence, mitotic recombination across the FRTs in cells trans-heterozygous for *UAS*> and Ø> transgenes is induced in the presence of a *nub.Gal4* driver that acts in the developing wing. Upon the appropriate segregation of the recombined chromatid arms at the 4-strand stage, this generates mutually exclusive populations of ligand and receptor expressing cells; the ligand expressing cells are marked by staining for the HRP epitope tag (blue in all the Figures), and the receptor expressing cells are marked "black" by the absence ligand expression (and when needed, red, by the mCherry epitope tag on the receptor). Signaling is monitored by assaying for ectopic expression of Cut protein from the Notch target gene *cut* in receptor expressing cells that abut ligand expressing cells along the MAPS ligand/receptor mosaic interface.

To induce mosaics by promoter swap, first or second instar larvae of the appropriate genotype (*hsp70.flp UAS.FSHα/hsp70.flp* or *Y*; *nub.Gal4/+; Ø*>*ligand/UAS*>receptor or *UAS*>*ligand/Ø*>*receptor*) were heat shocked at 36°C for one hour and wing discs from mature third instar larvae were dissected, fixed, stained for Cut and HRP expression and mounted for confocal microscopy [commercially available Mouse monoclonal anti-Cut (Mab Developmental

Studies Hybridoma bank 2B10, 1/100), and Rabbit anti-HRP (Abcam ab34885, 1/1000) antisera were used in combination with Alexa 488 and 633 conjugated labelled secondary antisera following standard protocols, e.g., as in(Langridge and Struhl 2017)]. The mCherry epitope tag was also readily visualized in these preparations, although only shown in Fig 5 and 6 (both the HA and HRP epitopes require rabbit monoclonal antisera for detection, precluding our use of the anti-HRP antisera to mark the "ligand" clones in this context). In general, the intensity of the mCherry signal was similar for all forms of the receptors analyzed and any variation did correlate with potency of receptor activation. Expression levels of the receptor were not further considered since we compared only relative levels of cleavage of the same receptor presented with different ligands. It is also possible that receptors scored refractory to cleavage were not strongly expressed, although that was not the case for those examined in more detail, including FSHR-Notch with the ΔNRR.

Quantification and Statistical Analysis

In all *Drosophila* MAPS experiments, most if not all the imaginal wing discs contained several mutually exclusive subpopulations of ligand and receptor expressing cells within each wing primordium. In all cases, the results of each ligand/receptor combination are presented by representative images as well as quantitative analysis. As noted in the main text, Cut expression is normally induced in "border" cells flanking the D/V compartment boundary in response to native DSL/Notch signaling which peaks in the vicinity of the boundary and declines in a graded fashion as a function of distance from the boundary (Blair 1997). Signaling between FSH-Dl and FSHR-Notch chimeras along MAPS mosaic borders located away from the D/V boundary induces Cut when the combined inputs of the native and chimeric ligand/receptor interactions reach the levels normally required to induce Cut in D/V border cells. Accordingly, the distances over which MAPS mosaic borders can induce Cut away from the boundary provide an indication of the strength of the signaling interaction between the chimeric ligand and

receptor.

As depicted in Figure 3A, we measured and averaged the distances from the D/V boundary over which ectopic Cut was detected along 1-3 MAPS mosaic borders in each of typically 10 or more discs for each genotype (power analysis>0.95, the numbers of discs analyzed for each ligand/receptor pair are presented in Fig 3 and 4). As disc size varies at least in part because of extra growth induced by ectopic Notch activation, we normalized the average of the distances obtained in each disc by dividing it by the width of the D/V compartment boundary within the prospective wing (the "Cut distance/DV width ratio"; Fig. 3A, y-axis).

Quantitation of the resulting ratios for each ligand/receptor pair are presented in Figures 3 and 4 in the form of boxplots in which each point depicts the average of the ratios measured for a single disc, and the mean, median, and 25-75% interquartile ranges of the ratios are depicted by a red triangle, a wide horizontal line, and the tops and bottoms of each box, respectively. Statistical comparisons of the results obtained for different ligand/receptor pairs were performed by Wilcoxon t-tests using *ggstatsplot* software (https://www.rstudio.com/).

Colabfold Structural Predictions

For the computational analysis of protein structures, the open-source software ColabFold(Nussinov et al. 2022) was employed (versions 1.3.0 and 1.5.2). The notebook "ColabFold: AlphaFold2 using MMseqs2" was used through the Google Collaboratory platform to run AlphaFold with the following parameters: use_templates = false, use_amber = true, msa_mode = "MMseqs2 (UniRef + Environmental)", model_type = "AlphaFold2-ptm", num_models = 5, model_order = [1, 2, 3, 4, 5], num_recycles = 6, rank_by = "plddt," max_msa = null, pair_mode = "unpaired + paired".

The amino acid sequences of the ligand-dependent domains of interest, were input to ColabFold for structure prediction using the specified parameters. No templates were used in the predictions. The AlphaFold algorithm generates five different structural models for each

individual input. The models are ranked from best to worst based on the overall predicted Local Distance Difference Test (pLDDT), AlphaFold's per-residue confidence metric. Along with the structural models, the Predicted Aligned Error (PAE) of each model is visualized on a matrix. This metric implies a measure of confidence in the relative positions of residue pairs. The best confidence model prediction was chosen in each case and displayed in Figure S1.

Figure Legends

Figure 1. Functional screen to identify force-sensitive cleavage domains *in vivo*.

A. Cartoons of *Drosophila* Delta (Dl)/Notch (N) and FSH-Dl/FSHR-Notch chimeras in which the ligand binding regions of Delta/N were replaced with functional heterologous binding regions. The Dl extracellular domain was replaced by the β-subunit of Follicle Stimulating Hormone (FSHβ) and coexpression of FSHα reconstitutes the composite FSH ligand (FSH). Reciprocally, the ligand-binding (EGF) portion of the N ectodomain was replaced by the FSH receptor ectodomain (FSHR).

B. The canonical FSHR-Notch receptor was modified by replacing the NRR with a single candidate sequence to produce 43 different FSHR-Notch receptors each with distinct juxtamembrane regions. For the screen FSH-Dl was used, but for later analyses FSH-Dl was modified by replacing the Delta intracellular domain (ICD) that targets FSH-Dl into the Epsin route with ICDs that exclude the ligand from that route, either through mutation of the lysines to arginine (FSH-Dl-KtoR) or removal of the intracellular domain entirely (FSH-Dl-ΔIC).

The coding sequence for each receptor was placed downstream of a UAS promoter followed by an FRT (>) recombination target sequence to form the *UAS>FSHR-Notch* transgenes, each of which was part of a vector with one or two of several eye (w+, Rap rough eye phenotype), wing (y+), bristle (y+) or cuticle color (y+) marker genes. Mixtures of *UAS>FSHR-Notch* transgenes with distinct markers were introduced at identical genomic docking sites (86Fb) by a single micro-injection. The FSH-Dl coding

sequence was placed downstream of an FRT, but no promoter (Ø), to form *Ø>FSHβ-Dl*, and inserted at the 86Fb genomic docking site.

C. To form FSH-Dl to FSHR-Notch signaling interfaces across which receptor activation can be assessed, Mosaic Analysis by Promoter Swap (MAPS) was used to induce mitotic recombination (red cross) in transheterozygous *UAS>FSHR-Notch/Ø>FSHβ-Dl* flies at an early stage of development, resulting in daughter cells that have swapped the promoter position to produce *UAS>FSHβ-Dl* and FSH-Dl expression in FSH-Dl cells adjacent to parental or recombinant *UAS>FSHR-Notch* cells (FSHR-Notch cells).

D. To assess FSHR-Notch cleavage most efficiently, G0 female flies transformed with a FSHR-Notch mixture were crossed with male flies from a ligand tester stock with the components for both UAS/Gal4 expression in the wing and the FSH-Dl ligand: *UAS FSHα* on the X chromosome, the nubbin-Gal4 driver on the second chromosome, and the *Ø>FSHβ-Dl* transgene. FSH-Dl/FSHR-Notch interfaces were induced by MAPS (see C) and receptor activation categorized by examining adult wing phenotypes resulting from the ectopic activation of Notch target genes. Ligand-dependent FSHR-Notch activation in the female G1 flies leads to ectopic wing margin (**D'**, left), whilst males have a wildtype wing (**D''**, left) since they do not inherit the X chromosome carrying the *UAS FSHα* trangene and do not express functional FSH-Dl. In contrast, constitutive FSHR-Notch activation does not require FSH-Dl, and as a result male G1 offspring have an abnormal wing phenotype, although to different degrees according to the extent of activation (**D''**, right). No receptor activation simply produces wildtype adult wings in the female G1 offspring (**D'**, right).

Figure 2. Three categories of receptor activation in the third instar imaginal wing disc.

A. MAPS generates mutually exclusive FSH-Dl/FSHR-Notch cell populations in the wing imaginal disc. The prospective wing (blue/black) is divided into cells expressing either FSH-Dl (the HRP epitope tag of FSH-Dl is stained blue) or receptor under Gal4/UAS control. Normally, peak levels of native DSL/N signaling induce expression of the N

target gene *cut* along the boundary between the dorsal (D) and ventral (V) compartments (Cut protein, yellow).

B. Patterns of receptor activation in the wing were divided into one of three main categories. Left, no cleavage of the candidate domain. Clones of FSH-Dl do not produce ectopic Cut at the interface with FSHR-Notch cells. Native Cut remains at the D/V boundary. Center, constitutive cleavage allows FSHR-Notch activation regardless of the presence of ligand, resulting in ectopic Cut produced in all FSHR-Notch cells whether they are close to FSH-Dl cells or not. Right, ligand-dependent activation induces cleavage of FSHR-Notch and ectopic Cut is produced only FSHR-Notch cells adjacent to FSH-Dl cells.

Table 1. List of domains in the ligand-dependent and constitutive categories. Columns describe the domain's abbreviated name, the identity of the native source protein and species, the size of the domain (#AA=number of amino acids), a brief description of the native source protein and the reason for its inclusion in the screen.

A. Table of candidate domains cleaved in response to ligand (green), including domains classed as 'hypersensitive' (chartreuse), i.e. intermediate in response between ligand dependent and constitutive. Note that FAT1 and DAG were identified as cleaved in response to ligand as part of the cell culture based SNAPs assay (Hayward, Aird, and Gordon 2019).

B. Table of candidate domains cleaved constitutively. Cdhr2 is cleaved constitutively, although it is reported as ligand-dependent in the SNAPs assay (Hayward, Aird, and Gordon 2019).

Domains that were not cleaved include FSHR-Notch lacking the NRR, EprinA2, *Drosophila* Fat, Filamin, Neuregulin, Starry Night, Dachsous, Junctional adhesion molecule A, prion related protein, human DSCAM, viral spike proteins, epithelial cellular adhesion molecule, KIAA0319, and Muc13. The last three domains were found not to be cleaved in the SNAPs assay (Hayward, Aird, and Gordon 2019).

Figure 3. Dependence of ligand-dependent receptors on Epsin-mediated ligand endocytosis.

A. Quantitation of signaling between FSH-Dl and FSHR-Notch receptors carrying domains cleaved in response to ligand. This was measured as the distance ectopic Cut extends away from the DV boundary relative to disc size (width of Cut expression at the DV boundary) and presented as a ratio of the two distances (left). Signal strength is depicted as boxplots (right) that show the "cut distance/DV width" ratio for each disc as a point, as well as the mean (cross), median (horizontal line), and 25%-75% interquartile ranges (tops and bottoms of the box) for each receptor/ligand combination. Statistical comparisons of the average Cut distance/DV width ratios for each ligand/receptor pair were performed by Wilcoxon tests (see methods); *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$. Each receptor was confronted with three different FSH-Dl variants (Fig 1A) with intracellular domains corresponding to the wildtype Dl domain (WT), the KtoR mutated Dl domain (KtoR) and a deletion of the intracellular domain (ΔIC). Representative images of these responses are shown on the far right.

As benchmarks, the response to these ligands of the canonical FSHR-Notch receptor with the *Drosophila* NRR (NRR) and FSHR-Notch with the LIN-12 NRR (LIN-12) is shown. The NRR receptor is strictly Epsin-dependent and does not show a significant response to versions of FSH-Dl that are excluded from the Epsin-route, indicating a high force tuning. In contrast, LIN-12 responds strongly to all three FSH-Dl ligands, indicating a low force tuning.

B. Receptors with a high dependence on Epsin-mediated ligand endocytosis (Class I) have no response to ligand excluded from the Epsin route, similar to the response of the canonical FSHR-Notch with the *Drosophila* NRR.

C. Receptors with a lower dependence on Epsin-mediated ligand endocytosis (Class II) have small but detectable responses to ligand excluded from the Epsin route.

Figure 4. Hypersensitive FSHR-Notch receptors are cleaved constitutively, and activation is enhanced by FSH-Dl on neighboring cells.

A. Quantitation of signaling between FSH-Dl and FSHR-Notch 'hypersensitive' variants as in Fig 3A.

B. Representative images showing Cut expression in MAPS mosaic wing discs for hypersensitive FSHR-Notch receptors paired with either FSH-Dl or FSH-Dl KtoR. Significant ectopic Cut is detectable in cells away from FSH-Dl cells (arrowhead, right image), but extends furthest from the DV boundary where the receptor cells meet ligand cells (arrow), quantified in A.

Figure 5. The ADAM protease Kuzbanian is required for the ligand-dependent cleavage of multiple distinct sequences.

A. Mitotic recombination using MAPS to remove detectable Kuz activity in sub-populations of FSHR-Notch cells. The expression of *UAS Kuz RNAi* (Kuz RNAi) in the wing disc reduces Notch activation sufficiently to abolish endogenous Cut at the DV boundary, whilst co-expression of *UAS KuzHA* restores Cut expression. *UAS Kuz RNAi* was positioned distal to the *UAS>FSHR-Notch* transgene and *UAS KuzHA* distal to the *Ø>FSHβ-Dl* transgene. The outcomes of mitotic recombination early in larval development include FSHR-Notch cells with functional Kuz (*UAS>FSHR-Notch, UAS Kuz RNAi / Ø>FSHβ-Dl*, *UAS KuzHA*) alongside FSHR-Notch cells with Kuz knocked-down (*UAS>FSHR-Notch, UAS Kuz RNAi / UAS>FSHR-Notch, UAS Kuz RNAi*) confronting FSH-Dl signal sending cells (either *UAS>FSHβ-Dl*, *UAS KuzHA/Ø>FSHR-Notch, UAS Kuz RNAi* or *UAS>FSHβ-Dl*, *UAS KuzHA/Ø>FSHβ-Dl*, *UAS KuzHA* each of which has equivalent signal sending ability). The capacity of the control and Kuz knock-down populations of FSHR-Notch cells to receive FSH-Dl signals can therefore be assessed side-by-side in the same region of the wing disc.

B. For each *UAS>FSHR-Notch* transgene, Cut expression was detected within control FSHR-Notch cells with UAS KuzHA and abut FSH-Dl cells (arrow). In the same wing, neighboring Kuz knock-down FSHR-Notch cells that express Kuz RNAi and not KuzHA

(outlined in yellow) do not express Cut where they are adjacent to FSH-Dl cells (arrowhead), indicating that receptor cleavage in each case requires Kuz. Note that where Kuz knock-down cells cross the D/V boundary they abolish endogenous Cut as expected.

Figure 6. The ADAM protease Kuzbanian is required for hypersensitive and constitutive cleavage of multiple distinct sequences

A. Mitotic recombination using MAPS to remove detectable Kuz activity in all FSHR-Notch cells for examination of ligand-dependent benchmarks and the hypersensitive receptors. Left: Control genetic scheme using the 'twin spot' MAPS paradigm, with a *UAS>FSHR-Notch/UAS>FSHβ-Dl* parental cell and twin spot daughter cells homozygous for either *UAS>FSHR-Notch* or *UAS>FSHβ-Dl*. To knock-down Kuz in all cells other than the *UAS>FSHβ-Dl* twin spots, *UAS Kuz RNAi* (Kuz RNAi) is placed distal to *UAS>FSHR-Notch.* The extent of ectopic Cut is assessed by comparing ectopic Cut in the control wing disc to ectopic Cut produced in the Kuz knock-down wing disc.

B. Genetic scheme for removing Kuz in transheterozygous *UAS>FSHR-Notch/UAS>FSHβ-Dl* within which the activity of constitutive receptors can be assessed. For convenience, *UAS Kuz RNAi* is distal to the ligand transgene and clones are not induced.

C. For FSHR-Notch benchmark receptors that are activated in response to ligand (left), ectopic Cut is produced where homozygous *UAS>FSHR-Notch* cells meet homozygous *UAS>FSHβ-Dl* or *UAS>FSHR-Notch/UAS>FSHβ-Dl* signal sending cells. In the Kuz knock-down wing disc, native Cut expression at the D/V boundary is undetectable and each receptor does not produce ectopic Cut within *UAS>FSHR-Notch/UAS>FSHβ-Dl* cells that meet ligand cells. In one case, FSHR-Notch with the LIN-12 NRR, Cut is produced in homozygous *UAS>FSHR-Notch* cells adjacent to ligand cells (inset).

Control wing discs expressing the hypersensitive receptors show extensive Cut activation in most FSHR-Notch cells, but this is eliminated in *UAS>FSHR-Notch/UAS>FSHβ-Dl* cells with Kuz knocked-down. Residual constitutive activation is

detectable in the case of homozygous *UAS>FSHR-Notch* cells in the case of FSHR-Notch with the Flagelliform domains (arrow).

D. Control wing discs expressing the constitutive receptors have extensive Cut activation in heterozygous *UAS>FSHR-Notch/UAS>FSHβ-Dl* cells close to the DV border. Ectopic Cut in these cells is reduced or eliminated when Kuz is knocked-down, with the exception of the FSHR-Notch receptors with the Delta and Dephrin domains.

Figure S1. Structural predictions of each of the candidate domains cleaved in response to ligand.

A. High confidence structural predictions of domains showing ligand-dependent cleavage. The 3D structure of the entire extracellular region of canonical FSHR-Notch was predicted using alphafold (collabfold, see materials and methods).

B. This prediction was repeated for receptors with each of the candidate domains, and in each image only the candidate domain is shown and the remaining receptor hidden. Each candidate has a distinct structure with few commonalities. PDB files for each case are available.

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Figure 1: Functional screen to identify force-sensitive cleavage domains *in vivo*

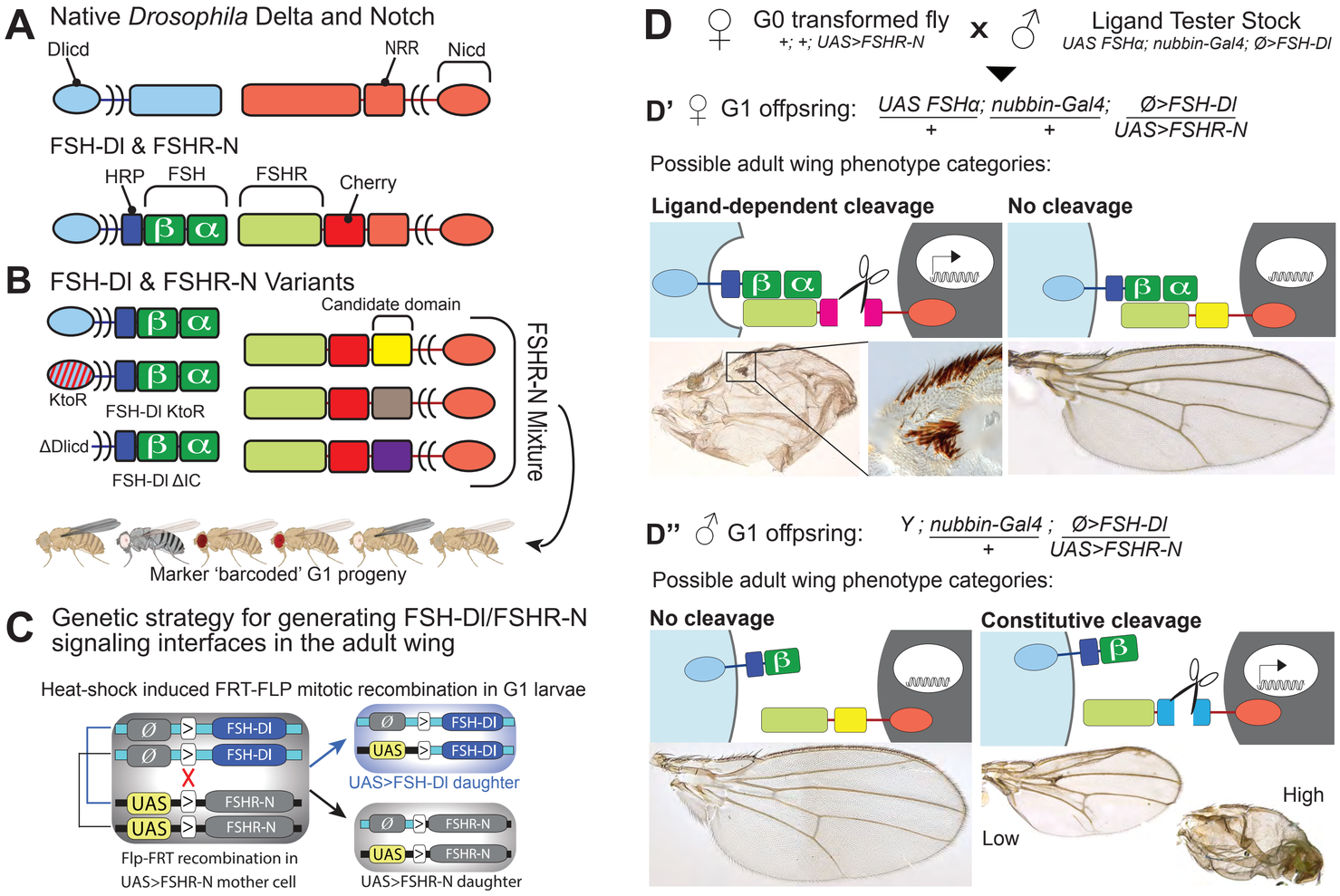


Table 1 List of cleaved candidate domains

A Candidate domains cleaved in response to ligand

Name	Domain Source	Species	Size (#AA)	Description	Reason for inclusion in screen
Dscam1	Down Syndrome Cell Adhesion Molecule 1	*Drosophila*	220	Neuronal guidance receptor	Evidence that ICD acts as a transcription factor
DCC	Deleted in Colorectal Cancer/ Frazzled	*Drosophila*	18	Neuronal guidance receptor	Evidence that ICD acts as a transcription factor
EphB2	Ephrin-type receptor B2	Mouse	110	Neuronal guidance receptor	Native receptor is cleaved in response to ligand binding
DEph	Ephrin-type receptor	*Drosophila*	250	Neuronal guidance receptor	Native receptor is cleaved in response to ligand binding
Venus GFP	Venus GFP	*Aequorea victoria*	242	Entire coding region of Venus GFP	Serendipitous result
Serrate	Serrate	*Drosophila*	264	Notch ligand	Cleaved by Kuz
FAT1	FAT1	Human	93	Atypical cadherin	SNAPS: Cleavage in response to ligand
DAG	Dystroglycan	Human	261	Cell adhesion and ECM binding protein	SNAPS: Cleavage in response to ligand
$NRR^{\Delta LNR}$	Notch variant	*Drosophila*	62	Variant of the NRR of Notch	LNRs removed, but S2 site remains
ephrinB	Ephrin B1 precursor ligand	Human	74	Neuronal guidance protein	Native ligand is cleaved in response to ligand binding
Flagelliform	Flagelliform F40	Spider	44	Spider silk – 40 PGGAG repeats	Biophysically demonstrated to stretch at pN forces

B Candidate domains cleaved constitutively

Name	Domain Source	Species	Size (#AA)	Description	Reason for inclusion in screen
Dephrin	Ephrin B ligand	*Drosophila*	211	Neuronal guidance protein	Native ligand is cleaved in response to ligand binding
Robo	Roundabout 1	*Drosophila*	371	Neuronal guidance receptor	DCC/Frazzled related protein
Nrg1	Neuregulin (Nrg1 type III)	Human	101	Neuronal guidance protein	ADAM10 substrate
Neogenin	Neogenin	Mouse	367	Neuronal guidance receptor	ADAM10 substrate
Eprhin B2L	Ephrin B2	Mouse	342	Neuronal guidance protein, juxtamembrane region and 2 fibronectin domains	Cleavage in response to receptor binding
Ephrin B2S	Ephrin B2	Mouse	110	Neuronal guidance protein, juxtamembrane region	Cleavage in response to receptor binding
EphB1	Ephrin B1 receptor	Human	124	Neuronal guidance receptor	Receptor cleavage in response to ligand binding
Delta	Delta	*Drosophila*	34	Notch ligand	Cleaved by Kuz
APP	Amyloid-beta precursor protein	Human	240	Neuronal repair	ADAM10 substrate
CD16	CD16	Human	194	Immune cell receptor	ADAM10 substrate
CD44	CD44	Mouse	461	Cell adhesion glycoprotein	ADAM10 substrate
Cdhr2	Cadherin-related family member 2	Human	356	Cell adhesion protein	SNAPS: Cleavage in response to ligand

Figure 2: Three categories of receptor activation in the third instar imaginal wing disc

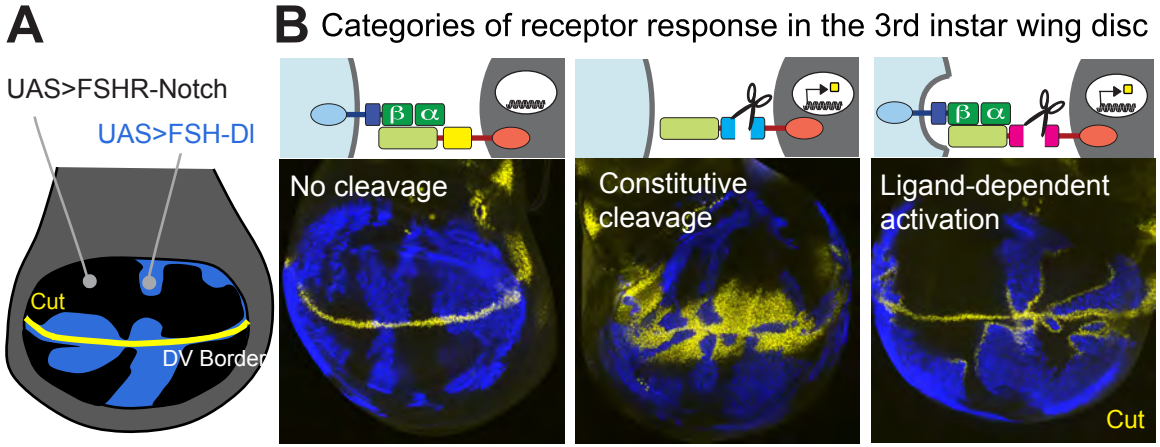
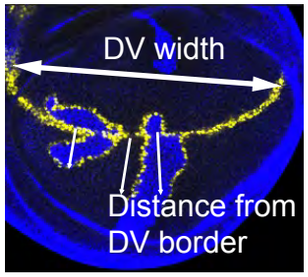
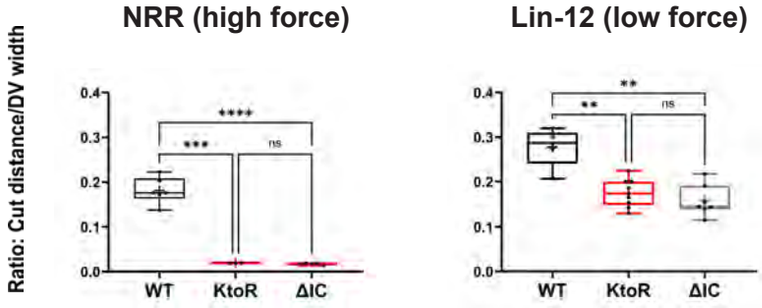
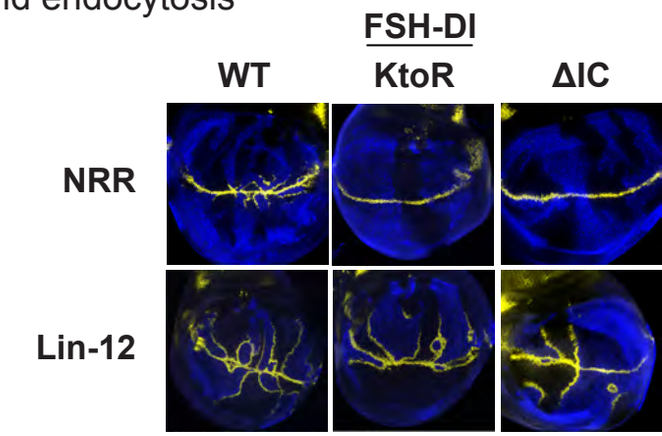


Figure 3: Dependence of ligand-dependent receptors on Epsin-mediated ligand endocytosis

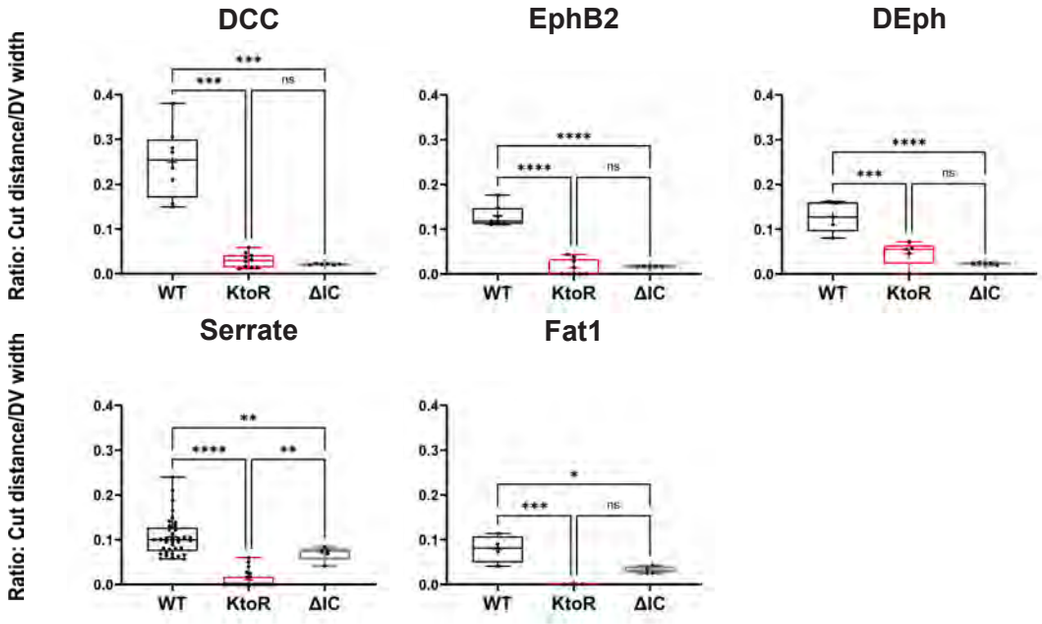
A Measurement of ectopic Cut and FHSR-Notch benchmark receptors

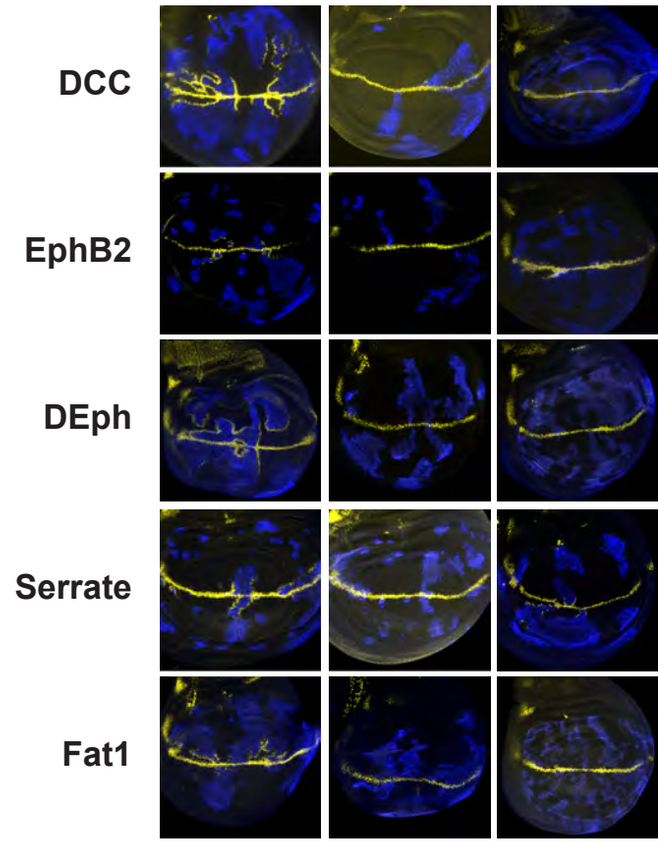




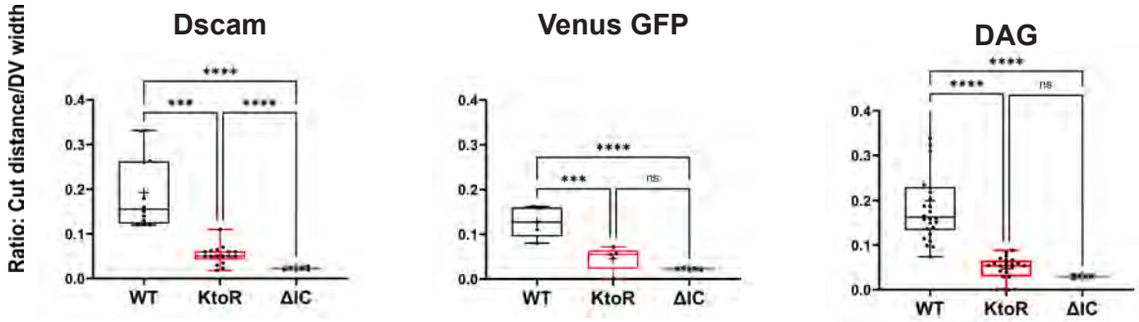


B Class I: Force sensing domains with high force tuning





C Class II: Force sensing domains with lower force tuning



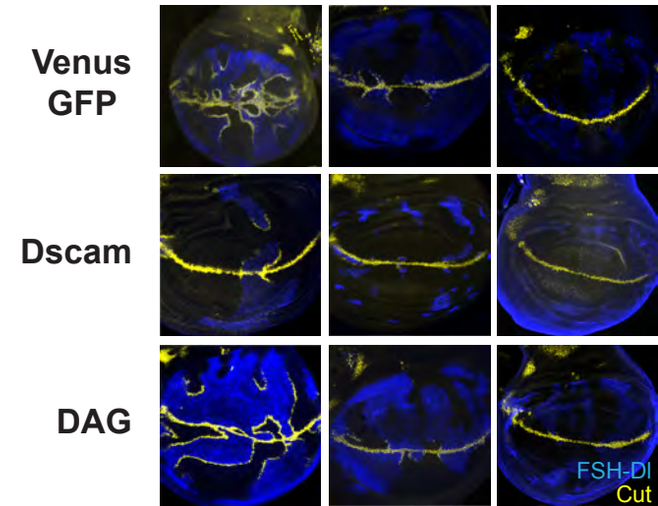


Figure 4: Class III: Hypersensitive FSHR-N receptors are cleaved constitutively and activation is enhanced by FSH-Dl on neighboring cells.

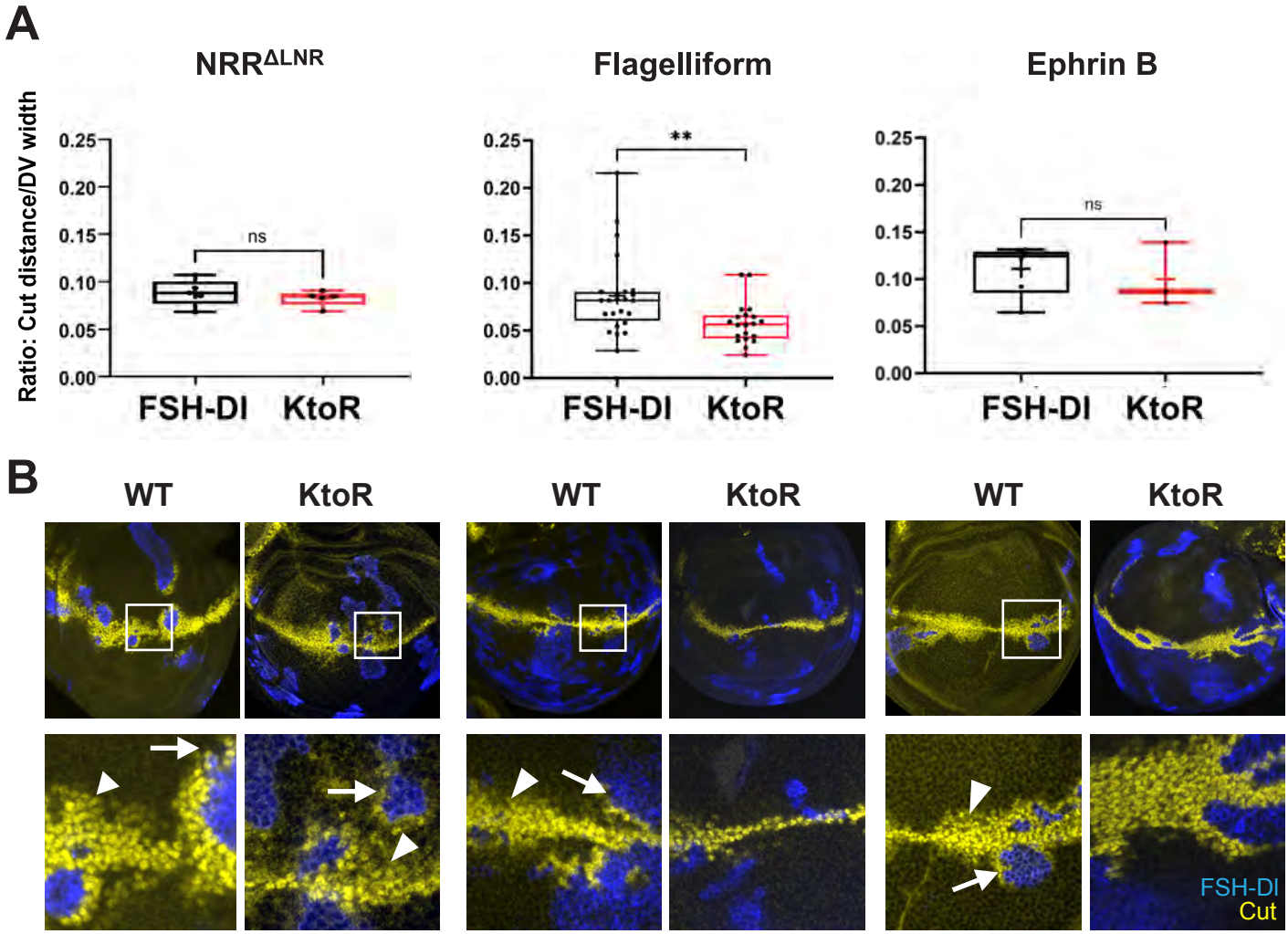
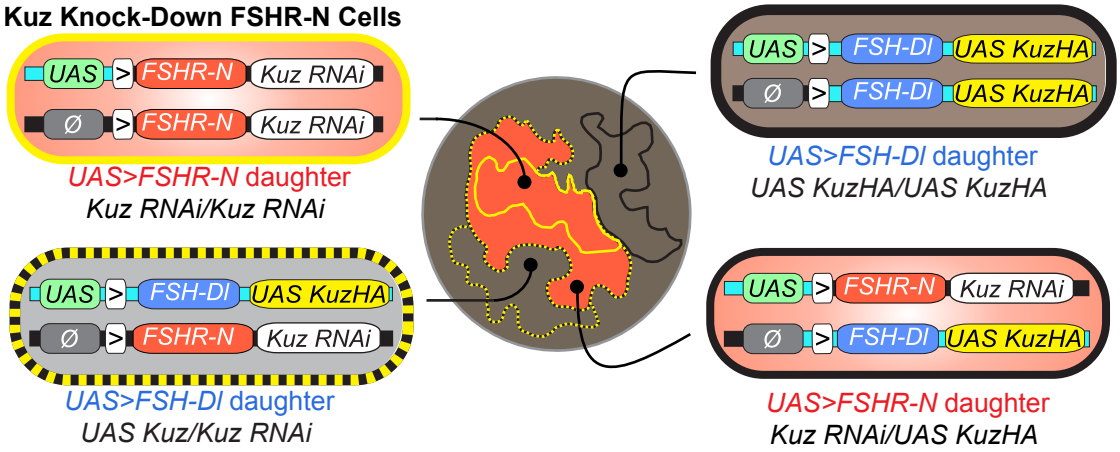


Figure 4: The ADAM protease Kuzbanian is required for the cleavage of candidate force sensors.

A Mitotic recombination using MAPS to remove detectable Kuz activity in sub-populations of FSHR-N cells.



B Kuz RNAi knock-down eliminates endogenous Cut expression and reduces or elimnates ectopic Cut expression

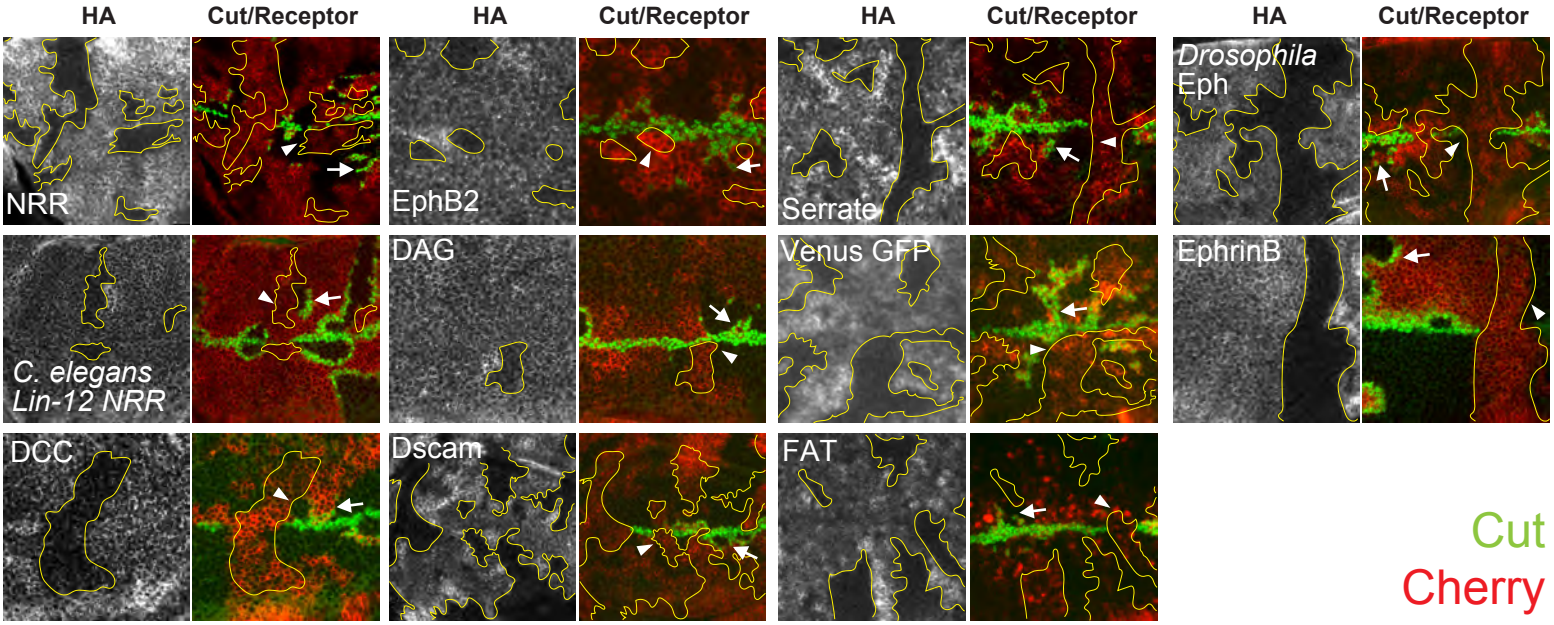
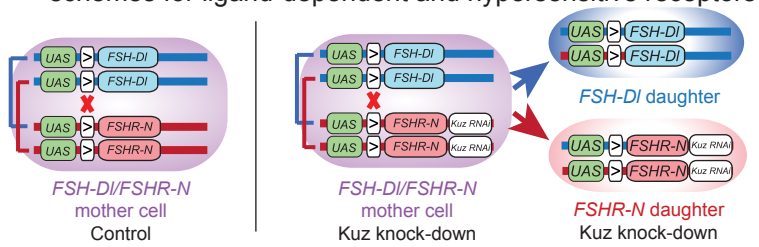
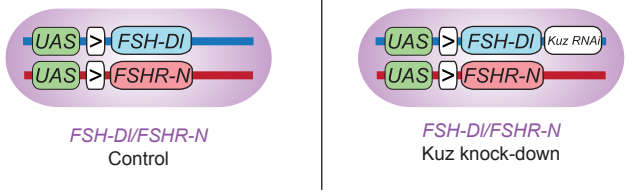


Figure 6: FSHR-Notch receptors with hypersensitive and constitutive activation require Kuz

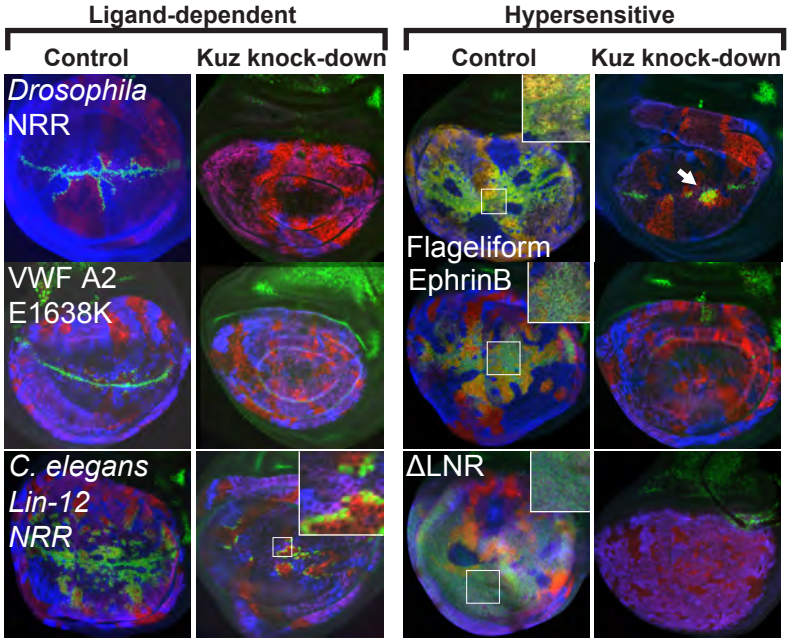
A Control and FSHR-Notch, Kuz RNAi knock-down genetic schemes for ligand-dependent and hypersensitive receptors



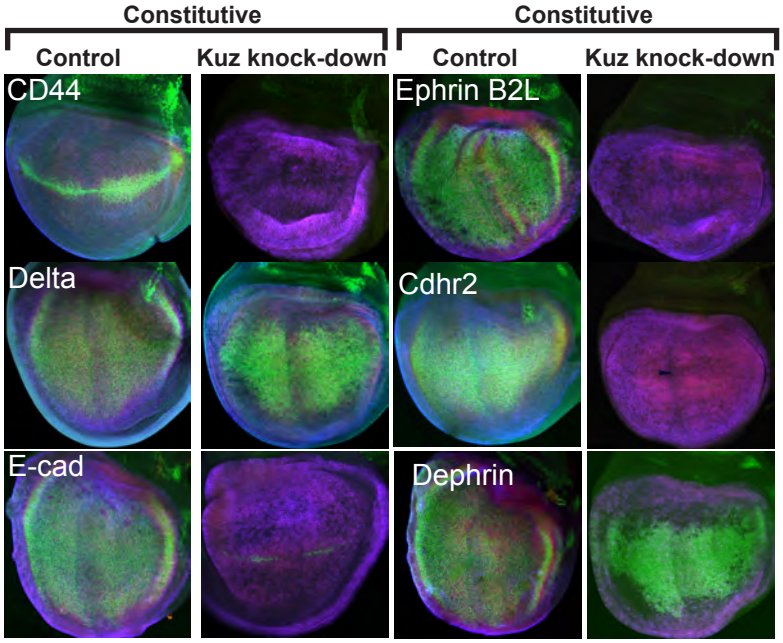
B Control and FSHR-Notch/FSH-DI, Kuz RNAi knock-down genetic scheme for constitutive receptors



C Kuz RNAi knock-down eliminates endogenous Cut expression and reduces or elimnates ectopic Cut



D Kuz RNAi knock-down eliminates endogenous Cut expression and reduces or elimnates ectopic Cut



FSH-DI
Cut
Cherry