Consequences of EGF-module mutations on Notch signalling and trafficking

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List of abbreviations

16: Abruptex allele 16

28: Abruptex allele 28

9B2: Abruptex allele 9B2

ACP and PCV: Anterior and Posterior wing Veins

AIP4: Atrophin-1 Interacting Protein 4

ANK: Ankyrin domain

AP3: Adaptor protein 3

Ax: Abruptex

AxE: Abruptex Enhancer

AxS: Abruptex Suppressor

Car: Carnation

COSMIC: Catalogue of Somatic Mutations In Cancer

Crb: Crumbs

CrbE: Crumb extracellular GFP-tagged

CSCs: Cancer Stem Cells

CSL: Conserved proteins from mammals, Suppressor of Hairless and Lag1

Dl: Delta

Dsh: Disheveled

DSL: Delta/Serrate/Lag-2 domain

DTX1: human Deltex 1

Dx: Deltex

E2: Abruptex allele E2

EGF: Epidermal Growth Factor

EGF*: EGF-like repeat number*

EGFR: Epidermal Growth Factor Receptor

ESCRT: Endosomal Sorting Complex Required for Transport proteins

et al.: *et alii*

FACS: Flow Cytometry analysis

FLP: Site-specific Flipase Recombinase

FRTs: Flipase Recombination Sites

Fz2: Frizzled2

GFP: Green Fluorescent Protein

GPI: Glycophosphatidylinositol-anchored proteins

HD: Heterodimerization Domain

HNSCCs: Head and Neck Squamous Cell Carcinomas

HOPS: Homotypic fusion and vacuole protein sorting

Itch: Itchy E3 Ubiquitin Protein Ligase

Kuz: Kuzbanian

KuzDN: Kuzbanian Dominant Negative

L*: wing Longitudinal vein number*

Lgd: Lethal Giant Discs

LNR: Lin12/Notch Repeats

M1: Abruptex allele M1

MVB: Multivesicular Body

N: Notch

NECD: Notch Extracellular Domain

NEXT: Next Extracellular Truncation

NICD: Notch Intracellular Domain

NRR: Negative Regulatory Region

PBS: Phosphate Buffered Saline

PEST: Proline, Glutamic acid, Serine and Threonine rich domain

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-Kinase

RAM: Recombination binding protein-Jk–Associated Molecule

RNAi: RNA interference

RTK: Receptor Tyrosine Kinase

S2 cells: Schneider-2 *Drosophila* cells

S2-Dl cells: Schneider-2 *Drosophila* Delta-expressing cells

SCCs: Squamous Cell Carcinomas

Ser: Serrate

Shi: *Drosophila* Shibire

Su(dx): Suppressor of Deltex

T-ALL: T-cell Acute Lymphoblastic Leukemia

TAD: Transactivation domain

TGFβR: Transforming Growth Factor-β Receptor

TM: Transmembrane domain

UAS: Upstream Activation Sequence

Wg: Wingless

WT: Wild-Type

Consequences of EGF-module mutations on Notch signalling and trafficking

Abstract

The Notch pathway is evolutionary conserved and involved in several key cellular functions that ensure tissue homeostasis in the adult organism. Such an important pathway requires a fine regulation which was found to be orchestrated by different regulatory mechanisms. Our group and others showed that the endocytic trafficking of Notch contributes to its regulation by sorting Notch into activation or degradation routes. Genetic alterations in Notch have been found in a number of diseases, such as different types of cancer. However, very often it is not known how mutations affect the Notch pathway thus making it difficult to specifically target Notch mutants. It is possible that a number of Notch mutants might alter specific regulatory mechanisms and it would be easier to specifically manipulate such mutants by targeting the regulatory steps they rely on. The study presented in this thesis aimed to functionally analyse mutations in the Abruptex (Ax) domain of Notch which spans EGF-like repeat 24-29 of the extracellular domain. The function of Ax domain and how *Ax* mutations affect Notch pathway have never been elucidated. Using *Drosophila melanogaster* as an *in vitro* and *in vivo* model of study, we showed a potential mechanism by which *Drosophila Ax* mutants alter the endocytic trafficking of Notch, ultimately affecting Notch signalling. We also showed that *Ax* mutants found in human cancers share *Ax*-like features, when reproduced in *Drosophila* Notch, and they can be functionally classified depending on their position in the Ax domain. Finally, we proposed that the Ax domain might mediate the binding of Notch with other proteins and we tested the effect of *Ax* mutants on potential binding partners. These findings provide new insights into the mechanism of *Ax* mutants, the function of the Ax domain and the relevance of *Ax* mutants in cancer.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

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A very dear 'thank you' goes to my family. I especially would like to thank my parents and grandparents for inspiring, supporting and understanding my path and for the person I have become today. This thesis is for you.

"Do not think of yourself, think of others. Think of the future that awaits you, think about what you can do and do not fear anything"

Rita Levi Montalcini

1. Introduction

Chapter1: Introduction

The Notch signalling pathway is evolutionarily conserved and plays a pivotal role in many aspects of development and tissue homeostasis by regulating cell-fate decisions. The Notch pathway is activated upon binding of the Notch receptor to its ligands exposed on neighbouring cells leading to the release, by regulated proteolysis, of the Notch intracellular domain that acts as a transcription factor. Additional regulatory mechanisms have been shown to be superimposed on the core Notch pathway and contribute to the fine regulation of Notch signalling. Consequently, Notch signalling results from the contribution of different regulatory mechanisms. The description of these mechanisms has gradually advanced, but further studies are needed to achieve a better understanding of Notch regulation in physiological and disease contexts.

1.1 Notch functional domains

The *Notch (N)* locus was first identified in *Drosophila melanogaster* about one hundred years ago when the "notching" phenotypes in the fly wings were characterized (Fig.1.1; Dexter, 1914; Morgan, 1917). Loss of function alleles were found to be haplo-insufficient and dominant by showing lethality when homozygous and causing a dominant "notching" wing phenotype when heterozygous with a wild type allele (Wharton *et al.,* 1985; Kidd *et al.,* 1986). The *Notch* gene was found to code for a single transcript encoding a type 1 spanning transmembrane receptor. The analysis of Notch mutants in *Drosophila* and other organisms has allowed the characterization of the functional domains of Notch and has opened the way for more complex studies, such as binding assays (Shimizu *et al.,* 1999; Pei and Barker, 2006) and structural analyses (Hambleton *et al.,* 2004; Cordle *et al.,* 2008), leading to a better understanding of the structure-function of Notch.

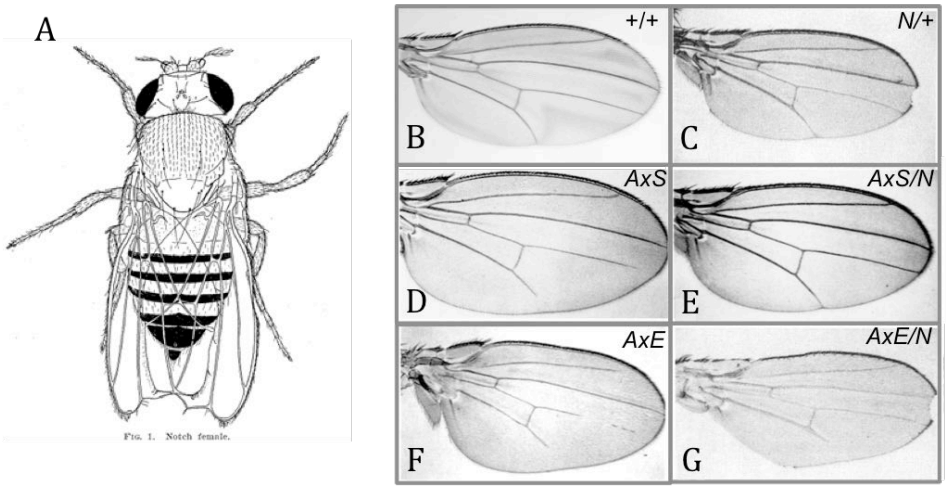


Figure1.1. Loss and gain-of-function *Drosophila* Notch alleles. "Notching" wing phenotypes in *Drosophila melanogaster* were identified one hundred years ago and led to the discovery of the *Notch* locus. (A) Reproduction of drawing by Thomas Hunt Morgan (1917) which first described the Notch haplo-insufficient mutant phenotype. (B-G) show female adult wings. (B) is a wild-type wing. (C) shows the "notching" wing phenotype resulting from haplo-insufficiency in a fly heterozygous for a loss-of-function allele N^{55e11} (N^{-}) and a wild-type allele. (D, F) The "gapping vein" wing phenotype of gain-of-function alleles belonging to the Ax suppressor class, (D) *9B2* (*AxS*) and (F) Ax enhancer class, *16* (*AxE*). (E) The "notching" phenotype is suppressed in *AxS/ N*$^{-}$ flies. (G) However, it is enhanced in *AxE/N*$^{-}$. (Modified from de Celis *et al.,* 1993; de Celis and Garcia-Bellido *et al.,* 1994).

Notch is highly conserved from invertebrates to humans (Fig. 1.2). One Notch receptor is encoded by the *Drosophila* genome, whereas two Notch paralogs were found in *Caernorhabditis elegans* (Lin12, GLP1) and four in mammals (Notch 1 to 4) (Fleming, 1998; Artavanis-Tsakonas *et al.,* 1999). Most Notch receptors are processed in the Golgi complex by a Furin-dependent cleavage that cuts at Site 1 (S1) which is in the extracellular domain in relative close proximity to the membrane. The two Notch fragments one consisting of the majority of the extracellular domain (NECD) and the other, a short extracellular region, the transmembrane domain and the intracellular domain (NICD) are non-covalently linked extracellularly through the heterodimerization

domain (HD) (Logeat *et al.,* 1998; Gordon *et al.* 2008). The NECD is composed chiefly of Epidermal Growth Factor (EGF)-like repeats the number of which varies among species and paralogs. Both *Drosophila* Notch, human Notch1 and Notch2 have 36 EGF-like repeats, while human Notch3 has 34 and Notch4 has 29 (Wharton *et al.,* 1985; Kidd *et al.,* 1986). Each EGF-like repeat contains six Cysteine residues that form three disulfide bonds. Some EGF-like repeats have a calcium-binding motif and might be involved in determining the stability of the receptor structure. Two key functional domains were identified in NECD, the ligand-binding and the Abruptex (Ax) domain. The first spans the EGF-like repeats 11-12 and is necessary for the trans-interaction of Notch with its ligands presented on adjacent cells. The specific function of the ligand-binding domain has been demonstrated by cell-aggregation assays between Notch-expressing cells and ligand-expressing cells (Rebay *et al.,* 1991).

The Ax domain is located in the EGF-like repeat 24 to 29 and it was first discovered through the identification of a class of dominant Notch mutant alleles in *Drosophila*. Mutations in the Ax domain produce a gain of function wing phenotype, characterized by gaps in the veins of the wing (Fig. 1.1), and increase Notch activation through a mechanism which is still not fully understood (Welshons, 1971; Portin, 1975; Foster, 1975; Kelley *et al.,* 1987; de Celis and Garcia-Bellido, 1994; de Celis and Bray, 2000). *Ax* mutants, which are homozygous viable, were divided in two classes depending on their phenotype when heterozygous with Notch null alleles. The enhancer class (*AxE*) enhances the notching wing phenotype caused by *Notch* haplo-insufficiency while the *AxS class* suppresses it (Fig.1.1). Interestingly, the heterozygous combination of *Ax* alleles belonging to the two different classes was found to be lethal. *Ax* mutants which are homozygous lethal were classified into a third class, named Ax lethal class (Foster, 1975; Portin 1975; Portin 1981; de Celis *et al.,* 1993; de Celis and Garcia-Bellido, 1994).

The function of the Ax domain is still unclear. It was first proposed that the Ax domain might be involved in cis-inhibition of Notch, which is a mechanism that prevents activation of Notch receptor by ligands expressed in the same cell (see § 1.2.2) (de Celis and Bray, 2000; Perez *et al.,* 2005). Pei and Baker (2008) showed that the Ax domain can interact with the ligand-binding domain of other Notch receptors, suggesting that Ax may be involved in receptor-receptor dimerization, however the biological meaning of such a process is not known. Finally, Ax mutants show a very strong genetic interaction with mutations in *deltex* (dx), a regulator of Notch endocytic trafficking (Xu and Artavanis-Tsakonas, 1990; Busseau, 1994; Diederich *et al.,* 1994), suggesting that the Ax domain might be involved in the endocytic regulation of Notch.

The EGF-like repeat region is followed by the Negative Regulatory Region (NRR) that consists of three Cysteine rich Lin12/Notch repeats (LNR) and the HD domain. The NRR function is to prevent unspecific activation of Notch by masking a key site (S2 site) for Notch cleavage (Gordon *et al.,* 2008). The Transmembrane domain (TM) marks the region of the receptor spanning the cell membrane. The NICD consists of the RAM (recombination binding protein-Jk–associated molecule) domain, the Ankyrin (ANK) domain and the transactivation domain (TAD), which are involved in the transcriptional activation of Notch target genes. The PEST domain (Proline, Glutamic acid, Serine and Threonine rich) is at the C-terminus of Notch and ensures the stability of NICD (Gordon *et al.,* 2008).

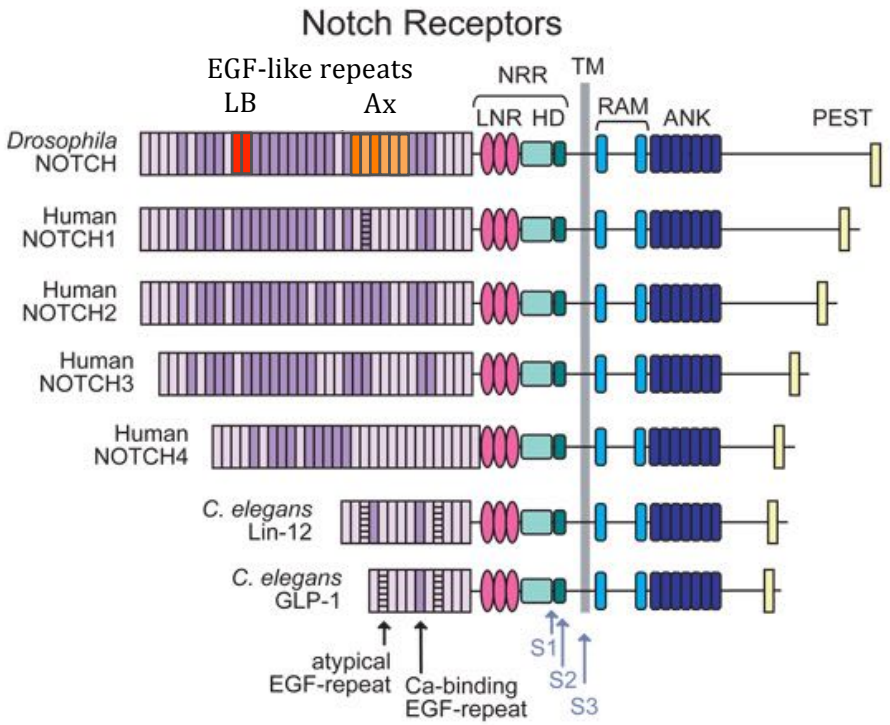


Figure 1.2. The domain organisation of Notch receptor is highly conserved across different species. *Drosophila*, *C. elegans* and humans have one, two or four Notch receptors, respectively. The extracellular region of Notch receptors consists of the EGF-like repeat region, which includes the Ligand-binding (LB) and Abruptex domain (Ax), and the Negative Regulatory Region (NRR), that consists of three Cysteine rich Lin12/Notch repeats (LNR) and the Heterodimerization domain (HD). The EGF-like repeat region varies in length in different Notch receptors and includes calcium-binding and non-calcium binding EGF-like repeats. The Transmembrane domain (TM) is the region spanning the cell membrane. The intracellular region consists of the Recombination binding protein-Jk–associated molecule domain (RAM), the Ankyrin domain (ANK), the transactivation domain (TAD) and the The Proline, Glutamic acid, Serine and Threonine rich domain (PEST). Notch receptor has three cleavage sites: Site 1 (S1) for receptor processing in the Golgi complex; Site 2 (S2) for cleavage by ADAM10/Kuzbanian; Site 3 (S3) for cleavage by γ-secretase (figure modified from Gordon *et al.,* 2008).

1.2 The Notch signalling pathway and its regulation

The core Notch pathway has a simple framework, which involves the interaction of Notch receptor with its ligands, followed by proteolytic cleavage of Notch and the release of NICD transcription factor. The Notch pathway is fundamental to animal development. Its mis-regulation leads to disease states including: Alagille syndrome which affects the liver and heart amongst other defects (MacDaniell *et al.,* 2006); Cadasil which affects the vasculature and can lead to strokes (Joutel *et al.,* 1996); and malignancies (see section § 1.3). It is therefore intuitive that there is a tight regulatory network to tune Notch signalling level and make it robust to pertubations. Many different mechanisms are involved in Notch regulation and these include: endocytic trafficking, cis-inhibition and cross-talks with other signalling pathways.

1.2.1 Notch ligand-dependent pathway

In the ligand-dependent Notch pathway, Notch is activated through the trans-binding with its ligands exposed on the surface of neighbouring cells. Notch ligands are type I transmembrane proteins and are evolutionary conserved. Two ligands, Delta and Serrate, are expressed in *Drosophila*, while three Delta-like ligands (DLL1, DLL3, DLL4) and two homologs of Serrate, Jagged1 and Jagged2 (JAG1 and JAG2) are expressed in humans. Notch ligands also have a domain with a characteristic number of EGF-like repeats and a Delta/Serrate/Lag-2 (DSL) domain, which is required for the interaction with the ligand-binding domain of Notch. Serrate and its homologs have an additional cysteine-rich domain (D'Suoza *et al.,* 2008; Gordon *et al.,* 2008; Cordle *et al.,* 2008).

The trans-interaction of Notch with its ligands triggers consecutive proteolytic cleavages in two sites, named S2 and S3 (Fig. 1.3) The S2 cleavage is mediated by the ADAM10 metalloproteinase, known as Kuzbanian (Kuz) in *Drosophila* (Lieber *et al.,* 2002; Hartmann *et al.,*

2002). How the S2 site is exposed to cleavage upon Notch-ligand interaction has not been elucidated yet. Some studies suggested that the ligand interaction might induce a mechanical force that causes a conformational change in the NRR domain and thereby unmasks the S2 site. An alternative model proposed that an allosteric interaction might be responsible for Notch receptor activation (Parks *et al.,* 2000; Gordon *et al.,* 2008). Upon S2 cleavage, NECD is trans-endocytosed with its ligand into the signal-sending cell. The Notch membrane-tethered intracellular domain, NEXT (Next Extracellular Truncation) is further cleaved at the S3 site within the TM by γ-Secretase complex, thus releasing the NICD, which translocates to the nucleus (Wolfe and Kopan, 2004). NICD forms a transcriptional complex with the DNA-binding protein CSL through the RAM domain and the ANK domain, facilitating the recruitment of the co-activator Mastermind and other co-activators to trigger the transcription of Notch target genes (Kopan and Ilagan, 2009; Choi *et al.,* 2012). CSL is an acronym of the names of conserved proteins from mammals (CBF1), *D. melanogaster* (Suppressor of Hairless) and *C. elegans* (Lag1).

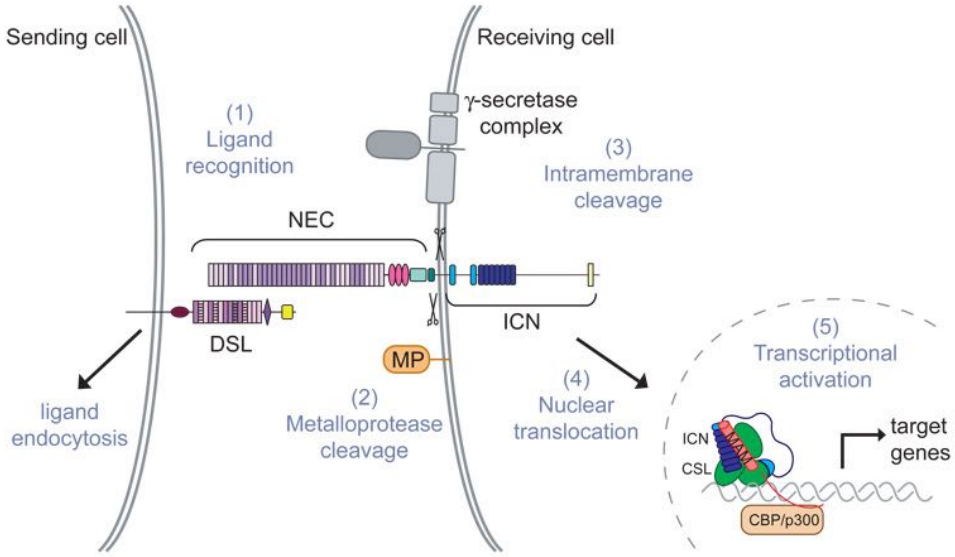


Figure 1.3. Notch ligand-dependent pathway. The trans-interaction of Notch with its ligand (1) leads to metalloprotease-mediated cleavage (MP) at S2 site (2), which allows the cleavage of Notch by the γ-secretase complex at site S3 (3), and release of Notch intracellular domain (ICN) and translocation to the

nucleus (4). In the nucleus, ICN forms a complex with CSL and Mastermind (MAM) which activates the transcription of Notch target genes (5) (figure from Gordon *et al.,* 2008).

1.2.2 Notch cis-inhibition

The interaction between Notch receptor and ligands on neighbouring cells (trans-activation) activates Notch signalling, whereas the interaction of Notch with ligands expressed in the same cell (cis-inhibition) inhibits Notch activation likely by preventing its interaction with other ligands (Fig. 1.4). A number of studies suggested that the Ax domain of Notch could play a role in cis-interactions, thus mutations in the Ax domain could make Notch less sensitive to cis-inhibition and in turn increase Notch activation (de Celis and Bray, 2000; Perez *et al.,* 2005). However, more recently it was shown that the EGF-like repeats in the ligand-binding region of Notch are instead necessary for the cis-binding with ligands, in addition to their requirement for trans-interactions (Becam *et al.,* 2010; Luca *et al.,* 2015), suggesting Ax domain might not be directly required in this process. It was also shown that the DSL domain of ligands mediates both trans and cis-interactions (Cordle *et al.,* 2008).

Cis-inhibition was found to be involved in the specification of Notch signal-receiving cells and signal-sending cells because it can modulate the availability of functioning Notch receptors and ligands on the cell surface (Sprinzak *et al.,* 2010; del Álamo *et al.,* 2011). Indeed, it was proposed that cis-interactions between Notch and ligands might not only inhibit Notch activation but also mutually inactivate the receptor and the ligand (mutual cis-inactivation) or in certain cases, only inactivate the ligand (ligand cis-inhibition by the receptor) (Fig. 1.4; Sprinzak et al., 2010; del Álamo et al., 2011). This conceptual idea has led to new hypotheses that can be tested on how receptor-ligand interactions modulate signalling.

The Notch receptor has been observed to dimerise *in vitro* (Pei and Baker, 2008; Luca *et al.,* 2015). It is not known whether this could contribute to the regulation of the cis and trans-interactions of Notch. For example, dimerization might be required for trans-activation or protect the receptor from cis-inhibition or on the contrary, might sequester the receptor. Interestingly, the Notch EGF-like repeats 21-30 were found to bind *in vitro* to the ligand-binding region of Notch, suggesting Ax domain might be involved in the receptor-receptor interaction (Pei and Baker, 2008).

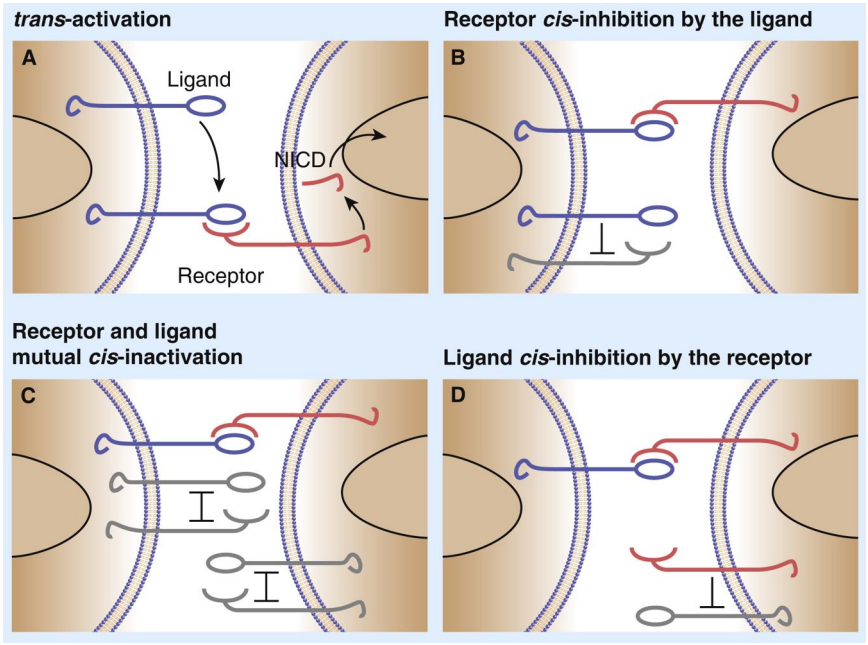


Figure 1.4. Ligand-receptor interactions. Ligands are indicated in blue, Notch receptors in red and inactivated ligands or receptors in grey. (A) Trans-activation in which Notch in a signal-receiving cell is activated upon interaction with a ligand on a signal-sending cell. (B) Cis-inhibition by the ligand whereby the ligand interacts and inhibits Notch receptor in the same cell. (C) Mutual cis-inhibition where the interaction of Notch receptor and ligand in the same cell leads to mutual inactivation of both the receptor and the ligand. (D) Cis-inhibition by the receptor on the ligand in the same cell such that the ligand can no longer send a signal (figure from del Alamo *et al.,* 2011)

1.2.3 Notch endocytic regulation

A growing body of evidence indicates that Notch can also be regulated and activated in a ligand-independent manner through its endocytic trafficking (Wilkin *et al.,* 2004; Wilkin *et al.,* 2008; Vaccari *et al.,* 2008; Yamada *et al.,* 2011; Hori *et al.,* 2011; Schneider *et al.,* 2013; Shimizu *et al.,* 2014). The first evidence of the endocytic regulation of Notch derived from the characterization of *Drosophila Shibire* (*Shi*), the homolog of human Dynamin protein, which is required for the scission of endocytic vesicles from the cell membrane. *Shi* mutants showed a phenotype similar to that of Notch loss-of-function mutants, suggesting that Dynamin and in turn endocytosis is required for Notch activation (Seugnet *et al.,* 1997; Parks *et al.,* 2000). Studies in *Drosophila* have described the endocytic trafficking of Notch and identified the regulators of this process.

A key regulator is the ring-finger ubiquitin ligase Deltex (Dx). Interestingly, Dx was first associated with Notch when loss of function *dx* mutations were found to dominantly suppress the phenotype of *Ax* mutants and the lethality caused by the combination of *Ax* mutants belonging to different classes (Xu and Artavanis-Tsakonas, 1990; Busseau *et al.,* 1994; Diederich *et al.,* 1994). Interestingly, these observations also suggest that Ax mutants might rely on the endocytic regulation of Notch, a possibility which has not been explored yet. Dx was generally observed to positively regulate Notch signalling (Gorman and Girton, 1992) and has been shown to induce the activation of Notch in a ligand-independent manner (Wilkin *et al.,* 2008; Hori *et al.,* 2011; Yamada *et al.,* 2011; Shimizu *et al.,* 2014). Dx directs Notch into a clathrin mediated endocytic route and retains Notch on the limiting membrane of late endosomes (Fig. 1.5), where the NECD is exposed to the luminal space, while NICD is facing the cytoplasm and can be proteolytically activated upon the fusion of late endosomes with lysosomes (Gupta-Rossi *et al.,* 2004; Wilkin *et al.,* 2008; Coumailleau *et al.,* 2009). In this way, Dx prevents Notch from being internalised into

the multivesicular body of late endosomes (MVB) and degraded. Dx exerts its function by binding to the ANK domain of NICD and mediating the mono-ubiquitination of Notch. It was shown that Dx-mediated activation of Notch requires specific proteins that are involved in the endosomal maturation and fusion of the late endosome to the lysosome. These include: the AP3 (Adaptor protein 3) complex, Rab5 and Rab7 GTPases, Trpml required for in the lysosomal-fusion of late endosomes as well as the HOPS Complex (Homotypic fusion and vacuole protein sorting) which is involved in early to late endosome movement as well as lysosome-late endosome fusion (Wilkin *et al.,* 2008; Shimizu *et al.,* 2014). The depletion of such proteins does not decrease the ligand-dependent activation of Notch, further highlighting the ligand-independent nature of the Dx-mediated activation. Similarly to the Notch canonical pathway, Notch activation in the Dx route relies on S3 cleavage by γ-Secretase complex. γ-Secretase was found to be more active in acidic environments (Pasternak *et al.,* 2003), like the lumen of endosomes Indeed, *Drosophila* mutants of the vacuole proton pump V-ATPase, which block the acidification of endosomes, and pharmacological inhibition of H+ ATPase in mammalian cells, were found to reduce Notch ligand-independent signalling (Vaccari *et al.,* 2008; Vaccari *et al.,* 2010; Kobia *et al.,* 2014). The ligand-dependent activation of Notch requires the unmasking of S2 and S3 sites, however this step might be bypassed in the endosomal activation. For example, the lysozymes in the MVB might proteolyse the NECD, or the acidic PH and ionic environment could destabilise the NRR and HD or trigger the dissociation of the NECD (reviewed by Steinbuck and Winandy, 2018).

The endocytic trafficking of Notch could lead either to activation or down-regulation of the pathway depending on the endosomal sorting of Notch (Wilkin *et al.,* 2004; Vaccari *et al.,* 2008; Shimizu *et al.,* 2014). Another key regulator of Notch endocytic trafficking is Suppressor of Deltex (Su(dx)), an HECT domain E3 ubiquitin ligase, that antagonises Dx and mediates Notch endosomal degradation (Fostier *et al.,* 1998;

Wilkin *et al.,* 2004; Shimizu *et al.,* 2014). As suggested by its name, *Su(dx)* mutants were found to suppress the *dx* mutant wing phenotype in flies. Su(dx) diverts post-endocytosed Notch from the Dx route (Fig. 1.5) or from a recycling route marked by Rab11-positive endosomes to degradation (Wilkin *et al.,* 2004; Shimizu *et al.,* 2014). Su(dx) sorts Notch into an endocytic route which is cholesterol-dependent and marked by Glycophosphatidylinositol (GPI)-anchored proteins (Shimizu *et al.,* 2014). In this route, Su(dx) favours the internalization of Notch into the MVB of late endosomes by polyubiquitination of Notch, leading to its degradation and downregulation (Wilkin *et al.,* 2004; Wilkin *et al.,* 2008). In the Su(dx) route Notch can also be mildly activated in early endosomes prior to internalisation into the MVB of late endosomes. This activation is mild but can be increased at low temperature when Su(dx) activity is decreased, due to its temperature-sensitive HECT domain, and more Notch is retained on the limiting membrane of endosomes (Shimizu *et al.,* 2014). This activation is ligand-independent, is mediated by Kuz and does not require lysosomal-fusion, contrary to the activation in the Dx route. It was proposed that Dx and Su(dx)-mediated routes, together with ligand-dependent pathway, contribute to the overall regulation of Notch and maintain the robustness of the pathway in different conditions. For example, it was shown that Notch signalling is kept tuned by the balance of the different routes across temperature variation in *Drosophila* (Shimizu *et al.,* 2014). If this system is conserved in mammalian cells, its function could ensure Notch regulation in response to environmental changes and cellular stress conditions. According to this emerging view, the sorting of Notch into different endocytic compartments largely contributes to Notch regulation, suggesting the overall Notch signalling could be the result of the summation of ligand-dependent and independent signals.

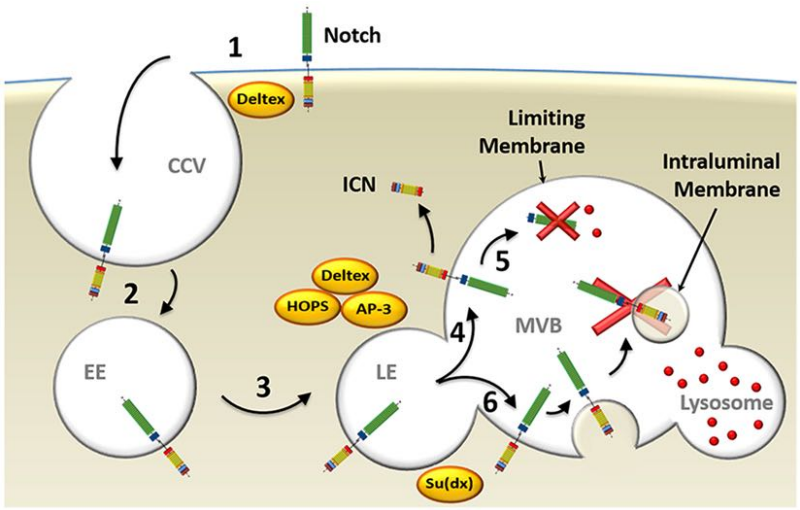


Figure 1.5. Notch endocytic regulation. Deltex favours the endocytosis of Notch receptor into clathrin-coated vesicles (CCV) (1) to early endosomes (EE) (2). Deltex directs Notch to late endosomes (LE) (3), together with adaptor protein 3 (AP-3) and homotypic fusion and vacuole protein sorting (HOPS) and keeps it on the limiting membrane of late endosomes (4). This prevents Notch from being internalised and degraded within the multivesicular body (MVB) and promotes its cleavage and activation (5). Suppressor of Deltex (Su(dx)) can redirect Notch into the multivesicular body (MVB) of late endosomes where Notch is degraded (6) (figure from Steinbuck and Winandy, 2018)

The endocytic trafficking of Notch is also coordinated by the Endosomal Sorting Complex Required for Transport (ESCRT) proteins. It was found that *Drosophila ESCRT* mutants, which block different steps of the endocytic trafficking preceding the fusion with lysosomes, increase the ligand-independent Notch activation (Vaccari *et al.,* 2008). This is an interesting study also because it showed that uncontrolled ligand-independent activation of Notch can occur at different stages of endosomal maturation and also prior to lysosomal-fusion. An interesting case is *lethal giant discs* (*lgd*), a tumour suppressor gene which encodes for a C2-containing protein interacting with Shrub, a subunit of the ESCRT III complex (Bryant and Schubiger, 1971; Jaekel and Klein, 2006). In *lgd* mutants, Notch was endocytosed and

ectopically activated in a ligand-independent and lysosomal-dependent manner in a fashion that resemble the activation of Notch in the Dx-mediated route. It was later found that this is because lgd function is to divert Notch to endosomal degradation by regulating Shrub (Hori *et al.,* 2011; Schneider *et al.,* 2013), thus suggesting ESCRT III complex contributes to the endosomal sorting of Notch.

Similarly, it was shown that the synergy between Shrub, Dx and Krz, a non-visual β-arrestin, modulates the sorting of Notch to endosomal degradation or activation (Mukherjee *et al.,* 2005; Hori *et al.,* 2011). Interestingly, in *shrub* mutants Notch was found to be activated in a ligand-independent manner in maturing endosomes similarly to *lgd* mutant. However, in *shrub* mutants Notch was activated in a lysosomal-independent manner through an unknown mechanism (Schneider *et al.,* 2013), suggesting different mechanisms of endosomal activation are possible.

Our understanding of the endocytic regulation of Notch has gradually advanced in *Drosophila*, but its characterisation in the mammalian system is still limited. A human homolog of *Drosophila* Dx, DTX1 was found to positively regulate Notch1 and Notch2 (Matsuno *et al.,* 1998; Yamamoto *et al.,* 2001) suggesting Dx function is conserved. DXT1 was also observed to inhibit Notch1 in certain contexts (Sestan *et al.,* 1999; Izon *et al.,* 2002). This is also the case in *Drosophila* where reductions in trafficking flux through the endocytic pathway to the lysosome, or the level of Su(dx) can convert Deltex from a positive to a negative regulator of Notch signalling. DTX1 may be a modulator of Notch signalling that can be regulated to be either positive or negative dependent on tissue type and context.

Su(dx) human homolog, Atrophin-1 Interacting Protein 4 (AIP4) and mouse homolog, Itchy E3 Ubiquitin Protein Ligase (Itch), were found to induce Notch ubiquitination and degradation similarly to Su(dx) (Qiu *et al.,* 2000; Chastagner *et al.,* 2006). The ubiquitin ligase c-Cbl was also

shown to mediate the degradation of Notch1 (Jehn *et al.,* 2002). In addition, the endocytosis of various receptors has been described in mammalian cells and shows similarities with Notch endocytic regulation, further suggesting that this is a conserved mechanism shared by different pathways. For example, the endocytosis into a clathrin or a lipid-raft endocytic route was found to regulate the signalling and fate of epidermal growth factor receptor (EGFR) (Sigismund *et al.,* 2005; Sigismund *et al.,* 2008; Capuani *et al.,* 2015) and transforming growth factor-β receptor (TGFβR) in mammalian cells (Di Guglielmo *et al.,* 2003; Mitchell *et al.,* 2004; He *et al.,* 2015).

A number of new regulators of *Drosophila* Notch endocytic trafficking have been recently identified. One of them is Crumbs (Crb), an evolutionary conserved transmembrane receptor involved in apicobasal polarity in epithelia (Tepass, 1990; Tepass, 2012; Letizia *et al.,* 2013; Rodriguez-Boulan and Macara, 2014). Crb was found to directly interact with Notch extracellular domain and prevent Notch endocytosis and ligand-independent activation at the apical membrane in *Drosophila* wing discs. Loss of Crb causes the endocytosis and Dx-dependent activation of Notch (Nemetschke and Knust, 2016). Interestingly, CrbRNAi flies show a similar gapping vein wing phenotype as Ax mutants (Nemetschke and Knust, 2016; Das and Knust, 2018). Another new regulator of Notch endocytic trafficking is cis-inhibition. Palmer *et al.,* 2014 showed that cis-inhibition can inhibit ligand-independent signalling in the *Drosophila* ovary cells and keep the pathway in a steady state by stabilising the receptor at the cell membrane.

1.2.4 Cross-talks between Notch and other pathways

Cross-talk between Notch and other pathways were also found to regulate Notch signalling. These cross-talks not only act at the transcriptional level by modifying the activation of Notch targets genes,

but might also occur outside the nucleus since upstream components of Notch and other pathways were found to directly interact.

An interesting example is the cross-talk between Notch and Wnt pathway in mammals or Wingless (Wg) pathway in *Drosophila*. In the Wnt pathway, Wnt, a secreted glycoprotein, binds to its receptor Frizzled (Fz) and to the co-receptor low-density-lipoprotein-receptor-related proteins-5/6 (Lrp5/6), inducing the stabilisation of its intracellular mediator β-catenin, which initiates the transcription of Wnt target genes. In the absence of Wnt, β-catenin is degraded by the Axin-based complex. The interaction of Wnt and the receptors triggers the activation of Disheveled (Dsh) which blocks the Axin complex and in turn stabilises β-catenin. The cross-talk between Notch and Wg was first observed in *Drosophila* in coordinating the acquisition of sensory organ precursor fate and lateral inhibition in the development of the peripheral nervous system (Brennan *et al.,* 1999; Couso and Martinez Arias, 1994). It was shown that Ax mutants disrupt these developmental processes. The Wnt/Notch cross-talk is antagonistic and was shown to modulate the regulation of gene expressions in different contexts (Hayward *et al.,* 2005; Hurlbut *et al.,* 2007; Fre *et al.,* 2009; Collu *et al.,* 2012). It was proposed that the Notch pathway can antagonise Wg pathway in *Drosophila* imaginal discs and Dx might be involved in this process (Ramain *et al.,* 2001). Also, Dsh was found to directly interact with NICD leading to Notch endocytic degradation (Hayward *et al.,* 2005; Munoz-descalzo *et al.,* 2011; Hemalatha *et al.,* 2016). Interestingly, Wg itself was found to interact with Notch receptor and it was proposed Wg might be a non-canonical Notch ligand, however the effect of this interaction is not known (Wesley, 1999; D'Suoza *et al.,* 2010).

The cross-talk of Notch with other pathways have also been proposed to be independent of ligands, suggesting some of these interactions might occur in endosomal compartments. For example, PI3K/AKT-dependent Notch activation was reported in dendritic cells and found to

be γ-secretase independent (Gentle *et al.,* 2012). Also, it was recently reported that the T-cell receptor complex/CD28 can activate Notch ligand-independent signalling in T-cells through PI3K (Steinbuck *et al.,* 2018). The molecular basis of this mechanism has not been identified yet but it was proposed this process relies on Notch endocytic trafficking and activation in the endosomes.

1.3 Notch in cancer

The Notch pathway ensures tissue homeostasis by modulating many important processes such as cell-fate decisions, proliferation, apoptosis, stem cells differentiation and maintenance. Notch function in mammals is highly dependent on the cellular context, thus the same signal can have opposite outcomes in different tissues or cell lineages even within the same tissue. Notch can either favour proliferation or apoptosis as well as self-renewal or differentiation in different contexts (Ranganathan *et al.,* 2011; Ntziachristos *et al.,* 2014; Aster *et al.,* 2016). For example, Notch promotes the self-renewal of stem cells in hematopoietic tissues, brain and muscles, but the differentiation of stem cells in the skin, breast and lung. Notch signal represses proliferation and promotes differentiation in prostate basal cells, whereas the same signal favours proliferation in prostate luminal cells (Andersson *et al.,* 2011; Valdez and Xin, 2013).

The alteration of such an important pathway has been associated with the development of several human diseases, including cancer (Mohr, 1919; Gridley, 2003; Louvi and Artavanis-Tsakonas, 2012; Penton *et al.,* 2012). Notch has a dual role in cancer and can either act as an oncogene or a tumour suppressor depending on the cellular context. Notch was found genetically altered in several tumours and mutations have been predicted to be either gain or loss of function depending on the role of Notch in the tissue of origin (Ntziachristos *et al.,* 2014; Aster *et al.,* 2016). However, the classification of Notch mutations in cancer is often

merely based on predictions that could be very inaccurate since the function and the consequences of cancer mutations are often unknown. Also, how Notch can exert its dual and opposite functions is still a matter of investigation. The dual role of Notch in carcinogenesis might be due to cross-talks with other pathways and interactions with the tumour microenvironment (South *et al.,* 2012; Louvi and Artavanis-Tsakonas, 2012; Ranganathan *et al.,* 2011). However, as previously discussed, many other regulatory mechanisms exist and may also influence Notch function in physiological or disease conditions.

1.3.1 Notch in hematopoietic tumours

The first evidence of the involvement of Notch in cancer derived from the identification of a rare chromosomal translocation in T-cell acute lymphoblastic leukemia (T-ALL) patients. Such chromosomal rearrangement were found in the *locus* that encodes human *Notch1* (Ellisen *et al.,* 1991). It was later found that more than half of the T-ALL patients have gain-of-function mutations that induce the constitutive activation of Notch1 pathway and causes T-cells to acquire a proliferative advantage, escape negative selection and undergo oncogenic transformation. The majority of these mutations are in the HD domain of Notch and increase the exposure of the S2 cleavage site, resulting in constitutive activation or increased sensitivity to the ligands (Weng *et al.,* 2004; Grabher *et al.,* 2006).

Gain-of-function mutations in Notch have been identified in other hematopoietic malignancies. These include: Chronic lymphocytic leukemia (Puente *et al.,* 2011; Fabbri *et al.,* 2011), Diffuse large B cell lymphoma (Lee *et al.,* 2009; Morin *et al.,* 2011), Burkitt lymphomas (Love *et al.,* 2012) and Non-Hodgkin lymphomas (Kiel *et al.,* 2012; Rossi *et al.,* 2012). Such mutations affect Notch1 and/or Notch2 and are clustered in the PEST domain of the receptor.

Other studies have shown that Notch could also act as a tumour suppressor in other hematopoietic malignancies (Rangarajan *et al.,*

2001; Nicolas *et al.,* 2003). For example, inactivation of members of the γ-Secretase complex or deletion of Notch1 or Notch2 *in vivo* leads to chronic myelomonocytic leukemia (CMML), suggesting that the inactivation of Notch could be one of the driving forces of this disease (Klinakis *et al.,* 2011). Inactivating mutations in Notch2 and other well-known myeloid mutations have also been found in patients affected by acute myeloid leukemia (AML). Remarkably, the re-activation of Notch in human AML samples leads to the arrest of AML growth and apoptosis (Klinakis *et al.,* 2011; Kannan *et al.,* 2013; Lobry *et al.,* 2013). Similarly, Notch seems to have a tumor suppressor role in B cells acute lymphoblastic leukemia (B-ALL) and its reactivation causes the arrest of the disease (Zweidler-McKay *et al.,* 2005; Kuang *et al.,* 2013).

1.3.2 Notch in solid tumours

Several studies have provided evidence for a key role of Notch signalling in solid tumours. However, such studies have also revealed that the role of Notch in solid tumours is remarkably complex and pleiotropic (Ranganathan *et al.,* 2011; Ntziachristos *et al.,* 2014; Aster *et al.,* 2017). In T-ALL and other hematopoietic cancers, it is quite clear how mutations in Notch affect its pathway. However, in solid tumours little is known about the meaning of alterations affecting Notch and their signalling outcome.

The involvement of Notch in solid tumours was first observed from experiments with mouse mammary tumour viruses (MMTVs). The integration of the virus into the "*Int 3*" locus led to the constitutive activation of Notch4 and tumour development (Gallahan *et al.,* 1987; Gallahan and Callahan, 1997). Induced activation of Notch1 was also found to cause mammary tumour formation in mice and transformation of human breast epithelial cells (Dievart *et al.,* 1999; Weijzen *et al.,* 2002; Stylianou *et al.,* 2006). Indeed, it is now known that Notch has an oncogenic role and is highly mutated in human breast cancers (Jiao *et al.,* 2012). However, it was shown that different signal dosages and

distinct Notch receptors might have opposite roles depending on the breast cancer subtypes (Colaluca *et al.,* 2008; Mazzone *et al.,* 2010; Robinson *et al.,* 2011). Further, Notch4 was found to be involved in the maintenance of breast cancer cells with stem-like properties, which are implicated in tumour-initiation and recurrence (Harrison *et al.,* 2010; Simoes *et al.,* 2015).

A remarkable number of mutations in Notch have been identified in squamous cell carcinomas (SCCs) in different tissues, suggesting the development of these tumours could be highly dependent on Notch. Loss of function mutations in Notch1 and Notch2 have been reported in cutaneous (60-75% of cases) and lung SCCs (5-12% of cases) indicating a tumour suppressive function of Notch. These mutations were reported to clustered in the EGF-like region and around the HD domain and are believed to affect the binding of Notch with its ligands or the receptor structure (Wang *et al.,* 2011). Mutations in the extracellular domain of Notch1 and a smaller number in Notch2, and Notch3 were also found in head and neck SCCs (HNSCCs) and predicted to be loss of function (Stransky *et al.,* 2011; Agrawal *et al.,* 2012). Mutations in Notch1 have a very high incidence in oral HNSCCs in Asian patients, suggesting Notch has a role in driving the disease (Song *et al.,* 2013; Sun *et al.,* 2014). The majority of the mutations were located in the Ax domain of Notch1, including a mutational hotspot (Song *et al.,* 2013; Zheng *et al.,* 2018). Notch mutations in HNSCCs were predicted to be loss of functions, however recent studies have pointed out that a significant number of these alterations could turn out to be gain of function (Zhao *et al.,* 2016) and this would be in agreement with the observation that Ax mutants are gain-of-function in *Drosophila*. It is also interesting that DTX1 was found to be downregulated in HNSCCs (Gaykalova *et al.,* 2017) and other tumours (Zhang *et al.,* 2010; Narayanappa *et al.,* 2016), suggesting DTX1 might have a tumour suppressor function in certain tissues.

In conclusion, Notch is clearly involved in the development of several tumours and mutations in functional domains of Notch have been identified in different cancers. Notch is a promising target for the treatment of several cancers, however, no Notch-targeting treatment is available as yet, although some Notch-targeting strategies are currently been tested. This is probably because it is still not possible to specifically target Notch in cancer cells, thus avoiding severe side effects resulting from the general inhibition or activation of Notch pathway in healthy tissues. A better understanding of the consequences of specific Notch mutations could greatly facilitate the design of effective and specific strategies to target Notch mutants in cancer.

1.4 Aims of the project

Notch is involved in many key cellular processes, from proliferation to differentiation, that ensure homeostasis in adult tissues. Alterations in such an important pathway have been linked to a number of human diseases including different types of cancer (Ranganathan *et al.,* 2011; Ntziachristos *et al.,* 2014; Aster *et al.,* 2017). Several cancer-associated mutations have been identified in the four human Notch receptors, but the consequences of these mutations on the pathway are often not clear, thus making it difficult to specifically target Notch mutants. Our group and others described that Notch can be regulated not only in a ligand-dependent manner, but also in ligand-independent fashion through its endocytic trafficking in different endocytic routes (Wilkin et al., 2004; Wilkin et al., 2008; Vaccari et al., 2008; Yamada et al., 2011; Hori et al., 2011; Schneider et al., 2013; Shimizu et al., 2014). Therefore, there are different ways in which Notch can be regulated in physiological conditions and misregulated in disease. It is possible that a number of Notch mutants rely on specific regulatory routes, thus it would be easier to specifically manipulate such mutants by targeting the regulatory routes or mechanisms they depend on.

My project aims were to understand how mutations in the Ax domain of Notch affect its signalling pathway (Fig. 1.6). *Ax* mutants are interesting because they affect a domain whose function is still unknown and might be involved in key steps of Notch regulation. Importantly, Ax like mutations have been found in the Ax domain of human Notch receptors and linked to a number of cancers.

Several *Drosophila* studies have shown that there is a strong genetic interaction between Ax and Deltex (Xu and Artavanis-Tsakonas, 1990; Busseau *et al.,* 1994; Diederich *et al.,* 1994), suggesting this domain could be involved in the endocytic regulation of Notch and this possibility had never been explored. Other evidence suggested that Ax might be involved in the cis-inhibition of Notch (de Celis and Bray, 2000; Perez *et al.,* 2005) or in receptor-receptor interactions (Pei and Baker, 2008) or in the interaction with other pathways (Brennan *et al.,* 1999; Couso and Martinez Arias, 1994) or regulators (Nemetschke and Knust, 2016). Therefore, I studied the *Ax* mutations to further elucidate the mechanism by which they act to modify Notch signalling output and potentially reveal new mechanisms of Notch regulation and mis-regulation in cancer.

Drosophila melanogaster, in which Notch was discovered and characterized, was used for the study of *Ax* mutants, taking advantages of its genetic and molecular amenability. Importantly, the use of *Drosophila* allowed me to perform both *in vitro* and *in vivo* studies of Notch mutants.

The analysis first focused on *Drosophila Ax* mutations previously identified through genetic screenings: *Ax* mutant *E2* and *16* from the enhancer class and *Ax* mutant *9B2* and *28* from the suppressor class (Fig. 1.6).

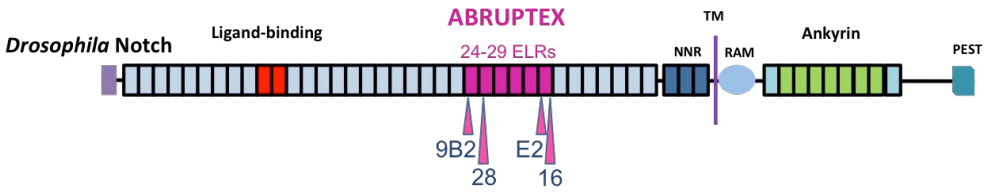


Figure 1.6. *Drosophila Ax* mutants. Ax domain identifies the region from EGF-like repeat 24 to 29 of *Drosophila* Notch receptor. *Ax* suppressor mutations, *9B2* (D948V) and *28* (N986I), are located in EGF24 and EGF25, respectively, whereas *Ax* enhancer mutations, *E2* (H1167Y) and *16* (G1174A), are in EGF29.

The first aim was to identify which regulatory steps are affected by *Ax* mutants. First, the phenotype of *Ax* mutant flies was analysed and second the signalling and localisation of *Ax* mutants were studied in Schneider-2 *Drosophila* cells (S2 cells) and *in vivo* in *Drosophila* wing larval tissue.

The second aim was to analyse *Ax* mutants found in cancer and test whether it is possible to functionally classify *Ax* mutants depending on their characteristics. To this purpose, cancer *Ax* mutations were introduced in *Drosophila* Notch receptor and their signalling and localisation determined in S2 cells.

Finally, the interaction of *Ax* mutants with other regulators was tested. The effect of a number of selected candidates on the signalling of wild type Notch and the mutants were assessed in S2 cells.

2. Methods

Chapter2: Methods

2.1 Molecular cloning

Ax *Drosophila* (pMT-16, pMT-28, pMT-M1) and cancer mutants (pMT-P915L, pMT-P919S, pMT-E890K, pMT-C945R, pMT-G1136V, pMT-G1215D, pMT-D1227G, pMT-E1270K) plasmids were prepared by mutagenesis of pMT-Notch wild-type (Shimizu *et al.,* 2014). The pMT (Invitrogen) is an expression vector that contains the *Drosophila* metallothionein gene promoter which can be induced by CuSO4 and contains Ampicillin resistance. GFP-tagged constructs (pMT-E2-GFP, pMT-9B2-GFP) were obtained by sub-cloning in pMT-Notch-GFP (prepared and validated by H. Shimizu and A. Smith) in which GFP was inserted downstream the Ankyrin domain.

2.1.1 Mutagenesis

The following primers were used for the mutagenesis of pMT-Notch:

Ax 16 G->R

Notch Ax 16172 FW tgctactgctcccagAgatacgcgggtagctat

Notch Ax 16172 RV atagctacccgcgtatcTctgggagcagtagca

Ax 28 N->I

Notch Ax 28 FW tgcgagacggacatcaTtgagtgcttgagtcag

Notch Ax 28 RV ctgactcaagcactcaAtgatgtccgtctcgca

Ax M1 C->Y

Notch Ax M1 FW cagaatggagccacgtAtagtcagtatgtgaat

Notch Ax M1 RV attcacatactgactaTacgtggctccattctg

hN1 P915L

Notch P954L FW gattgtgcctcgtttcTgtgccagaacggtgga

Notch P954L RV tccaccgttctggcacAgaaacgaggcacaatc

hN2 P919S

Notch P954S FW gattgtgcctcgtttTcgtgccagaacggtgga

Notch P954S RV tccaccgttctggcacgAaaacgaggcacaatc

hN1 C954R

Notch C993R FW tgcttgagtcagccgCgccagaatggagccacg

Notch C993R RV cgtggctccattctggcGcggctgactcaagca

hN1 G1136V

Notch G1175V FW tgctactgctcccaggTatacgcgggtagctat

Notch G1175V RV atagctacccgcgtatAcctgggagcagtagca

hN1 G1215D

Notch G1254D FW ccaccgggaacgatggAcatcatatgcgagatc

Notch G1254D RV gatctcgcatatgatgTccatcgttcccggtgg

hN3 E890K

Notch E987K FW gagacggacatcaatAagtgcttgagtcagccg

Notch E987K RV cggctgactcaagcactTattgatgtccgtctc

hN2 D1227G

Notch D1261G FW tgcgagatcaacaaggGtgattgcaaaccggga

Notch D1261G RV tcccggtttgcaatcaCccttgttgatctcgca

hN1 E1270K

Notch E1300K FW gagggcgacatcaacAagtgcctaagcaatccc

Notch E1300K RV gggattgcttaggcactTgttgatgtcgccctc

The primers were used in combination with the amplification primers listed below to generate the mutant fragments through a first round of PCR. In particular, the mutant FW primer was used with the RV amplification primer and vice versa.

-For mutations in the Ax domain (EGF-like repeats 24-29)

FW 5'-CTGCGCCAGCAATCGTTGC-3'

RV 5'-TCACACGGTCGTGGCAAGT-3'

-For mutations downstream Ax domain (EGF-like repeats 30-33)

NF8 FW 5'-TTGAACGGAGCCACCTGCCA- 3'

NR15 RV 5'-CGGCAAGACCGCTCTCCATT-3'

The PCR was performed using pMT-Notch as template and the Phusion-high fidelity DNA polymerase (NEB):

PCR		PCR Programme		
Primers(F+R)	0.5µl x 2	98°c	30s	
DNA (50ng)	0.5µl	35 cycles	98°c	10s
dNTPs	0.5µl		55°-65°C	10s
5xbuffer	10µl		72°c	30s-1min
H2O	37µl	72°c	5min	
Phusion	0.5µl			

The PCR product was run on a 1% agarose gel and purified using the gel extraction kit (Qiagen). The two mutant fragments was then amplified through a second round of PCR using the two fragments togheter as template, the amplification primers and Phusion polymerase. The DNA was run and purified from agarose gel.

2.1.2 Digestion and ligation

The mutant fragments and the vector pMT-N were digested at 37°C for 1 hour in 1X CutSmart buffer (NEB) using the restriction enzymes NheI (NEB) and BglII (Roche) for mutations in the Ax domain or BglII and NcoI (NEB) for mutations downstream of the Ax domain. The restriction enzymes NhEI and BglII (NEB) were also used to cut pMT-E2 and pMT-9B2 for sub-cloning in pMT-Notch-GFP. The fragments were ligated with the vector using the T4-ligase-based Quick ligase kit (NEB, 0.4µl enzyme in 2X ligation buffer in 10µl final volume) for 10min at room temperature. The ratio of vector and fragment for ligation was calculated using the NEBioCalculator (NEB).

2.1.3 DNA transformation and extraction

The constructs were transformed into competent *E. coli* cells (XL-10 Gold, Agilent technology) by incubating 20ng of DNA with 20µl of competent cells on ice for 30min. The mix was heat shocked for 30-45s in 42°c water bath and then incubated for 2min on ice. 400µl in Luria-Bertani (LB) medium containing no antibiotic were added to the mix and incubated for 1h at 37°C. The bacteria were then plated on LB agar plates containing ampicillin and incubated at 37°C o/n. Colonies grown on plates were screened by PCR using the myTAQ DNA polymerase (Bioline) and the primers previously used for amplification.

Colony PCR		5xbuffer	2µl
Primers(F+R)	0.1µl x 2	H2O	8µl
dNTPs	0.08µl	TAQ	0.05µl

Positive colonies were grown in a flask with new LB-ampicillin medium at 37°C over night. DNA was then extracted from bacteria using the Qiaprep Midiprep kit (Qiagen) and the presence of the correct mutation was checked in each construct by sequencing using the same primers as used for DNA amplification.

2.2 *In vitro* assays

2.2.1 Cell culture

Schneider's 2 cells (S2 cells, Invitrogen) and Delta-expressing S2 cells (S2-Dl, Drosophila Genomics Resource Center) were grown in P100 culture dishes (Corning) in Schneider's medium (Invitrogen) with 10% fetal bovine serum (FBS) (Hyclone) and 0.5% penicillin-streptomycin (Sigma) at 25°C. Cells were mechanically detached from the dish and S2 cells split 1:20 and S2-Dl 1:5 every three days.

2.2.2 Transfection

S2 cells were seeded at ~50% confluency in 24-well or 6-well dishes and transfected using Effectene kit (Qiagen). This kit contains the Enhancer reagent that favours the precipitation of DNA and the Effectene reagent that covers DNA with cationic lipids allowing a highly efficient transferring of DNA into the cells. The DNA plasmids were mixed with Enhancer reagent (DNA:Enhancer ratio of 1:8) and buffer EC provided in the kit to reach the final volume of 40ul in 24-well dish or 160µl for 6-well dish. The mix was incubated for 5 minutes at room temperature. Then 2µl or 8µl Effectene was added, for 24-well or 6-well dish, respectively and samples were incubated for 10 minute at room temperature. 100µl or 400µl of medium were added to the samples for 24-well or 6-well dish, respectively, and they were directly transferred into the wells where S2 cells were seeded. Cells were kept in the incubator at 25°C for two day to allow the DNA to transfect. For luciferase assays, cells were transferred to a 96-well white dishes and

$CuSO_4$ was then added into the medium to induce the expression of the transfected plasmids. For other assays the induction with $CuSO_4$ was done directly in the 24-well or 6-well dish where the cells were seeded. Cells were induced at 25°C for 24 hours before the assay. The final concentration of $CuSO_4$ used for induction was 1mM.

The plasmids used for S2 cell assays are: pMT-Notch and pMT-EYFP-Rab7 (Shimizu *et al*., 2014); pMT-E2, pMT-9B2 and pMT-Notch-V5 (a gift from H. Shimizu); pMT-Notch-GFP (a gift from H. Shimizu and A. Smith); pMT-E2-GFP, pMT-9B2-GFP, pMT-16, pMT-28, pMT-M1, pMT-P915L, pMT-P919S, pMT-E890K, pMT-C945R, pMT-G1136V, pMT-G1215D, pMT-D1227G and pMT-E1270K (designed and prepared in this thesis work); pMT-Dx (a gift from K. Matsuno); pMT-HA-Su(dx)(Flasza *et al*., 2006); pMT-Gal4 (DGRC); pUAST-GPI-GFP (a gift from S. Eaton); Notch Response Element:Firefly NRE:F (a gift from S. Bray); actin:Renilla Act:R (a gift from G. Merdes); pMT-Ser-V5 (Witheman *et al*., 2013); pMT-Dl-myc (DGRC, Klueg *et al,* 1998); pUAS-CrbE-GFP (a gift from Maria Llamas, Pellikka *et al.,* 2002); pMT-Fz2-V5 (a gift from J.P. Vincent); pMT-Kuz and pMT-KuzDN (Pan and Rubin, 1997); pTub-Wg (Addgene).

2.2.3 Luciferase reporter assay

S2 cells were transfected with 1 ng of pMT Notch plasmids and 10 ng of pMT-Dx or Su(dx). Every sample was transfected with 50 ng NRE-F and 25 ng act-R. These are two reporter constructs: Notch Response Element:Firefly (NRE:F) is activated by Notch and produces Firefly luciferase, while actin:Renilla (act:R) produces Renilla luciferase under the control of the actin promoter. Therefore, the amount of Firefly luciferase reflects Notch activity, whereas the Renilla luciferase acts as a control by reflecting the general transcriptional activity of the cells and transfection efficiency. For ligand-independent signaling, the cells were transferred into white 96-well plates and $CuSO_4$ was added two days post transfection. For ligand-induced signaling, S2-Dl cells and non-

transfected S2 cells, were seeded into white 96-well plate and the expression of Delta in S2-Dl cells was induced with $CuSO_4$ for 2 hours. Then cells were fixed with 4% formaldehyde in PBS for 30min, washed twice in PBS and twice in Schneider's medium for 30min. Transfected S2-cells were then transferred into the 96-well dish on top of the fixed S2-Dl cells for ligand-dependent signalling and S2 cells as a control. $CuSO_4$ induction was performed as usual. When the luciferase assay was performed at different temperatures, cells were incubated at 18°C, 25°C and 29°C during $CuSO_4$ induction. The temperature experiments were always performed in parallel at the three different temperatures. In the KuzRNAi assay, the dsKuzRNA (BKN23747 from the Sheffield University RNAi facility and based on the Heidelberg 2 BKN library, Horn *et al.*, 2010) was added one day after transfection in medium without serum for 1 day. Then complete medium was added and the cells were cultured for a further 2 days before $CuSO_4$ induction. Either dsGFP-RNA, as a control, or the dsKuzRNA was used.

In all experiments, luciferase activity was assayed with Dual-Glo Luciferase kit (Promega) 24 hours after induction. The kit provides the Dual-Glo solution, a reagent containing the substrate of Firefly luciferase, and Stop&Glo solution, containing a reagent that blocks Firefly luciferase and the substrate of Renilla luciferase. To perform the assay, medium was removed from the 96-well dish and 48µl of 50% Dual-Glo solution in PBS was added and the dish was incubated on a shaker for 10 minutes. The Firefly luciferase was then quantified by using a luminometer (Berthold). Then, 48µl of 50% Stop&Glo solution in PBS was added and the dish was incubated for 5 minutes on a shaker. The Renilla luciferase was quantified using the luminometer. The Firefly/Renilla ratio was calculated in order to normalize Notch activity (Firefly) by the general transcriptional activity, and transfection level of cells (Renilla). Three triplicates were always prepared for each sample.

2.2.4 Antibody uptake assay

S2 cells were transfected with 20ng of pMT-Notch plasmids, 100ng of pMT-empty vector or pMT-Dx or Su(dx) and markers. Glycophosphatydylinositol-anchored GFP was used as a marker to determine in which route Notch was trafficked: 40ng of pUAS-GPI-GFP were con-transfected with Notch together with 100 ng pMT-Gal4 to trigger GPI-GFP expression. Rab7-GFP was also used as a marker to labeled the limiting membrane of late endosomes and determine if Notch was on the limiting membrane or inside the lumen of endosomes: 40ng pMT-EYFP-Rab7 were co-transfected with pMT-Notch plasmids. The DNA amounts referred to transfection in 24-well dish and four times more were used for transfection in 6-well dish. Transfected and induced S2 cells were transferred from the 24-well dish on polylysine-coated coverslips. Then, cells were incubated with anti-Notch extracellular domain (anti-NECD) primary antibody (Diederich *et al*., 1994, C458.2H, DSHB, mouse concentrate, 1:100 diluted in medium) for 15 minutes on ice to slow down endocytosis and label Notch at the cell membrane. The cells were washed twice with ice-cold medium and then incubated with medium at 25°C for 1 hour to allow Notch endocytosis and chase the labeled Notch receptors. In time course experiments, cells were incubated at 25°C for 0, 5, 10, 30min or 1 hour depending on the time point. Afterwards, S2 cells were fixed in 4% formaldehyde in PBS for 30 minutes, rinsed in PBS, permeabilised in 0.2% Triton X-100/PBS for 15 minutes, and blocked in 5% skimmed milk/PBS over night. The cells were then incubated with anti-mouse secondary antibody (Alexa Fluor 568 donkey anti- mouse, Thermofisher, 1:500 diluted in 5% skimmed milk/PBS) for 1 hour in the dark. Cell preps were then washed twice in PBS and mounted in Vectashield with DAPI (Vector labs). An average of 100 vesicles (~20 cells) were scored for each sample and the assay was repeated three times for each sample. Images were captured using 100x oil immersion objective and Volocity software (Perkin Elmer) with an Orca-ER digital

camera (Hamamatsu) mounted on a M2 fluorescent microscope (Zeiss). Deconvolution using the 3 nearest neighbour algorithm on 0.5 μm optical sections was performed using Openlab software (Improvision) and processed in Photoshop (Adobe).

2.2.5 Surface staining

Surface and total Notch staining

Cells were seeded and transfected in 6-well dish using the same quantity of DNA for antibody uptake assay. Two samples were prepared, one for surface staining and one for total Notch staining. The surface staining samples were prepared using the antibody uptake assay procedure, with the exception that after incubation with primary antibody, cells were directly fixed in order to label only Notch at the cell membrane. Total Notch samples were prepared by first fixing and permeabilising the samples and then labelling with primary and secondary antibody. Images were taken using a M2 fluorescent microscope (Zeiss) with a 20X objective and processed using ImageJ. The intensity of surface Notch and total Notch staining were measured in individual cells using ImageJ. An average of 100 cells were scored for each sample and the experiment was repeated three times. The intensity of surface Notch was normalized by the intensity of total Notch in each sample.

Surface staining by Flow Cytometry

Cells were seeded and transfected in 6-well dishes using the same quantity of DNA for antibody uptake assay together with 80ng of pMT-GFP. Cells in medium were detached and collected into 1.5ml Eppendorf tubes, centrifuged at 3g at room temperature for 3min. The supernatant was removed. Anti-NECD primary antibody (Diederich *et al*., 1994, C458.2H, DSHB, concentrate mouse, 1:100 diluted in medium, final volume of 100μl,) was added to the cell pellet and mixed using the Grant-bio vortex for 5-10 secs. Samples were incubated on ice for 15min, then centrifuged at 1g for 1min at 4°C to remove the antibody.

Cells were fixed with 100µl of 0.1% Formaldehyde in PBS for 15min on ice. Then centrifuged to remove the fix solution and washed with 200µl of 5% donkey serum in PBS, vortexed and centrifuged. Secondary antibody (anti-mouse Alexa fluor 568, 100µl, 1:500 diluted in 5% donkey serum) was added, mixed by vortex and incubated for 30min in darkness. The samples were centrifuged at 1g for 1min at 4°c, to remove the antibody. Finally, the samples were washed with 200µl PBS twice and left in 400µl PBS for flow cytometry analysis (FACS). Samples were loaded in the flow cytometer (BD LSR Fortessa). One extra sample transfected with pMT-N wild-type was always prepared and labelled with secondary antibody only as a control (Fig. 2.1). Such control sample was used to threshold the FACS and identify the population of cells with Notch at the cell membrane (P square and red spots). Only single GFP positive cells (green spots) were considered and separated by the GFP negative cells (blue spots) in order to analyse only transfected cells. The percentage of cells in population P and their staining intensity were determined by FACS and reflect the localisation of Notch at the cell membrane. 10.000 cells were analysed by the FACS machine in every sample and the experiment was performed three times.

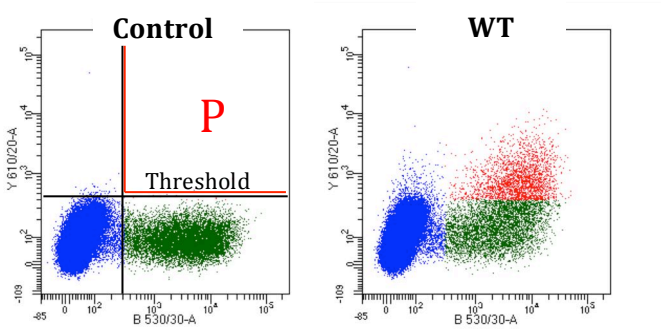


Figure 2.1. Flow cytometry analysis. The panel shows the diagrams obtained by FACS of the control sample and Notch wild-type sample. The control sample treated with secondary antibody only was used to threshold the FACS and determine population P. The percentage of cells and antibody staining intensity of P was determine in each sample.

2.2.6 Co-immunoprecipitation and Western Blot

S2 cells were seeded in 6-well plate and transfected with the same amount of DNA for uptake assay. For co-immunoprecipitation (co-IP), anti-GFP antibody conjugated beads (7µl/well, GFP-TRAP, Chromotek) were prepared and washed twice in lysis buffer. After the second wash, 40µl/well solution of beads were left in the tube. Lysis buffer was prepared with 100mM Tris-HCl (pH7.5), 150mM NaCl, 1mM CaCl2 and 1% Triton-X100. Proteasome inhibitor MG132 (used as 50µM, Sigma) and Complete™, Mini, EDTA-free Protease Inhibitor Cocktail (used as 1X Roche) were added before using the lysis buffer. Cells were lysed by adding 150µl lysis buffer, incubating on ice for 15min and periodically gently shaking the dish. Cell lysate was collected and centrifuged at maximum speed for 10min. For co-IP, 15µl of supernatant was used as total protein sample and the rest was incubated with 40µl beads on a rotating wheel at 4°C for 30min-1h. 4x Sample buffer (1X NuPage buffer + 1X NuPa sample reducing agent, Invitrogen) was mixed with the samples and the mixture was heated at 70°C for 10min. The protein samples were run on a 3-8% NuPage tris-acetate gel (Thermofisher) under constant voltage (130v) for 100min in running buffer (1X NuPage Tris-acetate SDS running buffer, Thermosfisher) and proteins were transferred to nitro-cellulose membrane under constant voltage (50-60v) for 90min in transfer buffer (1X NuPage transfer buffer + 20% methanol, Thermosfisher). The membrane was blocked with 5% skimmed milk in PBS for 1h and incubated with primary antibody in 5% skimmed milk in PBS at 4°C overnight. The membrane was washed 4 times in 0.05% PBS-Tween-20 and then incubated with secondary antibody diluted in 5% skimmed milk in PBS for 2hours at room temperature or overnight at 4°C. Then the membrane was washed 4 times in 0.1% PBS-Tween-20. The membrane was developed using the HRP substrate (SuperSignal west pico, Thermofisher) and photographic MR films (Carestream Kodak Biomax films, Sigma) or scanned using the Odyssey CLX machine (LI-COR). The pictures of the membranes were

processed and quantified using ImageStudio (LI-COR). Peanut protein was detected in all blots as housekeeping reference. The quantification values of the co-IP samples were divided by the values of the total protein samples which were normalised by their peanut values.

The primary antibodies used for Western Blot are: anti-NICD mouse (1:5000, Fehon *et al*., 1990; C17.9C6, concentrate DSHB), anti-GFP rabbit (1:5000; ImmunoKontac), anti-Myc tag rabbit (Abcam), anti-V5 mouse (Aviva systems biology), anti-Peanut mouse (DSHB, 1:5000). The anti-mouse and anti-rabbit HRP-linked secondary antibodies (Cell Signalling) were used for the photographic film development. The Goat anti-Mouse IgG H&L (IRDye® 800CW) preadsorbed (ab216772) and Goat Anti-Rabbit IgG H&L (IRDye® 680RD) preadsorbed (ab216777) (Abcam) were used for imaging using Odyssey CLX machine.

2.3 *In vivo* assays

2.3.1 Fly husbandry

The *Drosophila melanogaster* flies stocks were grown in vials containing standard maize based food prepared by the faculty media service of the University of Manchester. Live yeast (Sigma) was added to the vials before use. The fly stocks were maintained in the Fly Facility of the University of Manchester.

The fly stocks used for this thesis are: Ubi-GFP, FRT101;; mkrs, hsflp; yw, FRT101/FM7, E2, FRT101/FM7, 16, FRT101/FM7, 9B2, FRT101/FM7, 28/FM7, E2, dx^{152}, FRT101/FM7, 16, dx^{152}, FRT101/FM7, 9B2, dx^{152}, FRT101/FM7, dx^{152}, FRT101/FM7, y, w, E2, car^{1}, y, w, 16, car^{1}, y, w, 28, car^{1} and y, w, 9B2, car^{1} (were kindly prepared by M. Baron for this thesis); w, E2, dx^{ENU} (Baron lab stock).

2.3.2 Wing preparation

Adult wing preparation

The wings of anesthetised adult male flies were dissected using forceps and placed on a microscope slide with a drop of isopropanol. Once the wings were dry they were mounted with Gary's magic mountant (Canada balsam, methyl Salicitate, see Ashburner, 1989).

Pupal wing preparation

The wing expansion method (Lawrence *et al*., 2000) was used to examine the wing phenotypes of late pupal lethal flies. The flies were removed from the pupal case using dissecting forceps. The flies were then cooked in 10% NaOH in a tube for 1 hour at 60°C, which causes the wings to inflate. The flies were moved into a dissecting dish containing tap water and then placed on a microscope slide with a drop of water. The wings were removed from the fly body and left to dry on the microscope slide. The wings were mounted in Hoyer's medium.

The wings were photographed using a Zeiss Axioskop running Openlab software (Improvision).

2.3.3 Mutant clones in the wing disc

Negatively marked mutant clones were generated in the wing disc of *Drosophila* larvae using the FLP recombinase and its site-specific recombination sites, FRTs, system (Golic and Lindquist, 1989). This technique is based on the activity of the FLP, a yeast DNA recombinase, which is under the control of an heat shock promoter, which is activated by heat shock at 37°C. The FLP triggers the recombination between two FRT specific- recombination sites. If the FRT site recombination is induced between non-sister chromatids the chromosomal arm distal to the FRT site will be homozygous in daughter cells (Fig. 2.2). If the chromosomal arm distal to the FRT site contains the mutation of interest, homozygous cells for the mutations will be generated.

The clones in this study were produced by crossing *,FRT101/FM7 virgin flies with Ubi-GFP, FRT101;; mkrs, hsflp males. The offpring were heat shocked at second instar larval stage for 1 hour to produce the FLP recombinase. Discs of the genotype *,FRT101/Ubi-GFP, FRT101;; mkrs,hsflp/+ were dissected and examined for * homozygous cells (GFP-negative clonal spot) and GFP positive wild-type homozygous cells (GFP-positive clonal spot).

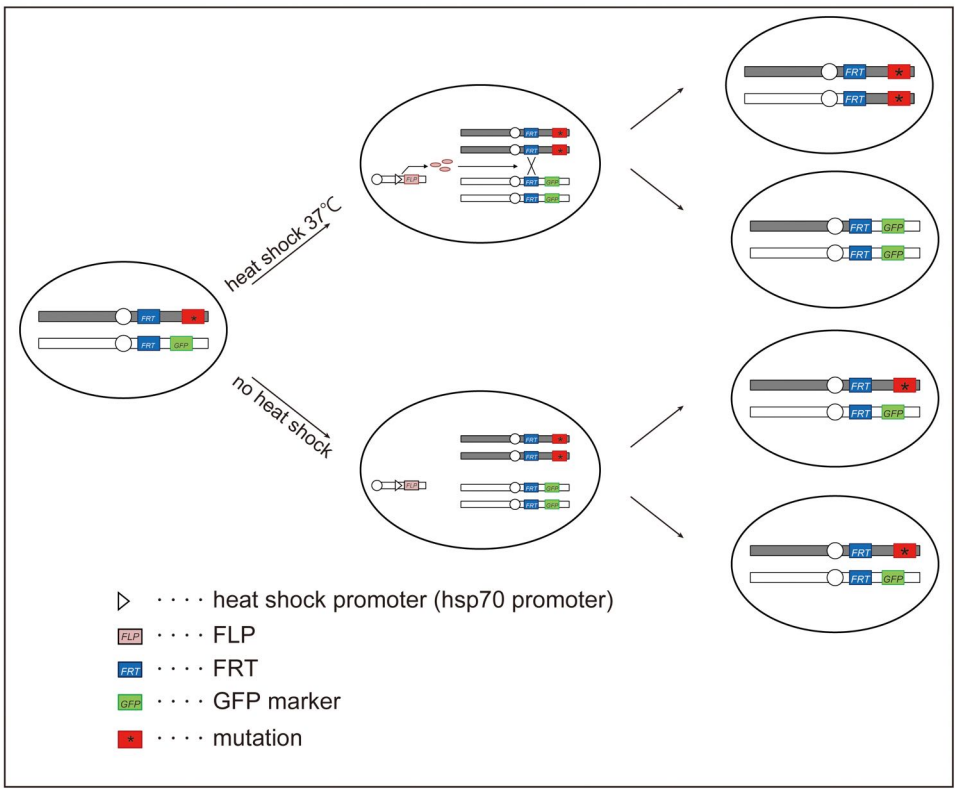


Figure 2.2. The FLP/FRT system. Heterozygous cells carrying one copy of the mutation of interest (red square) and one copy of GFP (green square) divide and give rise to two heterozygous daughter cells. If the expression of FLP (pink square) is triggered by heat shock the recombination between FRT sites (blue square) can occur among non-sister chromatids and lead to the generation of homozygous cells instead (from Aoyama *et al.*, 2018).

The flies were crossed at 25°C and kept at 18°C, 25°C or 29°C to make clones at different temperatures. The wing discs were dissected from third-instar larvae after 8, 4 or 3 days from heat shock depending on whether the larvae were grown at 18°C, 25°C and 29°C, respectively.

The wing discs were dissected in PBS, collected in a 1.5ul Eppendorf

tube and fixed in 4% formaldehyde in PBS for 20min on a rolling shaker. The wing discs were washed with 0.1% Triton-X 100 in PBS three times. The wing discs were blocked in PBS with 0.3% Triton-X 100 for 2h on a rolling shaker. Then washed with PBS three times and incubated with anti-NECD primary antibody (Diederich *et al.*, 1994, C458.2H, DSHB, mouse concentrate, 1:200 diluted in PBS with 4% donkey serum) and anti-GFP goat (1:500 diluted in PBS with 4% donkey serum, Abcam) overnight. The day after the wing discs were rinsed with 0.1% Triton in PBS for three times and washed with 0.1% Triton in PBS four times for 10min each. The preps were then incubated with anti-goat 488 and anti-mouse (1:500 diluted in PBS with 4% donkey serum, Thermofisher) overnight. The day after the wing discs were rinsed with 0.1% Triton in PBS for three times and washed with 0.1% Triton in PBS one time for 10min. The wing discs were incubated for Phalloidin-far red (Molecular probes, Thermofisher) for 1 hour and then rinsed with 0.1% Triton in PBS for three times and washed with 0.1% Triton in PBS three times for 10min. The wing discs were spin down, the supernatant was removed and DAPI Vectashield mounting medium (Vector labs) was added. Samples were stored at 4C overnight before being mounted and photographed. Images were captured using X63 oil immersion objective and Volocity software (Perkin Elmer) with an Orca-ER digital camera (Hamamatsu) mounted on a M2 fluorescent microscope (Zeiss). Deconvolution of 0.5 μm optical sections using 3 nearest neighbor algorithm was performed using Openlab software (Improvision) and processed in Photoshop (Adobe).

The vesicular Notch in the clones was quantified in ImageJ. A square section of equal size in the GFP+ and GFP- clonal spots of each clone was processed with the same settings in ImageJ and the number of Notch endosomal structures was measured using the 'analyze particles' function. The sections examined were 1.5μm below the apical membrane of the wing discs. The surface Notch was quantified by measuring the intensity of anti-NECD staining at the apical membrane

of the clones. The intensity was measured in a square section of equal size in the GFP+ and GFP- clonal spots of each clone using the 'intensity density' function of ImageJ. Five clones for each genotype at each temperature were examined for the quantification of the vesicular and surface Notch. In both quantifications, the values obtained in the GFP- and GFP+ spots were normalized by the values of the GFP+ spots.

2.4 Statistics

The statistical significance was determined by Student T test two tails equal variances in luciferase assays, surface staining assays and clones. The Fisher's exact test was used to compare the viabilities of E2/9B2 and E2/9B2, dx flies. The standard error was determined by Standard Error of the Mean which is equal to the Standard Deviation divided by the square root of the sample size (SEM=SD/SQRT(n)).

3. Functional analysis of *Drosophila Ax* mutants

Chapter 3: Functional analysis of *Drosophila Ax* mutants

3.1 Phenotypic analysis of *Ax* mutants

Abruptex (*Ax*) mutations are single amino acid changes in the EGF-repeats 24-29 region of the Notch receptor (Kelly et al., 1987). *Ax* mutants are dominant Notch alleles which show gain-of-function phenotypic characteristics, such as gaps in the wing veins and loss of bristles in the fly notum (Welshons, 1971; Portin, 1975; Foster, 1975; De Celis and Garcia-Bellido, 1994). All *Ax* mutants were found to produce a similar gain of function wing phenotype, however the veins affected and the extension of the vein gaps were different among the mutants. Temperature was found to modify the severity of *Ax* wing phenotypes in an allele-specific manner (Portin and Siren 1976, Portin 1977; De Celis and Garcia-Bellido, 1994). *Ax* mutants have been divided into different classes based on genetic interactions with the *Notch* locus. *Notch* null alleles are haplo-insufficient and this phenotype is characterised by notches in the wing margin. The combination of *Ax* mutants with *Notch* null alleles either enhances or suppresses the notching phenotype (Portin, 1975; Foster, 1975; Kelly et al., 1987; De Celis et al., 1993). This observation led to the classification of Ax alleles into two different categories, called enhancer (e.g. *AxE2, Ax16*) and suppressor (e.g. *Ax9B2, Ax28*) classes. *Ax* mutants which are homozygous lethal (e.g *AxM1*) were instead classified in a third class, named lethal class. It was also found that the heterozygous combination of suppressor/suppressor (e.g. *9B2/28*) or enhancer/enhancer (e.g. *E2/16*) was viable, while suppressor/enhancer combinations (e.g. *E2/9B2*) were found to be lethal, a phenomenon known as negative complementation (Welshons, 1971; Portin, 1975; Foster, 1975; Kelly et al., 1987). Mutations in *deltex (dx)*, a gene encoding for a ubiquitin ligase involved in Notch trafficking, were discovered through genetic screenings as mutations able to suppress the lethality of the negative complementation of *Ax* mutants (Xu and Artavanis-tsakonas, 1990; Busseau, 1994). Further, mutations in dx have been found to suppress

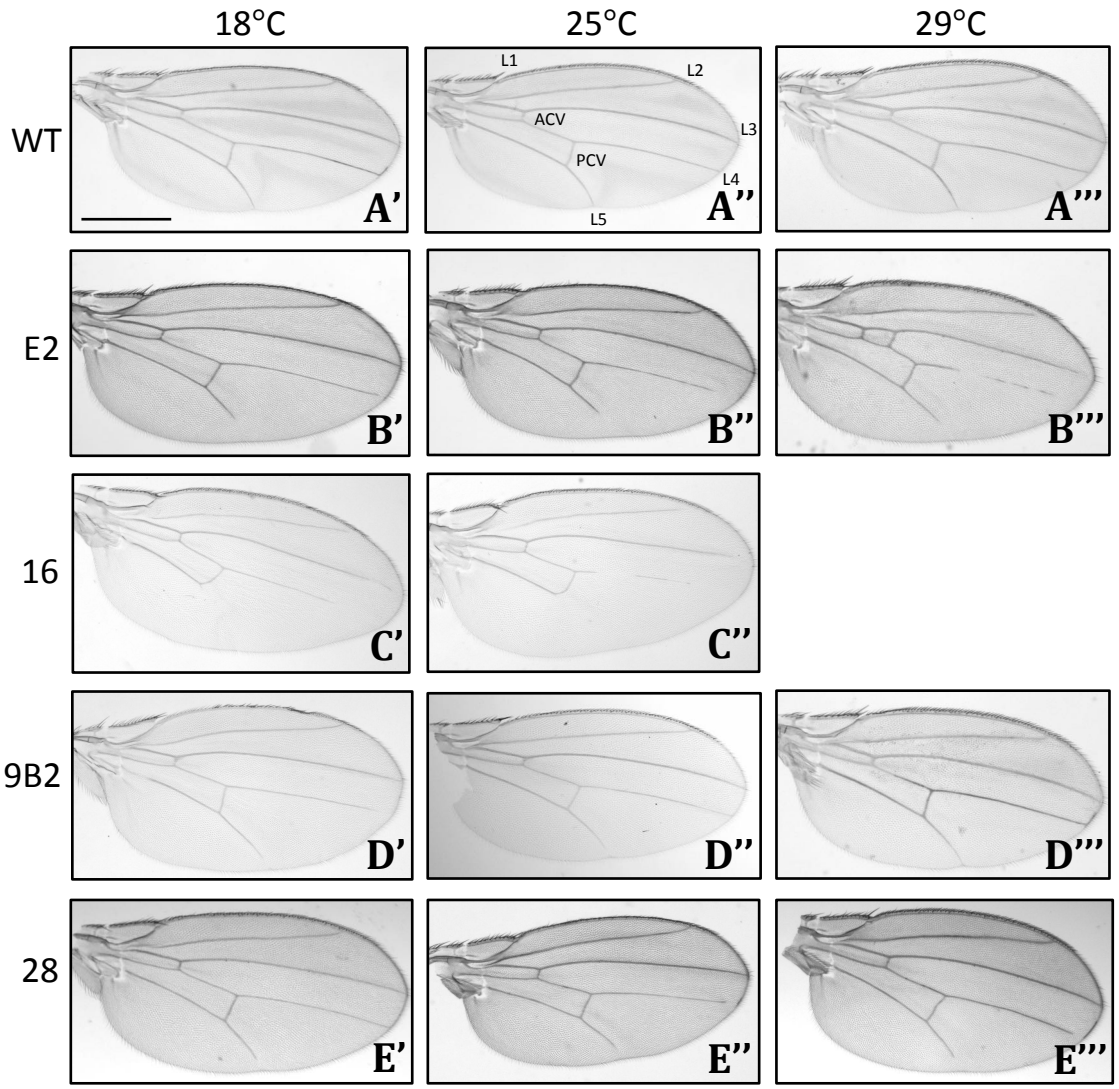
the Ax phenotype and enhance the *dx* mutant phenotype in *Ax* enhancer mutants, such as *E2*.

All the aforementioned studies showed that *Ax* mutants have a similar gain-of-function outcome on the Notch pathway, however how *Ax* mutants achieve this outcome and how they affect the Notch pathway are still open questions. The classification of *Ax* mutants, their phenotypic differences and the negative complementation are all observations that suggest different *Ax* mutants might affect the Notch pathway in different ways. Moreover, the genetic interactions of *Ax* and *dx* mutants suggest that Dx may have a role in the regulation of mutant Ax protein.

The aim of this chapter is to identify mechanistic differences in *Ax* mutants starting from the phenotypic analysis of *Ax* alleles and progressing to the molecular level. To this purpose the first section will aim to analyse the phenotypic differences between *Ax* alleles and their interaction with *dx*.

3.1.1 *Ax* mutants show a gain of function phenotype which is allele-specific

Notch has been found to be involved in *Drosophila* wing vein specification (Rebay et al., 1993; Johannes and Preiss, 2002) and mutations in Notch have been described to positively or negatively affect the wing vein specification depending on their effect on the Notch pathway. It was shown that *Ax* mutants cause a gain-of-function wing phenotype, characterised by shortening of longitudinal veins, which varies among different *Ax* alleles and with temperature. With the aim to dissect the phenotypic differences among *Ax* mutants, the wing phenotype of two enhancer mutants, *E2* and *16*, and two suppressors, *9B2* and *28*, were examined and compared at different temperatures. To do so, the wing phenotype of *Ax* males was scored at different temperatures based on number of veins shortened (Fig. 3.1F). Five longitudinal veins (from L1 to L5) and two cross-veins, anterior and



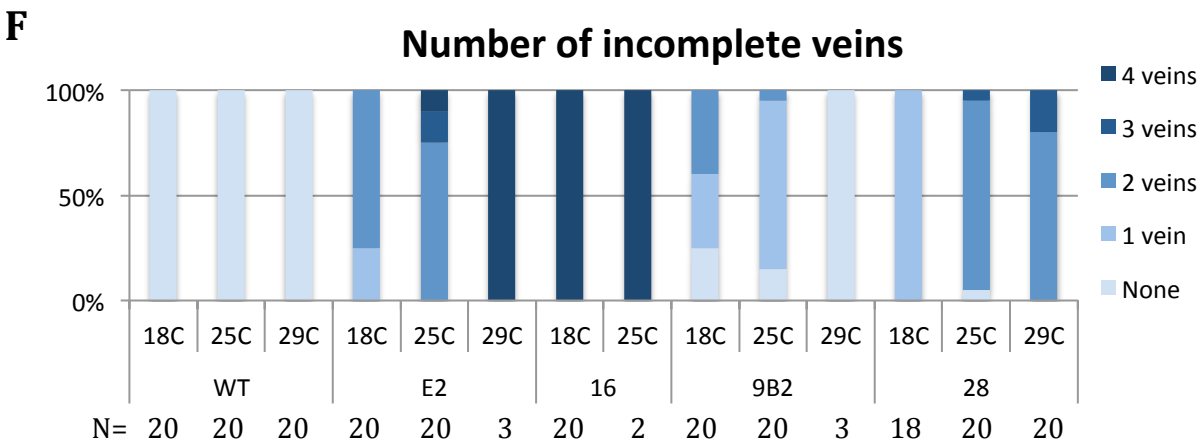


Figure 3.1. Wing phenotypes of Ax mutants at different temperatures. Wings of male flies are shown at 18°C (A'-E'), 25°C (A''-E'') and 29°C (A'''-E'''). (A) shows a yw wing and is the wild type (WT) reference. The Ax mutant wings are (B) E2, (C) 16, (D) 9B2 and (E) 28. Plot (F) shows the quantification of the phenotypes of Ax mutants and WT at different temperatures according to the number of incomplete veins between L2-L5. Scale bar(A): 500µm

posterior (ACV and PCV), are present in wild-type (WT) fly wings (Fig. 3.1A''). In order to compare the severity of phenotypes the veins L2-4 were examined for gapping frequency. At 25°C the phenotype of the enhancers was generally more severe than suppressors, that is two to four veins were gapped in *E2* and four veins incomplete in *16* which was nearly lethal at this temperature (Fig. 3.1B'', C''). At 25°C *9B2* phenotype showed shortening of one to two veins and *28* of two to three veins (Fig. 3.1D'', E''). In all genotypes, L5 tended to be the most sensitive vein, followed by L4 then L2 with L3 being the most robust.

At 18°C *E2* was milder than at 25°C and affecting one or two veins, such as L5 only or together with L4 (Fig. 3.1B'). *16* was shortening all four veins from L2 to L5 comparable to at 25°C, but it was viable at 18°C (Fig. 3.1C'). On the contrary, *9B2* phenotype appeared more severe compared to 25°C with one to two veins shortened, such as L5 only or together with L2 or L4 (Fig. 3.1D'). In the majority of cases *9B2* was also shortening the ACV and PCV.

Very few *9B2* and *E2* eclosed at 29°C and *16* was totally lethal. Three escaped males were collected from *9B2* and *E2*. Interestingly, *E2* showed a stronger phenotype with four veins shortened from L2 to L5 and an extra ACV (Fig. 3.1B'''). The appearance of extra veins has been associated with constitutive activation of Notch (Johannes and Preiss, 2002), suggesting the extra ACV in *E2* might be another sign of gain of function of Notch. On the contrary, *9B2* showed a WT wing phenotype, suggesting its additional activity in the wing veins is strongly reduced at this temperature (Fig. 3.1D''').

In summary, all *Ax* mutants showed a gain-of-function phenotype, which is characterised by shortening of the wing veins, suggesting the mutants have the same outcome on the Notch pathway. However, there were differences in the severity of the phenotype among different mutants and at different temperatures. *E2* and *16* had a clear phenotype at all temperatures. In particular, *E2* was milder at 18°C, stronger at

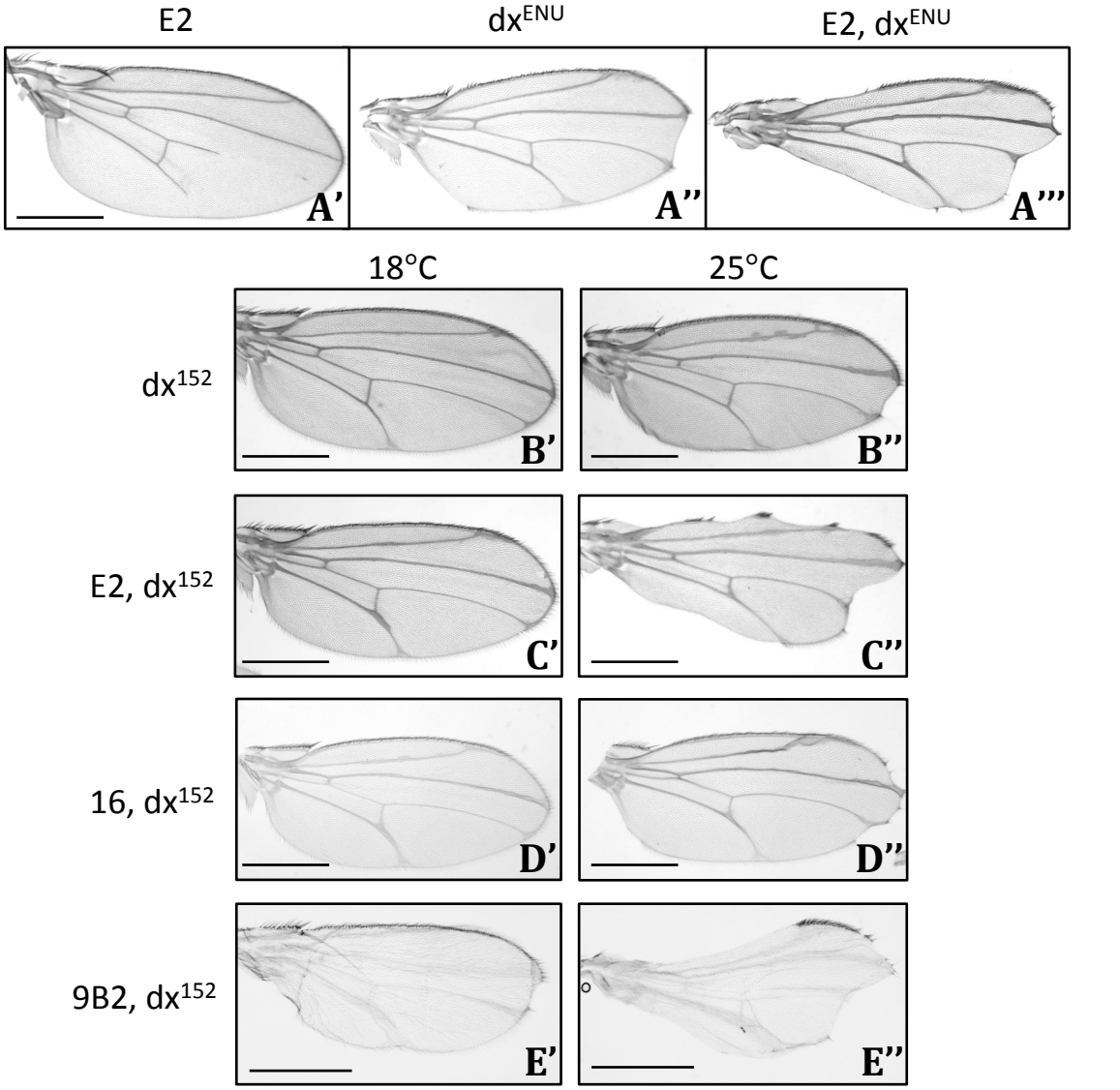
25°C and very strong and nearly lethal at 29°C, suggesting *E2* activation might intensify by increasing the temperature. *16* showed strong phenotype at 18°C and 25°C and it was nearly lethal at 25°C and lethal at 29°C, indicating that *16* might be stronger at high temperature similarly to *E2*. On the contrary, *9B2* wing phenotype was more severe at 18°C, milder at 25°C and with no wing phenotype and nearly lethal at 29°C, suggesting high temperature might decrease *9B2* activation.

The phenotypic differences observed in *Ax* mutants across different temperatures suggest *Ax* mutants might affect different routes and components of Notch regulation.

3.1.2 *Ax* mutants show a strong genetic interaction with *deltex* mutants

Mutations in *dx* have been shown to suppress the Ax phenotype and enhance the dx^{enu} mutant phenotype in *Ax* enhancer mutants (Xu and Artavanis-tsakonas, 1990; Busseau, 1994). dx^{enu} is a single amino acid change in the second WWE domain required to bind to Notch (Fuwa et al., 2006). To repeat what was shown in previous studies, dx^{enu}, *E2* flies were out crossed and their wings dissected (Fig. 3.2A). As previously shown, *E2,* dx^{enu} showed a strong loss of function wing phenotype, which was characterised by margin loss and vein thickening (Fig. 3.2A'''). What is remarkable is that a mutation in *dx* is capable of switching the strong gain of function phenotype of *E2* (Fig. 3.2A') into a strong loss of function, which is stronger than dx^{enu} (Fig. 3.2A'', F). However, in the previous studies, null alleles of *dx* were not used, therefore it was difficult to fully interpret the genetic interactions observed. Also, the effect of *dx* mutants was only tested on *Ax* enhancers and not on *Ax* suppressor alleles.

To better describe the interaction between *Ax* and *dx* mutants, *Ax* flies were combined with the *dx* null allele, dx^{152} (Fuwa et al., 2006), and their phenotype was examined at different temperatures. The phenotype of *Ax*,* dx^{152} males was scored based on wing margin loss



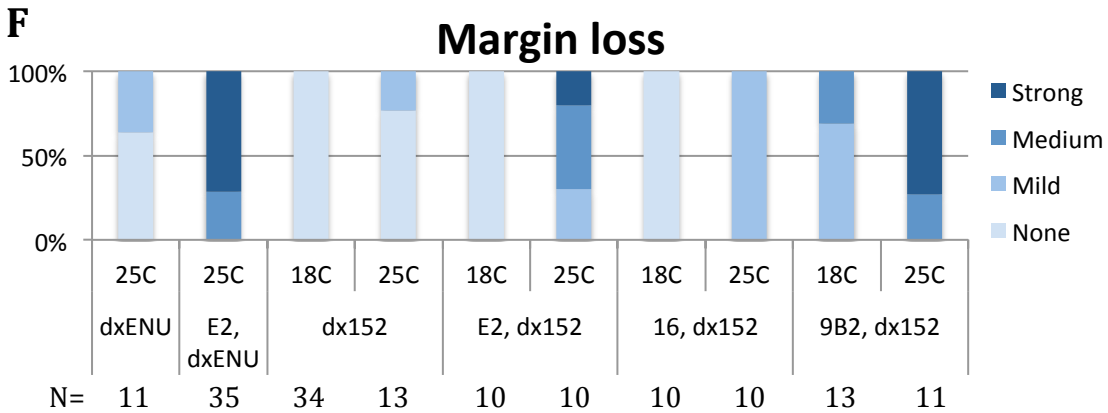


Figure 3.2. Wing phenotypes of Ax, and dx mutants. (A) shows confirmation of the strong genetic interaction between dx^{ENU} and E2. Panel A shows the male wing phenotype of (A') E2, (A'') dx^{ENU} and (A''') E2, dx^{ENU}. (B-E) shows a similar interaction of the dx^{152} null allele with both the enhancer and suppressor Ax classes at both 18°C and 25°C. The male wings shown are (B) dx^{152} , (C) E2,dx^{152}, (D) 16,dx^{152}, (E) 9B2, dx^{152} (were inflated from male pupae at stage P14). These were bred at 18C (B'-E') and 25C (B''-E''). Plot (F) shows the quantification of the phenotypes of Ax, dx and dx mutants at different temperatures according to the severity of margin loss. Scale bars: 500µm

(Fig. 3.2F). A strong loss of function phenotype was observed in *E2, dx^{152} as* well as *16, dx^{152}* and *9B2, dx^{152}* at 25°C similar to what was observed with *E2, dx^{ENU}* combination. Both margin loss and thickening of veins were stronger in *Ax*, dx^{152}* flies compared to *dx^{152}* (Fig. 3.2B''-E'', F) and the viability of *Ax*, dx^{152}* was markedly reduced. In particular, *9B2, dx^{152}* was lethal at the developmental pupal stage 14 (83-103 hours from pupation phase according to Bainbridge and Bownes, 1981) and its wing phenotype was examined by inflating the wings of the pupae as described in Lawrence et al., 2000 (Fig. 3.2E''). At 18°C the phenotypes of *dx^{152}* and *Ax*, dx^{152}* were milder (Fig. 3.2B'-E', F) and the viability was improved for the enhancer class combinations, though *9B2, dx^{152}* was still lethal. Increasing the temperature to 29°C resulted in lethality of all the *Ax*, dx^{152}* and *dx^{152}*. Taken together these observations indicate that all *Ax* mutants strongly interact with *dx* mutants suggesting that Dx may be involved in the production of the *Ax* mutant phenotype. It is noteworthy that all *Ax* mutants in combination with *dx^{152}* have the same dependency on temperature, which was observed to be different among *Ax* mutants (see § 3.1.1)

3.1.3 *carnation^{1}* reduces the gain-of-function wing phenotype of *Ax* mutants

The strong loss of function in *Ax, dx* flies might indicate that the gain-of-function of the mutants arises from Dx-dependent activation. To test if Dx function is to activate *Ax* mutants, *Ax* flies were combined with *carnation^{1}* (*car^{1}*), a hypomorphic allele, which reduces the function of the HOPS complex, thus inhibiting the lysosomal-dependent activation in the Dx-mediated route (Wilkin et al., 2008; Shimizu et al., 2014). The gain-of-function phenotype of all *Ax* mutants was reduced but not suppressed in *Ax, car^{1}* flies (Fig. 3.3). Also, the reduced viability of *16* flies was not improved in *16, car^{1}* flies. This suggests that lysosomal-dependent signalling might contribute to the Ax gain-of-function phenotype, but the achievement of the gain-of-function is likely more

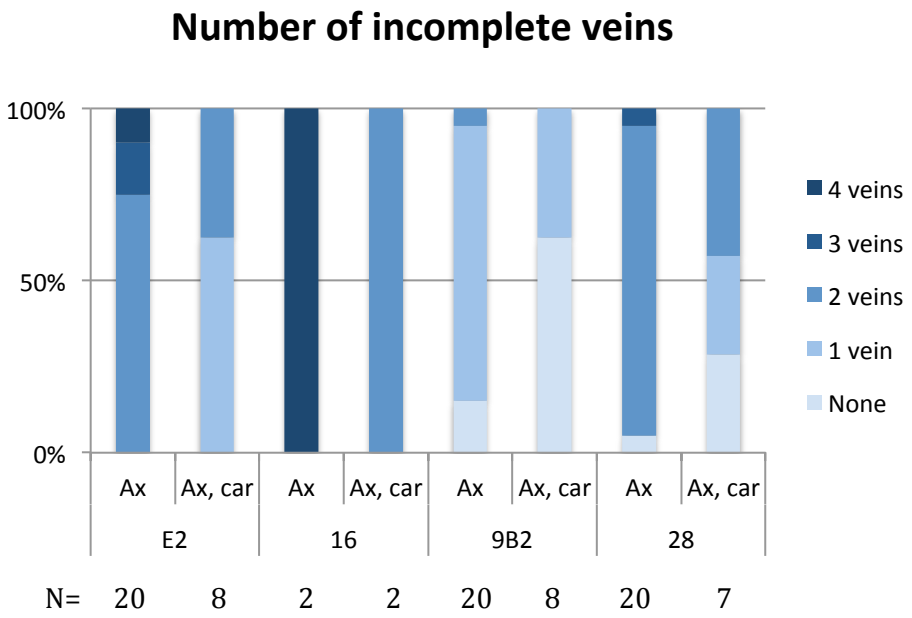


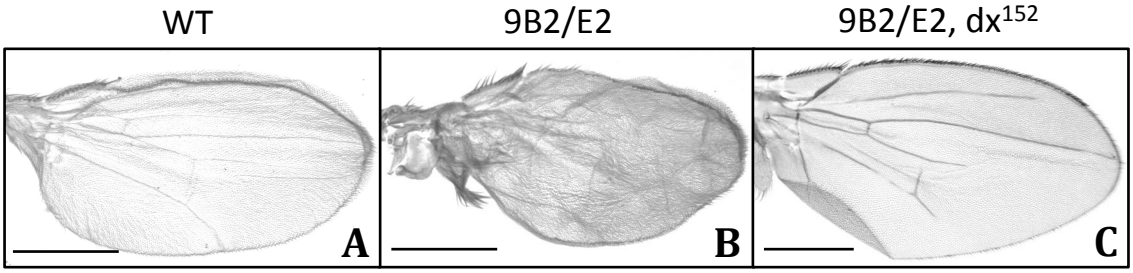
Figure 3.3. Scoring of Ax, car[1] mutant wing phenotypes. The plot shows the quantification of the phenotype of Ax and Ax, car[1] mutants at 25°C according to the number of incomplete veins.

complex than simply arising from increased Dx-mediated lysosomal-dependent signalling.

3.1.4 *deltex*^{152} rescues the negative complementation of *E2/9B2*

The heteroallelic combination of suppressor/enhancer *Ax* mutants was found to be lethal and to be rescued by mutations in *dx* (e.g. *dx*^{ENU}, Xu and Artavanis-tsakonas, 1990; Busseau, 1994). However, neither the phenotype of the lethal combination or of the rescued flies was described, making it difficult to identify the cause of the lethality and rescuing. To determine the phenotype and viability of these crosses, the negative complementation was repeated by crossing *9B2*/FM7 virgins with *E2* males, and the rescuing by crossing *9B2, dx*^{152}/FM7 virgins with *E2* males. The viability of the crosses was determined by scoring the ratio of *Ax/Ax* females and FM7 males, because FM7 males should have the same viability among different crosses. *9B2/E2* was lethal, while *9B2, dx*^{152}*/E2* was able to rescue the negative complementation and showed 5% viability (Fig. 3.4D). The difference between the ratio of *Ax/Ax* females and FM7 males in the two crosses was statistically significant ($P<0.05$). *9B2/E2* flies were found to reach the pupal stage 14 and their wing phenotype was determined by inflating the wings of the pupae together with the wing of WT pupae as a control. Interestingly, unlike WT, *9B2/E2* did not show any veins in the wing, (Fig. 3.4A, B), suggesting the combination of *9B2* and *E2* might give rise to a very strong gain of function of Notch, which is likely responsible for their lethality. The phenotype of *9B2, dx*^{152}*/E2* showed a strong Ax phenotype with shortening of 2 to 4 veins (Fig. 3.4C), similar to the one of the original *Ax* alleles. This suggests *dx*^{152} is able to reduce the strong gain of function produced by *9B2/E2*, thus rescuing its lethality.

Although both the suppressor and enhancer class show the same wing vein gapping phenotype, there are differences in their penetrance and temperature sensitivity. Both classes of mutation can switch from a gain to a loss of phenotype in combination with *dx* mutants. However, the



D

	Ax/Ax	FM7 / Y	Viability (%)
9B2/FM7 X E2/Y	0	112	0
9B2, dx^{152}/FM7 X E2/Y	5	101	5

Fisher's exact test P=0.0259

Figure 3.4. 9B2/E2 negative complementation and rescuing by a decreased dose of Dx. (A) WT adult female wing, (B) 9B2/E2 inflated wings from female pupae due to late pupal lethality, (C) 9B2/E2, dx^{152} adult female wing. Table (D) shows the number of Ax/Ax females and FM7 males in 9B2/ FM7 X E2/Y cross and 9B2, dx^{152}/FM7 X E2/Y and the viability of the progeny of the two crosses. The significance of the rescuing was measured by Fisher's exact test and the P value is indicated below the table. Scale bars: 500μm

best evidence that the two classes of *Ax* alleles are using two different mechanisms to activate Notch signalling comes from their trans-heterozygous combination. This shows a much stronger increase in Notch signalling at 25°C as compared to the homozygous *Ax* mutants. The most likely explanation for this is that the two classes of *Ax* alleles are increasing Notch signalling through two different mechanisms such that their effects are synergistic.

3.2 *Ax* mutants show allele specific differences in Notch signalling activation

Notch pathway is activated upon binding of Notch with its ligands, which causes proteolytic cleavages resulting in the Notch intracellular domain translocating into the nucleus and activating transcription of target genes. However, it is now known that Notch pathway is also regulated in a ligand-independent manner through different endocytic trafficking routes in which Notch receptor can either be activated or degraded (Shimizu et al., 2014; Hori et al., 2011; Wilkin et al., 2008; Vaccari et al., 2008; Wilkin at al., 2004; Hori et al., 2004). The overall Notch signalling is the result of the combination of ligand-dependent and independent routes, which act together to tune the pathway and maintain its robustness across different conditions. For instance, the Notch pathway was found to be resistant to temperature variation in *Drosophila* and this is because Notch regulatory routes have different temperature dependencies and they can compensate for each other (Shimizu et al., 2014).

According to what was observed in the phenotypic analysis of *Ax* mutants, the phenotypic differences in *Ax* alleles might reflect different ways in which *Ax* mutants affect Notch regulation. All *Ax* mutants showed a similar gain of function phenotype, suggesting they have the same outcome on the Notch pathway. However, Notch gain of function can result from different amounts of ligand-dependent and

independent signalling of Notch. Therefore, it is possible that different *Ax* mutants can produce a gain of function of Notch by using signalling contributions from different regulatory routes. This might explain why *Ax* mutants have different genetic interactions and their phenotype varies with temperature.

To test this hypothesis, the effect of *Ax* mutants on the different signalling routes of Notch were analysed in *Drosophila* Schneider 2 cells (S2 cells). Two endocytic routes have been found to give rise to ligand-independent Notch activation in S2 cells (Fig. 3.5; Shimizu et al., 2014). One route is mediated by Suppressor of dx (Su(dx)), a ubiquitin ligase which induces the internalisation of Notch into late endosomes leading to its degradation. Notch can still be activated in this route, when it is localised on the membrane of early endosomes. The second route is mediated by Dx, another ubiquitin ligase, which keeps Notch on the membrane of late endosomes, allowing Notch to be cleaved and activated. Using S2 cells, it is possible to singularly analyse the signalling contribution of the ligand-dependent and independent routes. When Notch is expressed in S2 cells, it produces a signal, defined as 'basal signalling', which is believed to arise from S2 cells endogenous Su(dx) route. Dx-mediated activation can be assayed by expression of Notch and Dx in S2 cells, which leads to the upregulation of the basal signalling. In addition, the ligand-dependent activation of Notch can be assayed by plating Notch expressing S2 cells onto Delta-expressing S2 cells (S2-Dl cells).

Temperature can also be used to dissect the contribution of the different routes to Notch activation in S2 cells, because Notch flux in ligand-dependent or independent routes can be modified by temperature (Fig. 3.5; Shimizu et al., 2014). In particular, Su(dx) activity is temperature-dependent because of its temperature-sensitive HECT domain which is required for ubiquitination and degradation of Notch (Wilkin et al., 2008; Shimizu et al., 2014). At 18°C the activity of Su(dx) HECT domain is reduced and Notch is retained on the limiting

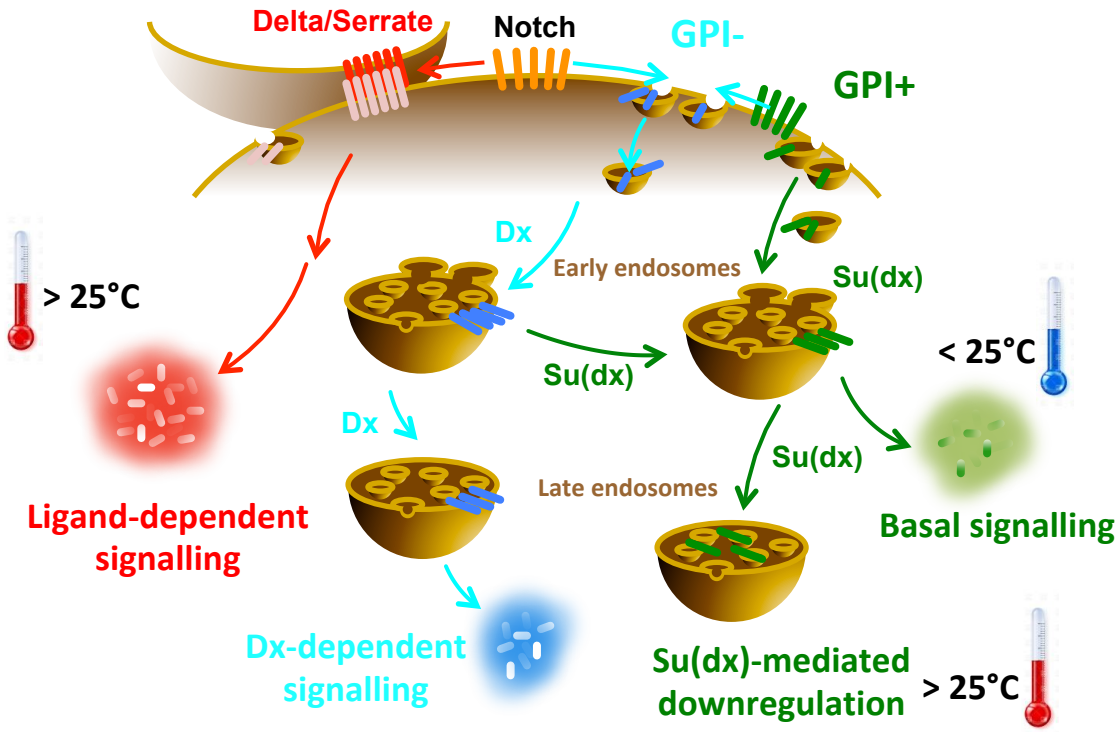


Figure 3.5. Notch regulatory routes in S2 cells. Ligand-dependent Notch pathway is indicated in red, while the ligand-independent routes, mediated by Dx and Su(dx) are in blue and green, respectively. Accordingly, Notch receptors are indicated in light red, blue and green depending on the route they are localised in. Notch can be endocytosed in the Dx route and kept on the limiting membrane of late endosomes, with the intracellular domain facing the cytosol, where it can be activated through a lysosomal-dependent mechanism. Notch can also be endocytosed in the Su(dx) route or shifted from the Dx route. In the Su(dx) route Notch is internalised into late endosomes and degraded. The different signalling routes have different temperature dependencies which are indicated by the thermometers. Ligand-dependent activation and Su(dx)-mediated downregulation of Notch are favoured at high temperature (>25C). The endocytosis of Notch in the Su(dx) route and the shift from the Dx route are also increased at high temperature. On the contrary, at low temperature (<25C) Su(dx) HECT domain is not active, thus Su(dx) cannot trigger the internalisation of Notch inside late endosomes. In this case, Su(dx) route leads to activation of Notch instead of degradation. Dx-mediated route is stable across different temperatures but it is favoured at low temperature when Su(dx) is less active. Su(dx) route occurs in glycophosphatidylinositol-protein-positive vesicles (GPI+) while Dx route is in GPI-protein-negative vesicles (GPI-), therefore GPI can be used as a marker to determine in which route Notch is trafficked. When Notch is expressed singularly in S2 cells it gives rise to a mild signalling, named basal signalling, which arises from the endogenous GPI+ route. If Notch is expressed singularly or co-expressed with Su(dx) it is trafficked in GPI+ vesicles and internalised into late endosomes, whereas when co-expression with Dx, Notch is in GPI- vesicles and on the limiting membrane of late endosomes. (Figure modified from Shimizu et al., 2014).

membrane of late endosomes and activated in this route instead of being degraded, thus increasing the basal signalling of Notch in S2 cells. At 25°C Su(dx) activity is balanced between activation and degradation of Notch and has a minor positive effect on basal signalling in S2 cells. On the contrary, at 29°C Su(dx) HECT domain activity is increased, more Notch is degraded and basal signalling is reduced. Also, the endocytosis of Notch in Su(dx) route is favoured at higher temperature. Dx-mediated signalling is stable at all temperatures and favoured at 18°C because of Su(dx) decreased activity. An increase in the ligand-dependent signalling can be observed at high temperature and this is compensated by the increased Su(dx)-mediated degradation. At low temperature ligand-dependent signalling is instead decreased together with Su(dx)-mediated degradation, thus compensating the lower signalling.

In this section, the signalling of *Ax* mutants will be assayed in the presence of ligands, Dx, Su(dx) or at different temperatures in S2 cells. In this way it will be possible to determine which signalling routes are affected by which *Ax* mutant.

3.2.1 *Ax* mutants downregulate Notch basal signalling

To analyse the signalling activation of *Ax* mutants in S2 cells, *E2, 16, 9B2* and *28* mutations were introduced in PMT-Notch, which is an inducible vector containing Notch WT cDNA (Shimizu et al., 2014). These constructs were transiently transfected in S2 cells using Effectene reagent kit (Qiagen). To test if the constructs were expressed in S2 cells, the protein expression was measured by western blot (Fig. 3.6B). The basal signalling of WT and *Ax* mutants was analysed by expressing the mutant constructs only in S2 cells and their signalling activation was measured by luciferase reporter assay (Dual Glo kit, Promega). The endogenous expression of Dx is low in S2 cells and they do not express ligands (Gelbart and Emmert, 2013), therefore this condition mimics a situation in which Notch is not exposed to Dx or ligands (Shimizu et al.,

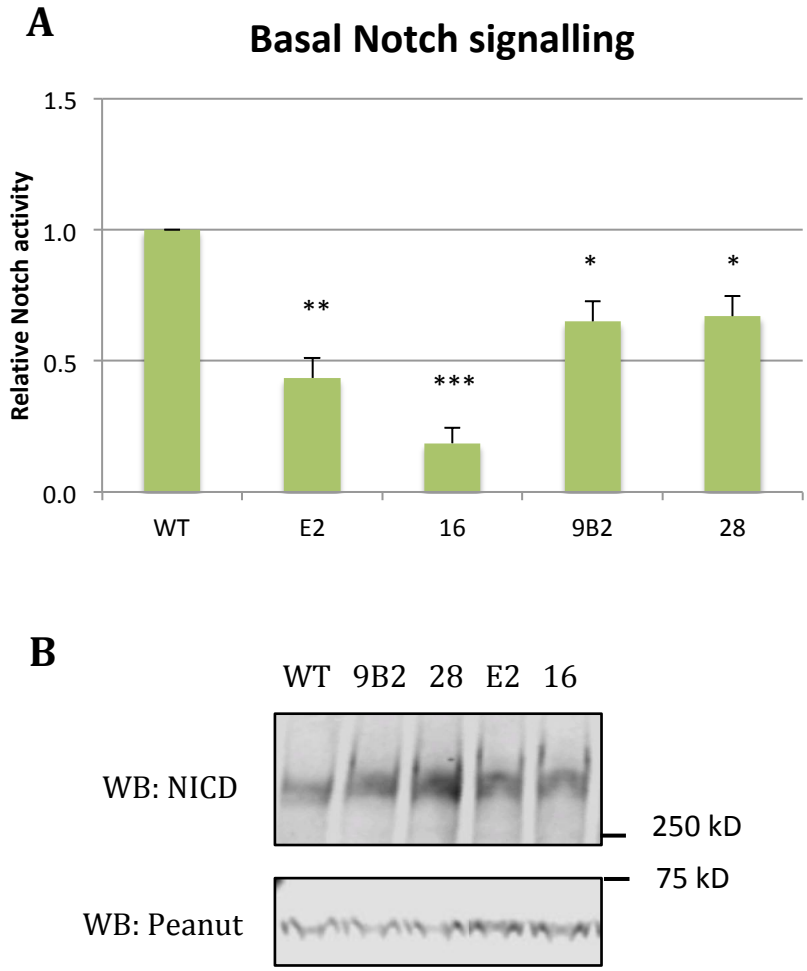


Figure 3.6. Basal signalling and protein expression of Ax mutants. WT, E2, 16, 9B2 and 28 were transfected in S2 cells. (A) Signaling activation was measured by luciferase reporter assay. Data are shown as means normalized relatively to WT expressed alone in S2 cells. The statistical significance was determined by Student T test and is relative to the basal signaling of WT (*p<0.05; **p<0.01; ***p<0.001). Error bars indicate SEM (n=3). (B) Protein expression was analysed by Western blotting, Notch was detected using anti-NICD antibody. Peanut protein expression was used as a control for cell expression and loading and detected with anti-Peanut antibody.

2014). Interestingly, the basal signalling of *Ax* mutants was found to be significantly downregulated compared to WT (Fig. 3.6A). The signalling reduction might reflect the strong loss of function phenotype observed in *Ax*, dx mutant flies (Fig. 3.2). The reduction was more severe in *E2* and *16* compared to *9B2* and *28*, thus underlying possible differences between enhancers and suppressors.

3.2.2 *Ax* mutants have a similar potential for ligand-dependent signalling as WT Notch

The ligand-dependent activation of WT and *Ax* mutants was assayed by culturing the transfected cells on top of fixed Delta-expressing S2 or non-transfected S2 cells as a control. The ligand-dependent signalling of *Ax* mutants was similar to WT, suggesting the mutants are able to respond to ligands (Fig. 3.7A). However, if the ligand-dependent signalling values were normalised by the basal signalling values, the fold change activation of *Ax* mutants was higher than WT given that the basal values of *Ax* mutants are lower (Fig. 3.7B).

3.2.3 *Ax* mutants show different Dx-dependent activation

Dx-dependent signalling was measured by co-expressing *Ax* mutants and PMT-Dx in S2 cells. *E2, 16* and *28* showed a similar Dx-dependent activation as WT, whereas Dx-dependent activation of *9B2* was significantly reduced (Fig. 3.8A). This suggests *9B2* might be differently regulated by Dx compared to *E2, 16* and *28*. The fold change activation showed an even higher Dx-dependent signalling in *16, E2* and *28* compared to WT and still a reduced signalling in *9B2* (Fig. 3.8B). Interestingly, the fold change activation is in agreement with the severity of the gain of function wing phenotype in *Ax* flies, which was generally more to less severe from *16* to *E2*, *28* and *9B2* at 25°C (Fig. 3.1).

Ax mutants were co-expressed with Su(dx) alone or together with Dx to test their response to Su(dx). At 25°C Su(dx) activity is not very strong and can induce a slight increase in the basal signalling of WT. This

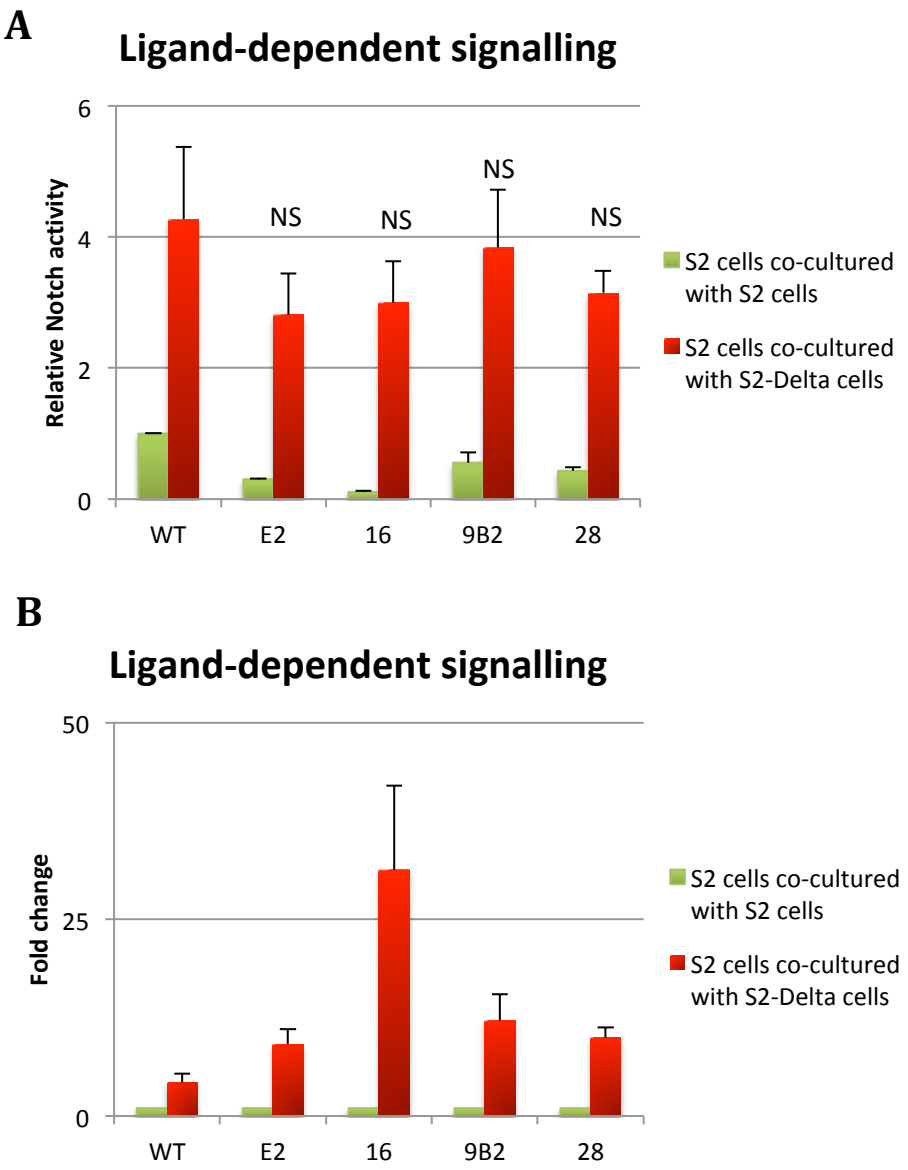


Figure 3.7. Ligand-dependent signalling of Ax mutants. WT, E2, 16, 9B2 and 28 were transfected in S2 cells. Transfected S2 cells were cultured alone or co-cultured with ligand-expressing S2-Dl cells. Signalling was measured by luciferase reporter assay. Plot (A) shows the basal signalling (green columns) and ligand-dependent signalling (red). Data are shown as means normalized relatively to WT expressed alone in S2 cells. The statistical significance was determined by Student T test and is relative to the basal signaling of WT (NS=non significant). Plot (B) shows the fold change of ligand-dependent activation. The ligand-dependent signalling data were normalised by the basal signalling of each sample. Error bars indicate SEM (n=3).

increase was also observed in the mutants but did not reach statistical significance in any sample (Fig. 3.8C). The combination of Dx and Su(dx) induced a decrease in the Dx-dependent signalling which was significant in all samples (Fig. 3.8C).

3.2.4 *Ax* mutants signalling show different temperature dependencies

At the beginning of the chapter, it was shown that the severity of *Ax* mutant phenotype *in vivo* varies with temperature (Fig. 3.1). Therefore, it was interesting to test if temperature can change the signalling of *Ax* mutants in cell culture. To test this idea, the basal and Dx-dependent signalling of *E2* and *9B2* were analysed at 18°C, 25°C and 29°C (Fig. 3.9). Notably *E2* basal signalling was significantly lower at each temperatures compared to WT and its Dx-dependent signalling was always not significantly different from WT. On the contrary, *9B2* Dx-dependent signalling was not significantly different compared to WT at 18°C, was significantly reduced at 25°C and strongly and significantly reduced at 29°C. *9B2* basal signalling was also not significantly different from WT at 18°C and 29°C, contrary to what was observed at 25°C.

E2 signalling was more stable across different temperatures, suggesting *E2* signalling might be less temperature sensitive. On the contrary, *9B2* signalling seemed more sensitive to temperature variation. *9B2* Dx-dependent signalling was reduced as temperature increases. *Ax* mutants do show different effects on the three routes of Notch pathway and in particular on ligand-independent signalling. In addition, the differences observed in *E2* and *9B2* across temperature variation might indicate that the nature and the dependencies of their signalling are different from WT and each other.

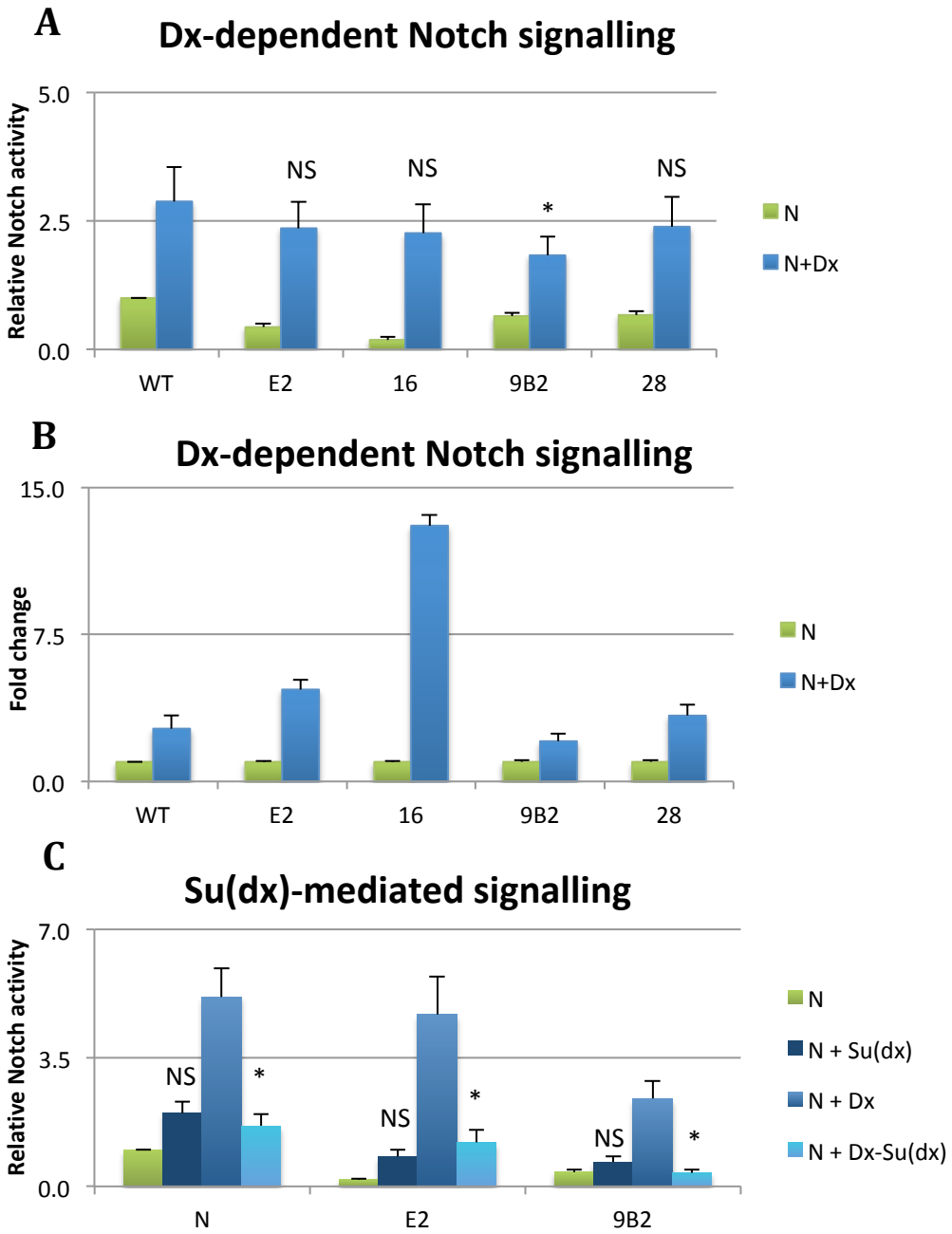


Figure 3.8. Ligand-independent signalling of Ax mutants. In plot (A) WT, E2, 16, 9B2 and 28 were transfected alone (green columns) or co-transfected with Dx (blue columns) in S2 cells and signaling was measured by luciferase reporter assay. Data are shown as means normalized relative to WT expressed alone in S2 cells. Plot (B) shows the fold change of Dx-dependent activation. In plot (B) the Dx-dependent signalling data were normalised by the basal signalling of each sample. In plot (C) WT, E2, 16, 9B2 and 28 were transfected alone (green columns) or co-transfected with Su(dx) (dark blue columns) or Dx (blue columns) or Dx+Su(dx) (light blue columns). Data are shown as means normalized relatively to WT expressed alone in S2 cells. The statistical significance was determined by Student T test and is relative to the basal signaling of WT in plot A. In plot (C) the significance of N+Su(dx) samples is relative to the basal signalling of each sample and the significance of N+Dx+Su(dx) samples is relative to the Dx-dependent signalling of each sample (NS=non significant; *$p<0.05$). Error bars indicate SEM (n=3).

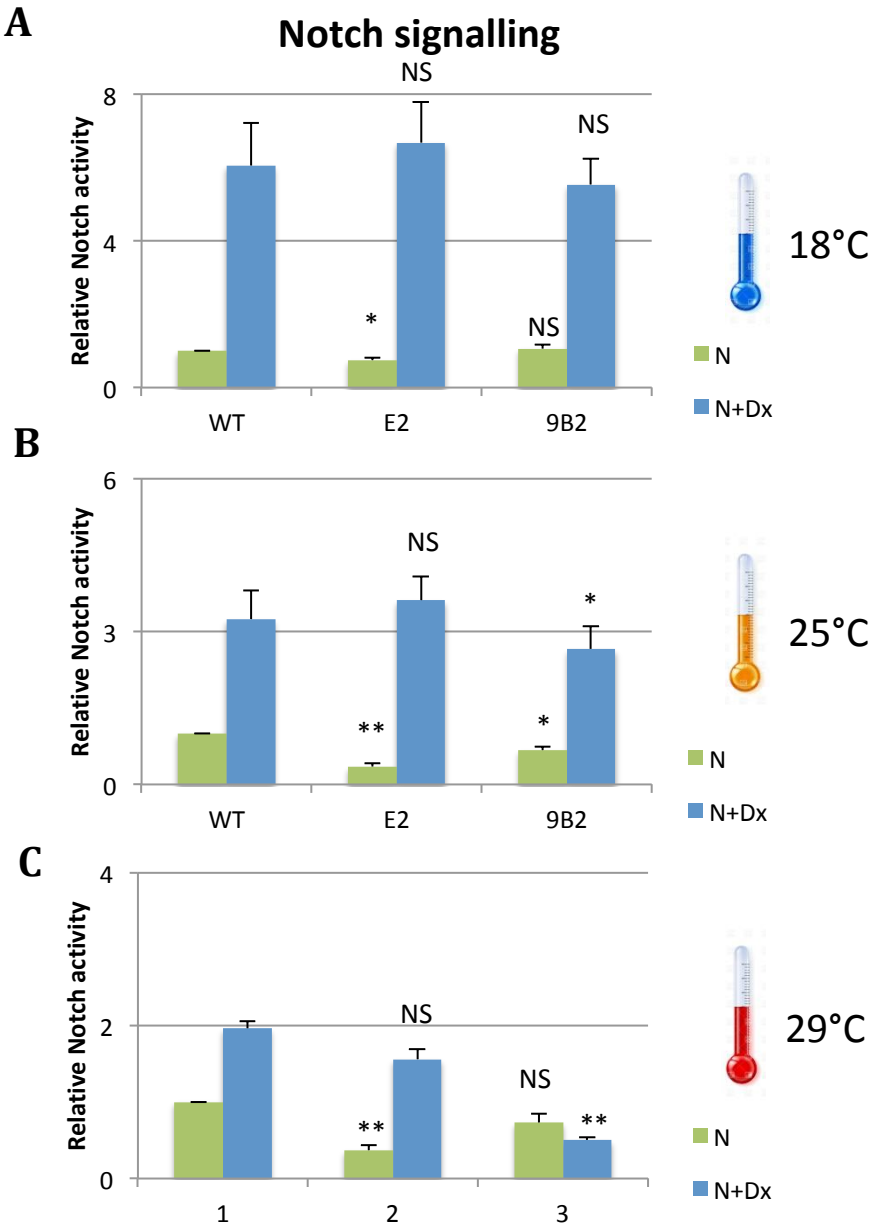


Figure 3.9. Ligand-independent signalling of Ax mutants at different temperatures. WT, E2, and 9B2 were transfected alone (green columns) or co-transfected with Dx (blue columns) in S2 cells and signaling was measured by luciferase reporter assay at 18C (A), 25C (B) and 29C (C). Data are shown as means normalized relatively to WT expressed alone in S2 cells at each temperature. The statistical significance was determined by Student T test and is relative to the basal signaling of WT at each temperature. (NS=non significant; *p<0.05; **p<0.01). Error bars indicate SEM (n=3).

3.3 *Ax* mutants affect Notch endocytic uptake and different steps of Notch endocytic trafficking

In the previous section, it was shown that *Ax* mutants have allele-specific effects on the ligand-independent signalling of Notch and that this suggests that they affect different steps of Notch regulation. One possibility is that the mutants are trafficked in different routes compared to WT Notch or that they alter Notch endocytosis, ultimately affecting the ligand-independent signalling of Notch. In particular, it would be interesting to understand why the basal signalling is reduced in *Ax* mutants and why *9B2* responds differently to Dx.

To address these questions, the cellular localisation of *Ax* mutants was compared with WT Notch in S2 cells. The trafficking of Notch receptor can be followed from the cell membrane to the endosomes by using anti-NECD antibody uptake assay. A number of markers can be used to determine in which route Notch is trafficked. Glycophosphatydylinositol-anchored proteins (GPI) marks the endosomes in the Su(dx) route, allowing one to distinguish whether Notch is in this route or in the Dx route which is not labelled by GPI (Fig. 3.5; Shimizu et al., 2014). Rab-7-GFP can be used to label the membrane of late endosomes, thus determine if Notch is on the late endosomal membrane and can be activated or is internalised into the endosomal lumen and degraded. When Notch is trafficked in the Dx-dependent route, it localises in GPI negative vesicles (GPI-) and is kept by Dx on the endosomal membrane. On the contrary, in the Su(dx) route, Notch is trafficked in GPI positive vesicles (GPI+) and is internalised into the late endosomal lumen. The latter is observed when Notch only is expressed in S2 cells or is co-expressed with Su(dx).

3.3.1 In the absence of Dx, both WT and 9B2 are trafficked into GPI positive vesicles while *E2*, *16* and *28* are trafficked into GPI negative vesicles.

To explain why the basal signalling is reduced in *Ax* mutants, the trafficking of the mutants was analysed by expressing *Ax* mutants only in S2 cells. GPI was used as a marker and the amount of Notch in GPI+

vs. GPI- vesicles was measured (Fig. 3.10F). As previously shown (Shimizu et al., 2014), when WT is expressed in S2 cells it localises mainly in GPI+ vesicles where it can give rise to the basal signalling. As expected, WT was mainly in GPI+ vesicles (Fig. 3.10A), however *E2*, *16* and *28* were mainly in GPI- vesicles (Fig. 3.10B, D, E). This is interesting because if Notch is in GPI- vesicles it cannot be activated in the absence of Dx and this could explain why the basal signalling of the mutants is reduced. *9B2* was mainly in GPI+ vesicles as WT, however it is worth to notice that a higher percentage of it was in GPI- compared to WT (Fig. 3.10C), although the difference was not significant. In this assay, the localisation of Notch was measured after 60 minutes of endocytosis, therefore it was not clear in which route *E2*, *16* and *28* were initially endocytosed. To test this, the assay was repeated for *E2* and WT at 5, 10, 30, 60 minutes of endocytosis (Fig. 3.10G). *E2* was observed to be mainly in GPI- vesicles at every time point, whereas WT was mainly in GPI+ vesicles, indicating *E2* is endocytosed and trafficked in GPI- vesicles and WT in GPI+ vesicles. The uptake assay was repeated for WT, *9B2* and *E2* at 60 minutes using Rab7 as a marker and the amount of Notch inside vs. on the limiting membrane of late endosomes was scored (Fig. 3.10K). Previously, WT was shown to be mainly inside Rab7-labelled late endosomes (Shimizu et al., 2014). WT, *9B2* and *E2* were mostly inside Rab7-labelled late endosomes and likely degraded (Fig. 3.10H-J). This indicates *E2* trafficking mainly leads to degradation even if it is in a different route and this is consistent with the reduction in basal signalling.

3.3.2 *9B2* strongly localises at the cell membrane while *E2*, *16* and *28* are less at the cell membrane in the absence of Dx

The trafficking results suggested that the reduction of Notch basal signalling in *E2*, *16* and *28* might result from trafficking to the wrong destination. However, this did not fully explain why *9B2* also reduced the basal signalling since its trafficking was similar to WT. A serendipitous observation that was made during the analysis of the trafficking assays is that the localisation of the *Ax* mutants at the cell

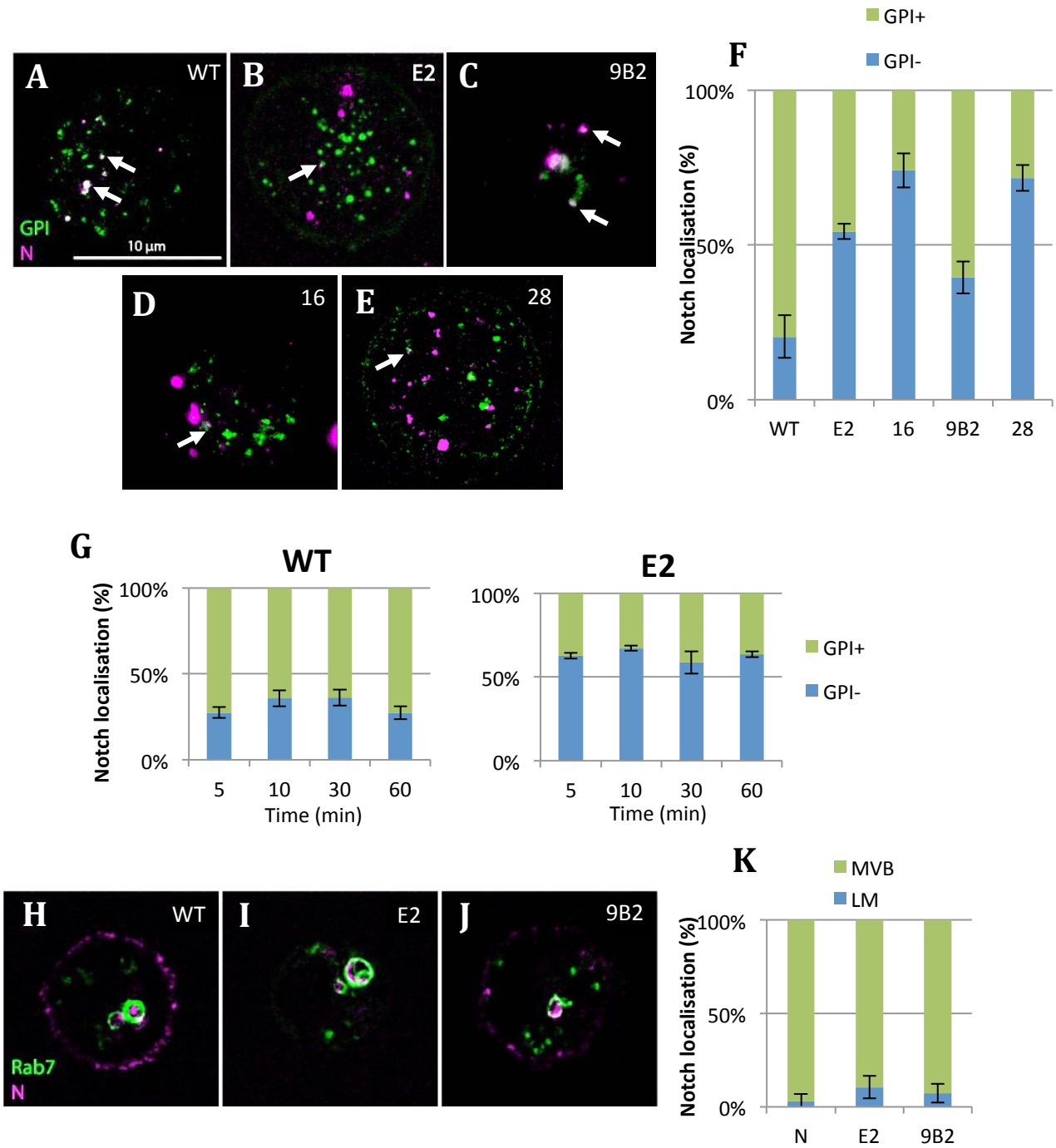


Figure 3.10. Localisation of Ax mutants in GPI+ vesicles and Rab7-labelled late endosomes in S2 cells following an-anti-Necd uptake assay. (A-E) shows the localisation of WT, E2, 9B2, 16 and 28 relative to GPI+ vesicles respectively at 60 minutes of endocytosis. White arrows indicate co-localisation of Notch and GPI-GFP. (F) shows a plot of the localisation of Notch within GPI+ or GPI- vesicles at 60 minutes of endocytosis. (G) shows a plot of the localisation of WT and E2 within GPI+ or GPI- vesicles at 5, 10, 30 and 60 minutes of endocytosis. (H-J) shows the localisation of WT, E2, 9B2 respectively, relative to Rab7-labelled late endosomes at 60 minutes of endocytosis. (K) shows the localisation of Notch on the limiting membrane or inside Rab7-labelled late endosomes. An average of 100 vesicles were scored for each sample looking at the co-localisation of Notch with GPI-GFP or in Rab7-labelled late endosomes at 60 minutes of endocytosis. Data are shown as percentage means (n=3). Error bars indicate SEM.

membrane seemed different compared to WT. To test whether the observation was right, Notch was labelled at the cell membrane by incubating the cells with anti-NECD antibody on ice and fixing the cells straight after labelling. First, the intensity of Notch at the cell membrane (surface Notch) was measured in individual cells using ImageJ and the results were normalised by the intensity of the total Notch staining (Fig. 3.11). Second, the same surface labelling method was used to label Notch at the cell membrane and then analysed by flow cytometry (FACS) (Fig. 3.12). The percentage of cells with Notch at the cell surface and the intensity of the surface Notch staining were measured in this assay. These measurements were found lower in *E2* and higher in *9B2* compared to WT (Fig. 3.12B, C), suggesting *E2* is less localised and *9B2* more strongly localised at the cell membrane. The surface staining intensity results were similar in both ImageJ (Fig. 3.11B) and FACS analyses (Fig. 3.12C). *16* and *28* were similar to E2 and showed a lower surface Notch staining and percentage in FACS (Fig. 3.12).

The surface Notch staining and FACS analysis were repeated for WT, *E2* and *9B2* in co-expression with Dx or Su(dx) (Fig. 3.13). Interestingly, Dx and Su(dx) reduced the surface Notch of WT and the mutants and the effect was generally stronger in response to Dx compared to Su(dx) (Fig. 3.13B, C). Since it is known that Dx and Su(dx) can induce the endocytosis of Notch, the amount of surface Notch could be an indication of endocytosis rate. If that is the case then *Ax* mutants surface localisation in the absence of Dx might reflect an effect on endocytosis, which could be increased endocytosis in *E2, 16, 28* and decreased endocytosis in *9B2*. Also, it is interesting that the strong surface staining of *9B2* was very significantly reduced by Dx and Su(dx), showing that *9B2* might be efficiently endocytosed in the presence of Dx and Su(dx).

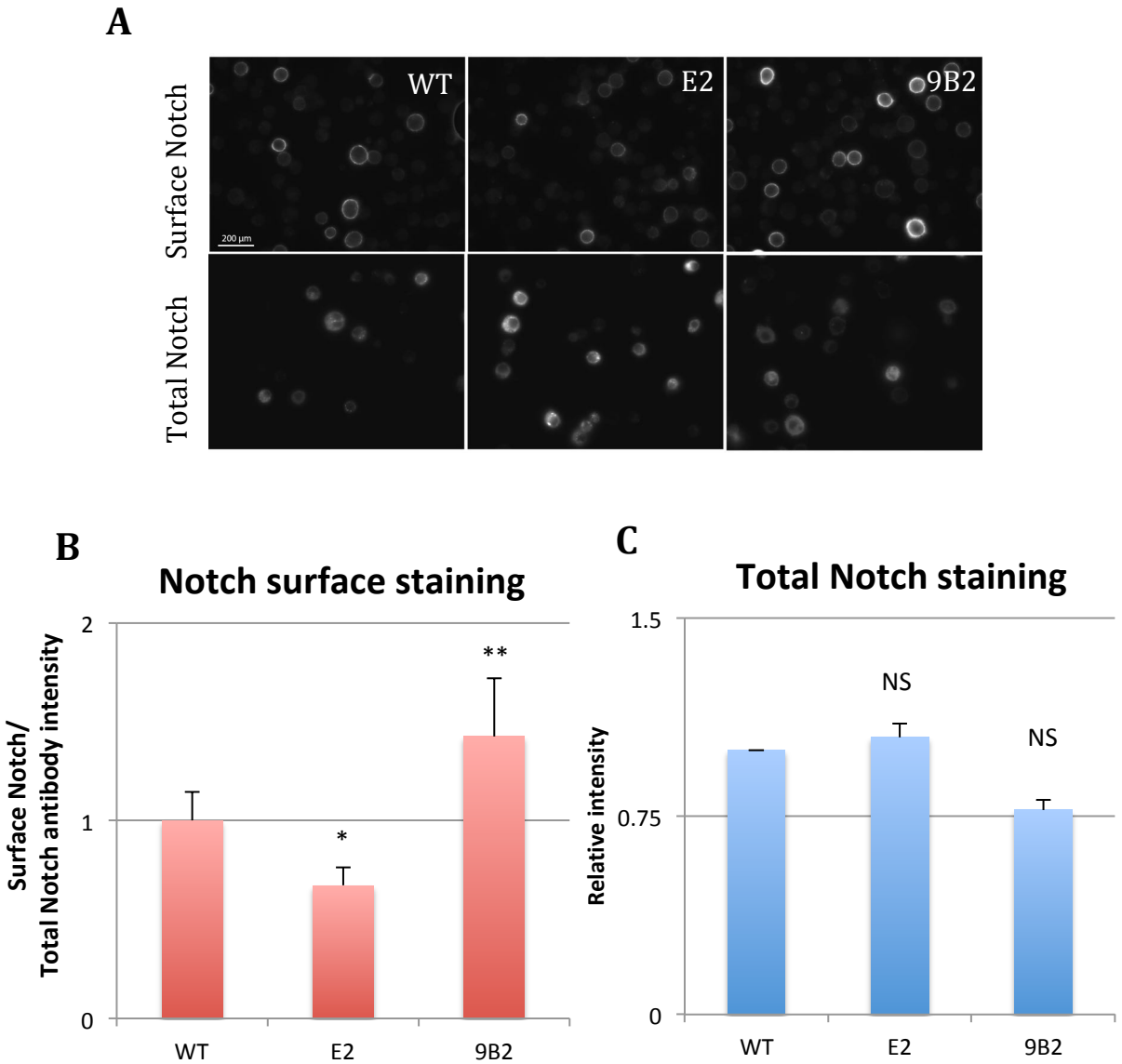


Figure 3.11. Surface and total Notch localisation of WT, E2 and 9B2 Notch constructs in S2 cells. WT, E2 and 9B2 were transfected in S2 cells. (A)Notch transfected S2 cells were either labelled with anti-NECD on ice, prior to fixation and secondary antibody detection in order to detect Notch on the plasma membrane (surface) or with anti-NECD detected post fixation and permeabilisation (total Notch staining). The intensity of surface Notch and total Notch staining were measured in individual cells using ImageJ. An average of 100 cells were scored for each sample (n=3). Plot (B) shows the mean intensity of surface Notch staining normalised by the mean intensity of total Notch staining. Plot (C) shows the mean intensity of total Notch staining. Data are shown as means normalized relatively to WT sample. The statistical significance was determined by Student T test and is relative to WT sample (NS=non significant; *p<0.05; **p<0.01). Error bars indicate SEM.

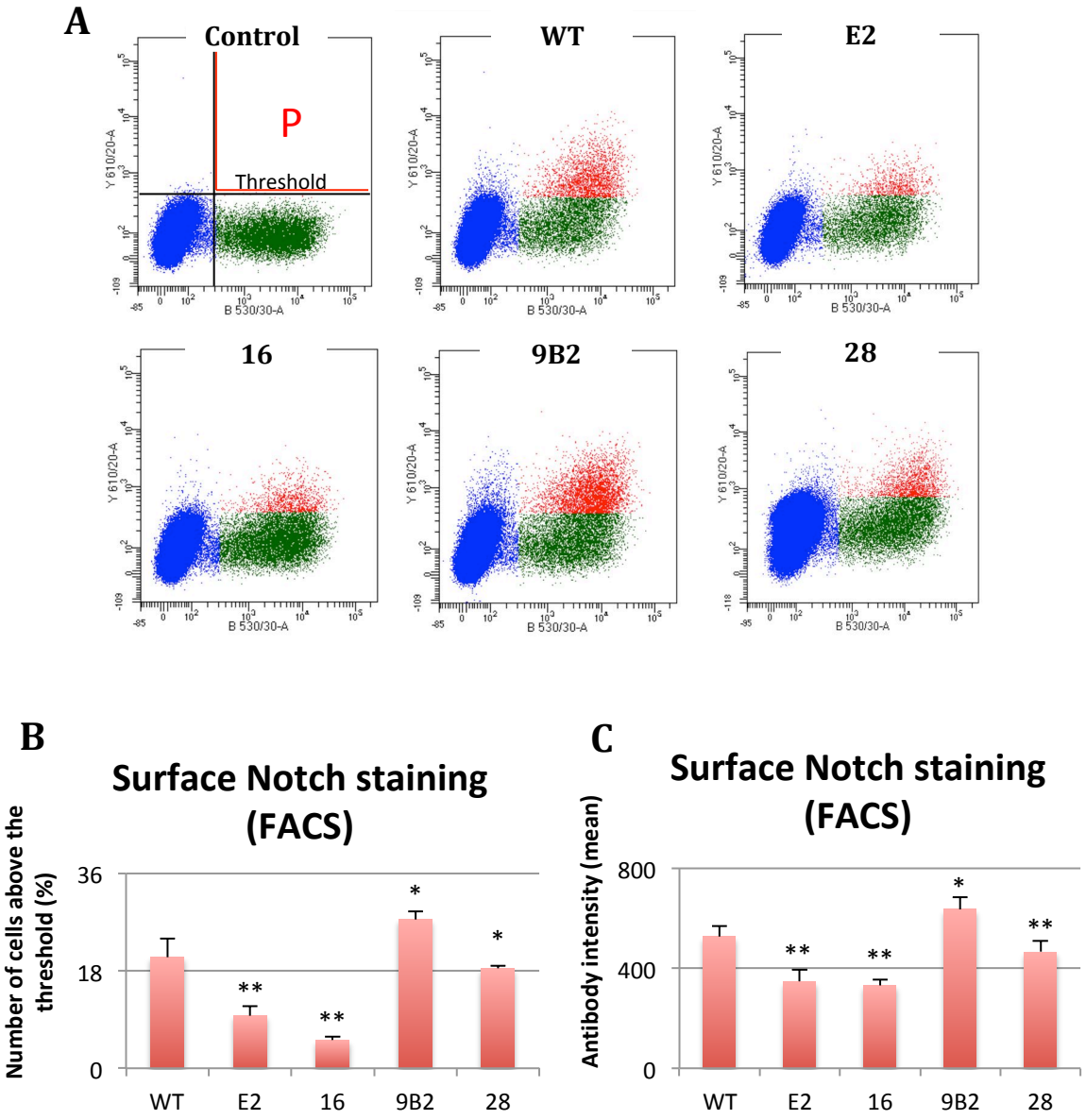


Figure 3.12. Localisation of Ax mutants at the cell membrane by FACS. (A) Shows FACS plots for WT, E2, 16, 9B2 and 28 co-transfected with PMT-GFP into S2 cells. All S2 cells (except the control sample) were labelled with anti-NECD on ice. All samples were then fixed and detected with secondary antibody in order to detect Notch on the plasma membrane (surface). The control was used to threshold the FACS and identify the population of cells with Notch at the cell membrane (P square and red spots). Only single GFP positive cells (green spots) were considered and separated by the GFP negative cells (blue spots). 10.000 cells were analysed by the FACS machine in every sample. The percentage of cells in population P and their staining intensity reflects the localisation of Notch at the cell membrane. Plot (B) shows the percentage of cells in population P, compared to the total number of GFP transfected cells above the threshold. Plot (C) shows the mean antibody staining intensity of P in all samples. The statistical significance was determined by Student T test and is relative to WT sample (*$p<0.05$; **$p<0.01$). Error bars indicate SEM. (n=3)

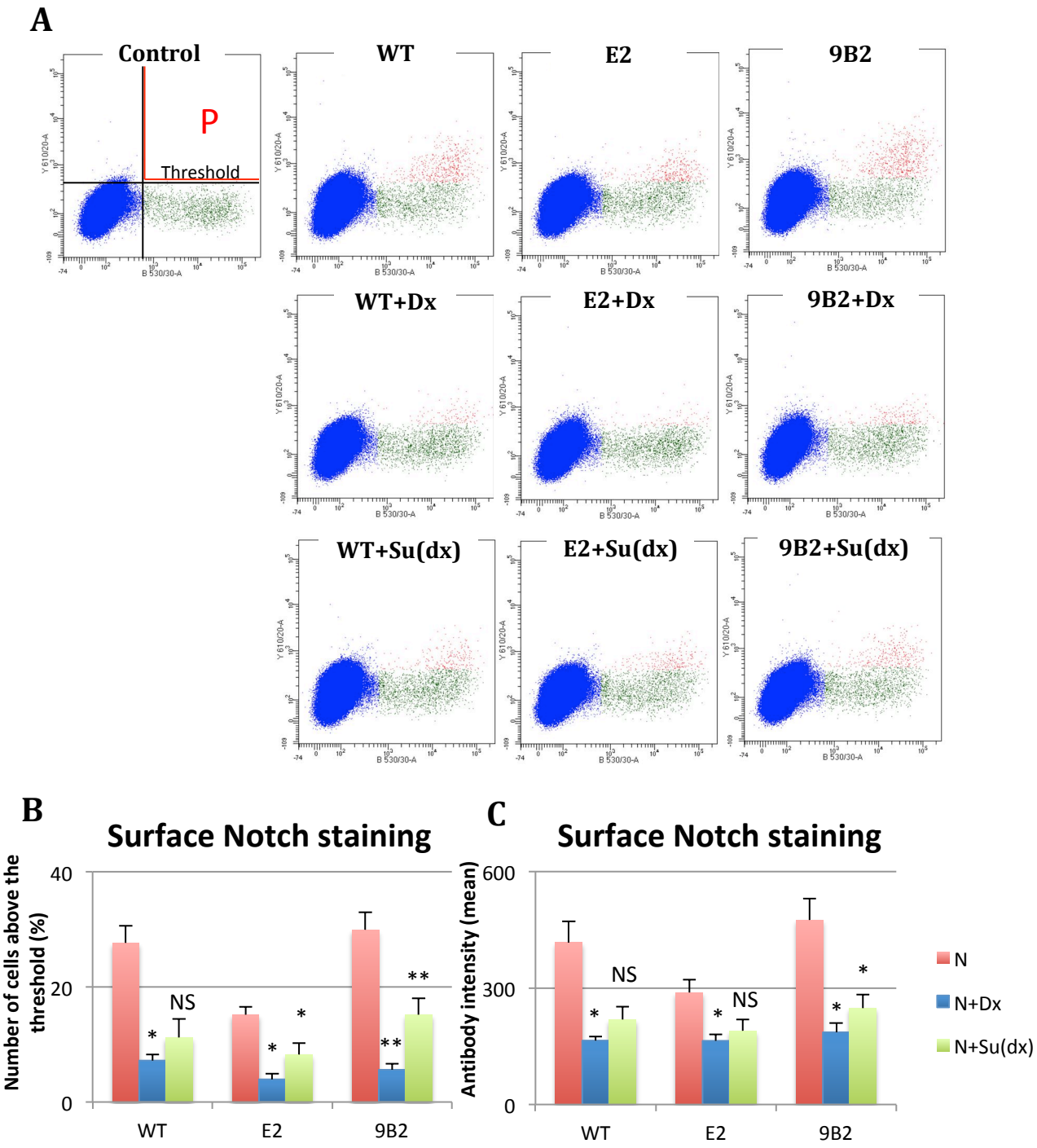


Figure 3.13. Localisation of Ax mutants at the cell membrane in the presence of Dx or Su(dx) by FACS. WT, E2, 16, 9B2 and 28 were co-transfected with Dx or Su(dx) and PMT-GFP in S2 cells. (A) The amount of Notch at the cell membrane was determined by FACS analysis. (B) shows the percentage of cells in population P as compared to the total GFP transfected cells above the threshold. (C) shows the mean antibody staining intensity of P in all samples. The statistical significance was determined by Student T test and is relative to WT only sample (NS=non significant; *p<0.05; **p<0.01). 10.000 cells were analysed by the FACS machine in every sample. Error bars indicate SEM. (n=3)

3.3.3 *E2* shows increased endocytosis which is independent of Dx

To test if *Ax* mutants affect Notch endocytosis when expressed alone in S2 cells, *E2* and *9B2* were labelled using anti-NECD antibody and the number of Notch endosomal structures were counted at 10, 30 and 60 minutes of endocytosis, (Fig. 3.14). Interestingly, it was observed that the number of Notch endosomal structures was significantly higher in *E2* after only 10 minutes of endocytosis (Fig. 3.14A, B), suggesting *E2* might increase Notch endocytic uptake in the absence of Dx. In all samples the number of Notch endosomal structures reached a peak at 30 mins of endocytosis and was decreased at 60 mins likely because of activation or degradation of Notch (Fig. 3.14C). However, the number of Notch endosomal structures was similar in *E2* at 10 and 30 mins, indicating that in *E2* the endocytic uptake was constantly high. On the contrary, in *9B2* the number of Notch endosomal structures reached a significantly lower peak at 30 mins compared to WT, suggesting *9B2* might have a lower endocytic uptake. This would explain why *9B2* accumulates at the cell membrane and reduces the basal signalling. However, it should be taken into account that degradation and activation of Notch are happening at the same time with endocytosis and they can affect the number of Notch endosomal structures, especially as we progress to later time points.

3.3.4 In response to Dx, all constructs were endocytosed in GPI- vesicles, 9B2 was later shifted to GPI+ vesicles whilst *E2* and WT were not

Since *9B2* showed a reduced Dx-dependent signalling, it was interesting to test if this effect is because *9B2* alters Notch trafficking. Therefore, WT, *E2*, *16, 28* and 9B2 were co-expressed with Dx and their trafficking was examined by uptake assay and using GPI as a marker (Fig. 3.15A-F). It was previously shown that, when WT is co-expressed with Dx in S2 cells, it is trafficked in GPI- vesicles and kept on the limiting membrane of late endosomes (Shimizu et al., 2014). As expected, WT was trafficked in GPI- vesicles and the same was true for *E2* and *16* (Fig. 3.15 A, B, D, E). However, *9B2* was surprisingly in GPI+ vesicles, suggesting

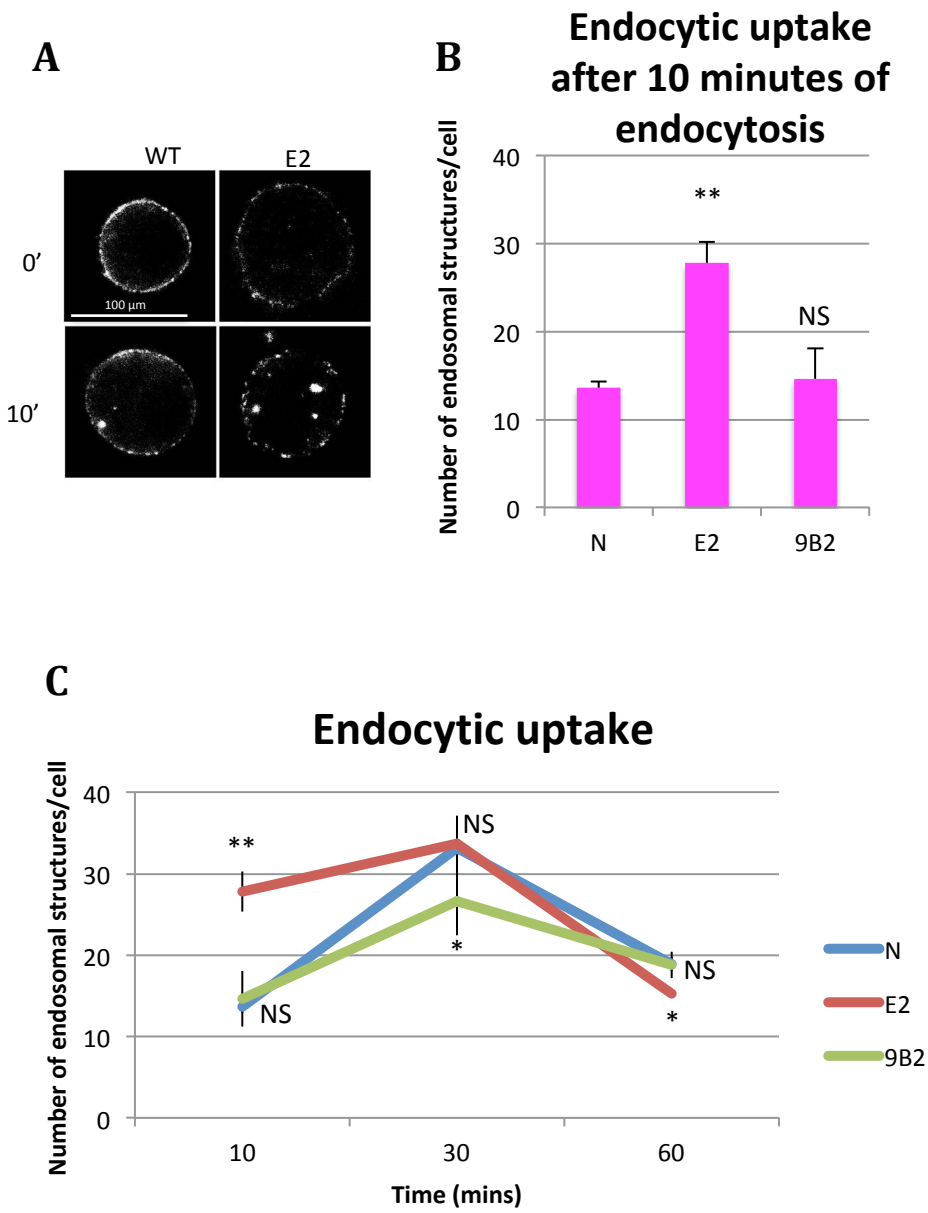


Figure 3.14. Endocytic uptake of Ax mutants. WT, E2 and 9B2 were transfected in S2 cells. Notch was labelled at the cell membrane using an anti-NECD antibody and endocytosis was induced for 10, 30 or 60 minutes. The number of endosomal structures containing Notch was scored in an average of 20 cells for each sample at each time point (n=3). (A) shows WT and E2 cells at 0 min (surface Notch staining) and 10 min of endocytosis. (B) shows the mean number of endosomal structures per cell at 10 minutes of endocytosis. (C) shows the mean number of endosomal structures per cell at 10, 30 and 60 minutes of endocytosis. The statistical significance was determined by Student T test and is relative to WT sample at each time point (NS=non significant; *p<0.05; **p<0.01). Error bars indicate SEM.
For M and m

9B2 is localised in a different route (Fig. 3.15C). *28* was mainly in GPI- vesicles like *E2* and *16,* however a consistent portion was in GPI+ vesicles like *9B2* (Fig. 3.15E). The experiment was repeated for *9B2* and WT at different time points of endocytosis to find out in which route *9B2* is endocytosed and trafficked (Fig. 3.15G). Interestingly, it was observed that *9B2* was mainly in GPI- vesicles after 10 mins of endocytosis and gradually more in GPI+ vesicles at 30 and 60 mins, whereas WT was constantly more in GPI- vesicles. This suggests that in response to Dx, *9B2* is endocytosed in GPI- vesicles, but then it is shifted to GPI+ vesicles. In this case the reduction in *9B2* Dx-dependent signalling might be due to the transfer of *9B2* from GPI- to GPI+ vesicles where *9B2* could be likely degraded inside late endosomes. To test this idea, the uptake assay was repeated using Rab7 as a marker to label the membrane of late endosomes (Fig. 3.15H-K). As expected, *9B2* was mainly inside the lumen of Rab7-labelled late endosomes (Fig. 3.15J) while WT and *E2* were on the limiting membrane of Rab7-labelled late endosomes (Fig. 3.15H, I). *9B2* is endocytosed in GPI- vesicles in response to Dx and then redirected to GPI+ vesicles and degraded inside late endosomes, which would lead to the reduction of Dx-dependent signalling. On the contrary, WT and *E2* are in GPI- vesicles and on the limiting membrane of late endosomes where they can likely be activated and this is in agreement with their signalling activation in response to Dx.

3.3.5 *Ax* mutants and WT Notch are trafficked in GPI+ vesicles in response to Su(dx)

The Su(dx)-mediated signalling of *Ax* mutants was similar to WT, therefore no difference in the trafficking was expected in this case. Indeed, in response to Su(dx), WT and the mutants were mainly in GPI+ vesicles, indicating Su(dx) sorts the mutants in to its route (Fig. 3.16). It is interesting that also *E2* was in GPI+ vesicles while it was clearly more in GPI- vesicles in basal and Dx-dependent trafficking.

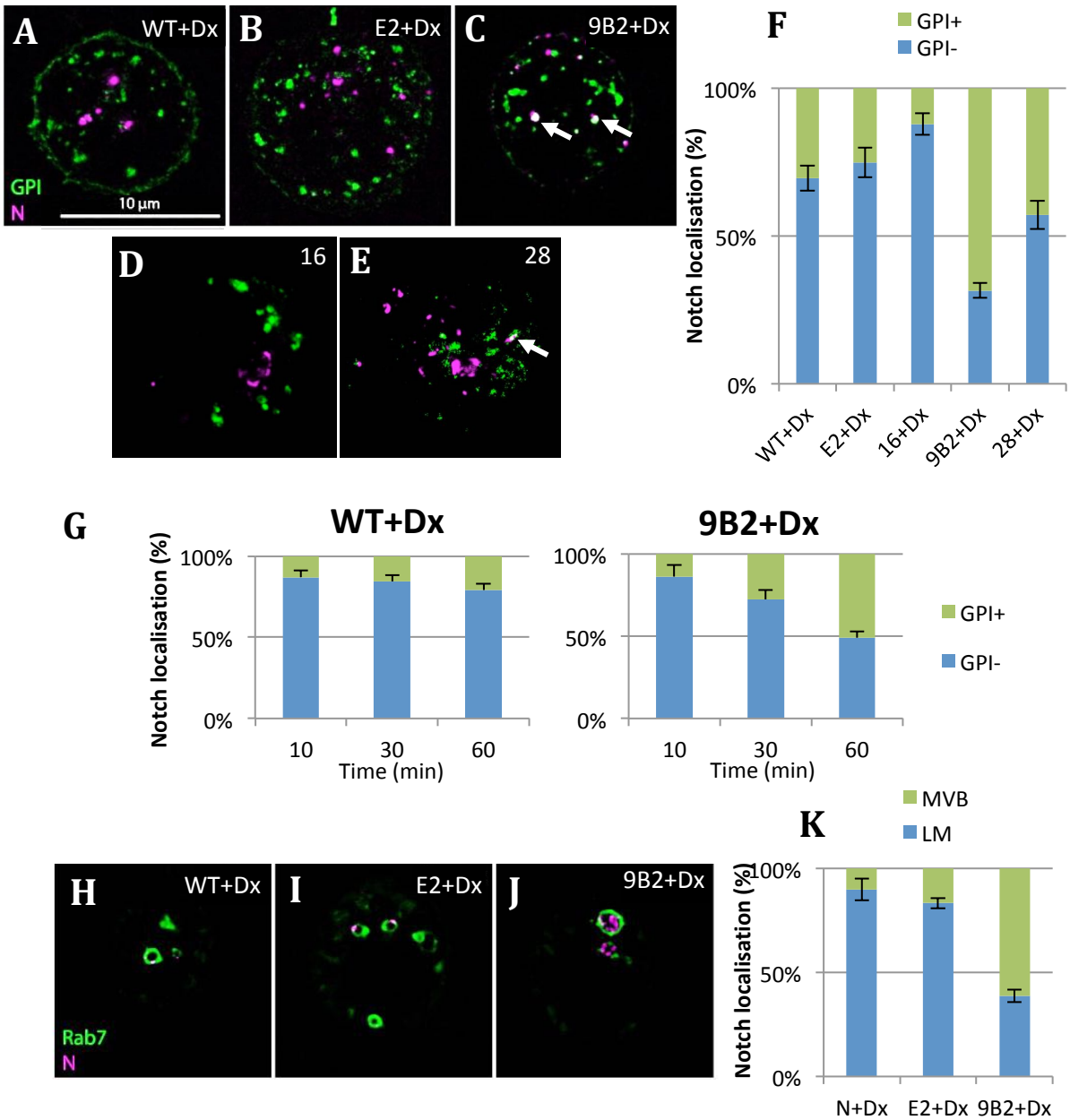


Figure 3.15. Localisation of Ax mutants in GPI+ vesicles and Rab7-labelled late endosomes in the presence of Dx in S2 cells following an-anti-NECD uptake assay. (A-E) shows the localisation in the presence of Deltex of WT, E2, 9B2, 16 and 28 respectively, relative to GPI+ vesicles at 60 min of endocytosis. White arrows indicate co-localisation of Notch and GPI-GFP. (F) plots the localisation of Notch within GPI+ or GPI- vesicles at 60 minutes of endocytosis. (G) shows a plot of the localisation of WT+Dx and 9B2+Dx within GPI+ or GPI- vesicles at 5, 10, 30 and 60 minutes of endocytosis. (H-J) shows the localisation in the presence of Deltex of WT, E2, 9B2 respectively, relative to Rab7-labelled late endosomes at 60 minutes of endocytosis.. Plots G show the localization of WT+Dx and 9B2+Dx within GPI+ or GPI- vesicles at 5, 10, 30 and 60 minutes of endocytosis. (K) shows the localisation of Notch on the limiting membrane or inside Rab7-labelled late endosomes. An average of 100 vesicles were scored for each sample looking at the co-localisation of Notch with GPI-GFP or in Rab7-labelled late endosomes at 60 minutes of endocytosis. Data are shown as percentage means (n=3). Error bars indicate SEM.

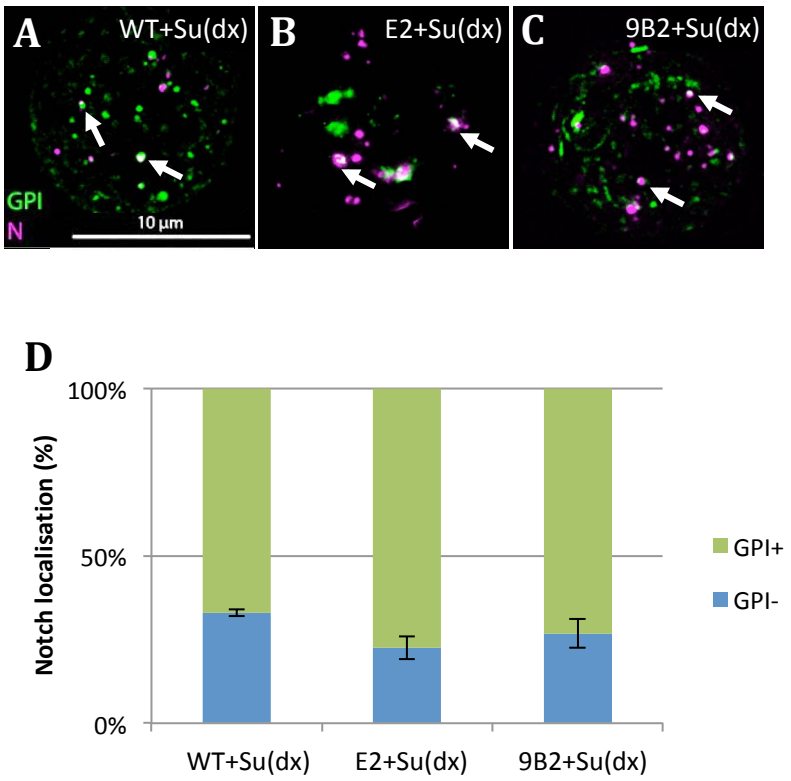


Figure 3.16. Localisation of Ax mutants in GPI+ vesicles and Rab7-labelled late endosomes in the presence of Su(dx) following an-anti NECD uptake assay with a 60 minute chase. (A-C) show the localization of WT, E2 and 9B2 respectively, in GPI+ vesicles in S2 cells at 60 minutes of endocytosis in the presence of Su(dx). White arrows indicate co-localisation of Notch and GPI-GFP. (D) plots the localization of Notch within GPI+ or GPI- vesicles at 60 minutes of endocytosis. An average of 100 vesicles were scored for each sample looking at the co-localisation of Notch with GPI-GFP at 60 minutes of endocytosis. Data are shown as percentage means (n=3). Error bars indicate SEM.

3.4 *Ax* mutants affect Notch localisation *in vivo*

The aim of this section will be to test the localisation of *Ax* mutants *in vivo* to confirm the observations that were made in S2 cells. Particular focus will be given to determine if *Ax* mutants affect the localisation of Notch at the cell membrane and in endosomal compartments and how this changes in a *dx* mutant background.

3.4.1 *Ax* mutants localise differently at the cell membrane and in endosomal compartments *in vivo*

To study the localisation of Notch *in vivo*, mutant clones were generated in the wing disc, which is the larval wing tissue of *Drosophila* larvae. Using this technique, it is possible to compare cells, which are homozygous for *Ax* mutant Notch (GFP-negative spot), with cells which are homozygous for WT Notch (GFP-positive spot) within the same wing disc (Fig. 3.18). The clones were produce using the FLP recombinase and its site-specific recombination sites, FRTs, system (Golic and Lindquist, 1989, see chapter 2). Clones were produced in the wing disc of yw, *E2*, *16* and *9B2* larvae and Notch was stained using an anti-NECD antibody. Phalloidin was used to label the tissue apical membrane to identify Notch at the cell membrane and exclude irregularities in the apical membrane of the tissue. The number of endosomal structures (vesicular Notch) and the staining intensity at the surface of the clones (surface Notch) were measured to account for differences in the localisation of Notch inside the cells and at the cell membrane (Fig. 3.17). These measurements were compared between the GFP+ and GFP- clonal spots. Since the mutants showed temperature-dependent differences in the phenotype and signalling (§ 3.1 and 3.2), the clones were produced at 18°C, 25°C and 29°C.

Yw clones did not show any significant difference in the vesicular and surface Notch between the GFP+ and GFP- clonal spots (Fig. 3.17, 3.18A) indicating that the clone technique does not cause any change in the localisation of Notch.

Mutant *E2* clones showed an increase in vesicular Notch at all temperatures, which reached statistical significance at 29°C (Fig. 3.17A-C, 3.18B). Surface Notch was not significantly changed (Fig. 3.17A'-C'), however the surface Notch staining appeared to be fragmented and dotty in the mutant clone, especially at 29°C and this was not observed in twin spot or in yw clones (Fig. 3.18B). This might indicate that the amount of Notch at the cell membrane is decreased. Taken together, the fragmented surface and the increase in vesicular Notch, suggests that *E2* has an increased rate of endocytosis. These results are in agreement with the observation that the *E2* phenotype was clear at all temperatures, though stronger at 29°C (Fig. 3.1) and that *E2* signalling was more stable than WT with temperature variation (Fig. 3.9).

Similarly, mutant *16* clones showed wild-type vesicular Notch at 18°C and a very significant increase in vesicular Notch at 25°C and 29°C compared to the twin spot (Fig. 3.17A-C, 3.18C). This might reflect the severity of *16* phenotype, which was increasing with temperature (Fig. 3.1). Also, similarly to *E2*, surface Notch was not significantly changed (Fig. 3.17A'-C'), but it appeared fragmented in *16* clones, especially at 29°C (Fig. 3.18C'").

Conversely, mutant *9B2* clones increased vesicular Notch at 18°C and 25°C, but not at 29°C (Fig. 3.17A-C, 3.18D). In addition, surface Notch was significantly increased at all temperatures, but the increase was milder at 29°C (Fig. 3.17A'-C'). It is possible that the increased vesicular and surface Notch in *9B2* clones account for an increased Notch signalling at 18°C and 25°C, which is abrogated at 29°C. At high temperature *9B2* might be endocytosed and degraded, thus reducing the amount of vesicular and surface Notch. This might support the observations that *9B2* Dx-dependent signalling was strongly decreased at 29°C (Fig. 3.9) and that *9B2* was lethal at 29°C likely because of decreased Notch activation (Fig. 3.1).

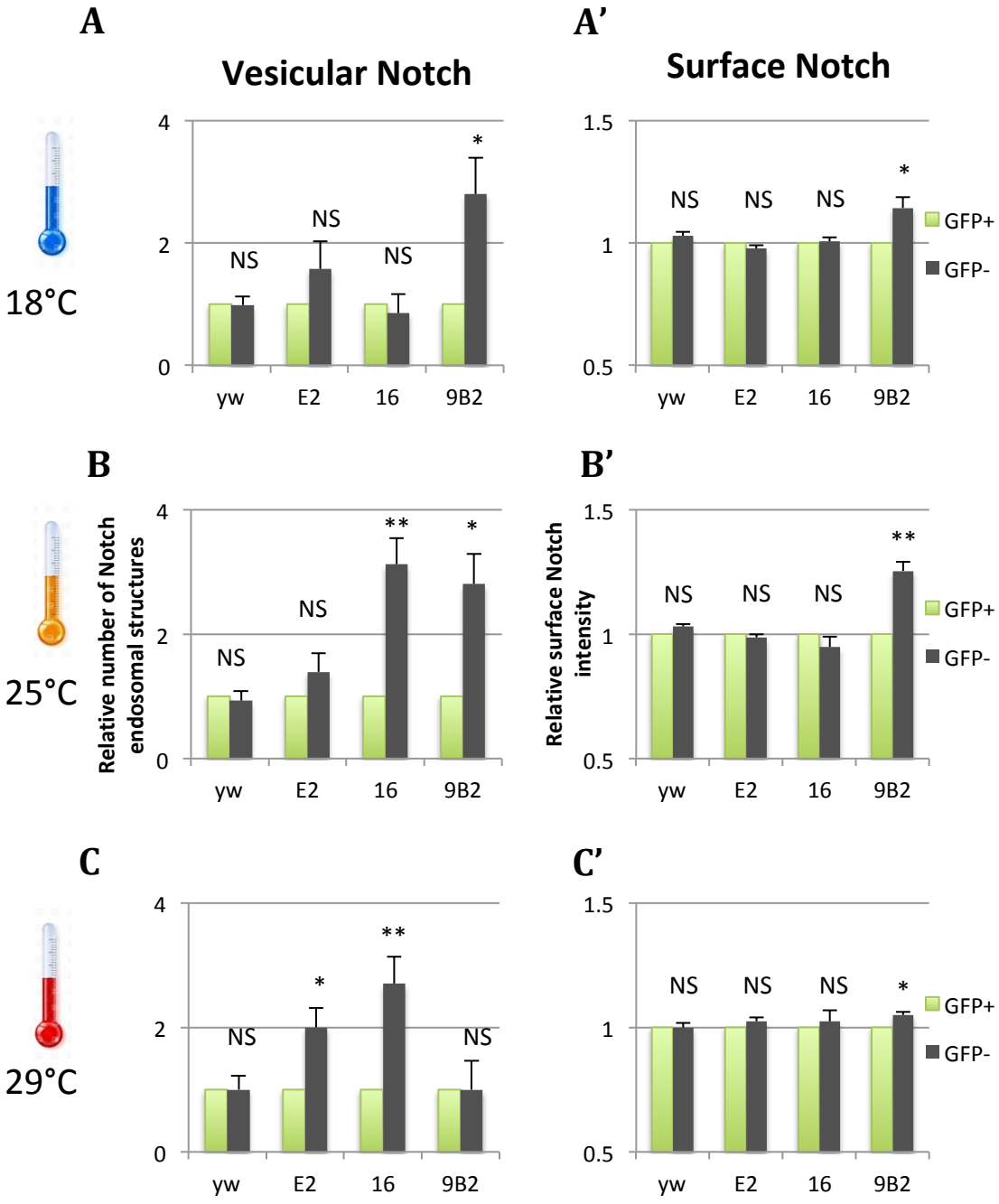


Figure 3.17. Quantification of vesicular and surface Notch in Ax clones relative to their twin spots. The number of Notch endosomal structures (vesicular Notch, A-C) and the intensity of Notch staining at the apical membrane (surface Notch, A'-C') were quantified in the GFP+ and GFP- clonal spots using ImageJ. The vesicular or surface Notch mean values in the GFP+ (green columns) or GFP- (black columns) spots were divided by the mean values in the GFP+ spots. The plots show the quantification of the clones at 18ºC (A, A'), 25ºC (B, B') and 29ºC (C, C'). The statistical significance was determined by Student T test and is relative to the GFP+ values of each sample (NS=non significant; *p<0.05; **p<0.01). Error bars indicate SEM (n=5).

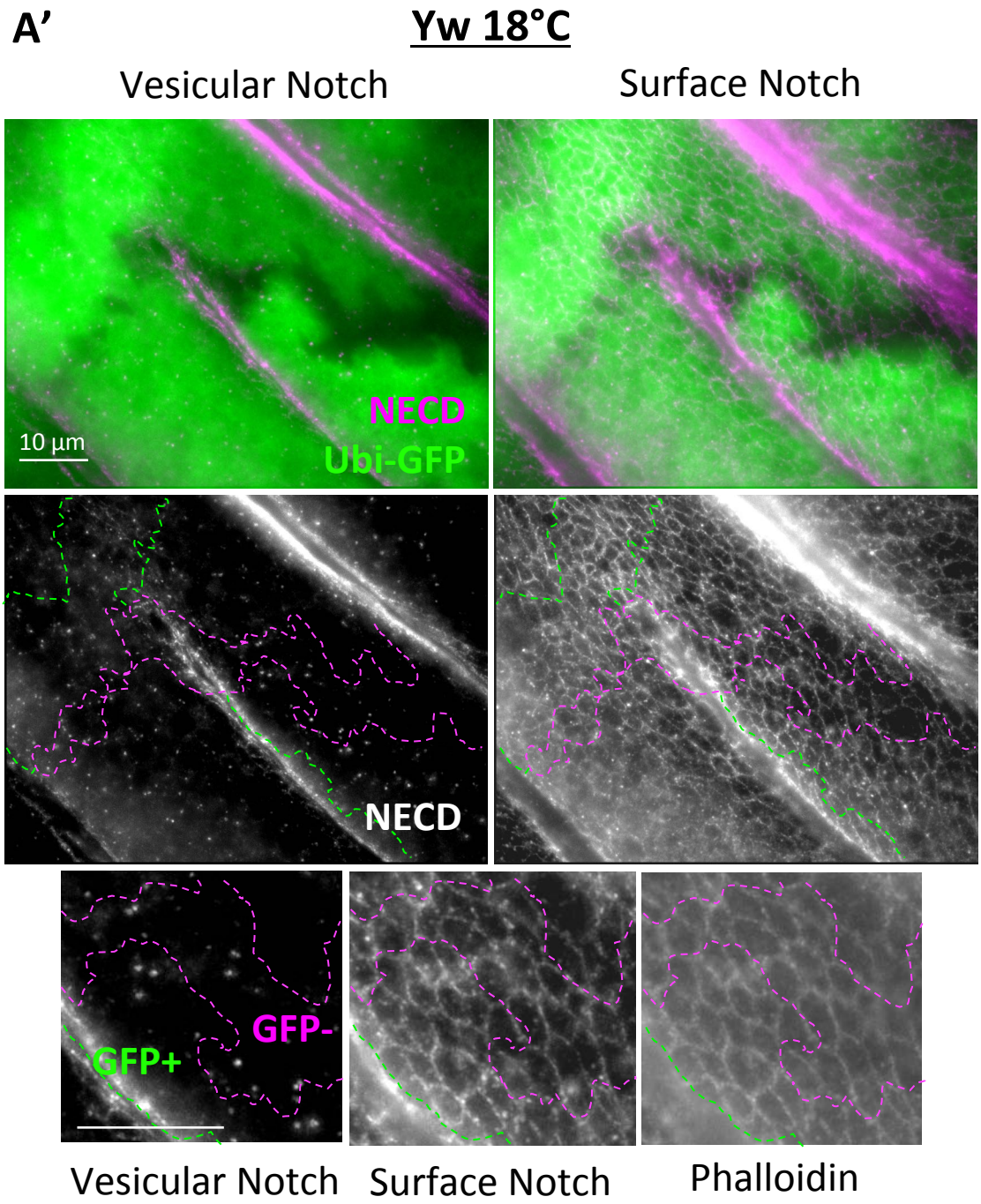
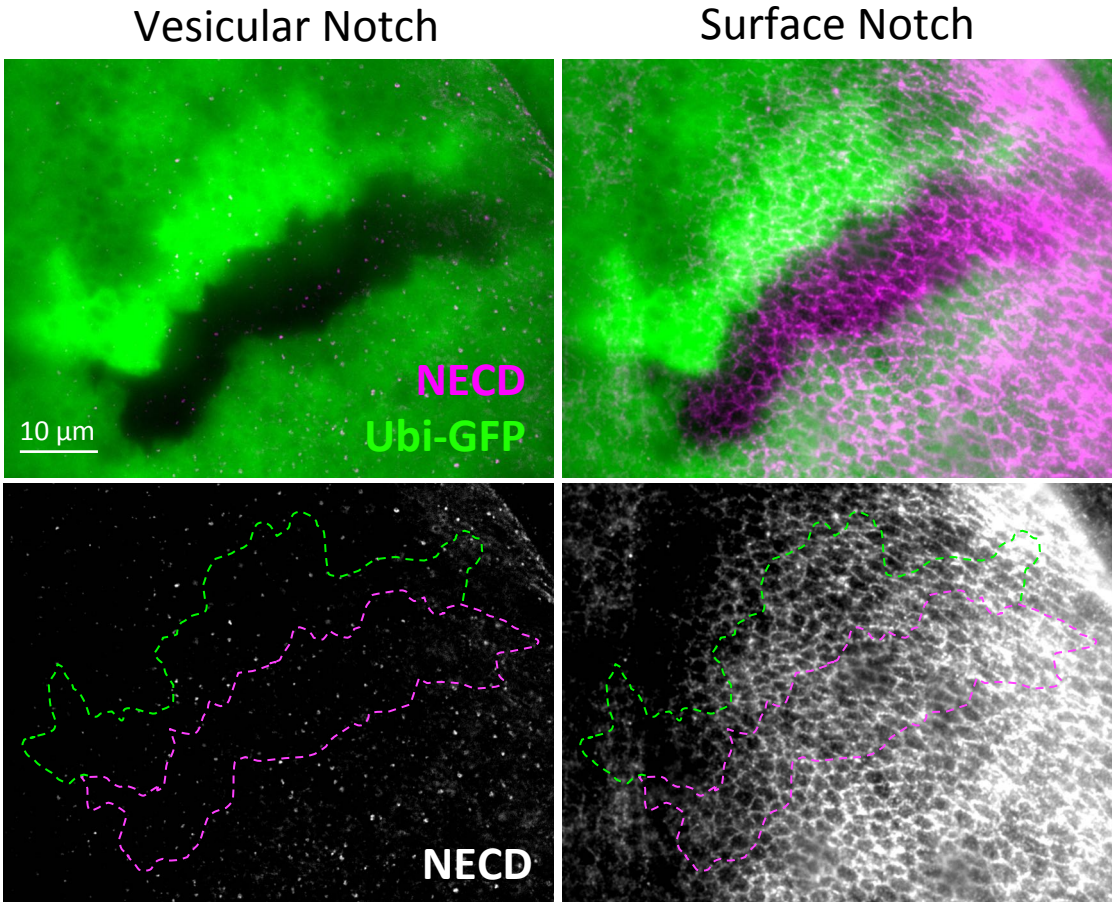
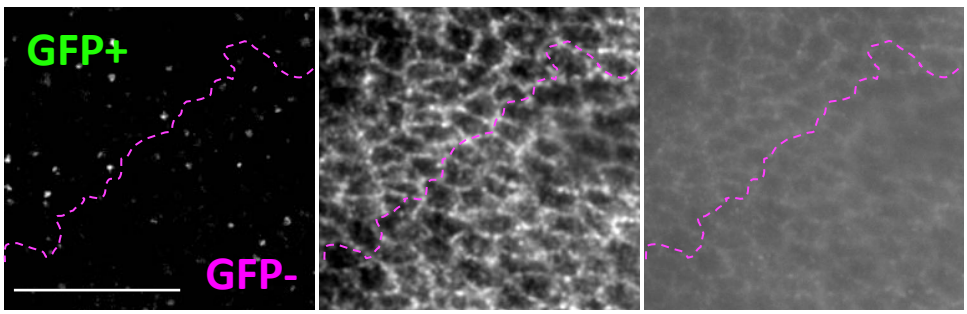


Figure 3.18. Ax mutant clones in the *Drosophila* wing disc using negatively labelled mutant clones compared to wild-type double GFP twin spot (see § 2.3.3). The panels in the current and following pages show (A) yw, (B) E2, (C) 16 and (D) 9B2 clones at (A’-D’) 18°C, (A’’-D’’) 25°C and (A’’’-D’’’) 29°C. The wing discs were fixed, permeabilised and stained for Notch using an anti-NECD antibody (purple), GFP using an anti-GFP antibody (green) and actin using phalloidin-far red to label the apical membrane. The GFP+ clonal spot (green dashed lines) is homozygous for WT Notch, while the GFP- spot (purple dashed lines) is homozygous for the mutant. The vesicular Notch panels show Notch endosomal structures at ~1.5 µm from the apical membrane. The surface Notch panels show Notch at the apical membrane. Phalloidin panels shows the staining at the same stack plane as surface Notch. Scale Bars correspond to 10 µm.

A’’

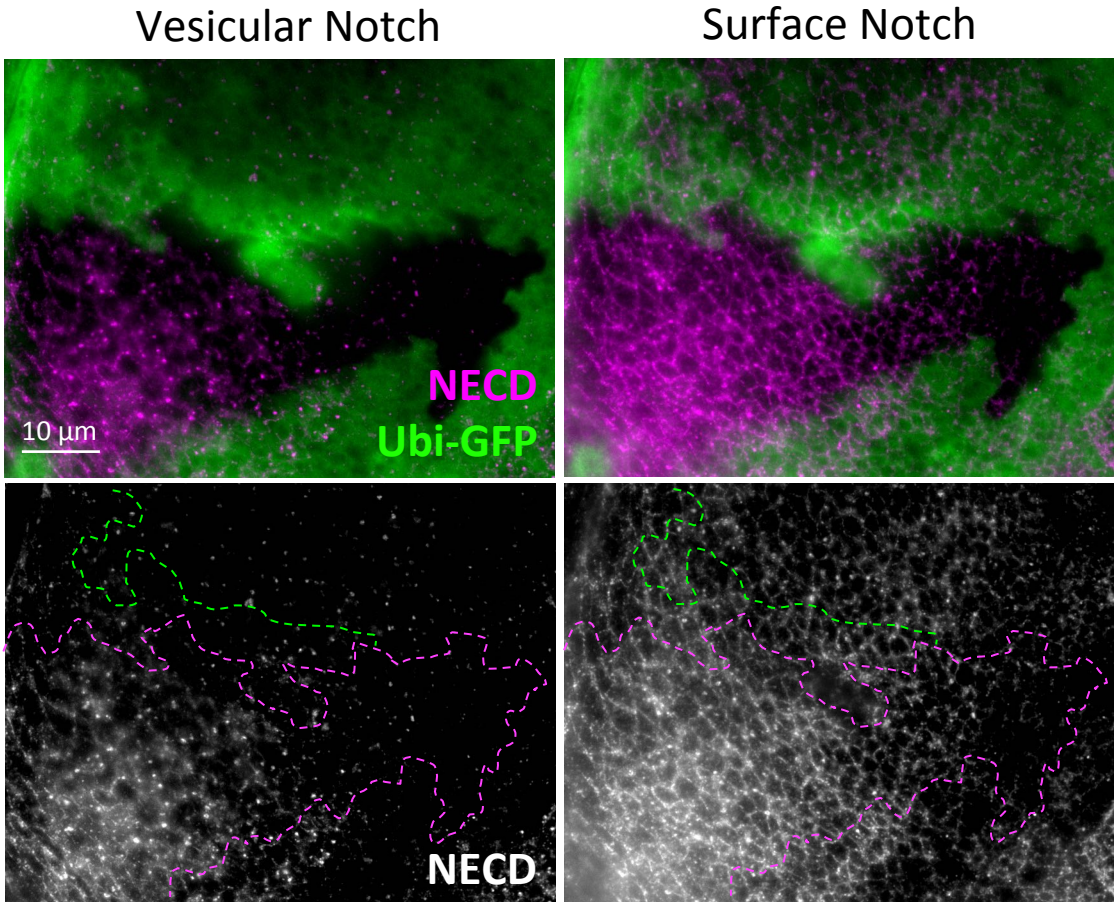
Yw 25°C

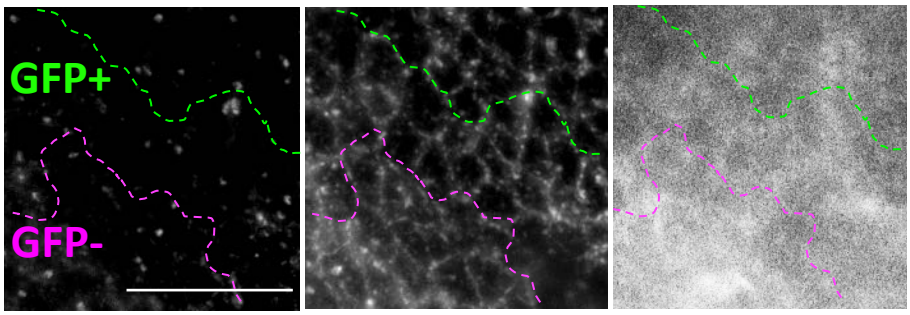

Vesicular Notch
Surface Notch
NECD
Ubi-GFP
10 µm
NECD


GFP+
GFP-
Vesicular Notch
Surface Notch
Phalloidin

A’’’

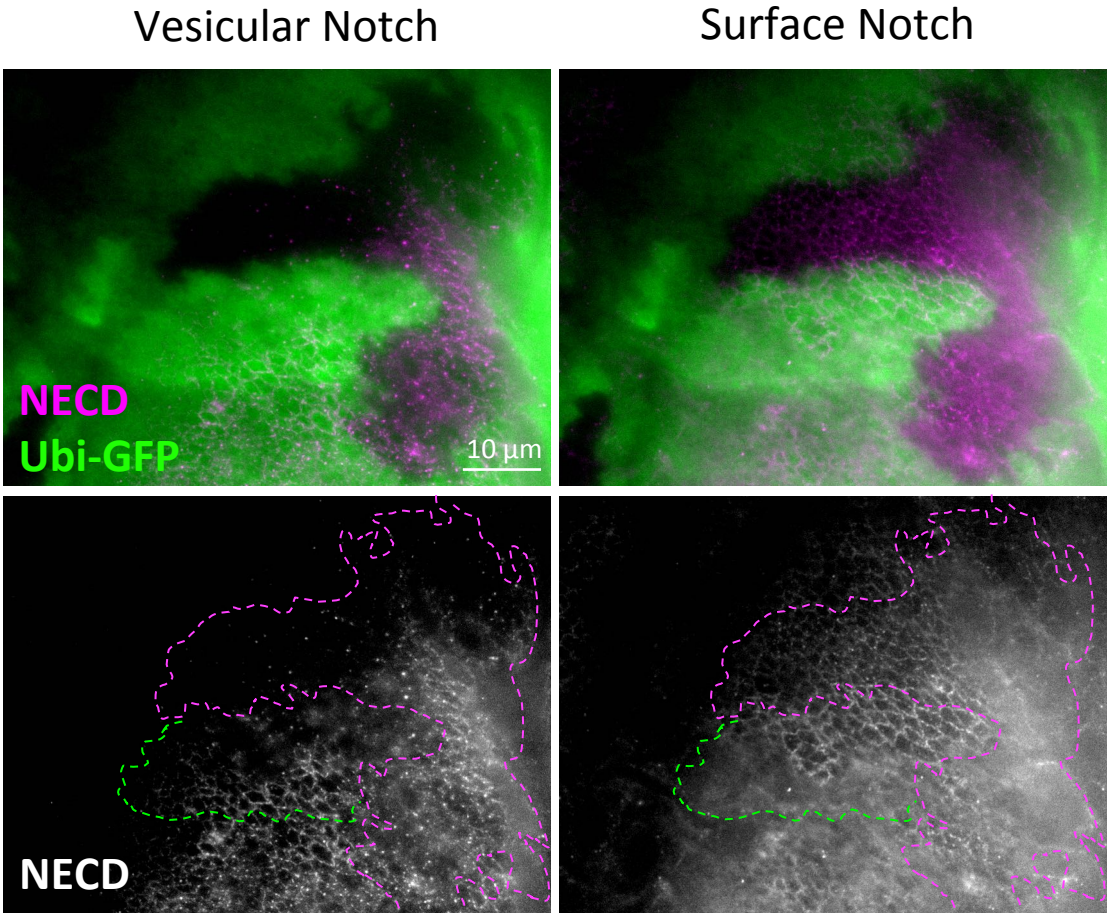
Yw 29°C

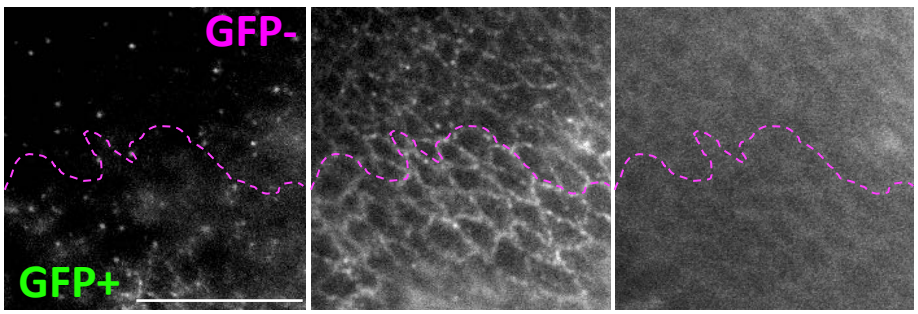

Vesicular Notch
Surface Notch
NECD
Ubi-GFP
10 µm
NECD


GFP+
GFP-
Vesicular Notch
Surface Notch
Phalloidin

B’

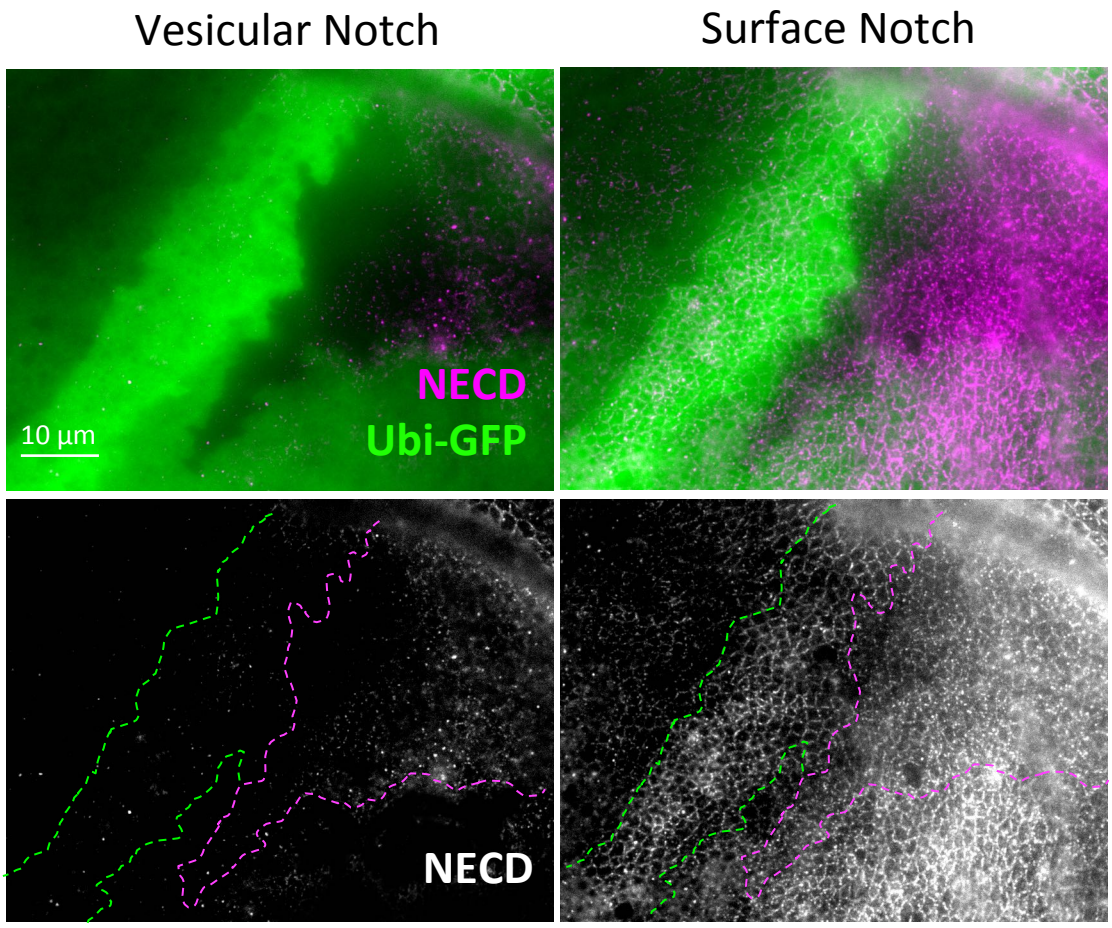
E2 18°C

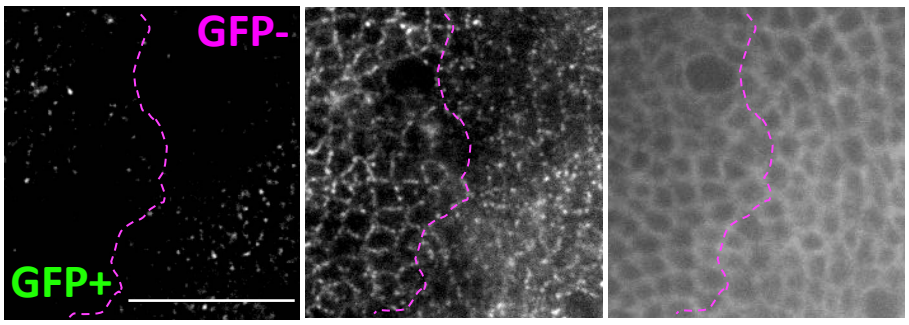

Vesicular Notch
Surface Notch
NECD
Ubi-GFP
10 µm
NECD


GFP-
GFP+
Vesicular Notch
Surface Notch
Phalloidin

B’’

E2 25°C


Vesicular Notch
Surface Notch
NECD
Ubi-GFP
10 µm
NECD

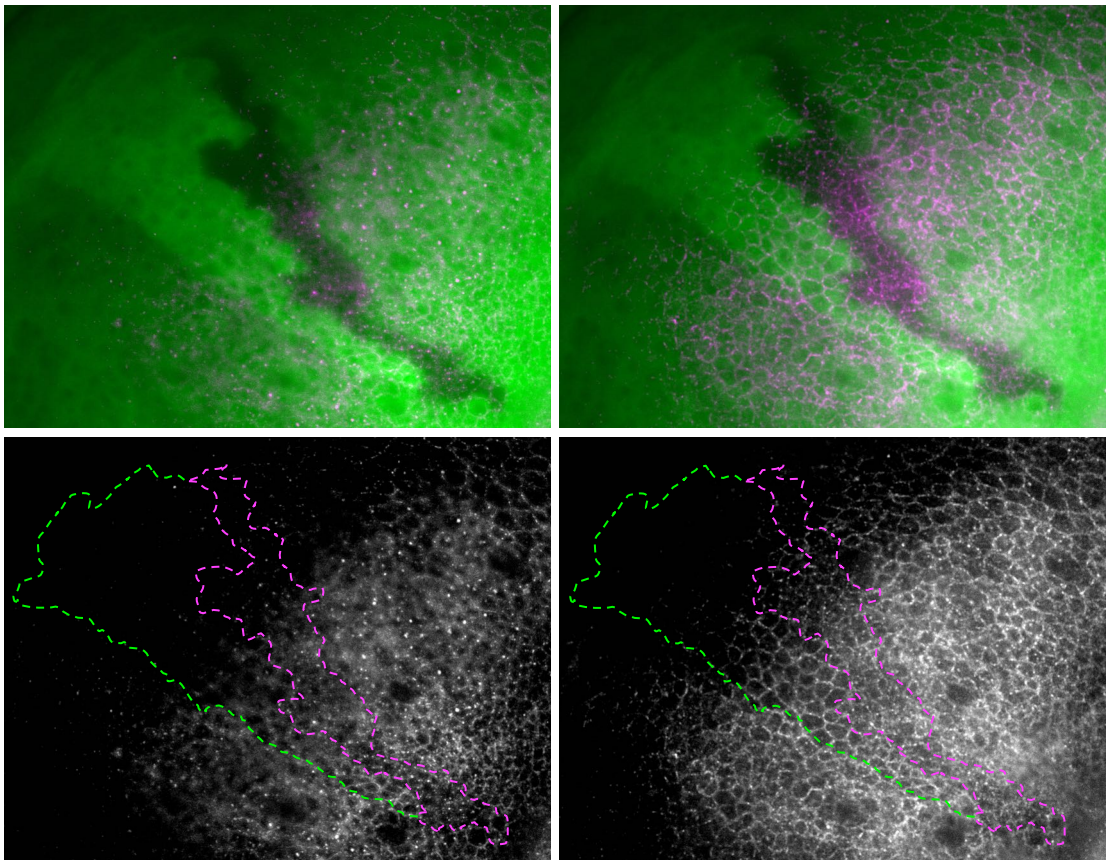

GFP-
GFP+
Vesicular Notch
Surface Notch
Phalloidin

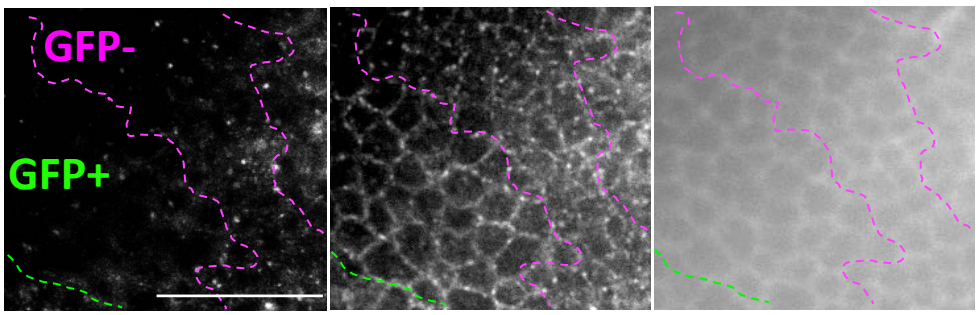
B’’’

E2 29°C

Vesicular Notch

Surface Notch

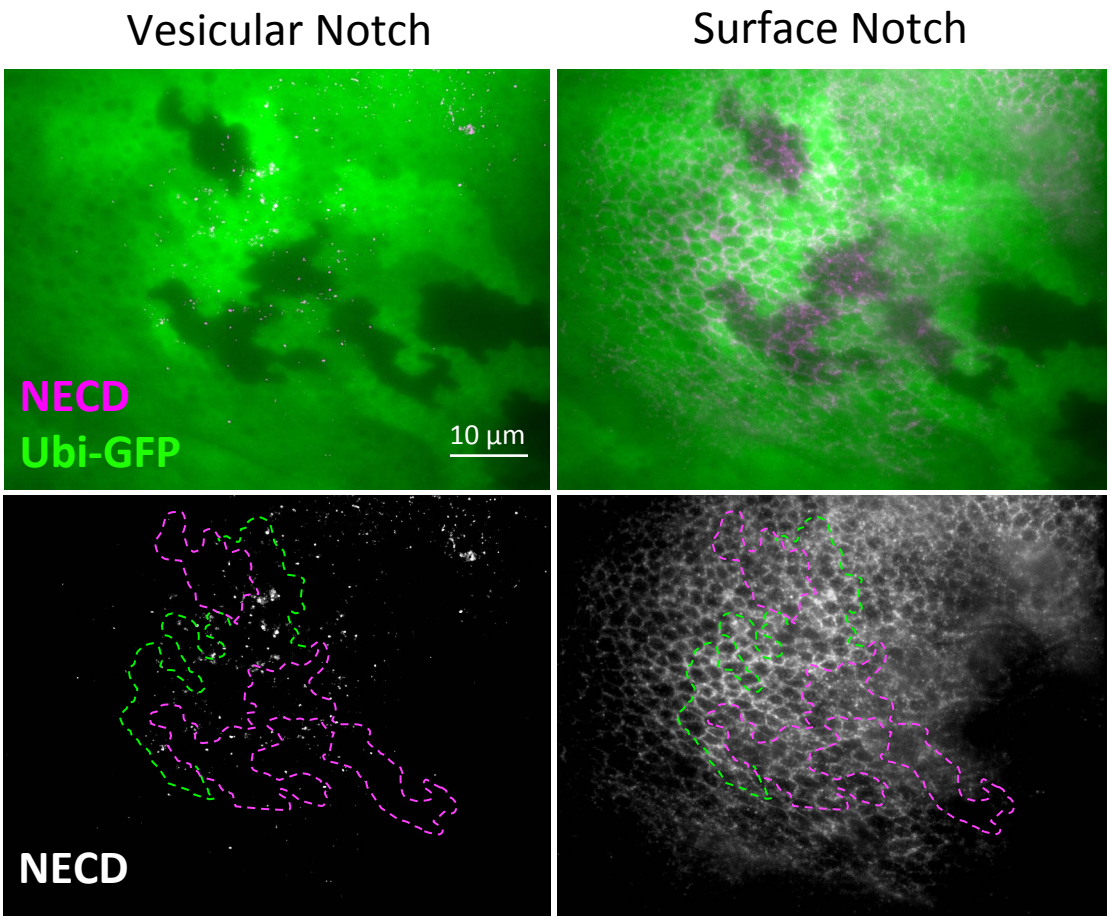


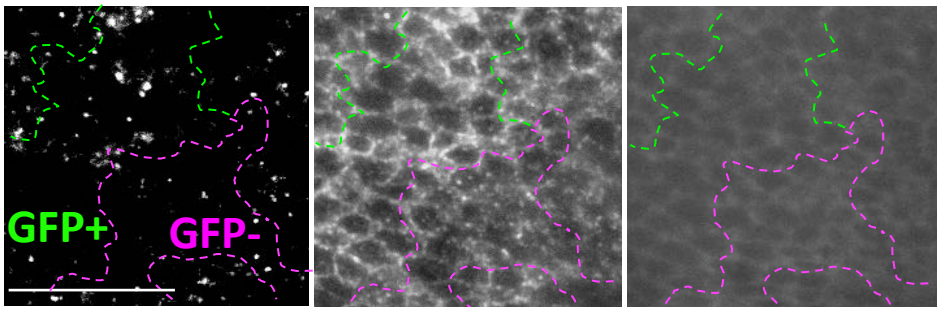


Vesicular Notch Surface Notch Phalloidin

C’

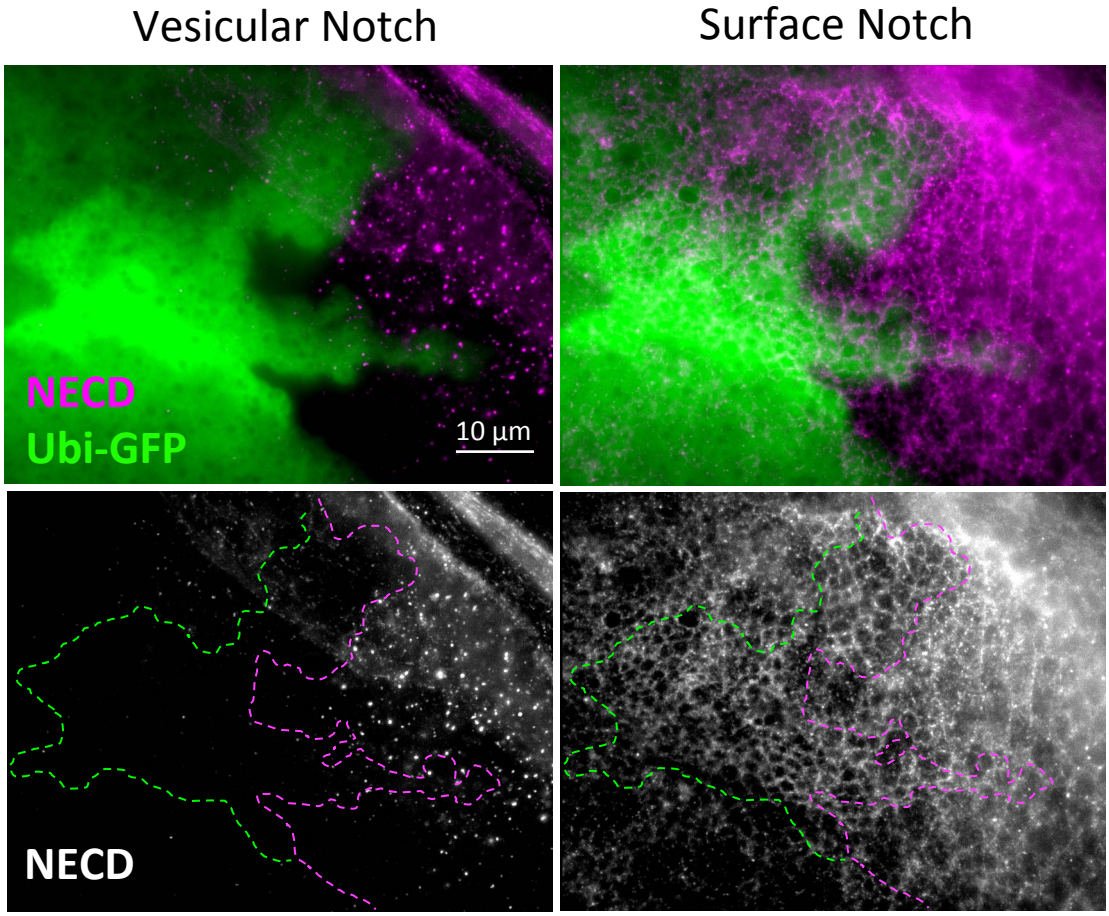
16 18°C

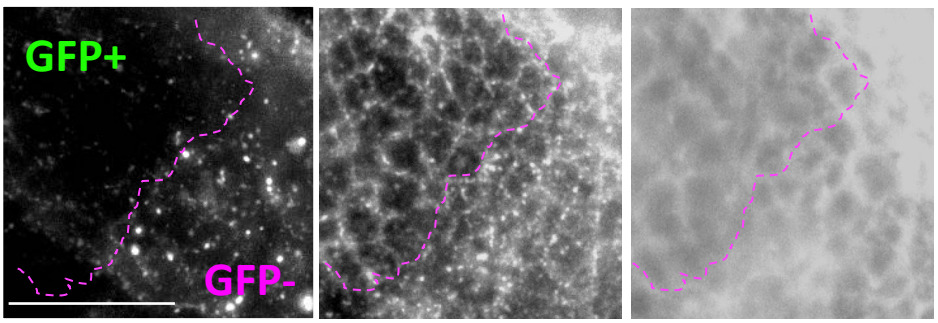

Vesicular Notch
Surface Notch
NECD
Ubi-GFP
10 µm
NECD


GFP+
GFP-
Vesicular Notch
Surface Notch
Phalloidin

C’’

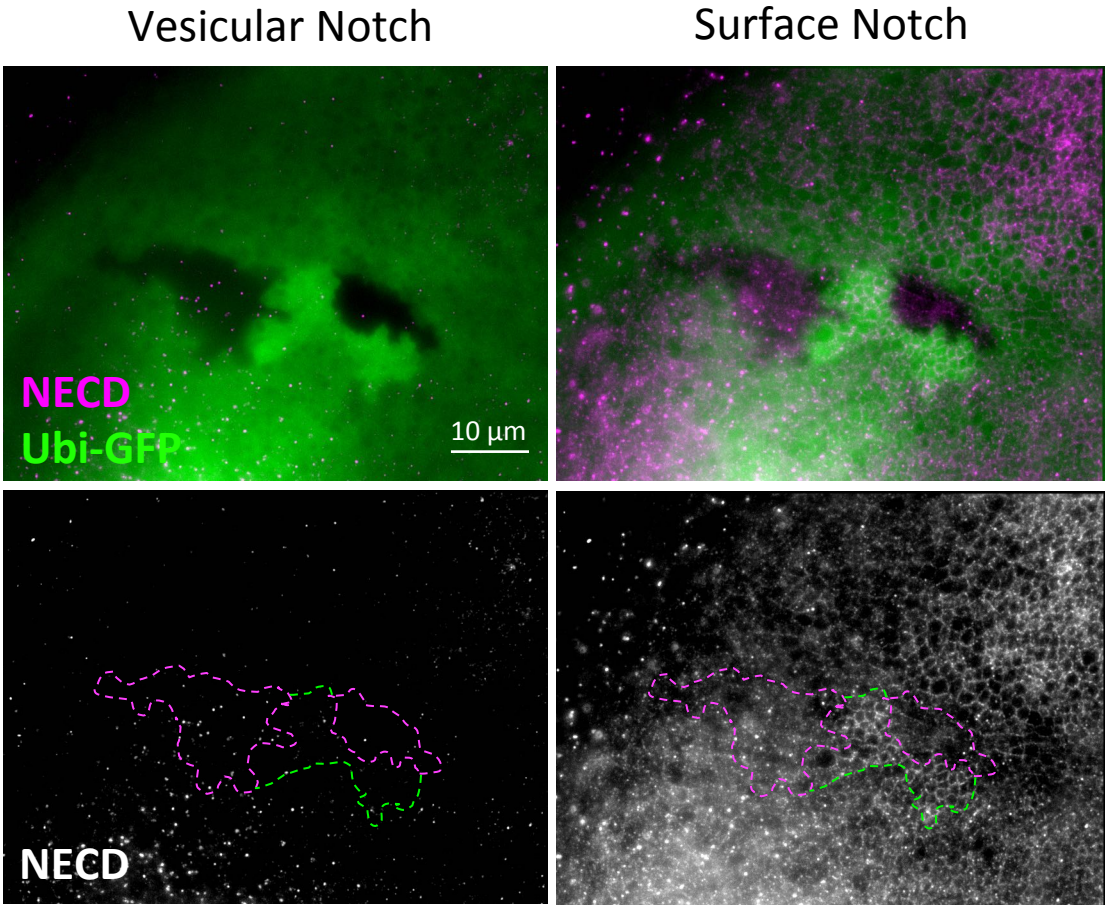
16 25°C

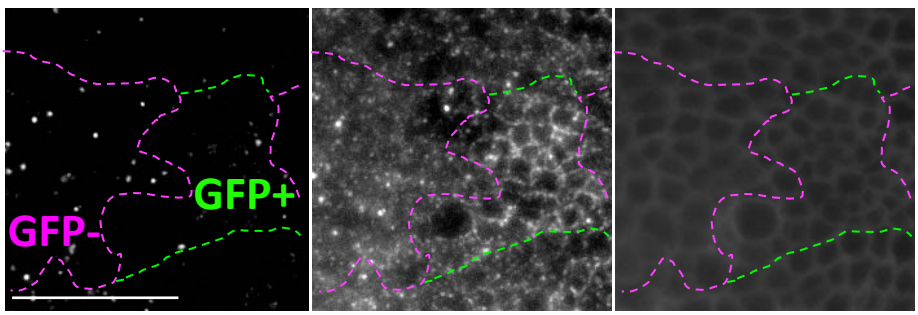

Vesicular Notch
Surface Notch
NECD
Ubi-GFP
10 µm
NECD


GFP+
GFP-
Vesicular Notch
Surface Notch
Phalloidin

C’’’

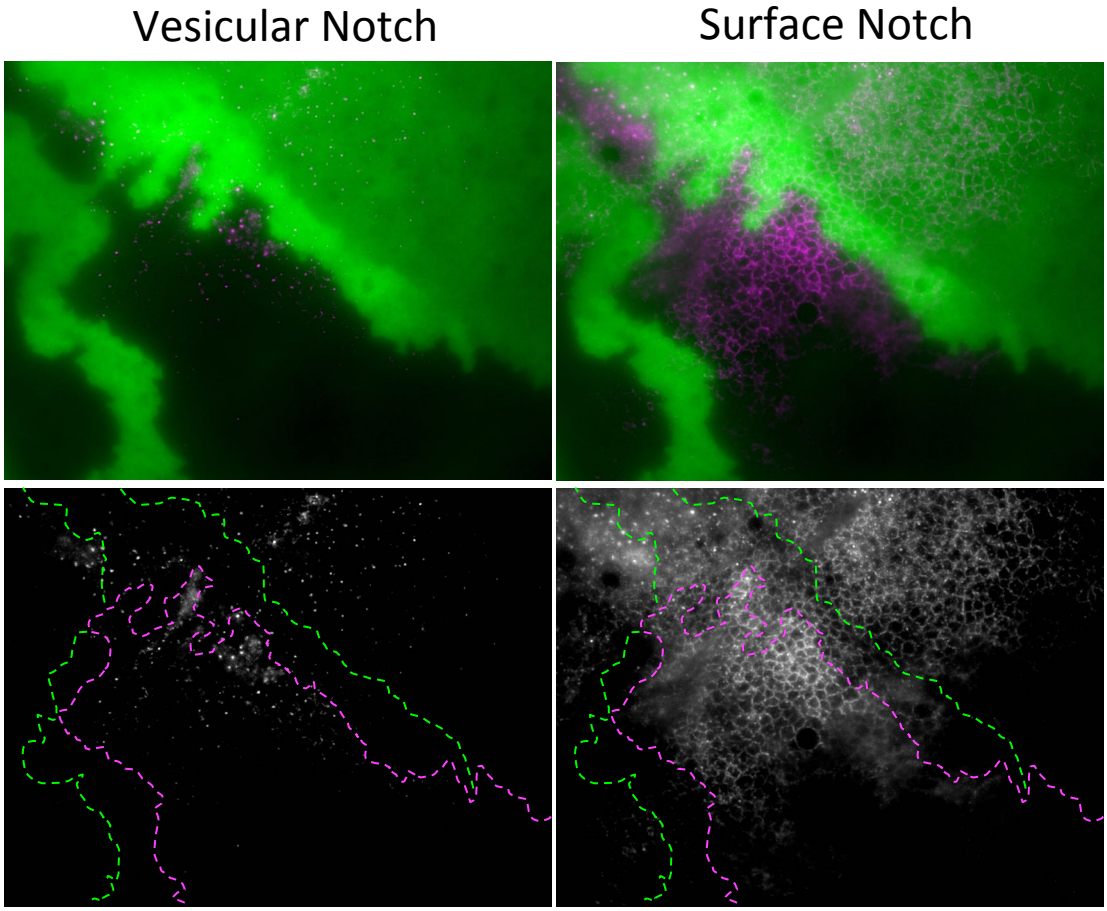
16 29°C

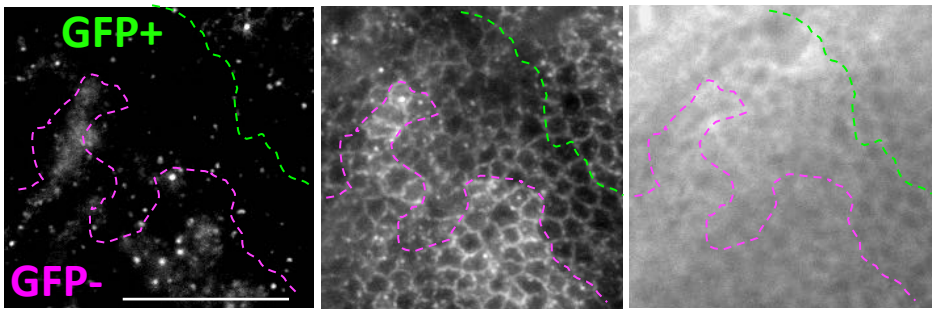

Vesicular Notch
Surface Notch
NECD
Ubi-GFP
10 µm
NECD


GFP+
GFP-
Vesicular Notch
Surface Notch
Phalloidin

D’

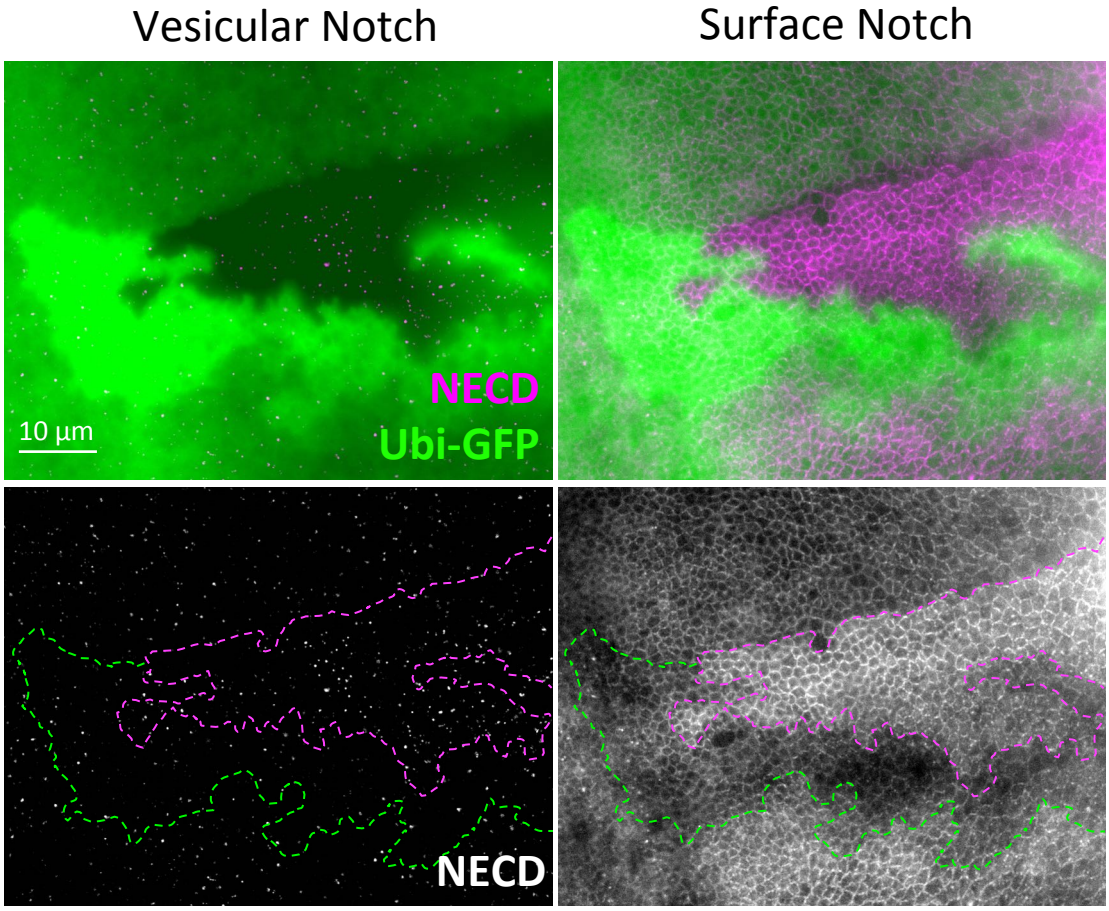
9B2 18°C

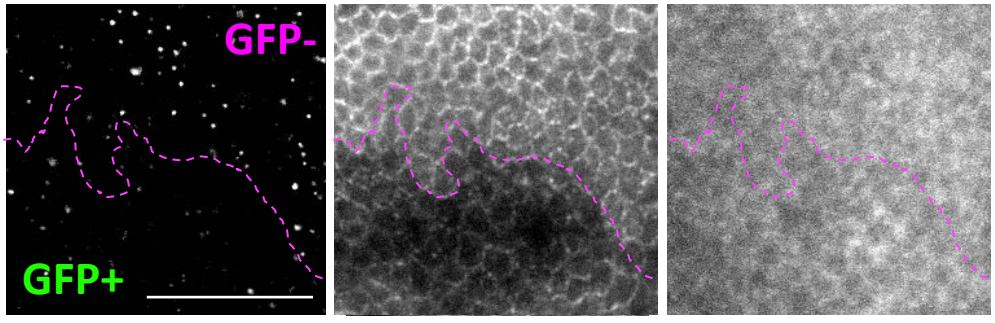

Vesicular Notch
Surface Notch


GFP+
GFP-
Vesicular Notch
Surface Notch
Phalloidin

D’’

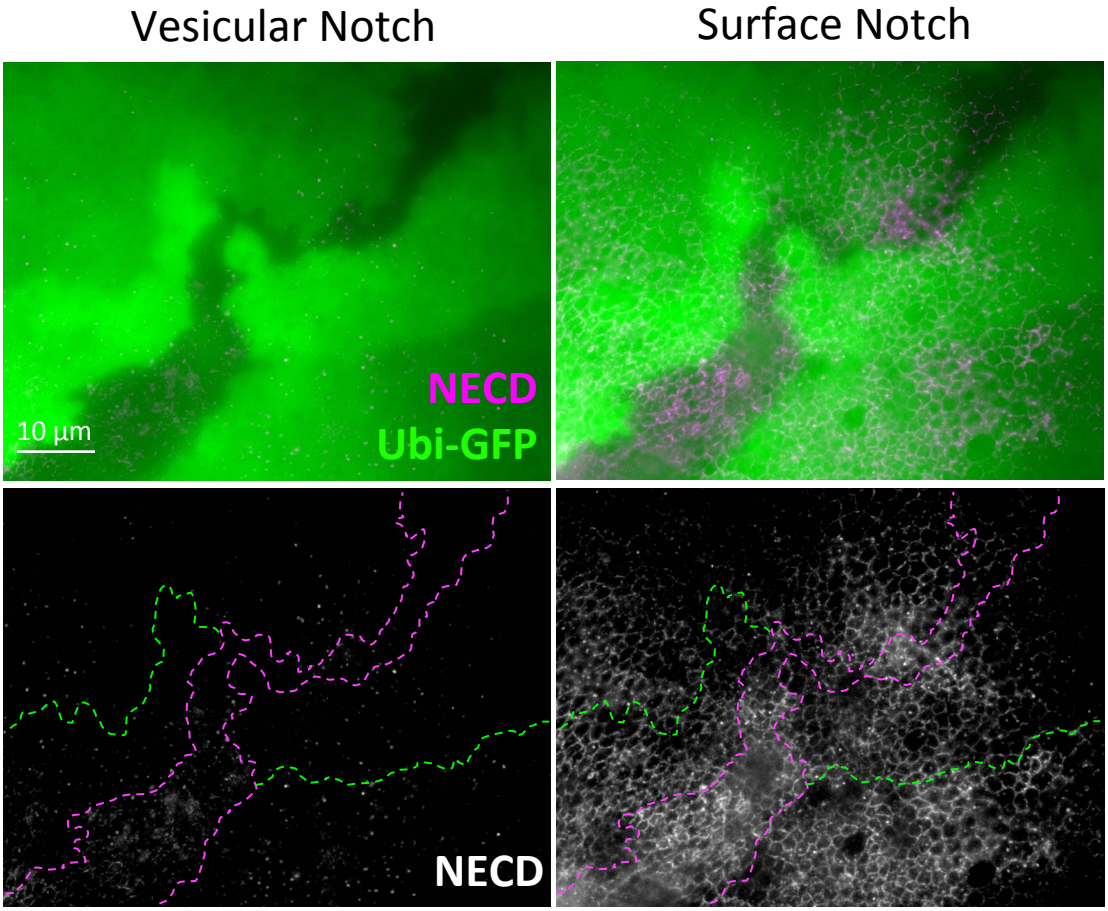
9B2 25°C

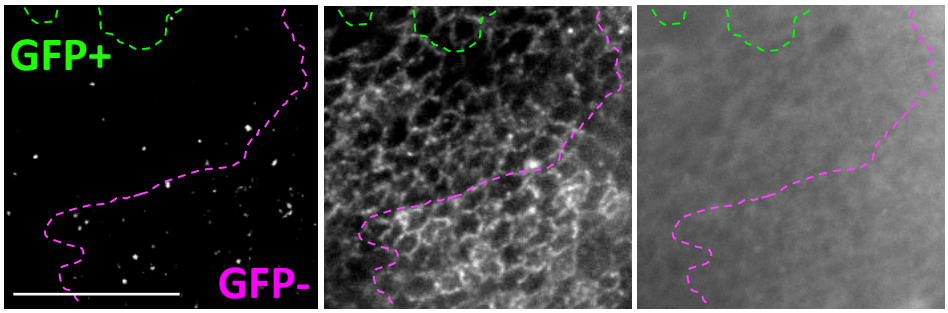

Vesicular Notch
Surface Notch
NECD
Ubi-GFP
10 µm
NECD


GFP-
GFP+
Vesicular Notch
Surface Notch
Phalloidin

D’’’

9B2 29°C


Vesicular Notch
Surface Notch
10 µm
NECD
Ubi-GFP
NECD


GFP+
GFP-
Vesicular Notch
Surface Notch
Phalloidin

3.4.2 *Ax* mutants show a different localisation at the cell membrane and in vesicular compartments in a *dx* mutant background *in vivo*

Ax mutants showed differences in the surface localisation and endocytic uptake in the absence of Dx in S2 cells (Fig. 3.12, 3.14). Therefore it was interesting to test if what observed *in vitro* was happening in the absence of Dx *in vivo*. For this purpose, the localisation of Notch was analysed in *Ax, dx^{152}* mutant clones at different temperatures produced using the FLP/FRT technique. Interestingly, at 25°C *E2, dx^{152}* clones showed an increase in vesicular Notch compared to *dx^{152}* clones (Fig. 3.19B) and this was in agreement with the increased endocytic uptake observed in *E2* in the absence of Dx in S2 cells (Fig. 3.14). Further, surface Notch was increased in *dx^{152}* clones, whereas the increase was milder and non-significant in *E2, dx^{152}* clones and stronger and very significant in *9B2*, *dx^{152}* clones (Fig. 3.19B'). This reflects the surface Notch localisation in the absence of Dx in S2 cells, which was lower in *E2* and higher in *9B2* compared to WT (Fig. 3.12).

Mutant *dx^{152}* clones showed non-significant changes in vesicular Notch at all temperatures, however the trend was toward a decrease at 18°C and 25°C and an increase at 29°C (Fig. 3.19A-C, 3.20A). Surface Notch was increased at all temperatures and this is likely due to a lower endocytic flux into the Dx-mediated route (Fig. 3.19A'-C'). This was particularly significant at 18°C probably because at low temperature Dx-mediated route is favoured, so a lower flux in this route might be exaggerated at 18°C. At 29°C, endocytosis is generally favoured, but still not enough to reduce the surface Notch.

Mutant *E2, dx^{152}* clones showed an increase in vesicular Notch compared to the twin spot at all temperatures, which was stronger to milder from 18°C to 29°C (Fig. 3.19A-C, 3.20B). Surface Notch was only significantly increased at 29°C and 18°C, however the increase was always mild and lower than that observed in *dx^{152}* clones (Fig. 3.19A'-

C'). This indicates that *E2* has an increased rate of endocytosis even in the absence of Dx. If *E2* is trafficked mainly in GPI- vesicles even without Dx, as shown in S2 cells (Fig. 3.10), then this explain why its endocytosis is increased at 18°C where Dx-mediated route is favoured. Nevertheless the endocytosis of *E2* at 18°C might lead to its accumulation in the Dx-mediated route, but not activation.

Vesicular Notch was also increased in *16, dx*^{152} clones compared to the twin spot and this was inversely correlated with increasing temperature, no change to surface Notch was observed (Fig. 3.19A-C, 3.20C). This increase in vesicular Notch was similar, though milder, to what was observed in *E2, dx*^{152} clones (Fig. 3.19A'-C').

9B2, *dx*^{152} clones showed no significant changes in vesicular Notch compared to the twin spot (Fig. 3.19A-C, 3.20D) but a very strong accumulation of surface Notch at all temperatures, which was clearly stronger than in *dx*^{152} clones (Fig. 3.19A'-C'). A number of Notch endosomal structures can be seen in the deconvolved pictures of *9B2, dx*^{152} clones in the mutant spot, however, the quantification of vesicular Notch did not detect a significant difference compared to the twin spot. This could be because the background staining, arising from the very strong surface Notch, might enhance the brightness of the vesicular Notch.

In summary, it was shown that *in vivo*, in mutant clones, *E2* increased vesicular Notch and this effect was even stronger in the absence of Dx. Similar observations were made for *16*, however the behaviour of this mutant was similar but not identical to E2. *9B2* increased the localisation of Notch at the cell membrane, especially in the absence of Dx. *9B2* also, increased vesicular Notch, but this effect was suppressed at high temperature or in the absence of Dx. Taken together these observations suggest that *E2* and *16* are more prone to endocytosis which might have a positive effect on Notch signalling in physiological conditions and have a negative effect in the absence of Dx. *9B2* might

also increase Notch endocytosis and this might have a positive effect on Notch, however this increase is suppressed in the absence of Dx and might negatively impact on Notch. Antibody uptake assays in the clones might be required to test the endocytosis of the mutants and support these observations. Finally, it is interesting that the localisation of Notch in the *Ax* mutant clones or in *Ax, dx*^{152} mutant clones showed distinct temperature dependencies and this was already observed in the wing phenotypes at the beginning of the chapter (Fig. 3.1, 3.2) In conclusion, the mutants showed an allele-specific localisation of Notch *in vivo* and this is consistent with the differences observed in the phenotype, signalling and trafficking of *Ax* mutants.

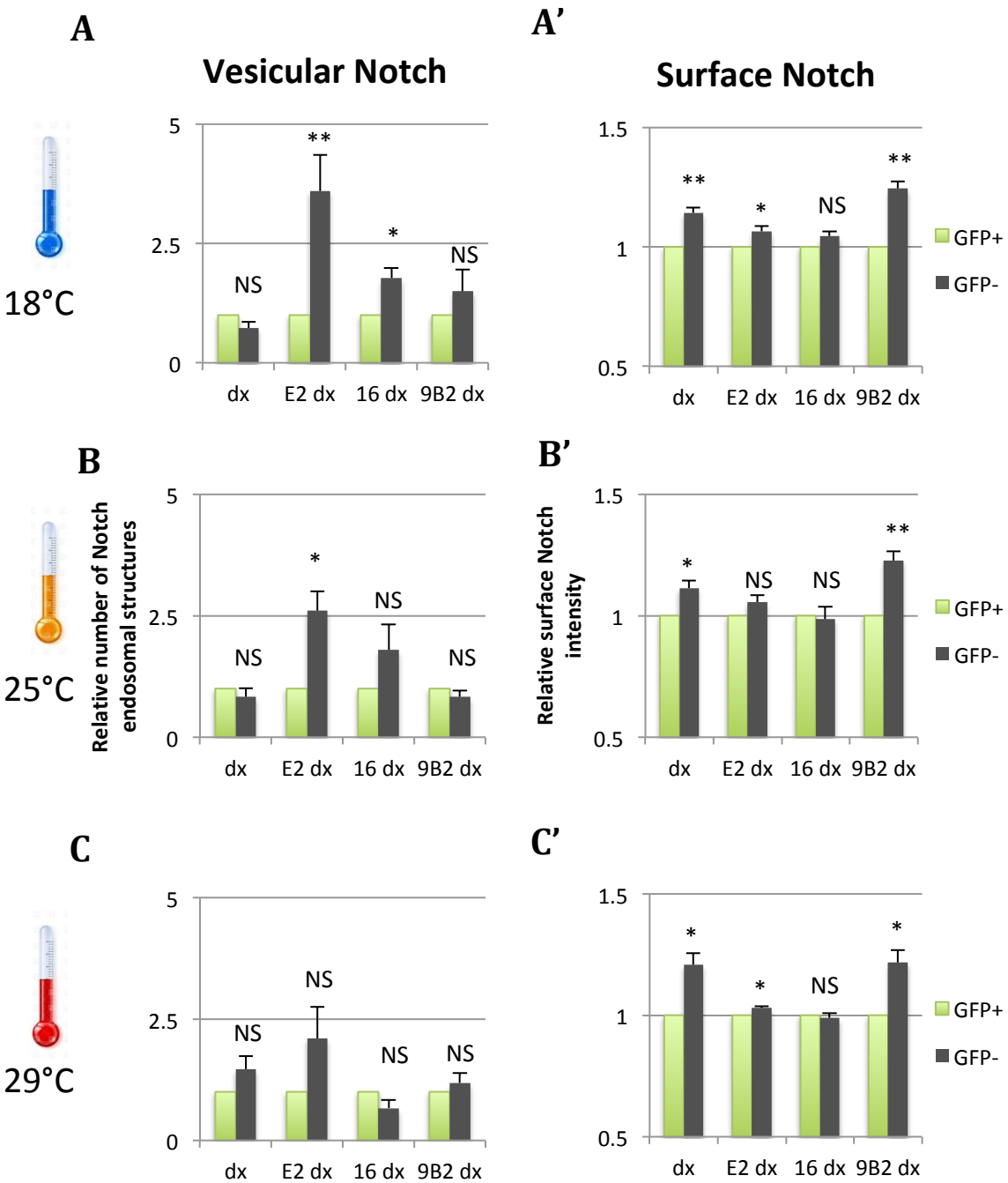


Figure 3.19. Quantification of vesicular and surface Notch in Ax, dx^{152} clones relative to their twin spots. The number of Notch endosomal structures (vesicular Notch, A-C) and the intensity of Notch staining at the apical membrane (surface Notch, A'-C') were quantified in the GFP+ and GFP- clonal spots using ImageJ. The vesicular or surface Notch mean values in the GFP+ (green columns) or GFP- (black columns) spots were divided by the mean values in the GFP+ spots. The plots show the quantification of the clones at 18°C (A, A'), 25°C (B, B') and 29°C (C, C'). The statistical significance was determined by Student T test and is relative to the GFP+ values of each sample (NS=non significant; *p<0.05; **p<0.01). Error bars indicate SEM (n=5).

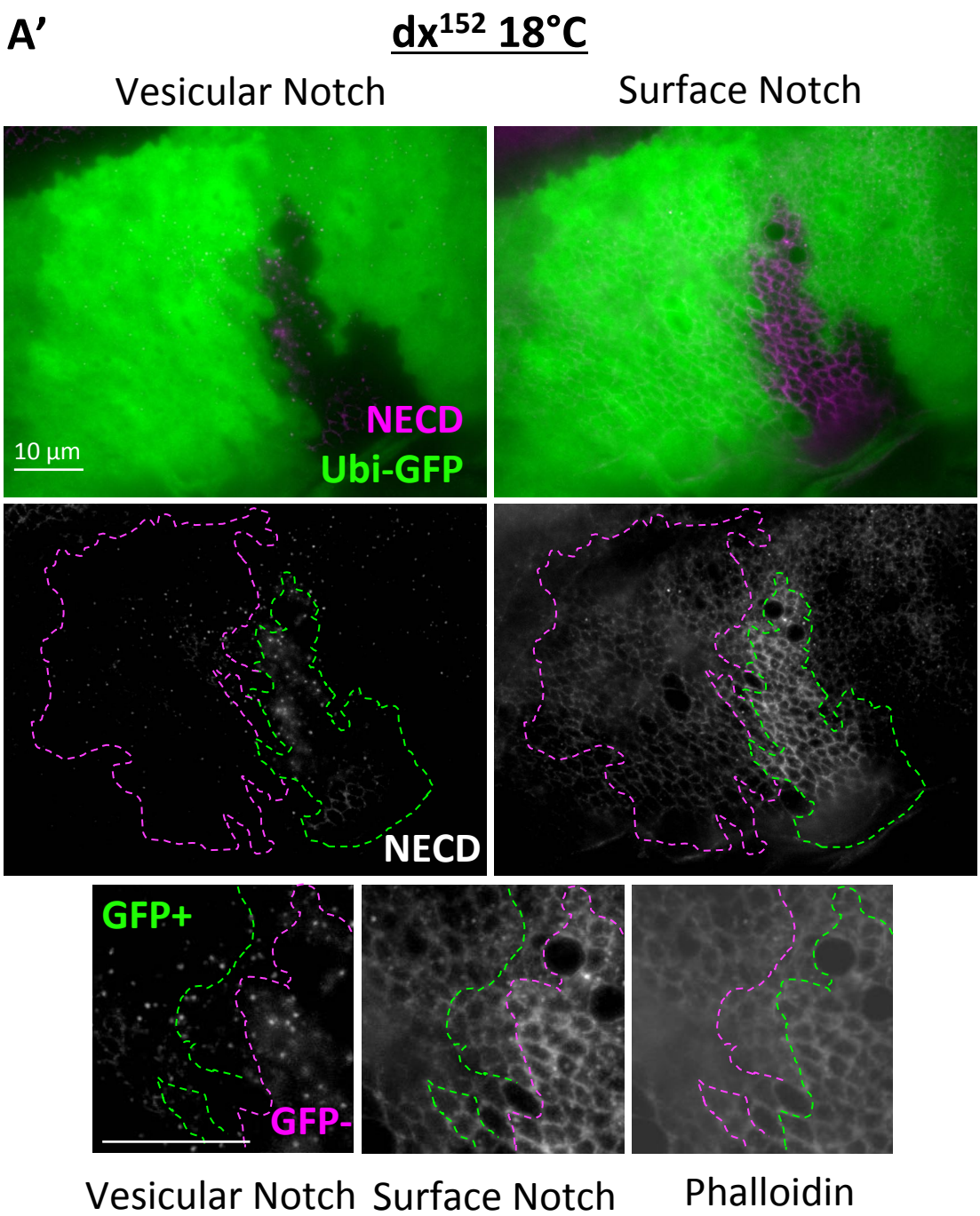
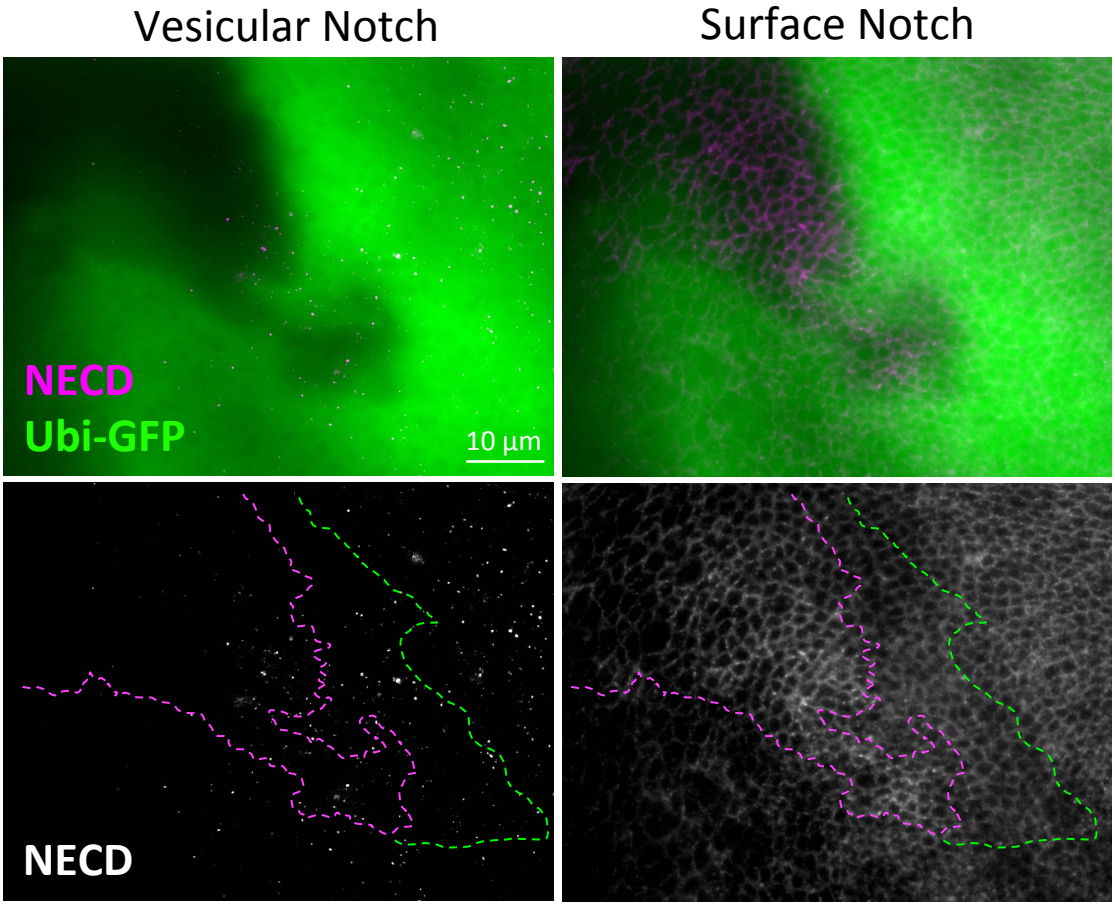
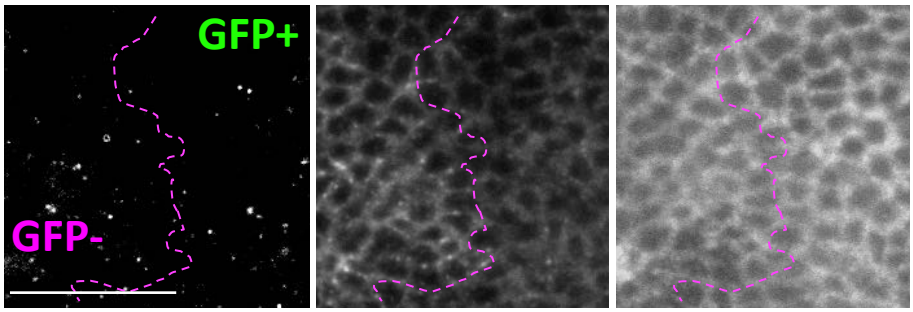


Figure 3.20. Ax, dx^{152} mutant clones in the *Drosophila* wing disc using negatively labelled mutant clones compared to wild-type double GFP twin spot (see § 2.3.3). The panels in the current and following pages show (A) dx^{152}, (B) E2, dx^{152}, (C) 16, dx^{152} and (D) 9B2, dx^{152} clones at (A’-D’) 18°C, (A’’-D’’) 25°C and (A’’’-D’’’) 29°C. The wing discs were fixed, permeabilised and stained for Notch using an anti-NECD antibody (purple), GFP using an anti-GFP antibody (green) and actin using phalloidin-far red to label the apical membrane. The GFP+ clonal spot (green dashed lines) is homozygous for WT Notch, while the GFP- spot (purple dashed lines) is homozygous for the mutant. The vesicular Notch panels show Notch endosomal structures at ~1.5 µm from the apical membrane. The surface Notch panels show Notch at the apical membrane. Phalloidin panels shows the staining at the same stack plane as surface Notch. Scale Bars correspond to 10 µm.

A’’

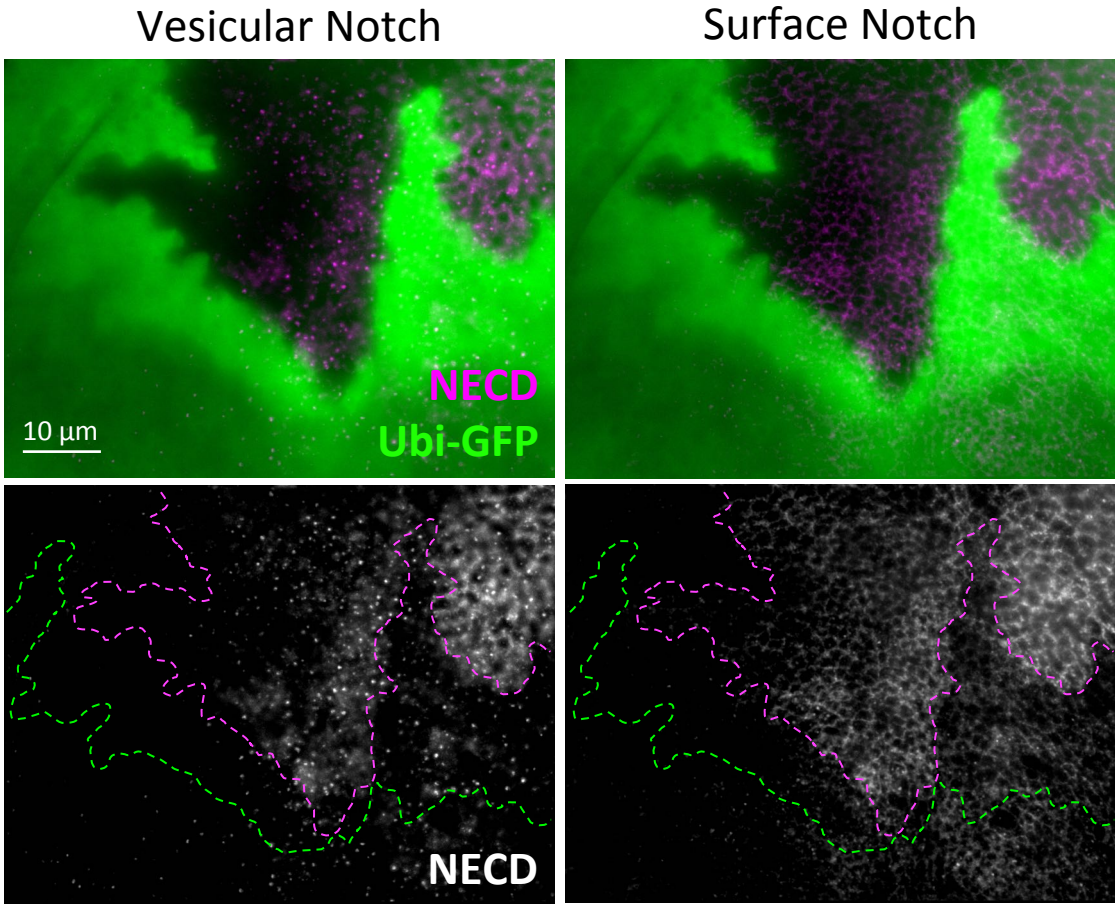
dx^{152} 25°C

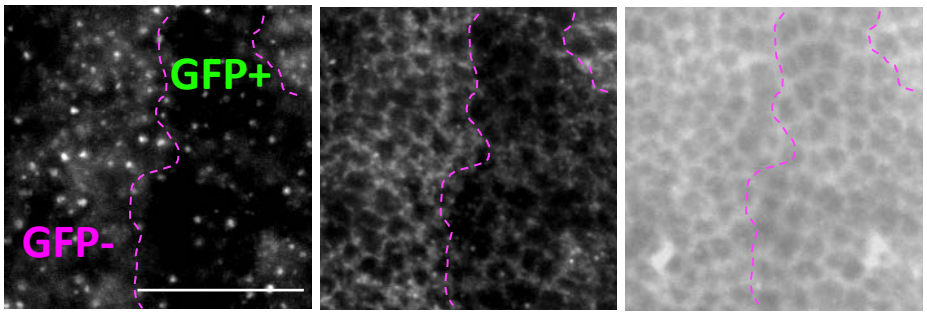

Vesicular Notch
Surface Notch
NECD
Ubi-GFP
10 µm
NECD


GFP+
GFP-
Vesicular Notch
Surface Notch
Phalloidin

A’’’

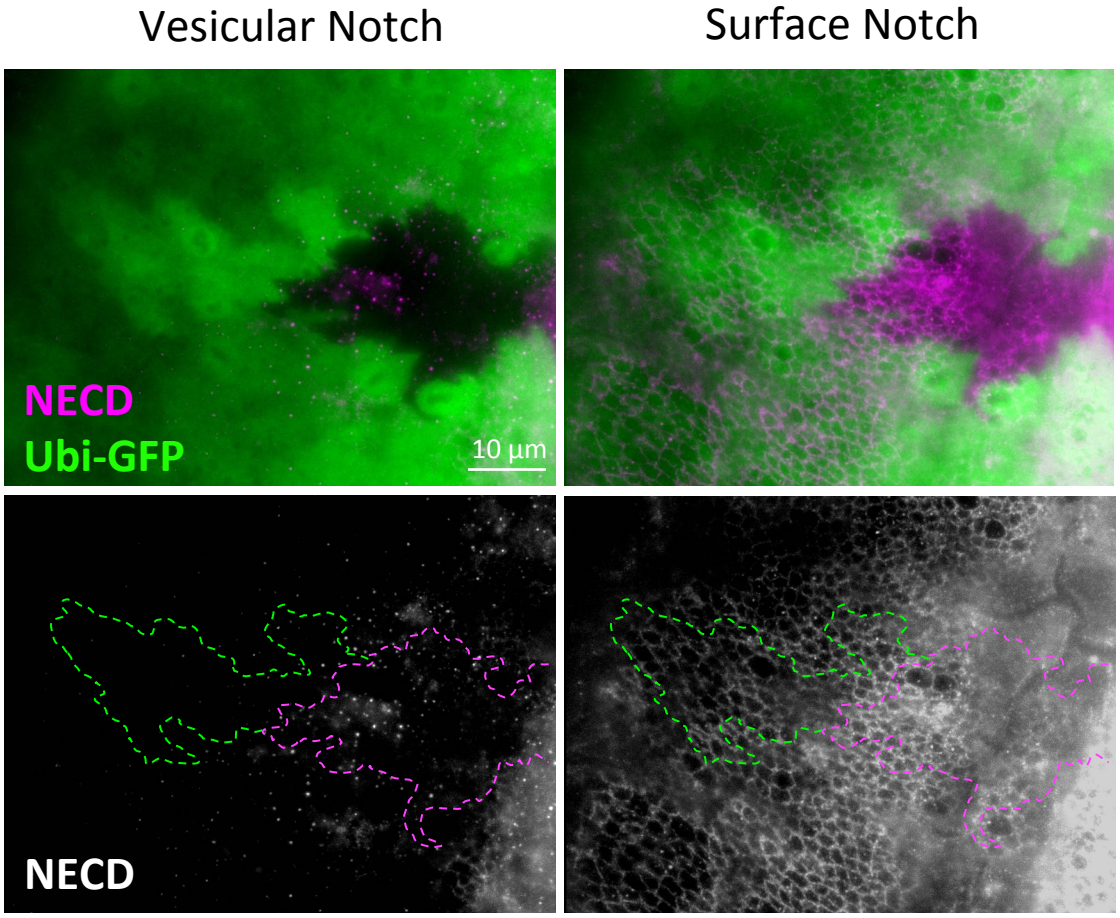
dx^{152} 29°C

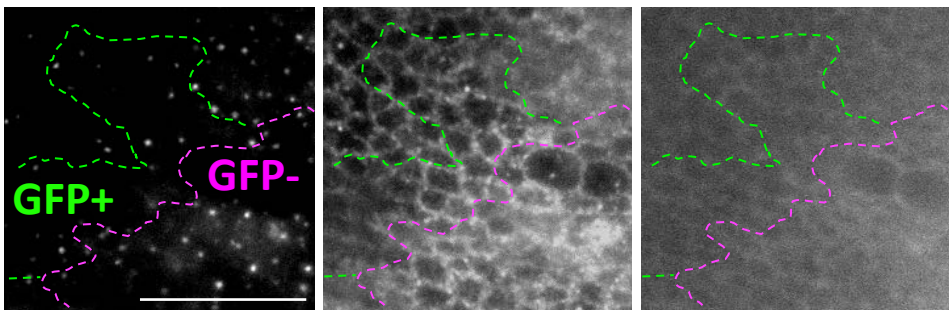

Vesicular Notch
Surface Notch
NECD
Ubi-GFP
10 µm
NECD


GFP+
GFP-
Vesicular Notch
Surface Notch
Phalloidin

B’

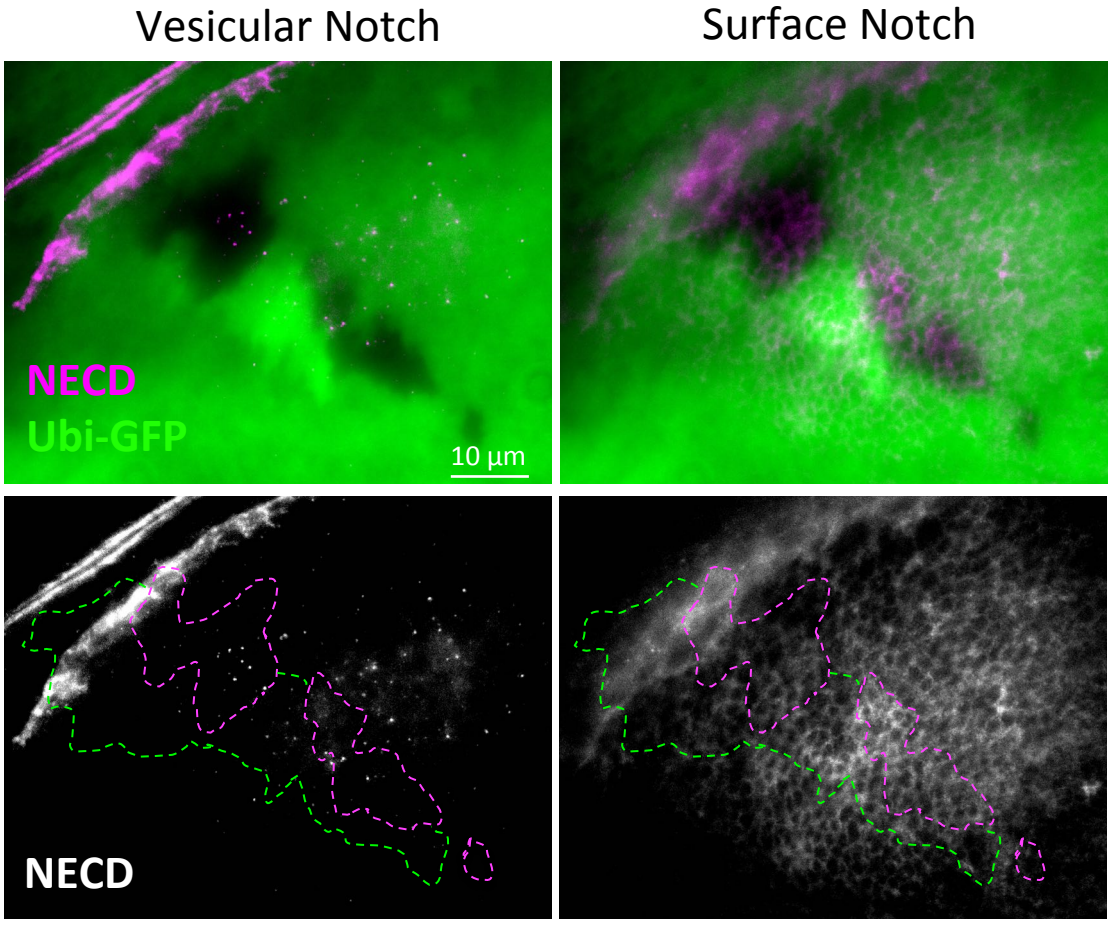
E2, dx^{152} 18°C

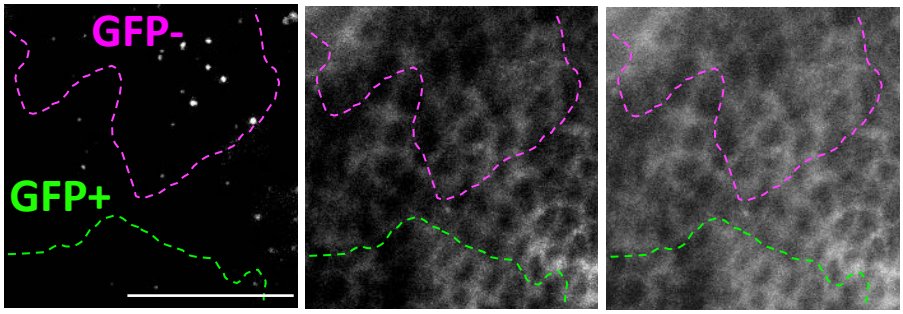




B’’

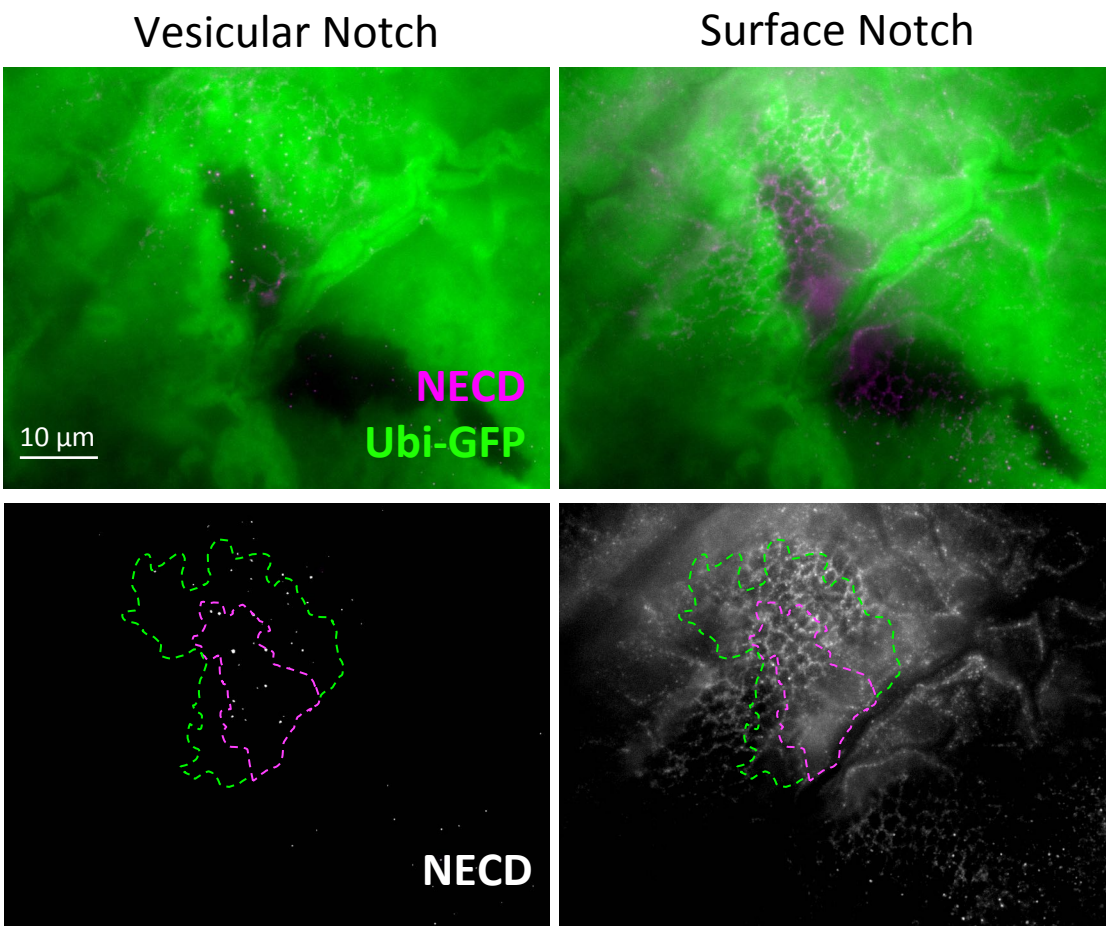
E2, dx^{152} 25°C

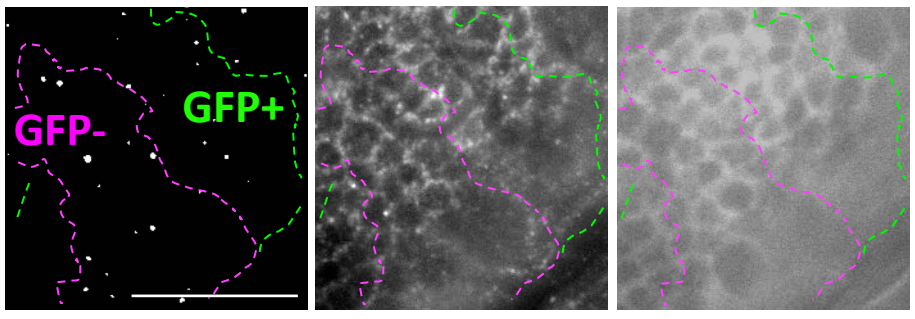




B’’’

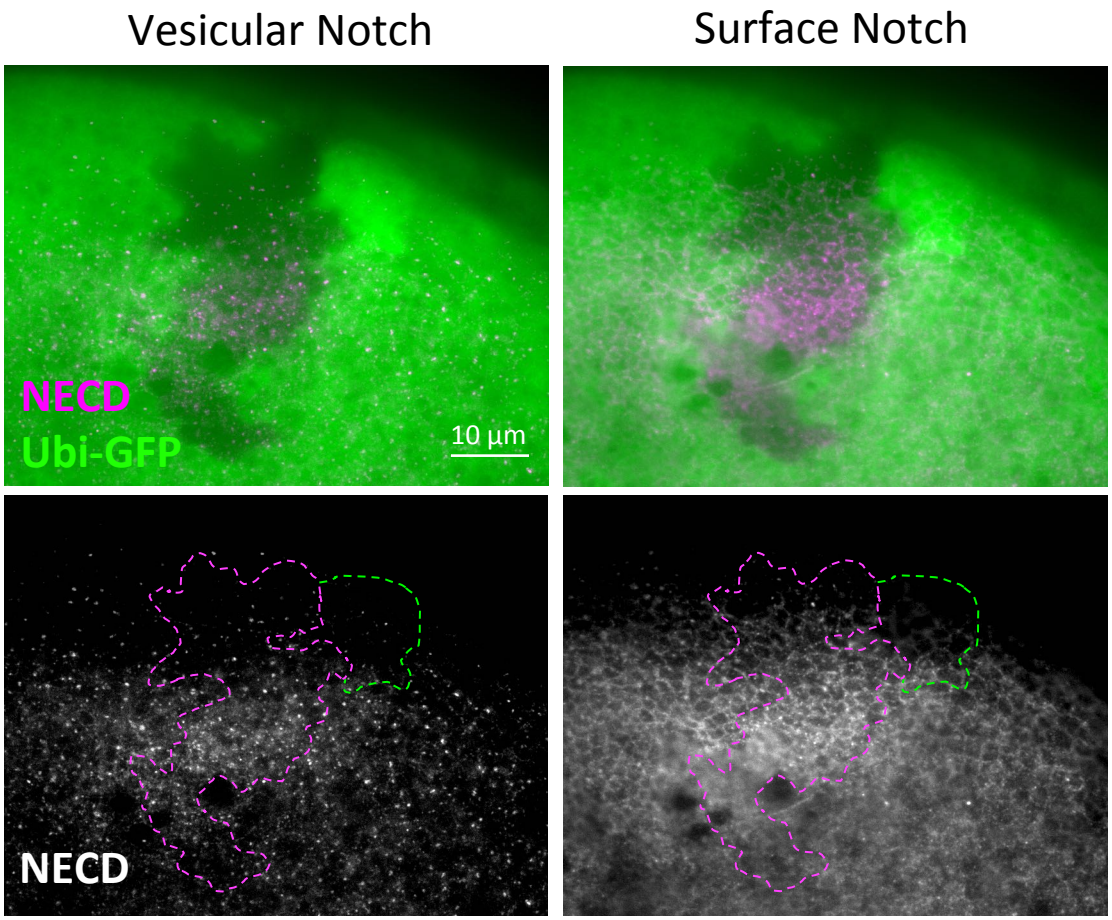
E2, dx^{152} 29°C

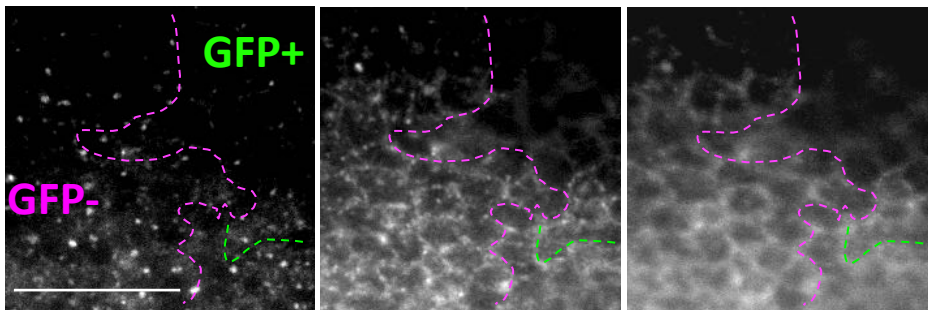




C’

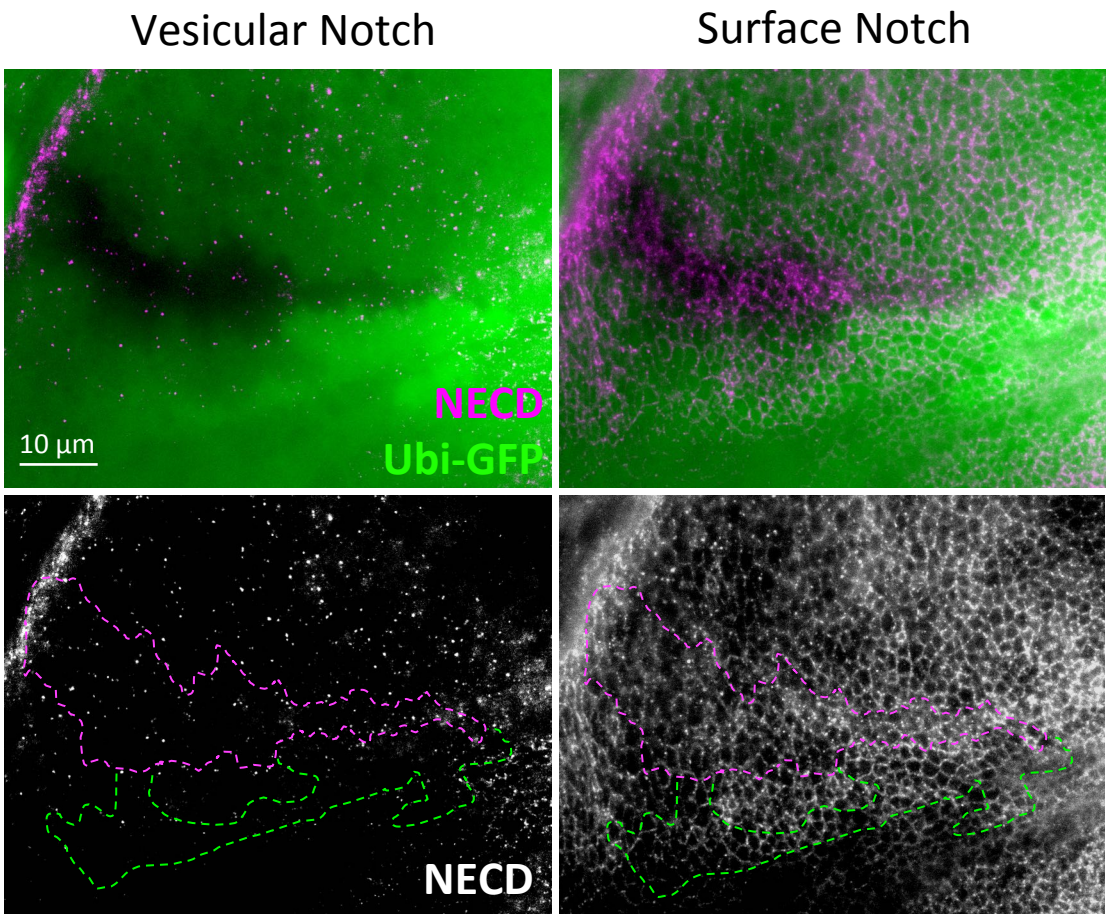
16, dx^{152} 18°C

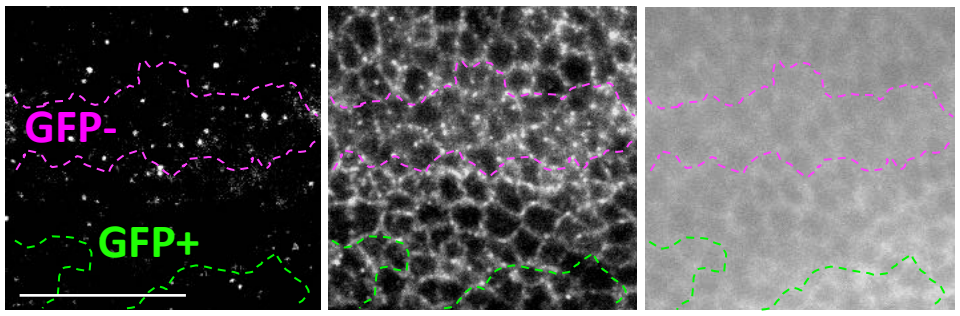




C''

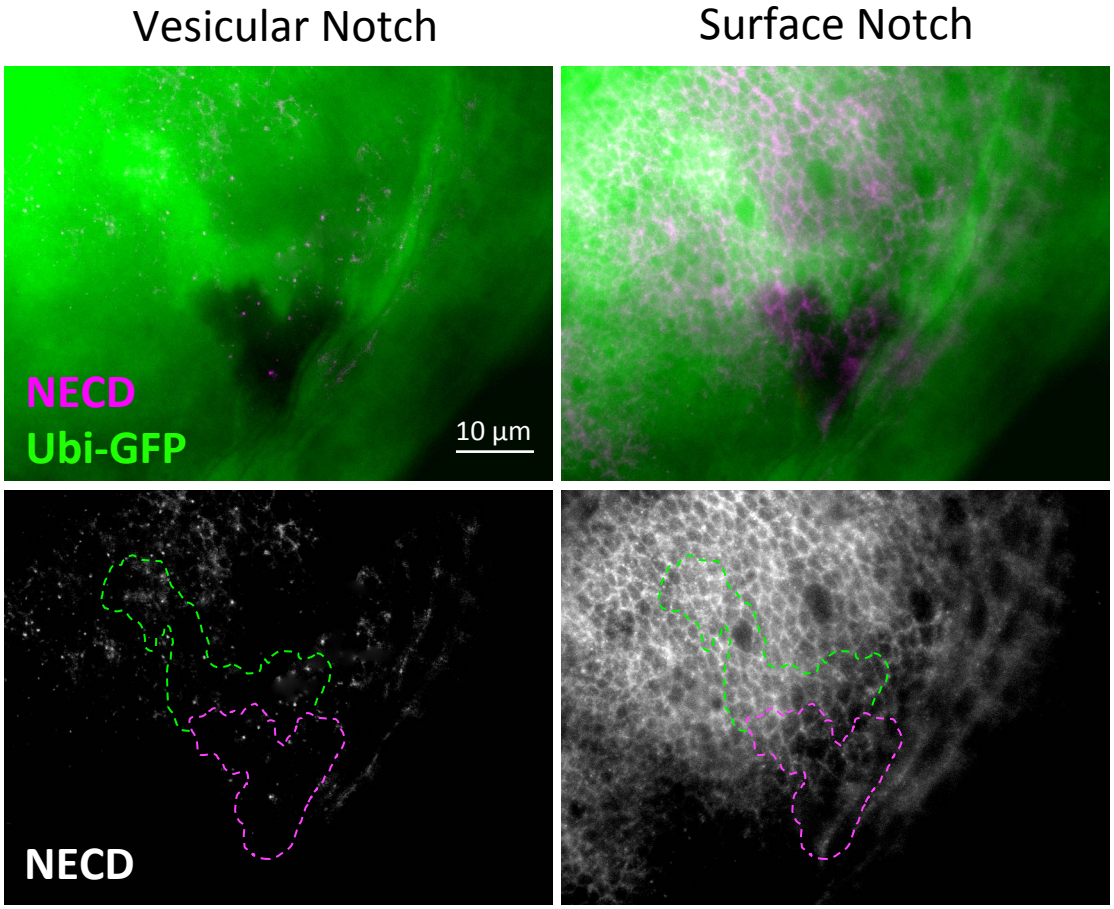
16, dx^{152} 25°C

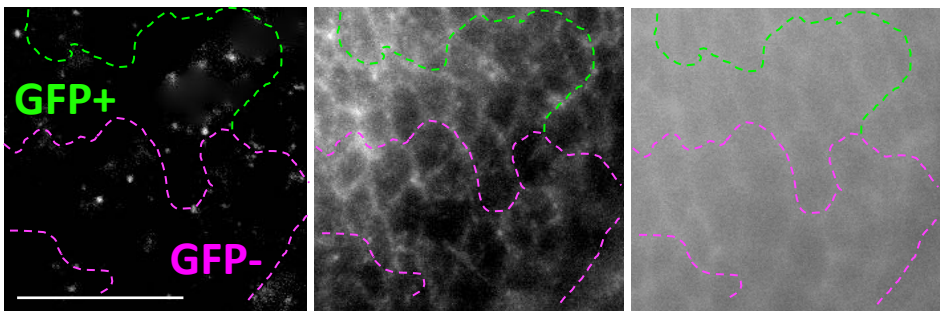




C’’’

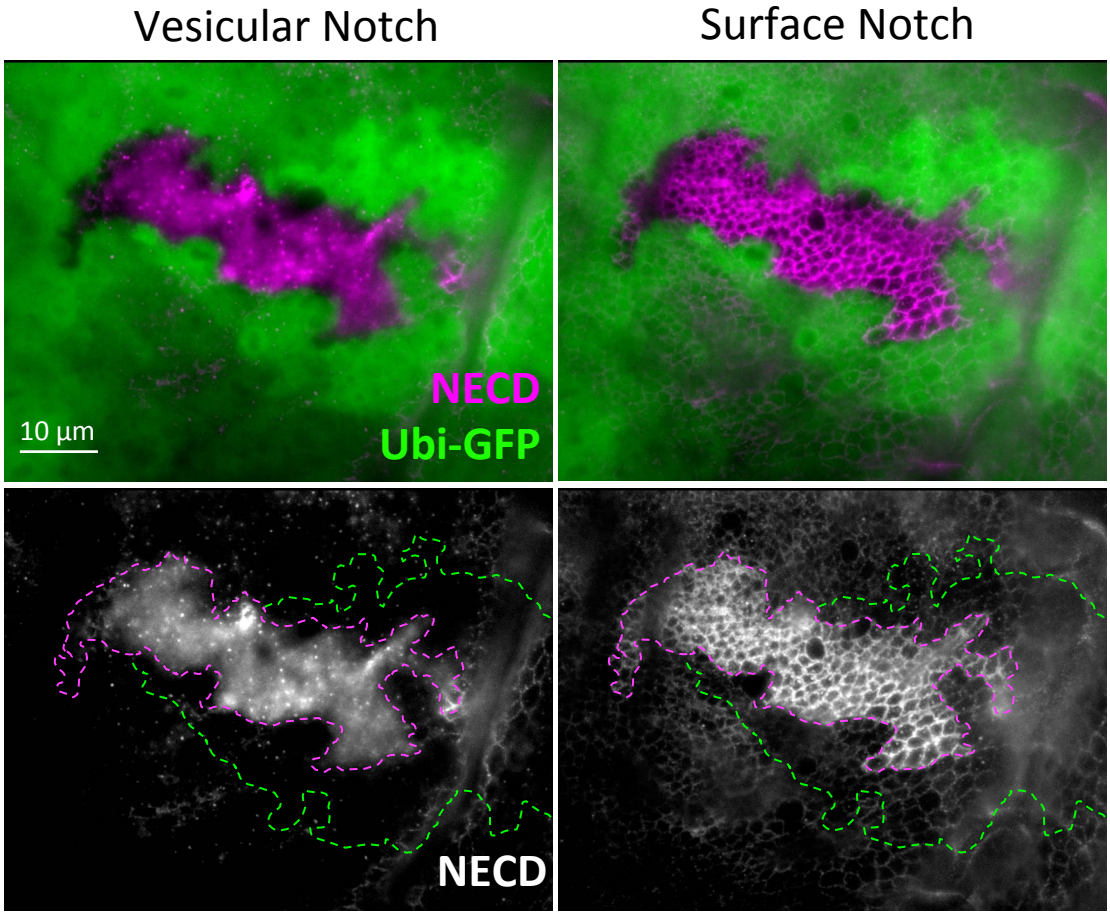
16, dx^{152} 29°C

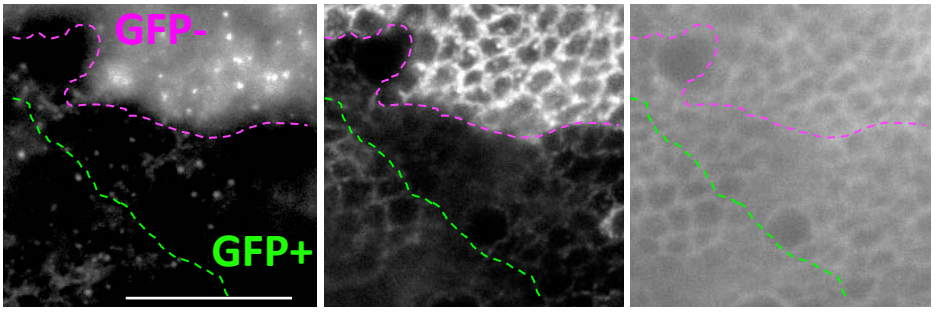




D’

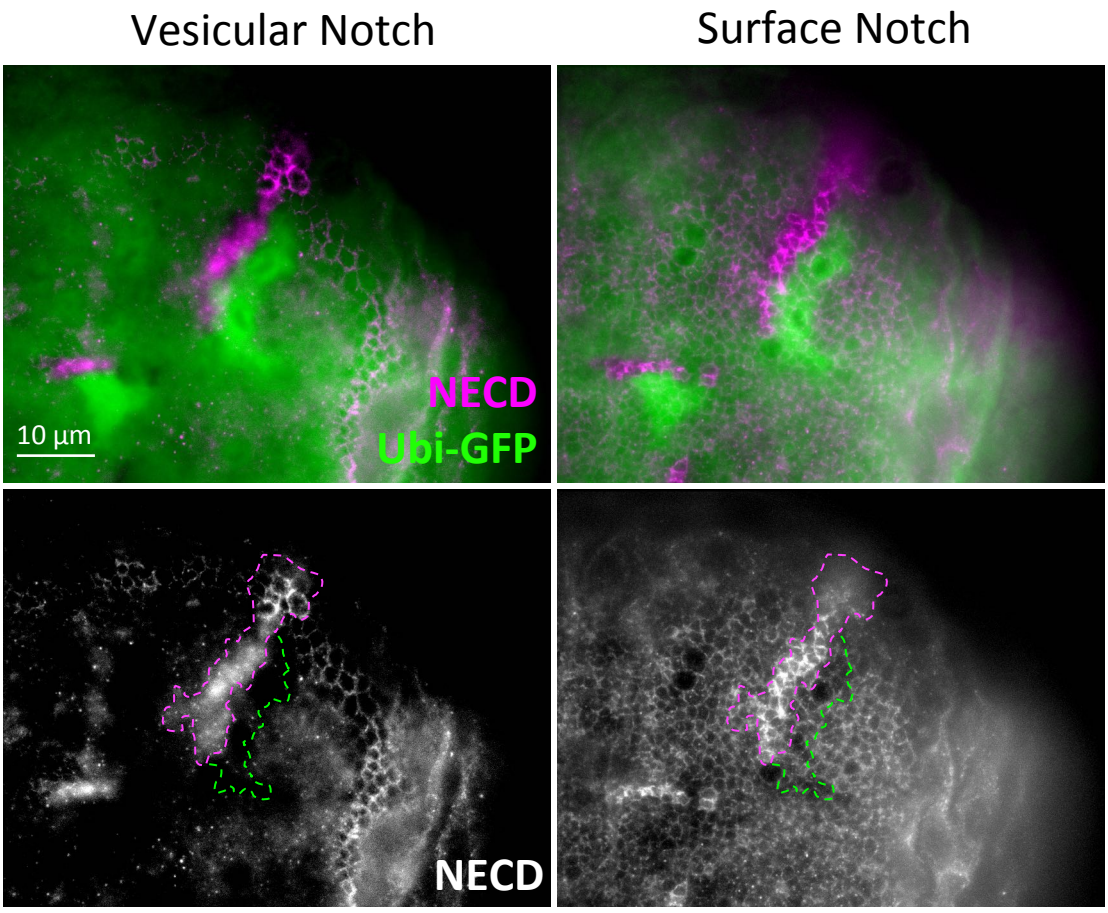
9B2, dx^{152} 18°C

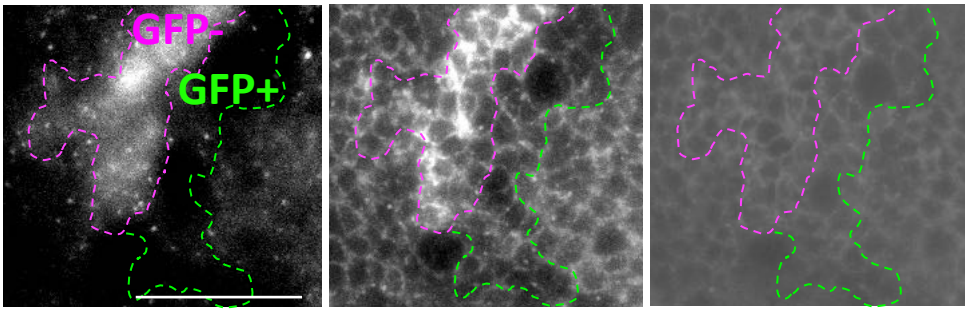




D’’

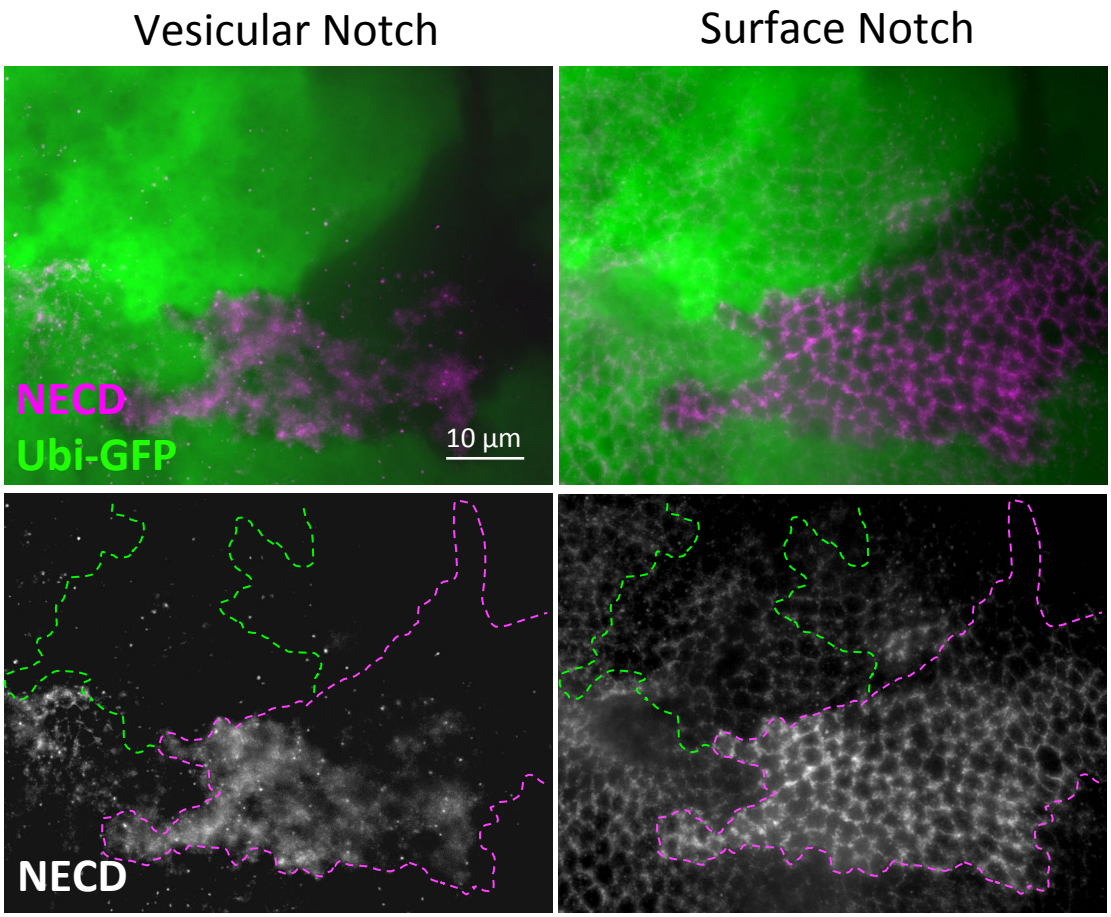
9B2, dx^{152} 25°C

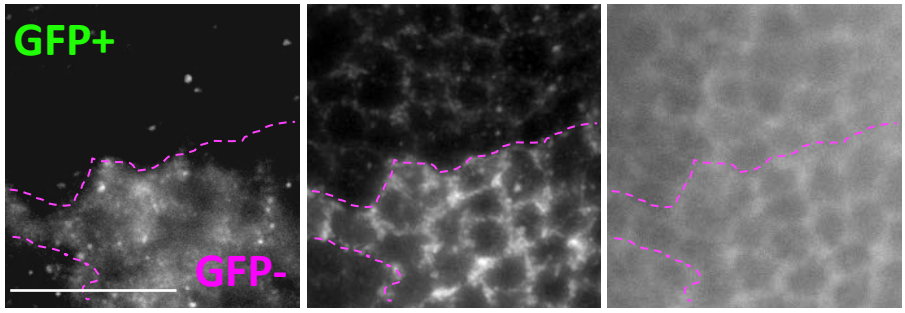




D’’’

9B2, dx^{152} 29°C





3.5 Discussion

It has been demonstrated that *Ax* mutants of the two different classes, suppressors and enhancers have phenotypic differences *in vivo* and different temperature dependencies. For instance, the wing vein gapping phenotype of *E2* increases with rising temperature, whilst that of *9B2* decreases. Further, the hetero-allelic combination of enhancer(*E2*)/suppressor(*9B2*) had a much-increased Notch signal than the two mutants as homozygotes. The simplest explanation for this synergistic interaction was that the two *Ax* mutations were increasing Notch signalling *via* different mechanisms. Furthermore, the *in vivo* work showed that as with the dx^{enu} (a point mutant in the Notch binding domain; Fuwa et al., 2006), the dx^{152} (null mutant; Fuwa et al., 2006) also interacted with the *Ax* alleles of both classes, to switch them from gain-of-function to strong loss-of-function. This suggested that Dx may be a key protein through which the *Ax* mutants were having their effects.

In vivo and in S2 cells, the Dx route requires an active HOPS complex. The hypomorphic allele of carnation, car^{1}, compromises the function of the HOPS complex, inhibiting the lysosomal-dependent activation in the Dx-mediated route (Wilkin et al., 2008; Shimizu et al., 2014). To test if Dx function is to activate *Ax* mutants in a lysosomal-dependent manner, flies were generated mutant for both *Ax* and car^{1}. The gain-of-function phenotype of *Ax* mutants was reduced in *Ax,* car^{1} flies. However, there was not the dramatic switch from high to low Notch activity seen with *dx* mutants suggesting that the cause of the Ax phenotypes was more complex than purely increased Dx-mediated lysosomal-dependent signalling.

The *Drosophila* S2 cell culture system was crucial in helping to unravel the mechanisms through which the *Ax* mutants act. Firstly, alterations in Notch signalling were identified in different *Ax* alleles in S2 cells. This led to the idea that *Ax* mutants might have different signalling

potentials because they affect different steps of Notch trafficking. Using different assays in S2 cells, it was shown that *Ax* mutants alter the trafficking and endocytosis of Notch. Interestingly, it was observed that different *Ax* alleles affect different signalling and trafficking routes and this might explain the effect of *Ax* mutants on Notch signalling.

In S2 cells, endocytosed wild-type Notch is predominantly trafficked from the plasma membrane through GPI+ endosomes when Dx is not present, switching to GPI- endosomes when Dx is available. *E2* was shown to increase the localisation of Notch in endosomal compartments in S2 cells but trafficked mainly to GPI- vesicles in both the presence and absence of Dx. This was a clear and robust difference between wild-type and E2 receptor trafficking in S2 cells. However, E2 was still able to be trafficked in the GPI+ route in S2 cells when Su(dx) was co-expressed. Therefore, E2 can still be down-regulated *via* the Su(dx) route when endocytosed through this route.

From anti-NECD uptake assays, it was clear that there was an increased Notch flux in the GPI- route with E2 as compared to wild-type. This agreed with the findings that there was less E2 at the cell surface in S2 cells compared to wild-type as measured by microscopy and FACS analysis. As the GPI- route is less temperature sensitive, this may explain why *E2* ligand-independent signalling is stable across different temperatures in S2 cells and likewise that the *E2* phenotype in adult flies is clear at all temperatures. Furthermore, in clones in the larval wing disc, E2 was found to be more heavily localised to endosomal structures at all temperatures tested.

From these observations alternative models are proposed that are not mutually exclusive, as to how E2 may change Notch activation. Firstly, *E2* gain of function results in increased endocytic flux in the GPI- route. E2 does not require Dx to enter this route, although it still can respond strongly to it (Fig. 3.21.A1).

A second outcome of E2 being more on the GPI- route may be to prevent most of it from being degraded in the GPI+ Su(dx)-mediated route, ultimately leading to a higher signalling (Fig. 3.21.A2). The latter is supported by the observation that *E2* has a stronger phenotype and increased localisation in endosomal compartments *in vivo* at 29°C, when Su(dx)-mediated degradation is favoured, suggesting E2 can counteract this degradation better than wild-type.

However, the increased flux of E2 in the GPI- route might not be enough to sustain its signalling in the absence of Dx. Indeed, in S2 cells, without Dx, the endocytic uptake of *E2* was still increased and the majority of *E2* was still trafficked in the GPI- route, but this lead to reduction in the basal signalling. *In vivo*, a lack of Dx in combination with *E2* led to a strong loss-of-function instead of gain-of-function phenotype. In *E2, dx^{152}* clones there was more endocytic Notch in the mutant clone as compared to the wild-type twin spot at all temperatures. This might indicate that in the absence of Dx, *E2* might accumulate in the GPI- route without being activated (Fig. 3.21.B1) or be transferred and degraded in the Su(dx) route (Fig. 3.21.B2). The relative increase in endocytic Notch in *E2, dx^{152}* as compared to its twin spot diminished with rising temperature, suggesting that E2 without Dx cannot completely counteract Su(dx) and, as Su(dx) activity increases with the temperature, so does E2 degradation. This is also supported by the observations that in flies E2 gain-of-function is enhanced by reduction in *Su(dx)* (Fostier et al., 1998) and further that the depletion of one copy of *Su(dx)* can rescue the loss-of-function phenotype of *E2, dx* flies (Busseau, 1994; Dr. M. Baron, personal communication).

Therefore, Dx function might be to prevent *E2* from being degraded by Su(dx), by enhancing the propensity of E2 to be trafficked in the GPI- route. However, how E2 might be activated in the GPI- route in the presence of Dx is still an open question since blocking of lysosomal-dependent activation was not enough to suppress E2 gain-of-function.

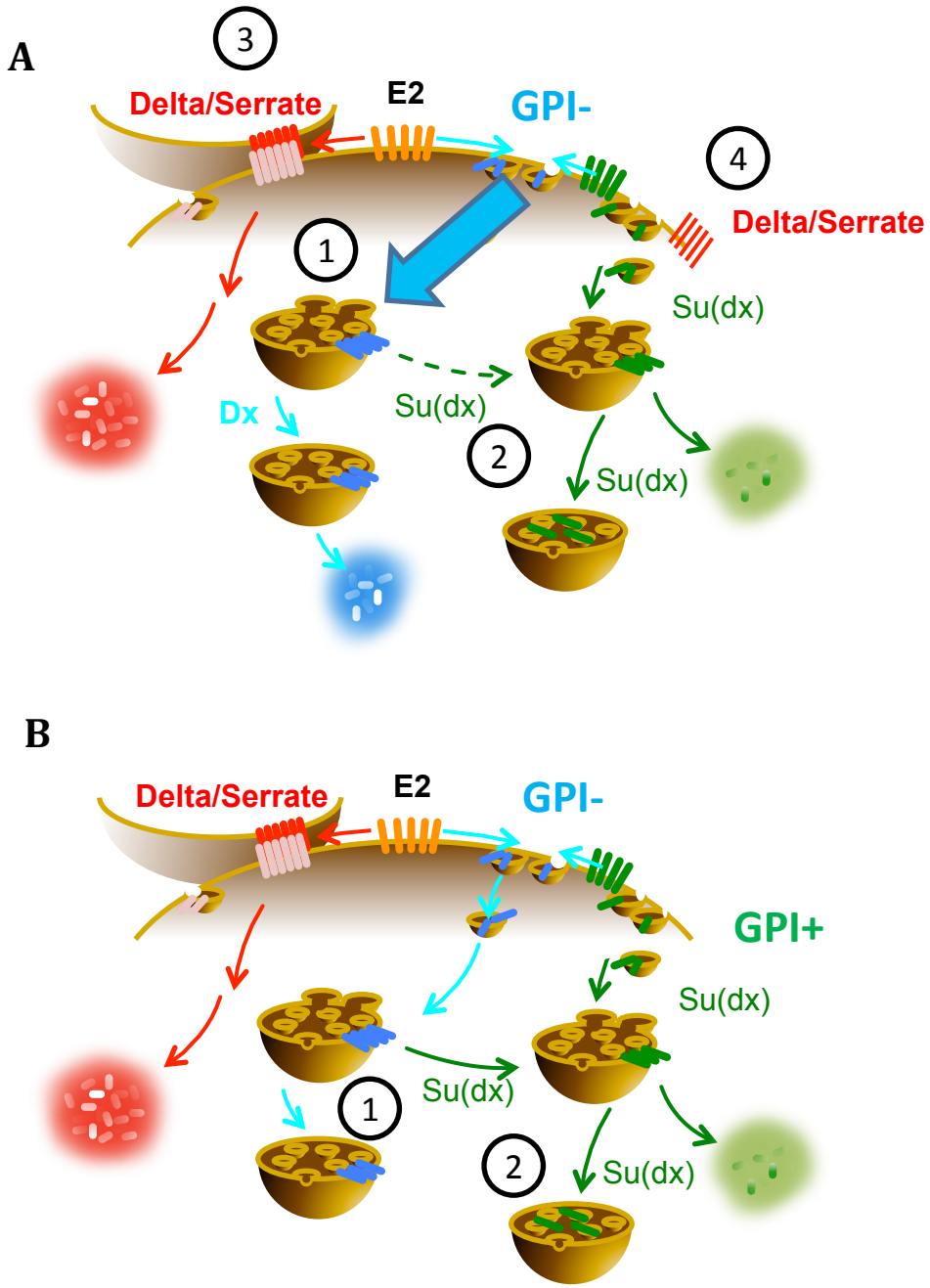


Figure 3.21. Alternative models for E2 regulation. (A) model which E2 increases Notch flux into the GPI- route (1A), or prevents Notch from being shifted into the Su(dx) route (2A), is trans-activated by ligands (3A) and has less cis-interaction with ligands, thus leaving more ligands available for trans-activation of Notch in neighboring cells (4A). (B) shows a model of E2 regulation in the absence of Dx in which E2 increases Notch flux into the GPI- route and it is accumulated and degraded in the GPI- route (1B) or GPI+ route (2B). In both (A) and (B) ligands and ligand-dependent pathway are shown in red, Dx-mediated route in blue and Su(dx)-mediated route in green.

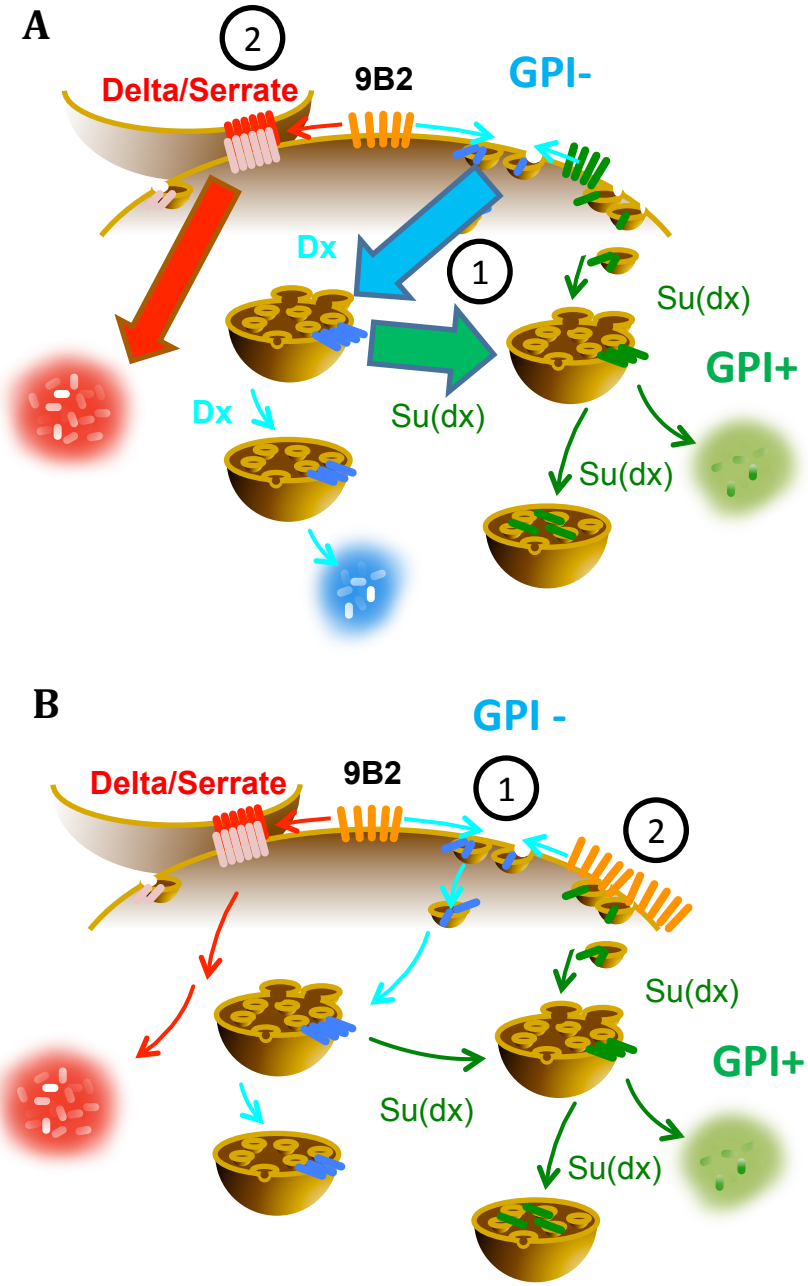


Figure 3.22. Alternative models for 9B2 regulation. (A) shows a model of 9B2 regulation in which 9B2 is endocytosed into the GPI- route and is shifted into the GPI+ route (1A), thus it is constantly kept at the right level at the cell membrane and is trans-activated by ligands (2A). (B) shows a model of 9B2 regulation in the absence of Dx in which 9B2 endocytosis is decreased (1B) and 9B2 accumulates at the cell membrane (2B) and might cis-interact more with ligands, thus reducing the availability of ligands for trans-activation of Notch in neighboring cells. In both (A) and (B) ligands and ligand-dependent pathway are shown in red, Dx-mediated route in blue and Su(dx)-mediated route in green.

It is possible E2 might be activated at a different stage of endosomal maturation and this might somewhat depend on the presence of Dx.

Finally, *E2* can respond normally to ligand-dependent signalling (Fig. 3.21.A3). However, because of decreased *E2* localisation at the cell surface, there may be a reduction in the cis-interaction of Notch with its ligands thus leaving more ligands available for ligand-dependent trans-activation on neighbouring cells (Fig. 3.21.A4). The *E2* gain-of-function *in vivo* may thereby be the result of both ligand-independent and ligand-dependent signalling. It is interesting that, if the fold induction is considered (see Fig. 3.7B and 3.8B), E2, and also 16 and 28, shows an increased ligand-dependent and Dx-dependent signalling in S2 cells.

Analogous conclusions can be drawn for the other enhancer class member *16*, which showed similar though somewhat more pronounced features to *E2*. These included: increased localisation in endosomal compartments; trafficking in the GPI- route; and decreased localisation at the cell membrane.

In S2 cells, *9B2* endocytic trafficking was not the same as wild-type nor E2. Without Dx, like wild-type it was endocytosed into GPI+ endosomes. Further, like wild-type, it was efficiently endocytosed in response to Dx into the GPI- route. However, there the similarity ended as *9B2* was then shifted into the GPI+ route and likely degraded (Fig. 3.22.A1), which would explain the reduced Dx-dependent signalling observed. This may also explain why *9B2* Dx-dependent signalling is suppressed at 29°C, a temperature in which the degradation of Notch in GPI+ route and its shift from GPI- route are favoured. Similarly, this might explain why the endosomal localisation of 9B2 *in vivo* is reduced at 29°C.

In S2 cells and *in vivo*, 9B2 in the absence of Dx, accumulates at the cell membrane. This may be due to a lower rate of endocytic uptake and may result in the reduction of the basal signalling (Fig. 3.22.B1). It is possible that *9B2* by accumulating at the cell membrane might cis-interact with ligands in the same cell and in turn reduce the availability

of ligands, to activate Notch in the adjacent cells (Fig. 3.22.B2). This effect might be in part responsible for the strong loss of function of Notch in *9B2, dx*^{152} flies which has a very strong accumulation of Notch at the surface.

If *9B2* trafficking leads to the reduction of signalling, the gain-of-function of *9B2* is unlikely to arise from the ligand-independent activation of Notch. Given that *9B2* is prone to accumulate at the cell membrane, Dx function might be to remove the excess of *9B2* from the cell membrane. Ultimately, *9B2* gain-of-function might arise from a constant ligand-dependent signalling *via* the action of Dx ensuring *9B2* level at the cell surface is not too high to prevent signalling (Fig. 3.22.A2). In line with this idea, if the fold induction is considered, 9B2 shows an increase in the ligand-dependent signalling, but not in Dx-dependent signalling in S2 cells (see Fig. 3.7B and 3.8B).

28 showed similar features to *E2* and *16*, however its basal signalling reduction was like *9B2* and it was partially in GPI+ vesicles in response to Dx similarly to *9B2*. It is possible that *28* might have a slightly different mechanism compared to *E2*, *16* and *9B2*.

The effect of *9B2* and *E2* on ligand-dependent activation in neighbouring cells needs to be tested in mutant clones using a Notch reporter such as NRE-GFP. From the results so far, it would be expected that there would be more activation in wild-type cells adjacent to the *E2* clonal spot than in a wild-type clone. Conversely, for 9B2 it would be expected that there would be less activation in wild-type cells adjacent to a *9B2*, *dx* clonal spot.

If Ax mutants are affecting signal sending *in vivo*, then this could be modelled in S2 cells using the luciferase signalling assay. To do this, the signal donor cells could be transfected with E2+Dx+Dl to see if this increases the Notch signal. Or, the signal donor could be transfected with 9B2+Dl to test if this reduced the Notch signal.

In conclusion, *Ax* mutants were shown to change the localisation and trafficking of Notch in S2 cells and *in vivo* in the wing disc. This impaired localisation of Notch can be responsible for the alterations in the signalling activation of the mutants and ultimately giving rise to the upregulation of Notch and *Ax* gain-of-function phenotypes. Interestingly, the regulation of *Ax* mutants might strongly depend on Dx and this might explain the strong genetic interaction between *Ax* mutants and *dx*. *Ax* mutants share similar phenotypes, thus have the same outcome on Notch pathway, but affect Notch in different ways, possibly underlying different molecular mechanisms. Therefore, the *Ax* mutants analysed in this chapter might represent different functional sub-classes with distinct characteristics into which the mutants can be classified.

4. Functional classification of *Ax* cancer mutants

Chapter 4: Functional classification of *Ax* cancer mutants

4.1 Screening of *Ax* cancer mutants

Mutations in Notch have been found in several types of cancer and associated with the development of the disease (reviewed by Ranganathan *at al.,* 2011; Ntziachristos *et al.,* 2014; Aster *et al.,* 2016). Interestingly, a number of mutations were found in the Ax domain of human Notch receptors in different cancer types, for example in head and neck squamous cell tumours (Song *et al.,* 2013; Zhao *et al.,* 2016; Zheng *et al.,* 2018). In the previous chapter it was shown that *Drosophila Ax* mutants affect Notch localisation and signalling. It is possible that *Ax* mutations found in cancer have a similar effect on Notch pathway. Therefore the aim of this chapter was to test whether *Ax* cancer mutants share similar functional features with *Drosophila Ax* mutants. It is also of particular interest to test if *Ax* cancer mutations can be functionally classified and if their outcome can be predicted. The first classification of *Drosophila Ax* mutants divided them into suppressor, enhancer and lethal classes and was based on their genetic interactions with the *Notch* locus (Portin, 1975; Foster, 1975; Kelly *et al.,* 1987; de Celis *et al.,* 1993). In addition, the functional characterization in chapter 3 showed that the mutants might represent different functional sub-classes.

The *Drosophila Ax* mutations, *E2, 16, 28* and *9B2,* are located in different EGFs and at the opposite ends of the Ax domain (Fig. 4.1A). Therefore it is tempting to speculate that *Ax* mutations might have distinct effects on the Notch pathway not only depending on which amino acid they affect within a EGF repeat but also depending on their position in the Ax region. For this reason, *Ax* cancer mutations in conserved amino acids were selected based on whether they affect the same EGF in which *Drosophila Ax* mutations are located or the same residues or whether they are placed in the same region across the domain (Fig. 4.1). The cancer mutations chosen were listed in the Catalogue Of Somatic

Mutations In Cancer (COSMIC). Interestingly, the majority of the cancer mutations (86%) reported in the Ax domain were affecting either: the negatively charged amino acids at the beginning of the EGFs (Aspartic acid or Glutamic acid); the conserved Proline residue before the second Cysteine of the EGF; the conserved Glycine residues, especially the ones between the fifth and sixth Cysteine residues (Fig. 4.1B, red amino acids). In addition, a consistent number of cancer mutations were localised in the EGF repeats just downstream of the Ax domain (EGF 30-33). Cancer *Ax* mutant P915L (Notch1, colon cancer), P919S (Notch2, head and neck squamous cell carcinoma and melanoma) and E890K (Notch3, lung squamous cell carcinoma) were selected as predicted *Ax* suppressors. This is because P915L and P919S are in the same EGF as *9B2* (EGF 24) and E890K affects a similar negatively charged residue as *9B2* (D948V) and is in EGF 25 like *28* (N986I). G1136V (Notch1, breast cancer) was chosen as predicted enhancer since it is in EGF 29 like *E2* (H1167Y) and *16* (G1174A) and changes the same Glycine as *16*. Also, G1215D (Notch1, breast cancer) or G1219D (Notch2, small cell lung cancer) was selected in EGF 31 and predicted to be enhancer-like since it affects the same Glycine residue as *16*, but in an EGF just outside the Ax domain. D1227G (Notch2, head and neck squamous cell carcinoma) and E1270K (Notch1, breast cancer) were selected as suppressors because, like *9B2*, they affect the negatively charged residues at the beginning of the EGF repeat although they are located downstream of the Ax domain. Finally, a number of cancer mutants were affecting Cysteine residues like the *Drosophila Ax* mutants belonging to the lethal class. Therefore, C954R (Notch1, breast cancer), a cancer mutant affecting the second Cysteine in EGF 25, was chosen together with the lethal *Drosophila Ax M1* (C999Y), which affects the third Cysteine in EGF 25. The selected mutations were introduced into *Drosophila* Notch in order to look for signalling and localisation similarities or differences with *Drosophila Ax* mutants in S2 cell culture.

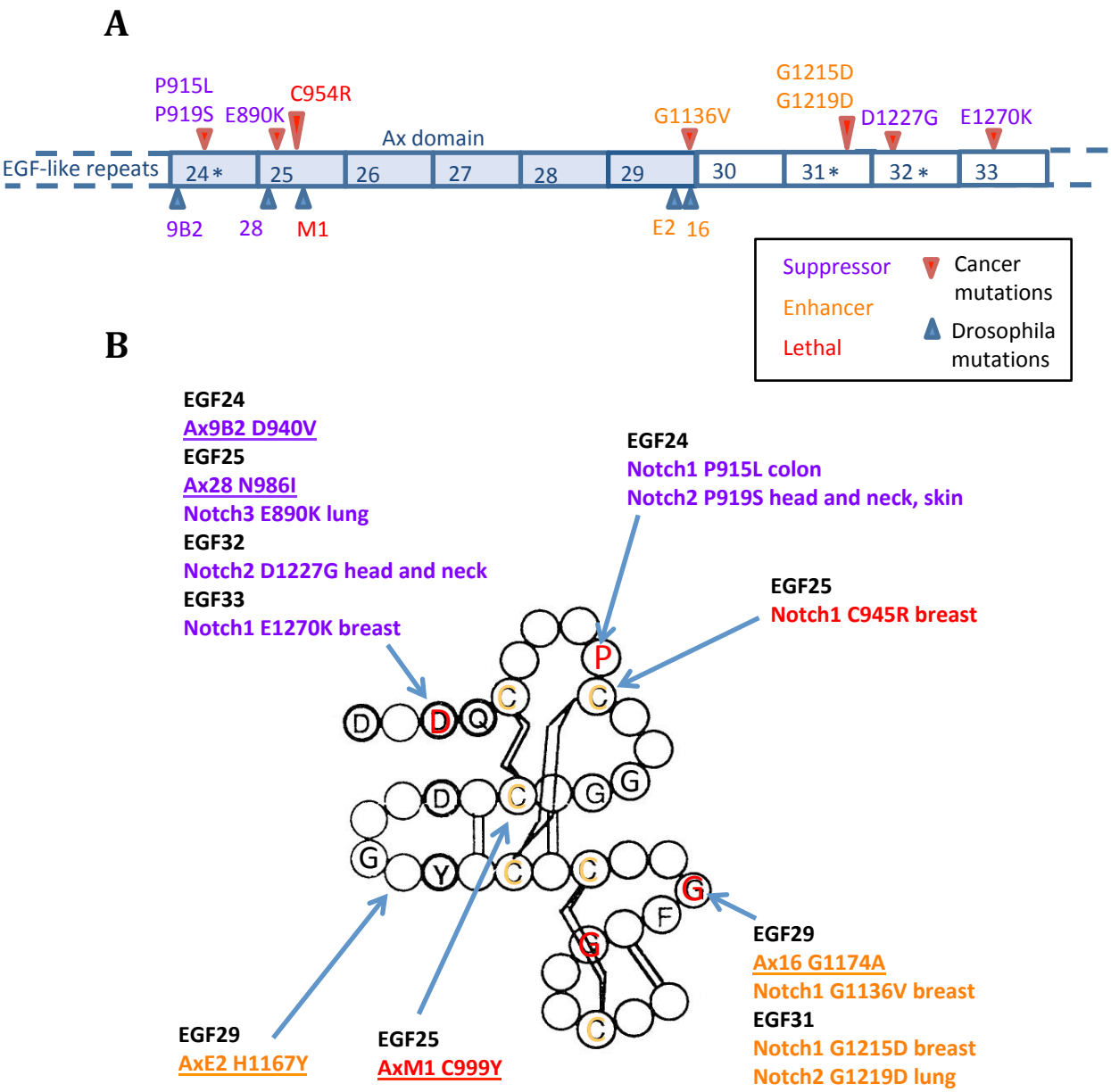


Figure 4.1. Position of Ax mutations in the Ax region. (A) shows the position of Ax cancer mutants (red triangles) and *Drosophila* Ax mutants (blue triangles) in the Ax domain (light blue rectangles) and the neighboring EGFs (white rectangles). The EGF number is indicated in each EGF and the calcium-binding EGFs are marked by the asterisks. (B) shows a schematic representation of a typical Notch EGF module. The amino acid conserved among different EGFs and the six Cysteines (in yellow) are shown in one-letter code. The amino acids that are more frequently affected in Ax cancer mutants are shown in red. The position of the Ax mutations in the EGF is indicated by the arrows and the EGF in which they are located is indicated above the mutant. *Drosophila* Ax mutants are underlined and their amino acid change is shown. For each Ax cancer mutant the human Notch receptor, the amino acid change and tissue of origin are indicated. In (A) and (B) predicted suppressors, enhancers and lethal mutants are indicated in purple, orange and red, respectively.

4.2 *Ax* cancer mutants reduce Notch basal signalling

One of the main characteristics of *Drosophila Ax* mutants is the ability to reduce the basal signalling in S2 cells. To test if cancer *Ax* mutants share this feature, the mutants were introduced into PMT-Notch and expressed in S2 cells. The protein expression of the mutant constructs was analysed by Western Blot and showed that all the constructs were produced (Fig. 4.2B). Their basal signalling was measured by luciferase reporter assay by expressing the mutants in S2 cells. Notably, all cancer mutants, except the Proline mutants, reduced the basal signalling (Fig. 4.2A). E890K showed a similar reduction as *9B2* and *28*, while the other mutants showed a more severe reduction which was similar to *16*. On the contrary, the Proline mutants increased the basal signalling, suggesting they might be fuctionally different from the other mutants, although the increase was not statistically significant. It is interesting that both *Drosophila* and cancer mutants located at the end of the Ax region more strongly reduced the basal signalling compared to the ones at the beginning excluding *M1* and C954R. This may be an indication that the position of the mutants in the Ax region determines some of their features.

4.3 *Ax* cancer mutants show different signalling responses

It was shown that *9B2* significantly reduces the Dx-dependent signalling whereas the other *Drosophila Ax* mutants are activated by Dx. To test the Dx-dependent signalling of the cancer mutants, they were co-expressed with Dx in S2 cells and signalling was measured by luciferase reporter assay. The cancer mutants E890K, P915L, P919S responded to Dx like WT and *28* (Fig. 4.3A). Mutant G1136V and D1227G also showed a consistent activation in response to Dx, which was not significantly different compared to WT, however slightly reduced likely because of the strong reduction in their basal signalling. Notably, Dx-dependent activation was strongly suppressed in *M1*, C954R, G1215D and E1270K.

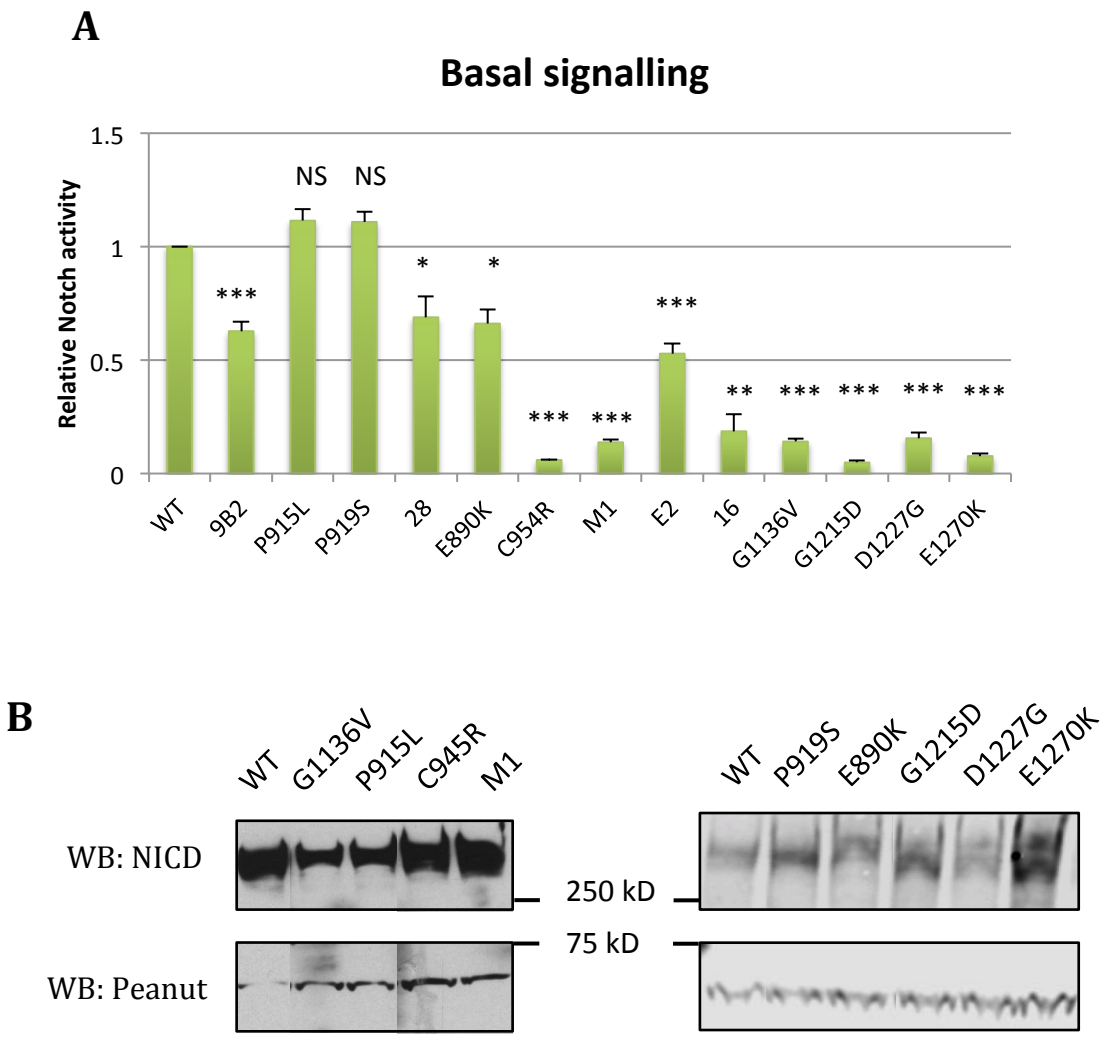


Figure 4.2. Basal signalling and protein expression of Ax cancer mutants. (A) Signaling activation was measured by luciferase reporter assay. Data are shown as means normalized relatively to WT expressed alone in S2 cells. The statistical significance was determined by Student T test and is relative to the basal signaling of WT (NS=non-significant; *p<0.05; **p<0.01; ***p<0.001). Error bars indicate SEM (n=3). (B) Protein expression was analysed by WB, Notch was detected using anti-NICD antibody. Peanut protein expression was used as a control for cell expression and loading and detected with anti-Peanut antibody.

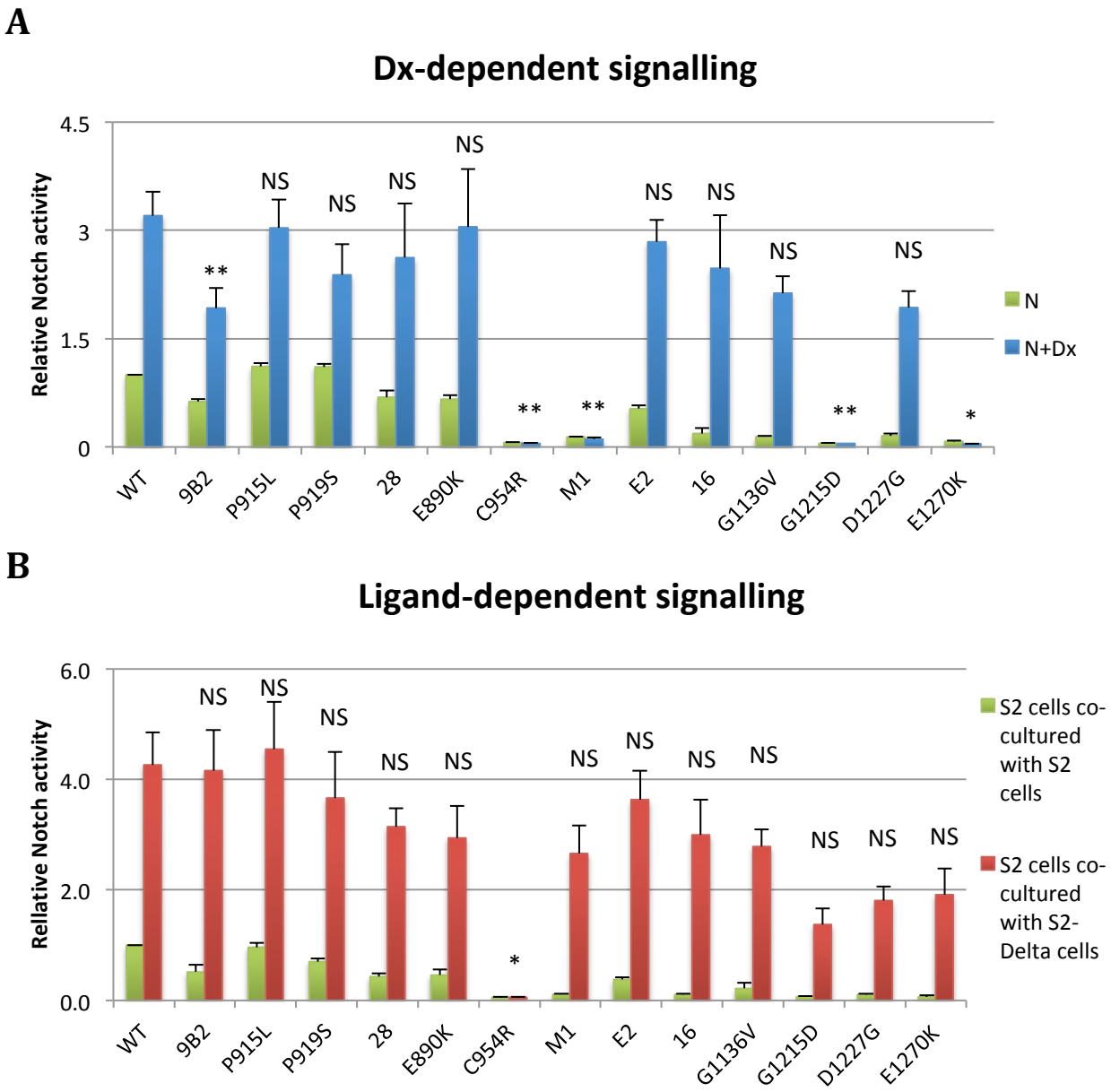


Figure 4.3. Dx-dependent and ligand-dependent signalling of Ax cancer mutants using a luciferase reporter assay. (A) Ax mutants were transfected alone (green columns) or co-transfected with Dx (blue columns) in S2 cells. (B) transfected S2 cells were cultured alone (green columns) or co-cultured with ligand-expressing S2-Dl cells (red columns). Data are shown as means normalized relatively to WT expressed alone in S2 cells. The statistical significance was determined by Student T test and is relative to the basal signaling of WT (NS=non-significant; *p<0.05; **p<0.01). Error bars indicate SEM (n=3).

Drosophila Ax mutants were shown to normally respond to ligands. To test the ligand-dependent signalling of the cancer mutants, the cells transfected with the mutants were co-cultured with S2-Dl expressing cells and signalling activation was measured by luciferase reporter assay. Cancer mutants were also shown to respond to ligands and their signalling was not significantly different from WT, although in some of them a lower signalling was observed (Fig. 4.3B). It is remarkable that *M1*, G1215D and E1270K suppressed the basal and Dx-dependent signalling, but were able to respond to ligands. On the contrary, C954R also suppressed the ligand-dependent signalling.

The signalling response of the cancer mutants shows that they share some features with *Drosophila Ax* mutants. For example, *28* and E890K show similar basal signalling as *9B2*, but a comparable Dx-dependent signalling as *E2* and *16*. The Proline mutants are characterized by increased basal signalling, a feature which was not represented in the *Drosophila Ax* mutants. *E2*, *16*, G1136V and D1227G strongly reduce the basal signalling but still efficiently respond to Dx. Finally, *M1*, G1215D and E1270K suppress basal and Dx-dependent signalling but respond to ligand-dependent signalling. *9B2* and C954R showed unique features. Mutants with similarities might share the same mechanism and represent different functional classes.

4.4 *Ax* cancer mutants affect Notch localisation at the cell membrane

Another defining characteristic of *Drosophila Ax* mutants is the increased or decreased localisation at the cell membrane compared to WT Notch. The cancer mutants showed a reduction in surface Notch both in the percentage of cells with Notch at the cell surface (Fig. 4.4A) and the intensity of surface Notch staining (Fig. 4.4B) measured by FACS. Similarly to what was observed in the basal signalling, the reduction in surface Notch was stronger in the mutants at the end of the Ax region compared to the ones at the beginning, except for C954R. The

two Proline mutants showed a similar reduction to each other. In E890K the reduction was similar to *28*, although the reduction did not reach statistical significance. The reduction was strong in G1136V and D1227G and very similar to *16* and *E2*. Surface Notch was also very strongly decreased in C952R, G1215D and E1270K. It is very interesting that *M1*, which strongly reduces the basal signalling, showed a similar surface level of Notch as WT.

In E890K, G1136V and D1227G surface Notch reflected the same trend as their basal signalling and this is what was observed in *28*, *E2* and *16* mutants. It is possible that all these mutants share a similar mechanism of reduction of the basal signalling. This might also be the case of C954R, G1215D and E1270K. However these mutants generally showed a lower potential for signalling and a very strong reduction in surface Notch. Thus it is not excludable that these mutants have a defect in reaching the cell membrane. Surface Notch was also reduced in the Proline mutants, however this might underline a different mechanism since their basal signalling was increased. On the contrary, M1 showed a normal surface Notch localisation even if its basal signalling was strongly reduced, suggesting M1 might also have a different mechanism. *9B2* was the only mutant to increase surface Notch and decrease basal signalling, suggesting it has a distinct mechanism (see chapter 3).

4.5 *Ax* cancer mutants change Notch trafficking

In the previous chapter, it was shown that *Drosophila Ax* mutants affect Notch trafficking in the GPI- and GPI+ routes and this can be linked with the differences in the signalling response and localisation at the cell membrane. It was shown that P919S, E890K, G1136V and D1227G cancer mutants reduce surface Notch but they have different basal signalling responses. This might result from different mechanisms and lead to different signalling outputs. For this reason, the localisation of these mutants was analysed in the absence or presence of Dx using an uptake antibody assay and GPI as a marker (Fig. 4.5). In the absence of

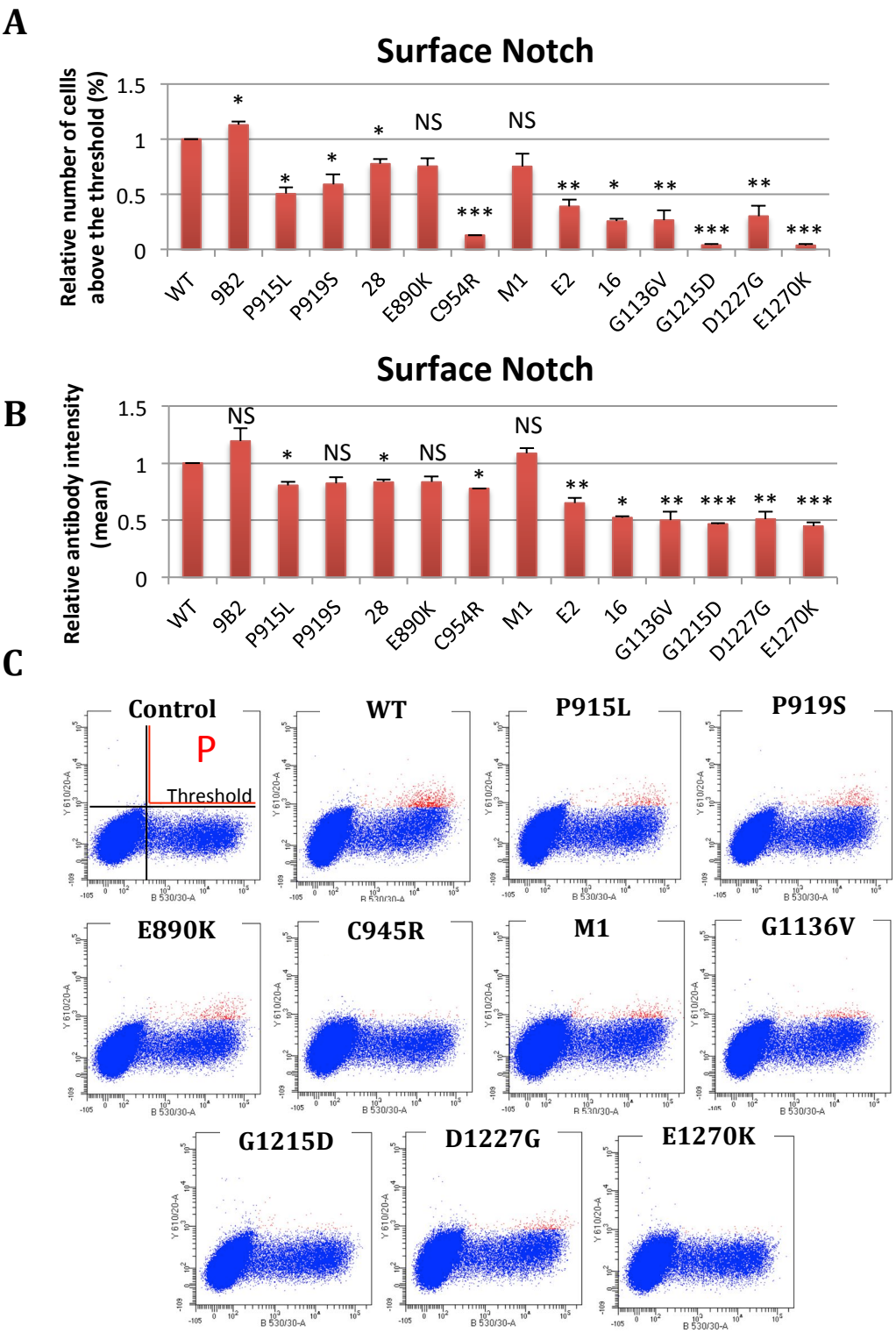


Figure 4.4. Localisation of Ax cancer mutants at the cell membrane by FACS analysis. FACS plots for WT and mutant Notch constructs co-transfected with pMT-GFP into S2 cells (see Method chapter2). (A) shows the percentage of cells in population P compared to the total number of GFP transfected cells above the threshold. (B) shows the mean antibody staining intensity of P in all samples. (C) shows the FACS traces and P populations. The statistical significance was determined by Student T test and is relative to WT sample (*p<0.05; **p<0.01). Error bars indicate SEM. (n=3)

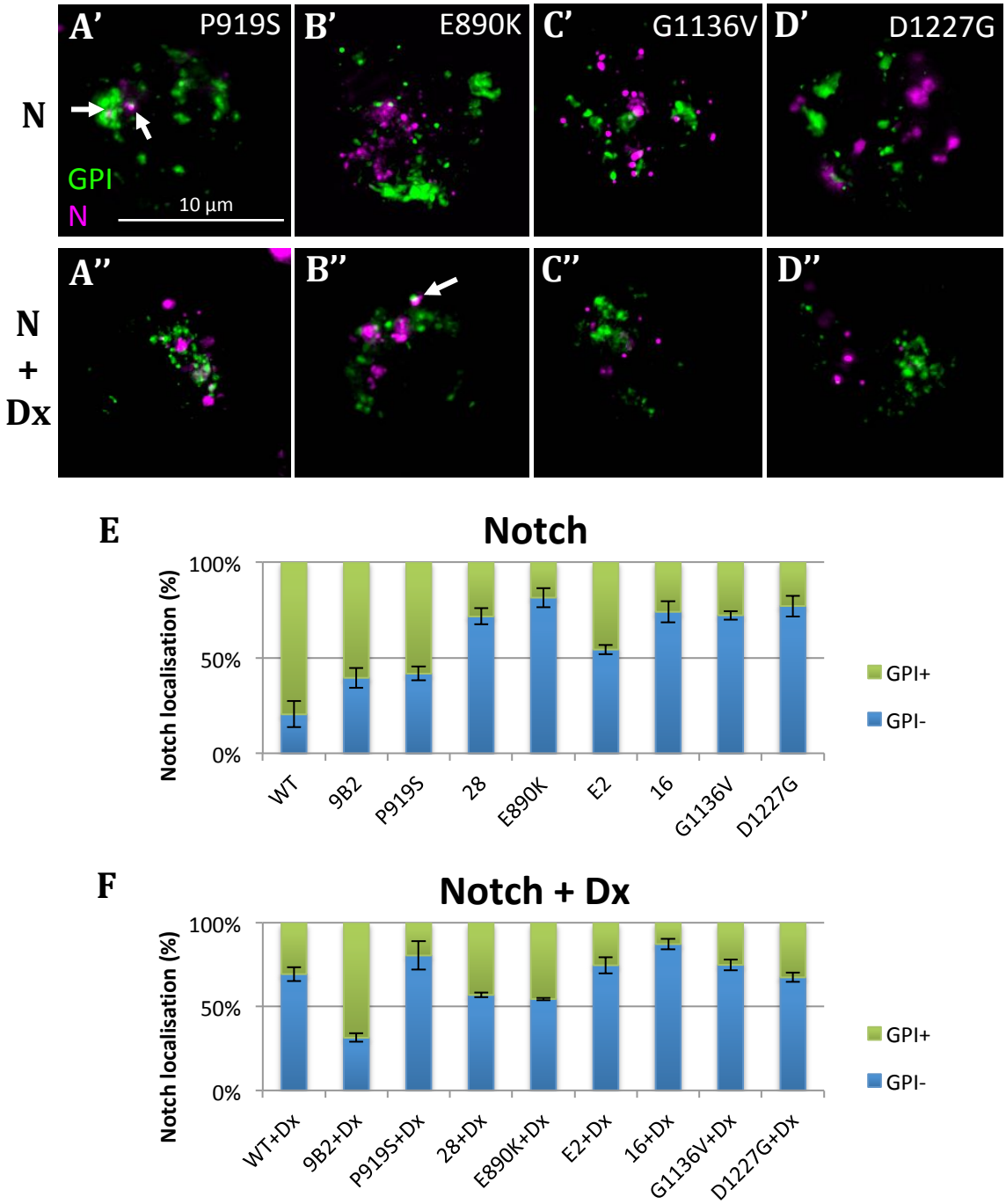


Figure 4.5. Localisation of Ax cancer mutants relative to GPI+ vesicles in S2 cells at 60 minutes of endocytosis. (A-D) show P919S, E890K, G1136V and D1227G, respectively relative to GPI+ vesicles in the absence (A'-D') or in the presence of Dx (A''-D''). Notch was labelled with anti-NECD antibody and its trafficking was tracked by antibody uptake assay. pUAS-GPI-GFP was expressed to mark GPI+ vesicles and pMT-Gal4 was expressed to trigger its expression. The white arrows indicate co-localisation of Notch and GPI-GFP. Plot (E) and (F) show the localization of Notch within GPI+ or GPI- vesicles in the absence or presence of Dx. An average of 50 vesicles were scored for each sample looking at the co-localization of Notch with GPI-GFP. Data are shown as percentage means (n=3). Error bars indicate SEM.

Dx, WT Notch is mainly in GPI+ endosomes. However, the constructs E890K, G1136V and D1227G were mainly in GPI- vesicles as *28*, *E2* and *16* (Fig 4.5B'-D', E). This is in agreement with the reduction in the basal signalling and surface Notch, which were common features of these mutants. On the contrary, P919S was mostly in GPI+ vesicles like WT (Fig. 4.5A', E) and this might reflect the increase in the basal signalling which was a unique feature of Proline mutants. It is interesting that the ratio of GPI- and GPI+ vesicles in P919S was similar to *9B2*, even if the two mutants likely have two different mechanisms of action. In the presence of Dx, the variants P919S, G1136V and D1227G were mainly in GPI- vesicles as WT, *E2* and *16* (Fig. 4.5A'', C'', D'', F). Interestingly, E890K was also mostly in GPI- vesicles, although a consistent portion was in GPI+ vesicles similar to *28*, This suggests that E890K and *28* might have a comparable trafficking route to one another which is t different to the other mutants (Fig. 4.5B'', F).

4.6 Discussion

Mutants localised across the Ax region showed signalling and localisation differences, but also common features. This indicates that the mutants could be functionally classified depending on their characteristics and this classification could implement the traditional one of *Drosophila Ax* mutants. A number of functional classes could be identified based on the characteristics of the mutants (Fig. 4.6). One class could be represented by the Proline mutants, P915L and P919S which showed increased basal signalling but reduced surface Notch and normal trafficking. This could be a new class which was not present in the traditional classification. *28* and E890K could represent a class of mutants that reduce basal signalling and surface Notch and affect trafficking. A feature that distinguishes these mutants is that they were mainly in GPI- vesicles with Dx, but with a large portion (about 43% and 45%) of them on GPI+ vesicles, similarly to *9B2*. This can be one of the common functional features of suppressors *Ax* mutants and might explain the classification of *28* and *9B2* in the same traditional class.

9B2 was the only mutant to increase Notch at the cell membrane and might represent another functional class. Since *9B2* is at the very beginning of the Ax domain, it is possible that mutations located in the region upstream of the Ax domain might be similar to *9B2*; in a similar manner to how D1227G shares properties with *E2* and *16* and is located in the downstream region. *E2*, *16*, G1136V and D1227G might fit in the same functional class, which consists of mutants that strongly reduce the basal signalling and surface Notch, but still retain a strong Dx-dependent signalling potential and increased trafficking into the GPI-route. This class might represent the traditional enhancer class. However, it was previously pointed out that *E2* and *16* show similar, but not identical, features. Therefore *16* might represent another sub-class which has a stronger effect on Notch compared to *E2*. Finally, *M1*, C954R, G1215D and E1270K could be grouped in a class of mutants that suppress the basal and Dx-dependent signalling. This class is heterogeneous and consists of mutants: that respond to ligand-dependent signalling and have a normal surface localisation, like *M1*; respond to ligand-dependent signalling, but have a reduced surface localisation, like G1215D and E1270K; and those that suppress ligand-dependent signalling and reduce surface Notch, like C945R. The traditional lethal class was represented by Cysteine mutants only, but it is possible that mutants affecting other residues might share the same characteristics, such as G1215D and E1270K.

Interestingly, some of the features of the mutants seem to be linked to the position of the mutants in the Ax region. For example, *Ax* mutants located at the end of the Ax region showed a stronger reduction in the basal signalling and surface Notch compared to the ones at the beginning of the domain. Another example is that *9B2*, *28* and E890K affect similarly located residues but *9B2* is in EGF 24, a calcium-binding EGF, and is located in the calcium-binding domain, whereas *28* and E890K are in EGF 25, which is not a calcium-binding EGF. The nature of the EGF they are located in might be the main difference between these

mutants and might result in their functional differences. Remarkably, D1227G affect a similarly located mutated residue as *9B2* in the calcium-binding EGF 32, but D1227G shows *E2*-like instead of *9B2*-like features. This might suggest that the same amino acid change in different EGFs in the Ax region might have different effects on Notch depending on the position of the EGF. Similarly, E1270K in EGF 33 and G1215D in EGF 31 affect the same residues as E890K in EGF 25 and G1136V in EGF 29, respectively, but they do not show *9B2* or *E2*-like features. P915L and P919S are the only mutants which increase the basal signalling. It would be interesting to test if the same change within a different EGF leads to the same outcome or whether their effect is due to their location in EGF 24.

These observations indicate that, not only the amino acid affected or its position within the EGF module, but also the exact EGF in which the *Ax* mutant are located might have a major impact on its functional features. It is possible that the EGFs in the Ax region might have different functions and identify functional sub-domains. For example different EGFs in the Ax region might mediate the interaction of Notch with different proteins. Therefore, EGF 29 and 32 might define an '*E2*-like' sub-domain (Fig. 4.6A) which might mediate the interaction with a particular protein partner and mutations in this sub-domain might affect the interaction and in turn Notch localisation and signalling. A broader screening of *Ax* mutations might help in understanding the function of the domain and the different EGFs within the Ax region.

These results suggest that *Ax* cancer mutants have *Ax*-like features and they could be functionally classified depending on their position in the Ax region. This means that a cancer mutation located in a certain EGF of the Ax region is likely to have a certain outcome. Therefore it could be possible to make functional predictions depending on the position of the mutation. In the screening presented in this chapter, little of emphasis was placed on which human Notch the mutant was from or which kind of tumour, but this can be implemented in a future analysis.

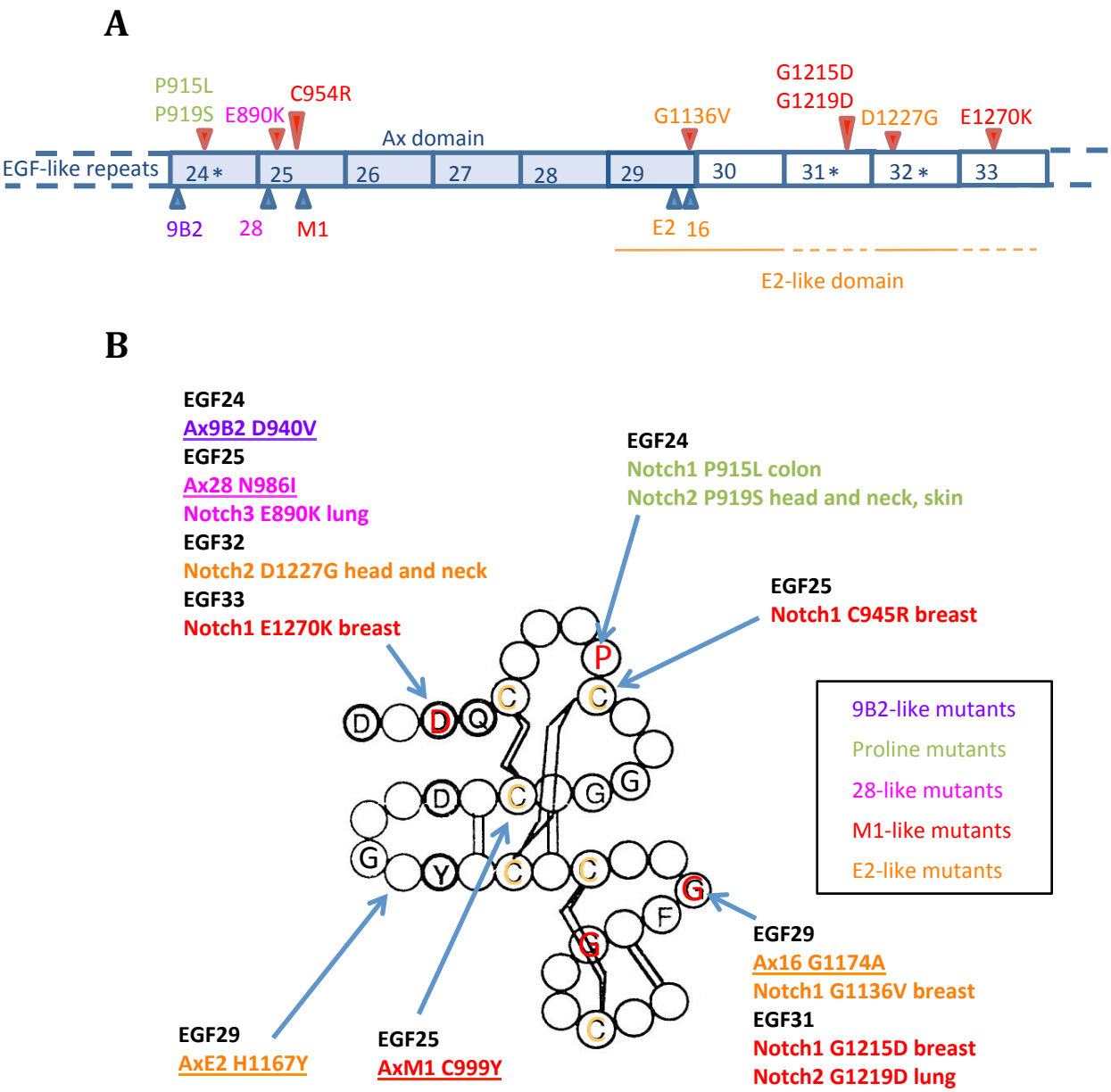


Figure 4.6. Functional classification of Ax mutants. Mutants belonging from different classes are indicate in different colours. 9B2-like mutants are indicated in purple, Proline mutants in green, 28-like mutants in pink, M1-like mutants in red and E2-like mutants in orange. (A) shows the position of Ax mutants in the Ax domain (light blue rectangles) and the neighboring EGFs (white rectangles). The EGF number is indicated on each EGF and the calcium-binding EGFs are marked by the asterisks. The E2-like sub-domain is indicated by the orange line. (B) shows a schematic representation of a typical Notch EGF module. The position of the Ax mutations in the EGF is indicated by the arrows and the EGF in which they are located is indicated above the mutant.

5. Screening of protein candidates for Notch Ax domain interaction

Chapter 5: Screening of protein candidates for Notch Ax domain interaction

In chapter 3 and 4, it was showed that several mutations around the Ax domain of Notch affect the signalling, endocytosis and trafficking of Notch in an allele specific manner.

It is very intriguing that mutations in the extracellular domain of Notch have such a significant impact on the regulation of intracellular trafficking and it would be interesting to understand how does this outside-in mechanism work. One hypothesis is that Ax domain might be involved in the interaction with other protein partners and the disruption or strengthening of these interactions is responsible for the *Ax* mutants effect on the Notch pathway.

One possibility is that *Ax* gain of function arises from a disruption of a cis-inhibition mechanism, which is the cis-interaction of Notch receptor with its ligands. It has been reported that *Ax* mutants are less sensitive to cis-inhibition *in vivo* in the wing disc (de Celis and Bray, 2000) and *in vitro* in S2 cells (Perez *et al.,* 2005). In both studies *Ax* mutants showed a decreased sensitivity to Delta-mediated cis-inhibition but generally no difference to Ser-mediated cis-inhibition. This observation was also supported by more recent evidence that Ser does not affect Notch activity in *Ax* mutant clones in wing discs (Becam *et al.,* 2010). However, in all the aforementioned studies the effect of cis-inhibition was only tested in *AxM1*, a member of the lethal class, or in the heteroallelic combination of *Ax16/Ax28* or *AxM1/Ax16*, but this has never been tested in homozygous viable *Ax* mutants, like *E2* and *9B2*.

It is also possible that Ax mutants upregulate Notch by affecting its interaction with Crumbs (Crb), an evolutionary conserved transmembrane receptor involved in apicobasal polarity in epithelia (Tepass, 1990; Tepass, 2012; Letizia *et al.,* 2013; Rodriguez-Boulan and Macara, 2014). Interestingly, Crb has been shown to prevent Notch endocytosis and ligand-independent activation by direct interaction of

its extracellular domain with Notch at the apical membrane in *Drosophila* wing discs. Further, loss of Crb leads to endocytosis and ligand-independent activation of Notch. Importantly, CrbRNAi flies showed an *Ax*-like phenotype in the fly wings (Nemetschke and Knust, 2016; Das and Knust, 2018), suggesting loss of Crb might mimic the effect of *Ax* mutants like *E2* by favouring Notch endocytosis and ligand-independent signalling. On the contrary, it was shown that loss of Crb can rescue the wing phenotype of *AxM1* (Nemetschke and Knust, 2016) and this might indicate Crb is required for *AxM1* gain of function. Since *E2* and *9B2* mutants affect the localisation at the cell membrane and endocytosis of Notch this could be the result of the impaired interaction with Crb.

Another possibility is that *Ax* mutants affect the interaction of Notch with Kuzbanian (Kuz), a metalloprotease, member of the ADAM family which mediates the S2 cleavage of Notch in ligand-dependent (Pan and Rubin, 1997; Lieber *et al.,* 2002) and ligand-independent activation (Shimizu *et al.,* 2014). The latter occurs in GPI+ endosomes and as part of a basal signalling mechanism identified in S2 cells (Delwig and Rand 2008; Shimizu *et al.,* 2014). One possibility is that Kuz might have an effect on Notch other than simply promoting cleavage itself, perhaps by interacting to affect protein trafficking decisions. Therefore, it is possible that *E2* and *9B2* show a reduced basal signalling and altered trafficking because the mutations perturb the interaction with Kuz.

Ax mutants might also affect the cross-talk between Wingless (Wg) and Notch pathway, ultimately changing Notch ligand-independent trafficking. The cross-talk between Wg pathway in *Drosophila*, or Wnt in mammals, and Notch pathway is well established and shown to be involved in the regulation of gene expressions in different contexts (Hayward *et al.,* 2008; Fre *et al.,* 2009; Collu *et al.,* 2012). While the cross-talk was firstly believed to result from common transcriptional effectors, it is now known that Notch and Wg can also interact upstream and this is independent on transcription regulation. Notably, it has been

proposed that Notch pathway can antagonise Wg pathway in *Drosophila* imaginal discs and this effect might be dependent on Dx or more generally on Notch trafficking (Ramain *et al.,* 2001; Hayward *et al*., 2005; Hayward *et al.,* 2008; Munoz-descalzo *et al.,* 2010). Additionally, recent evidence showed that Wg and its receptor Frizzled2 (Fz2) can be endocytosed and activate Wg pathway in endosomes in *Drosophila* wing discs (Hemalatha *et al.,* 2016). Therefore Wg might interact with Notch not only at the cell membrane but also in the endosomes. These observations suggest that the endocytic trafficking machinery might play a role in the interaction between the two pathways. Interestingly, *Ax* mutants were found to enhance the loss-of-function phenotype of *wingless* and *Ax* phenotype can be partially rescued by gain-of-function of Wg (Couso and Martinez Arias, 1994; Hayward *et al.,* 2008). It has also been proposed that the Ax region might be involved in the interaction between Notch and Wg signalling (Brennan *et al.,* 1999; Hurlbut *et al.,* 2007). Since Ax mutants affect Notch ligand-independent signalling, it is possible this is because E2 and 9B2 alter the effect of Wg on Notch signalling.

An alternative explanation to be considered is that Notch may dimerise and this may have a regulatory outcome on Notch trafficking. A number of papers showed that Notch can form receptor dimers, although the dimerization has only been observed *in vitro* in crystal structures of human Notch1 (Luca *et al.,* 2015) or deduced from *in vitro* binding assays of *Drosophila* Notch (Xu *et al.,* 2005; Pei and Baker, 2008) and its physiological function remains unknown. Interestingly, it was shown that *Drosophila* Notch EGF-like repeat 21-30 region, which includes the Ax domain, is able to bind to the ligand-binding domain of other Notch receptors and compete with Dl for the binding to the Notch ligand-binding domain (Xu *et al.,* 2005; Pei and Baker, 2008). This indicates that the Ax region might be involved in the formation of Notch-Notch dimers and leads to the idea that *Ax* mutations might alter the dimerization. Another observation supporting this idea comes from the

negative complementation of *Ax* mutants. The heteroallelic combination of 'suppressor' *Ax* mutants with 'enhancer' *Ax* mutants has been found to be lethal, but the reason for this lethality was previously unknown (Foster, 1975; Portin 1975; Kelley *et al.,* 1987; Grushko *et al.,* 2004). The data presented in chapter 3 indicate that the combination of *E2* and *9B2* leads to a stronger gain-of-function of Notch, which is ultimately lethal. It is possible that the strong gain of function is the result of an additive effect of *E2* and *9B2* signalling or it can result from the direct interaction of the two receptors, which might cause an impaired trafficking and increased activation of Notch.

The aforementioned hypotheses were examined in this chapter and the effect of the selected regulators on WT Notch, *E2* and *9B2* will be tested in S2 cells with the aim of describing the mechanism behind *Ax* mutants.

5.1 Delta and Serrate cis-inhibit WT-Notch and *Ax* mutants

It was proposed that *Ax* mutants are less sensitive to Dl-mediated, but not Ser-mediated, cis-inhibition and this might contribute to their gain of function (de Celis and Bray, 2000, Perez *et al.,* 2005; Becam *et al.,* 2010). Therefore, the cis-inhibition mediated by Ser or Dl was tested for *E2* and *9B2* to determine if *Ax* mutants have a different sensitivity to cis-inhibition and cis-interactions.

To test Ser and Dl-mediated cis-inhibition on ligand-dependent Notch signalling, WT, *E2* and *9B2* were co-expressed with Ser or Dl in S2 cells and cultured on fixed S2-Dl cells to stimulate Notch ligand-dependent signalling (Fig. 5.1A-B). The signalling activation was analysed by luciferase reporter assay. The ligand-dependent signalling was significantly reduced in both WT Notch and the mutants, suggesting *E2* and *9B2* are cis-inhibited by Ser and Dl as efficiently as WT.

To test if cis-inhibition affects the ligand-independent signalling, Ser or Dl were co-expressed with WT, *E2* and *9B2* only or with Dx (Fig. 5.2A-D). The basal and Dx-dependent signalling were then measured by

luciferase reporter assay. It was observed that Ser reduced both basal and Dx-induced signalling of WT Notch and both of the mutants, although only the effect on basal signalling reached statistical significance (Fig. 5.2A-B). Dl also caused a small reduction in the basal signalling of WT and the two mutants but the effect was only statistically significant for WT (Fig. 5.2C). Dl expression also reduced Dx-induced signalling (Fig. 5.2D). Interestingly, this effect appeared to be stronger for *9B2* compared to WT and it was the only difference between a mutant and WT which reached statistical significance.

These results suggest Ser and Dl expression tend to reduce Notch signalling and *9B2* seems to significantly increase the cis-inhibitory effect of Dl on Dx-dependent signalling.

The difference observed in Dl-mediated cis-inhibition might derive from an increased affinity of *9B2* for the cis-interaction with Dl. For this reason, the binding of WT, E2 and 9B2 with Dl or Ser was tested by co-immunoprecipitation (Fig. 5.3A-D). For this assay, a C-terminus GFP-tag was introduced in WT, E2 and 9B2 and used to pull-down Notch. A tagged version of Ser or Dl, Ser-V5 (Witheman *et al.,* 2013) and Dl-Myc (Klueg *et al.,* 1998), was co-expressed with WT Notch-GFP, E2-GFP and 9B2-GFP. Notch-GFP constructs were pulled-down using anti-GFP antibody conjugated beads and Ser-V5 or Dl-Myc was detected by western blot using anti-V5 and anti-Myc antibodies, respectively. We observed no difference in the amount of Ser (Fig. 5.3A-B) or Dl (Fig. 5.3C-D) that was pulled-down, suggesting that E2 and 9B2 have the same affinity for Dl or Ser binding as WT. However, the experiment was not performed in the presence of Dx and this might make a difference to the outcome.

5.2 Crumbs downregulates Notch ligand-independent signalling of WT and Ax mutants

The interaction of Crb with Notch can prevent Notch endocytosis and ligand-independent activation. Inhibition of Crb produces an *Ax*-like

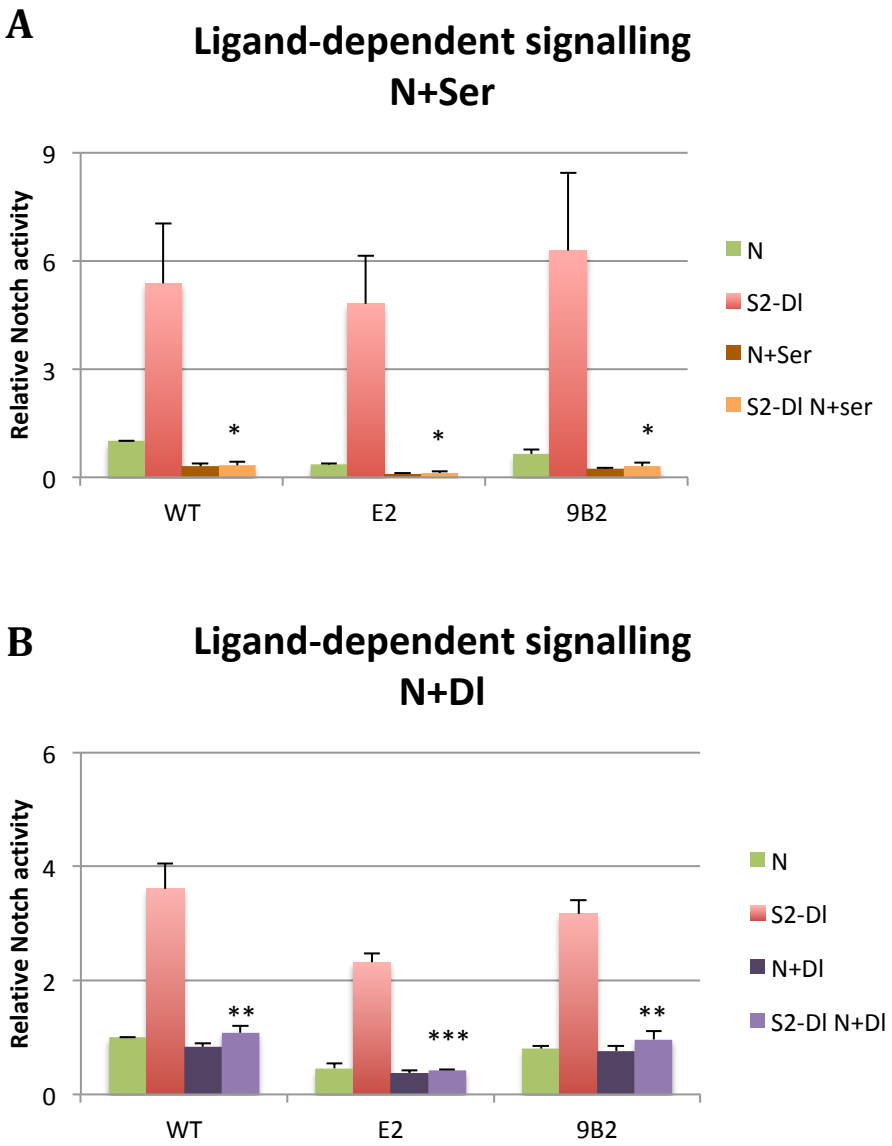


Figure 5.1. Serrate and Delta-mediated cis-inhibition on ligand-dependent signalling. S2 cells were co-transfected with Ser or Dl and WT, E2 or 9B2. Transfected S2 cells were cultured alone or co-culture with ligand-expressing S2-Dl cells. Signaling was measured by luciferase reporter assay. Plot (A) shows the basal signalling (green and dark red columns) and ligand-dependent signalling (red or orange columns) without or with co-expression of Ser. Plot B shows the basal signalling (green and dark purple columns) and ligand-dependent signalling (red or purple columns) without or with co-expression of Dl. Data are shown as means normalized relatively to WT expressed alone in S2 cells. The statistical significance was determined by Student T test and is relative to the ligand-dependent signaling for S2-Dl N+Ser and S2-Dl N+Dl of each sample (*$p<0.05$; **$p<0.01$; ***$p<0.001$). Error bars indicate SEM (n=3).

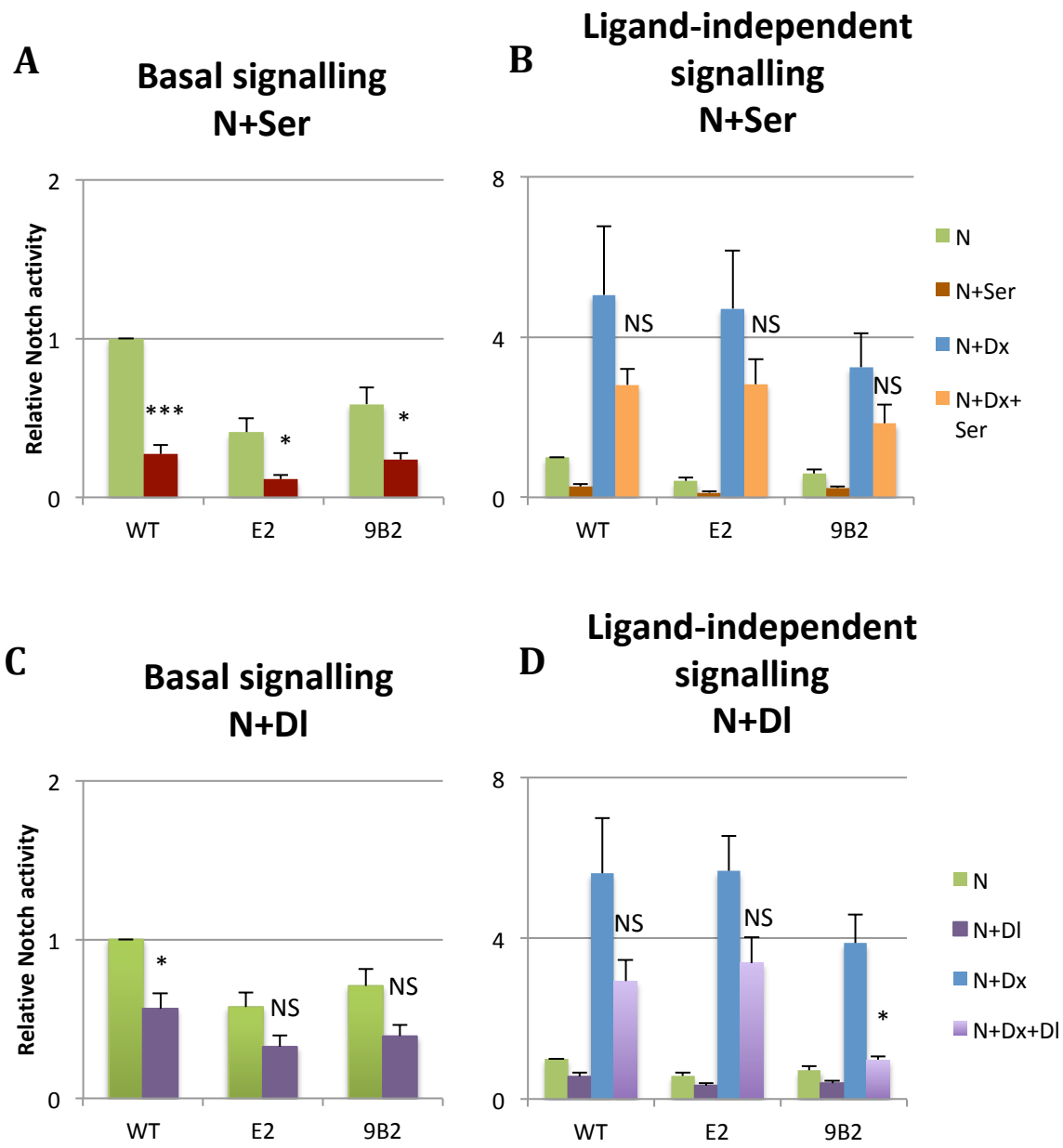


Figure 5.2. Serrate and Delta-mediated cis-inhibition on ligand-independent signalling. WT, E2 or 9B2 were co-transfected with Ser or Dl and with or without Dx. Signaling was measured by luciferase reporter assay. Plots (A) and (C) show the basal signalling without or with Ser (green and dark red columns) or Dl (green and dark purple columns). Plots (B) and (D) shows the basal signaling and Dx-dependent signaling without or with Ser (blue and orange columns) or Dl (blue and purple columns). Data are shown as means normalized relatively to WT expressed alone in S2 cells. The statistical significance was determined by Student T test and is relative to the basal signaling of each sample for N+Ser and N+Dl or Dx-dependent signaling of each sample for N+Dx+Ser and N+Dx+Dl (*p<0.05; ***p<0.001). Error bars indicate SEM (n=3).

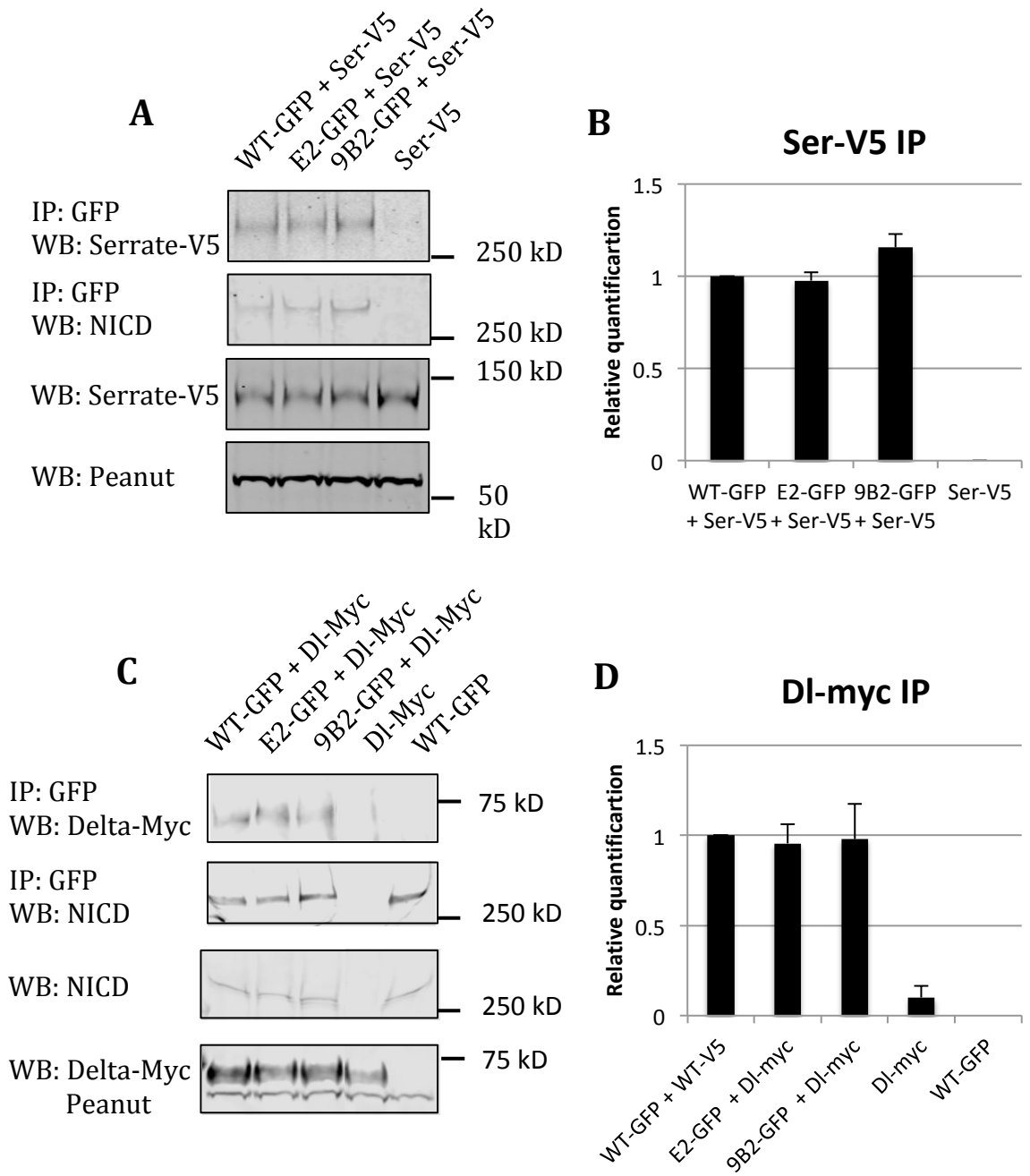
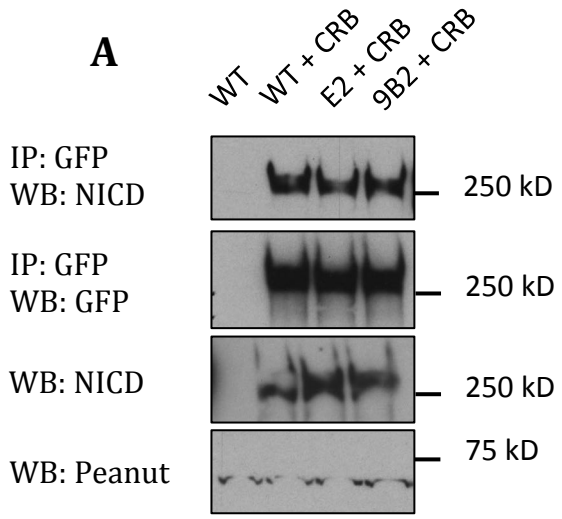


Figure 5.3. Interaction of WT, E2 or 9B2 with Serrate or Delta. WT-GFP, E2-GFP or 9B2-GFP were co-transfected with Ser-V5 (A) or Dl-Myc (C). WT-GFP, E2-GFP or 9B2-GFP were pulled down using anti-GFP conjugated antibody beads and Ser or Dl were detected using anti-V5 (A) or anti-Myc antibodies (C), respectively. Notch was detected using anti-GFP or anti-NICD antibodies. Ser-V5 only, Dl-myc only or WT-GFP only samples were used as controls. Peanut expression was detected using anti-Peanut antibody as cell expression and loading control. Plot (B) shows the WB quantification of Ser-V5 or Dl-myc co-IP values normalized by Ser-V5 and peanut. Plot (D) shows the WB quantification or Dl-myc input and peanut. Error bars indicate SEM (n=2).

wing phenotype and induces the endocytosis and ligand-independent activation of Notch (Nemetschke and Knust, 2016; Das and Knust, 2018), thus mimicking *Ax* mutants. In the wing disc, where Crb is endogenously expressed, *Ax* mutants might affect the interaction of Notch with Crb and cause an effect on endocytosis and ligand-independent signalling. It is possible that E2 might have less affinity for Crb binding and this is why E2 is less localised at the cell membrane and more endocytosed, whereas 9B2 might have a stronger affinity for Crb and accumulate at the cell membrane.

Crb and Notch were co-immunoprecipitated in S2 cells to test if E2 and 9B2 have different affinity for Crb binding (Fig. 5.4A-B). S2 cells do not endogenously express Crb (Gelbart and Emmert, 2013; Flybase FB2018_04), therefore a UAS-Crb construct in which the intracellular domain of Crb has been substituted with GFP (CrbE)(Pellikka *et al.,* 2002) was expressed. CrbE was pulled down using anti-GFP antibody-linked beads and WT, E2 and 9B2 were detected with anti-Nintra antibody. It was observed that E2 and 9B2 do not affect the binding of Notch with CrbE, although the tendency was toward a decrease especially with E2.

Since Crb has an effect on Notch endocytosis and in turn, on ligand-independent signalling, it was interesting to test if Crb also affects the signalling of Ax mutants. For this purpose, Notch ligand-independent signalling by luciferase assay upon co-expression of CrbE and Notch in S2 cells (Fig. 5.5A-B). First, different amounts of CrbE were co-expressed with WT and its signalling activation was measured by luciferase assay (Fig. 5.5A). 2ng, 5ng or 10ng of CrbE, and 2ng, 10ng or 20ng of pMT-Gal4, were expressed, respectively, to trigger the expression of CrbE. It was observed that the overexpression of CrbE reduces the basal signalling of WT in a dosage dependent manner and the reduction becomes significant with 10ng of CrbE, but does not affect the Dx-dependent signalling. This suggests CrbE might have a similar effect on WT basal signalling as Ax mutants. However, when 10ng of



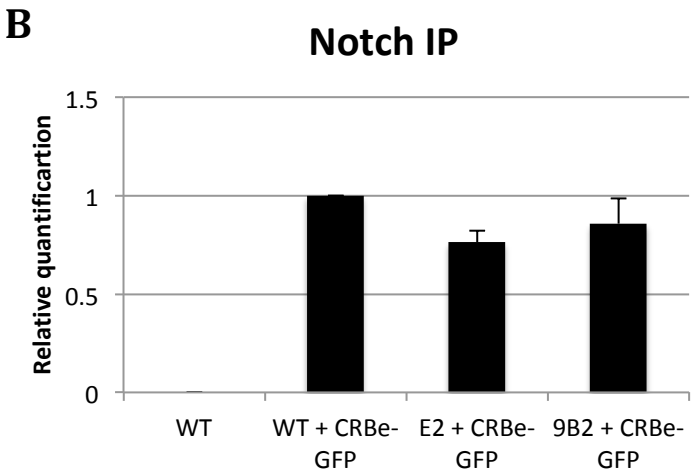


Figure 5.4. Interaction of WT, E2 or 9B2 with CrbE. WT, E2 or 9B2 were co-transfected with CrbE-GFP which was pulled down using anti-GFP conjugated antibody beads. Notch was detected using anti-NICD antibody. CrbE-GFP only sample was used as negative control. Peanut expression was detected using anti-Peanut antibody as cell expression and loading control. Plot B shows the WB quantification of Notch co-IP values normalized by Notch input and peanut. Error bars indicate SEM (n=2).

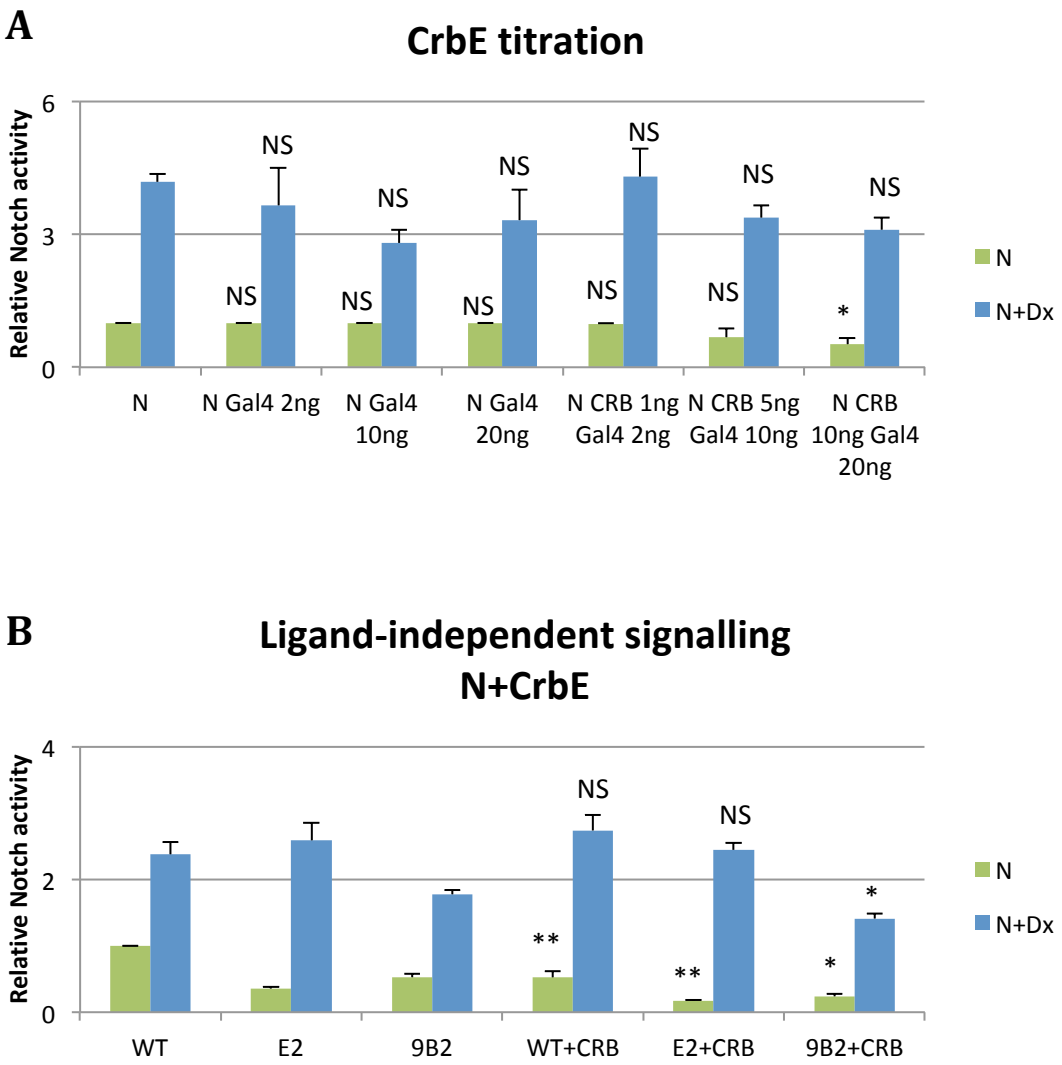


Figure 5.5. CrbE effect on ligand-independent signalling. In plot (A), WT was co-transfected with 2ng, 10ng, 20ng of Gal4 singularly or together with 1ng, 5ng or 10ng of CrbE with or without Dx. In plot B, WT, E2 or 9B2 were co-transfected with 20ng of Gal4 only or together with 10ng of CrbE with or without Dx. Signalling was measured by luciferase reporter assay. Data are shown as means normalized relatively to WT expressed alone in S2 cells (A) or WT+20ng Gal4 (B). The statistical significance was determined by Student T test. In plot (A), the significance of N+Gal4 samples is relative to the WT N only signaling; the significance of N+Gal4+CRBe samples is relative to N+Gal4. In plot (B), the significance of N+Gal4+CrbEe samples is relative to the N samples (*$p<0.05$; **$p<0.01$). Error bars indicate SEM (n=3).

CrbE were co-expressed with E2 and 9B2 (Fig. 5.5B), CrbE also reduced the basal signalling of E2 and 9B2, indicating that the mutants have the same response compared to WT. Intriguingly, Dx-dependent signalling was significantly reduced in 9B2, but not in E2 and WT when co-expressed with CrbE, suggesting 9B2 might be more sensitive to Crb and this is in agreement with the initial hypothesis.

5.3 Kuzbanian regulates WT Notch and *Ax* mutants

Kuz mediates the ligand-independent activation of Notch in GPI+ endosomes in S2 cells (Delwig and Rand 2008; Shimizu *et al.,* 2014) and might also have an effect on Notch trafficking. One hypothesis is that *E2* and *9B2* might be less responsive to Kuz-mediated activation and this causes the reduction in their basal signalling. Also, since 9B2 is redirected from GPI- to GPI+ endosomes in response to Dx, the reduction of Dx-dependent signalling in 9B2 might be due to a lower Kuz-dependent activation.

Since Kuz is endogenously expressed in S2 cells, Kuz was inhibited by RNAi and E2 and 9B2 signalling activation was measured by luciferase assay (Fig. 5.6A-B). It was expected that if 9B2 or E2 are less responsive to Kuz, then reducing the expression of Kuz by RNAi would have no further effect on their signalling. The experiment showed that inhibition of Kuz significantly downregulates the basal signalling of WT, as expected (Fig. 5.6A). However, the basal signalling of E2 and 9B2 was also reduced, and the size of the reduction was not statistically significant. Further, Dx-dependent signalling was not significantly affected (Fig. 5.6B). It is interesting that 9B2 Dx-dependent signalling is not reduced by Kuz RNAi. This might indicate that 9B2 is not shifted to GPI+ endosomes in the absence of Kuz or 9B2 Dx-dependent signalling is produced when 9B2 is still in GPI- endosomes.

To test the effect of Kuz on ligand-independent signalling, Kuz or a dominant negative form of Kuz, KuzDN, which lacks the proteolytic activity (Pan and Rubin, 1997; Lieber *et al.,* 2002; Shimizu *et al.,* 2014)

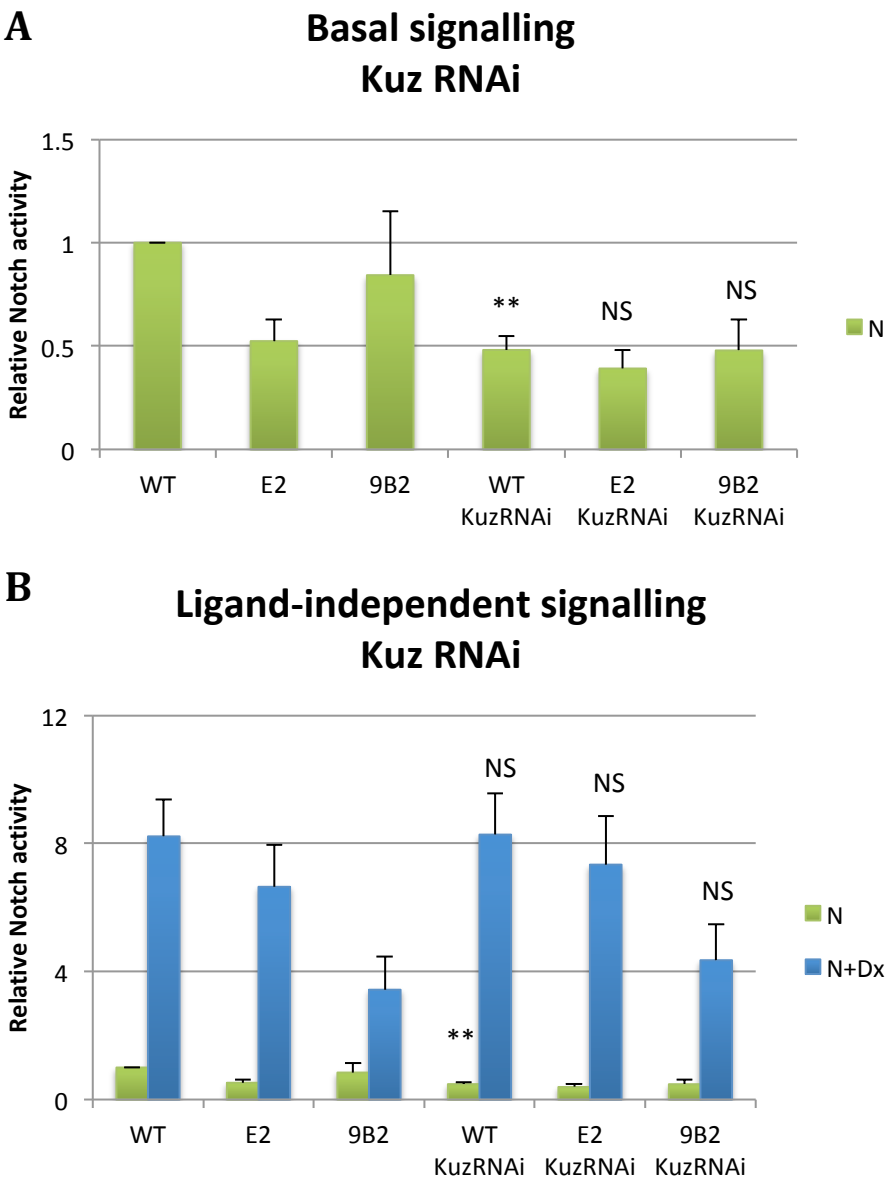


Figure 5.6. Effect of Kuz inhibition by RNAi on ligand-independent signalling. S2 cells transfected with WT, E2 and 9B2 were incubated with GFP RNAi as a control and Kuz RNAi. Signaling was measured by luciferase reporter assay. Plot (A) shows the basal signalling only, while plot (B) shows both basal and Dx-dependent signaling (green and blue columns, respectively). Data are shown as means normalized relatively to WT expressed alone in S2 cells incubated with GFP RNAi. The statistical significance was determined by Student T test and is relative to the basal or Dx-dependent signalling of each sample incubated with GFP RNAi (**$p<0.01$). Error bars indicate SEM (n=3).

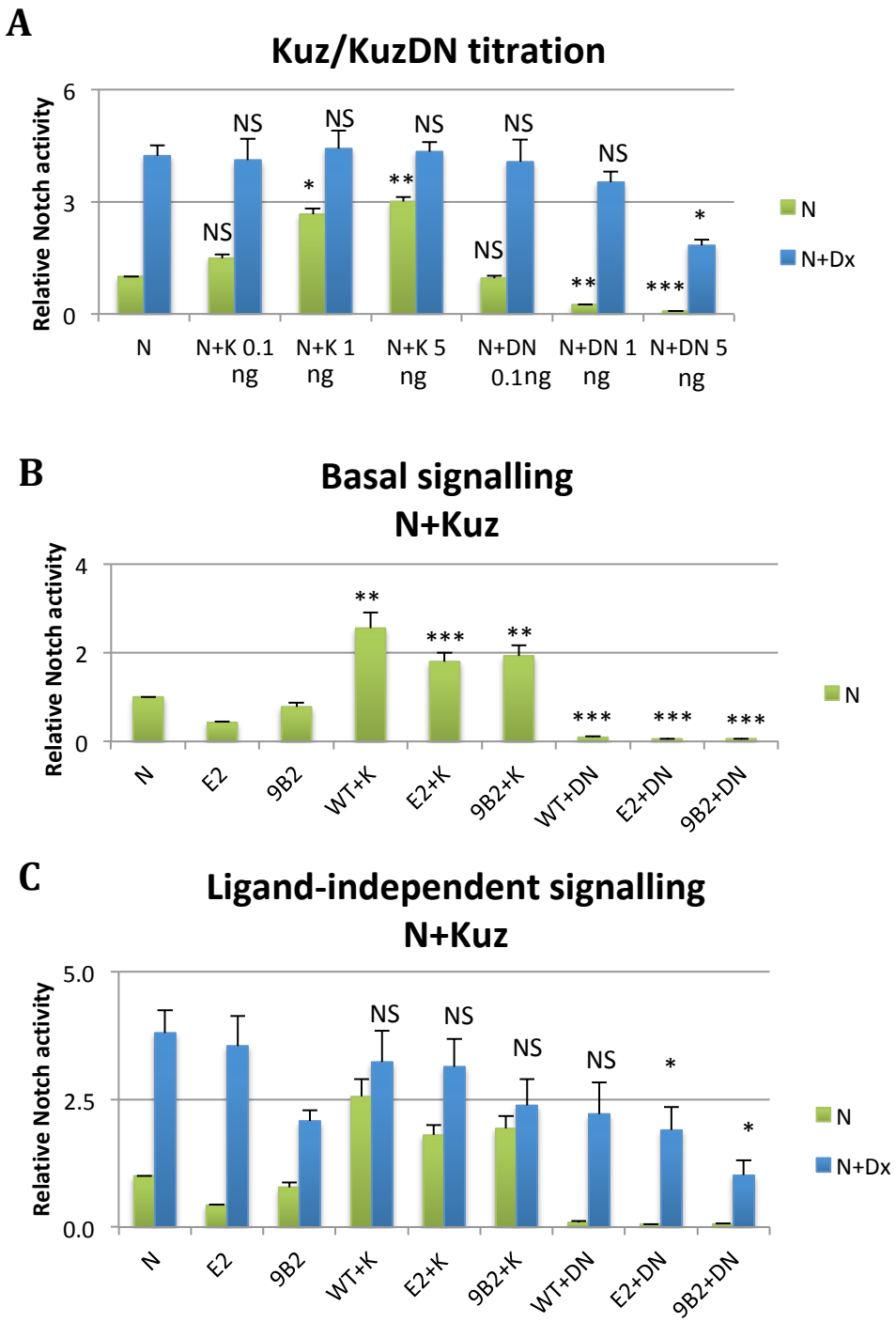


Figure 5.7. Kuz and KuzDN effect on ligand-independent signalling. In plot (A), WT was co-transfected with 0.1 ng, 1 ng or 5 ng of Kuz, K, or KuzDN, DN, with or without Dx. In plot (B) and (C), WT, E2 or 9B2 were co-transfected with 5ng of Kuz, or KuzDN, without or with Dx. Signaling was measured by luciferase reporter assay. Data are shown as means normalized relatively to WT expressed alone in S2 cells. The statistical significance was determined by Student T test. In plot (A), the significance is relative to WT basal signaling or WT Dx-dependent signaling. In plot (B) and (C), the significance is relative to the basal signaling of each sample for N+Kuz and N+KuzDN or Dx-dependent signaling of each sample for N+Dx+Kuz and N+Dx+KuzDN (*p<0.05; **p<0.01; ***p<0.001). Error bars indicate SEM (n=3).

were co-expressed with Notch (Fig. 5.7A-C). First, different amount of Kuz or KuzDN, 0.1ng, 1ng or 5ng, were co-expressed with WT (Fig. 5.7A). Kuz overexpression caused an increase in the basal signalling, while KuzDN reduced the basal and Dx-dependent signalling of WT and both effects were dosage-dependent. The expression of 5 ng Kuz or KuzDN were then tested on E2 and 9B2 (Fig. 5.7B-C). It was expected that Kuz overexpression would affect only WT if E2 and 9B2 were signalling independently from Kuz. However, it was observed that Kuz overexpression also causes an increase in the basal signalling and KuzDN a decrease in the basal signalling of the mutants (Fig. 5.7B). Notably, Dx-dependent signalling was also reduced in WT and significantly reduced in E2 and 9B2 by KuzDN (Fig. 5.7C).

These results indicate that E2 and 9B2 respond to Kuz or KuzDN in a similar manner as WT.

5.4 Wingless and Frizzled2 affect WT and *Ax* mutants

Notch and Wg have been found to cross-talk in different contexts (Hayward *et al.,* 2005; Munoz-descalzo *et al.,* 2010; Collu *et al.,* 2012). Also, Ax domain might be involved in the interaction between the two pathways and perhaps in the direct interaction of Notch and Wg (Brennan *et al.,* 1999; Hurlbut *et al.,* 2007).

To test if *E2* and *9B2* affect the cross-talk between the Wg and Notch pathway, Wingless (Wg), or its receptor, Frizzled2 (Fz2), were overexpressed in S2 cells and Notch signalling activation was measured by luciferase assay (Fig. 5.8A-B). In this experiment, a secreted version of Wg, pTub-Wg (Ching *et al.,* 2008), or Frizzled2 V5-tagged, pMT-Frizzled2-V5 (Sawala *et al.,* 2015), were expressed in S2 cells. First, WT Notch was co-expressed with different amounts of Wg or Fz2 (1ng, 5ng or 10 ng) to test the effect of Wg and Fz2 on Notch ligand-independent pathway in S2 cells (Fig. 5.8A). A dosage-dependent decrease in the basal signalling of WT was observed and it reached statistical significance with 10 ng of Wg or Fz2. Dx-dependent signalling was

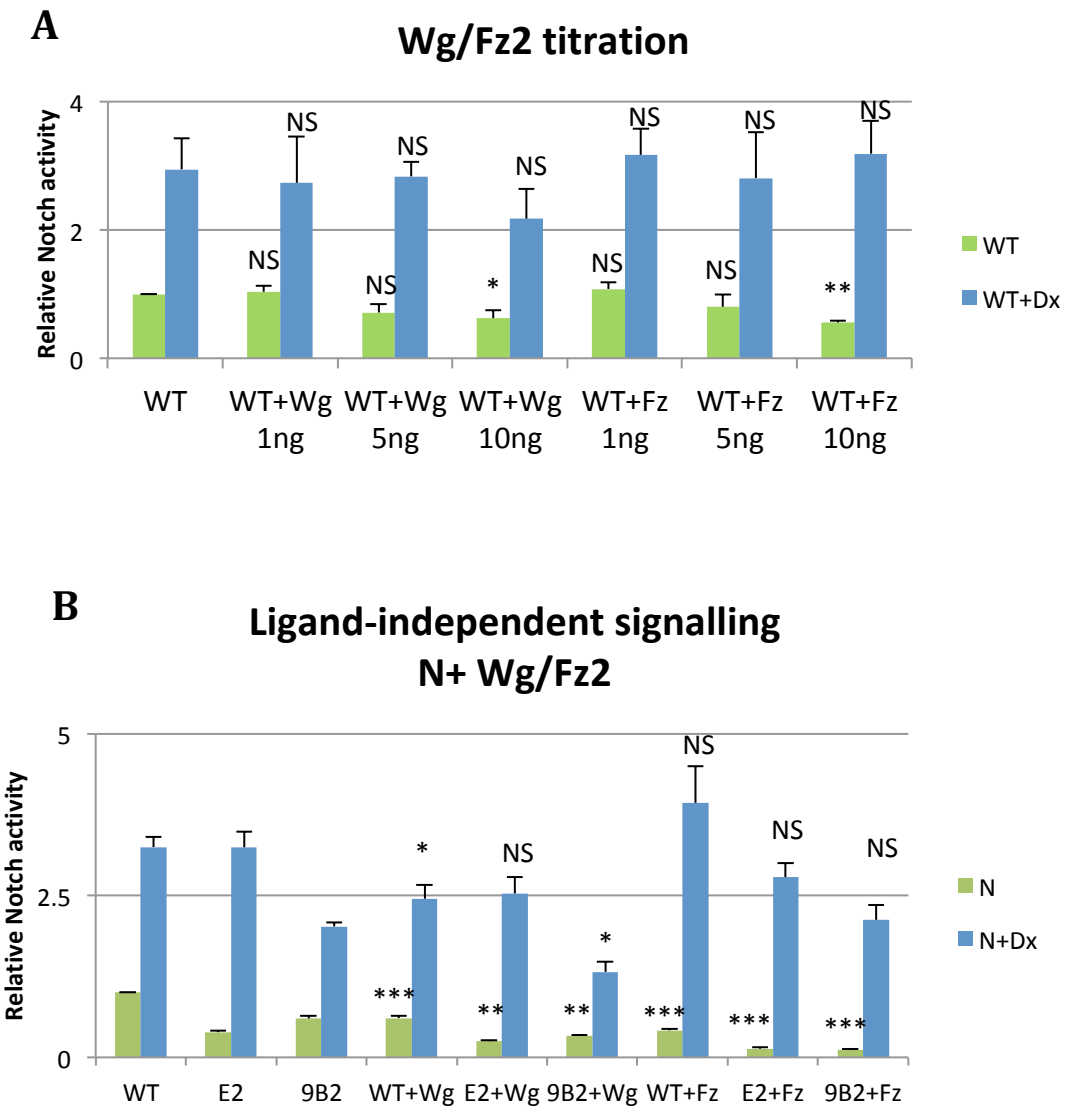


Figure 5.8. Wg and Fz2 effect on ligand-independent signalling. In plot (A), WT was co-transfected with 1 ng, 5 ng or 10 ng of Wg or Fz2 with or without Dx. In plot (B), WT, E2 or 9B2 were co-transfected with 10 ng of Wg or Fz2 without or with Dx. Signaling was measured by luciferase reporter assay. Data are shown as means normalized relatively to WT expressed alone in S2 cells. The statistical significance was determined by Student T test. In plot (A) the significance is relative to WT basal signaling or WT Dx-dependent signaling. In plot (B) the significance is relative to the basal signaling of each sample for N+Wg and N+Fz2 or Dx-dependent signaling of each sample for N+Dx+Wg and N+Dx+Fz2 (*p<0.05; **p<0.01; ***p<0.001). Error bars indicate SEM (n=3).

reduced by 10ng Wg although the reduction was not significant. The co-expression of Wg or Fz2 with E2 and 9B2, also significantly reduced the basal signalling of the mutants like in WT (Fig. 5.8B). The Dx-dependent signalling was reduced by Wg in WT and the mutants and the reduction was statistically significant for WT and 9B2. These data show that Wg and Fz2 can antagonise Notch ligand-independent signalling and this is not prevented by E2 and 9B2.

5.5 *Ax* mutants dimerise with WT Notch

It has been proposed that the Ax domain might be involved in the dimerisation of Notch (Xu *et al.,* 2005; Pei and Baker, 2008). According to this idea, it is possible that mutations in the Ax domain might alter the dimerization of Notch. To test the dimerization between *Ax* mutants and WT Notch, GFP-tagged versions of WT, E2 and 9B2 were co-immunoprecipitated, using anti-GFP antibody-linked beads, with WT Notch V5-tagged, which was detected using an anti-V5 antibody (Fig. 5.9A-B). WT-V5 was pulled down by WT, E2 and 9B2, suggesting that the mutants do dimerise with WT. This also indicates that WT Notch dimerises in S2 cells. Interestingly, WT and the mutants pulled down a similar amount of WT-V5, however 9B2 showed a tendency toward an increase for WT-V5 binding.

5.6 Discussion

The effect of *Ax* mutants on Notch pathway might result from the alteration of the interaction with other protein partners, which normally interact with Notch through the Ax domain. In this chapter, a number of potential regulators of Notch were identified and their interaction with WT Notch and *Ax* mutants were described in S2 cells.

5.6.1 *Ax* mutants localisation affects cis-inhibition

It was proposed that *Ax* mutants gain of function results from their lower sensitivity to cis-inhibition (de Celis and Bray, 2000, Perez *et al.,* 2005). In this chapter, it was shown that *E2* and *9B2* do not affect Ser-

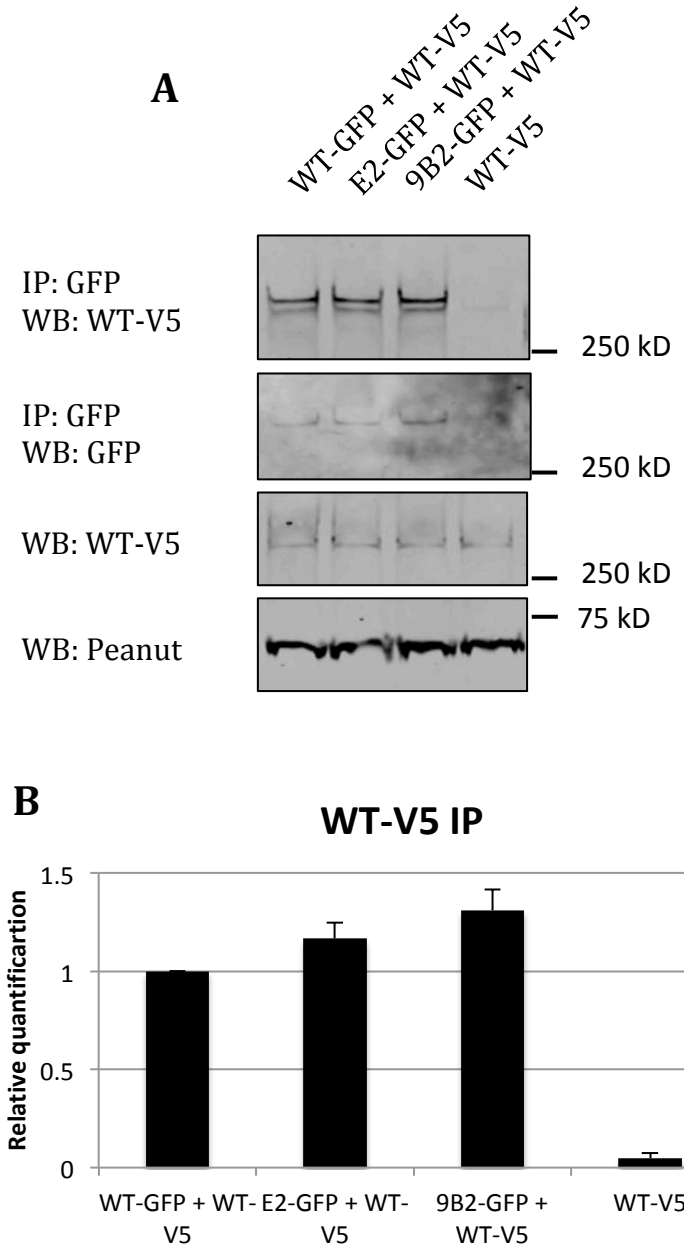


Figure 5.9. Dimerisation of WT, E2 or 9B2 with WT. WT-GFP, E2-GFP or 9B2-GFP were co-transfected with WT-V5 and pulled down using anti-GFP conjugated antibody beads. WT-V5 was detected using anti-V5 and Notch was detected using anti-GFP. WT-V5 only sample was used as control. Peanut expression was detected using anti-Peanut antibody as cell expression and loading control. Plot B shows the WB quantification of WT-V5 co-IP values normalized to WT-V5 input and peanut. Error bars indicate SEM (n=2).

mediated cis-inhibition nor the binding with Ser and this is in agreement with what previously observed for *AxM1* (Perez *et al.,* 2005; Becam *et al.,* 2010). However, Dl-mediated cis-inhibition showed a different effect on the ligand-independent signalling of *Ax* mutants. This effect seems generally milder in the basal signalling of both mutants and stronger in 9B2 Dx-dependent signalling compared to WT. It is possible this is due to the different localisation of *Ax* mutants at the cell membrane rather than a different binding affinity since the mutants did not affect the binding with Dl. In chapter 3, it was shown that 9B2 tends to accumulate at the cell membrane when Dx is not expressed. Similarly, Dl might retain 9B2 at the cell membrane counteracting Dx-driven endocytosis and leading to a decrease in the Dx-dependent signalling. Another possibility is that 9B2-Dl complex might increase the trafficking of 9B2 in the GPI+ route and this might explain why 9B2 Dx-dependent signalling was suppressed by Dl-mediated cis-inhibition. On the contrary, Dl might not be able to retain E2 at the cell membrane because E2 is more endocytosed compared to WT and its endocytosis is independent on Dx. Similar ideas were proposed in the study by Palmer et al., 2014, which showed that cis-inhibition of Notch ligand-independent signalling can occur in the *Drosophila* ovary. This study proposed that the ligands might sequester Notch at the membrane or increase Notch sensitivity to degradation, or increase the stability of Notch heterodimer through its endocytic trafficking. Therefore it is possible that ligands might divert or block Notch trafficking when cis-interacting with Notch and the mutants might affect this process.

These ideas could be tested using Notch surface labelling and trafficking assays in S2 cells in the presence of Dl. Also, it would be interesting to test if Dx might change the rate of interaction of the mutants with Dl and this could be tested by co-immunoprecipitation of Notch and Dl in the presence of Dx. In conclusion, E2 and 9B2 differently respond to Dl-mediated cis-inhibition although this might be the result and not the cause of E2 and 9B2 effect on Notch localisation.

5.6.2 Crb might contribute to the effect of *Ax* mutants on Notch

It was previously shown that Crb can prevent Notch endocytosis and ligand-independent activation by direct interaction with Notch. Further, loss of Crb produces an *Ax*-like phenotype in the fly wings (Nemetschke and Knust, 2016; Das and Knust, 2018). Therefore, it is possible *Ax* mutants upregulate Notch by affecting the interaction of Notch with Crb *in vivo*. Here, it was shown that Crb overexpression reduces the basal signalling of WT Notch in S2 cells, thus mimicking the effect of *Ax* mutants. This might be because Crb interacts with Notch and blocks its endocytosis in S2 cells as observed in the wing discs (Nemetschke and Knust, 2016). Crb also reduces the basal signalling of E2 and 9B2, however, Dx expression can counteract the effect of Crb on the signalling of WT and E2. This effect might be even stronger in E2 *in vivo* if E2 relies more than WT on Dx function. Indeed, E2 showed a tendency towards a decrease binding of Crb, and Dx might enhance this effect. Interestingly, Crb significantly reduces 9B2 Dx-dependent signalling. It is possible that Crb can retain more 9B2 at the cell membrane even in the presence of Dx, although this might be due to the intrinsic tendency of 9B2 to accumulate at the cell membrane and not to its affinity for Crb binding. In line with this idea, Crb might promote 9B2 accumulation at the cell membrane and this might ultimately favour 9B2 ligand-dependent signalling. This could be tested using Notch surface labelling and trafficking assays in S2 cells in the presence of Crb and *in vivo* in the wing discs in the presence or absence of Crb. According to these observations, it cannot be excluded that Crb might contribute to or favour the effect of *9B2* and *E2 in vivo* in those tissue in which Crb is endogenously expressed, like the wing disc.

5.6.3 Kuz might be involved in Notch trafficking

Ax mutants effect on Notch might be because of their interaction with Kuz, which mediates the ligand-independent activation of Notch in S2 cells (Delwig and Rand 2008; Shimizu *et al.,* 2014) and might also be

involved in Notch trafficking. The data presented in this chapter show that Kuz expression is able to upregulate Notch basal signalling in a ligand-independent manner in S2 cells and does not affect Dx-dependent signalling, as previously shown (Shimizu *et al.,* 2014). Interestingly, KuzDN, but not Kuz RNAi, is able to reduce the Dx-dependent signalling of WT and the mutants, suggesting Kuz might be able to redirect Notch from GPI- to GPI+ endosomes. Generally, Kuz seems to regulate WT, E2 and 9B2 in a similar way, suggesting it is unlikely to be responsible for the effect of E2 and 9B2 on Notch pathway. However, it is interesting that KuzDN, but not Kuz RNAi, reduces 9B2 Dx-dependent signalling which is supposed to arise from GPI+ endosomes. This might indicate that Kuz promotes 9B2 transfer from GPI- to GPI+ endosomes. Alternatively, this might mean 9B2 is activated when it is still in GPI- endosomes. Also, KuzDN and Kuz reduce and increase the basal signalling of E2, respectively, and this might be because Kuz shifts E2 from GPI- to GPI+ endosomes. Based on these observations, Kuz might not only mediate the cleavage of Notch, but also be involved in Notch trafficking. However, signalling assays are not sufficient to prove these hypotheses, especially because Kuz is involved in the cleavage and activation of Notch, making it difficult to fully interpret signalling readouts. Therefore, trafficking assays in S2 cells in the presence or absence of Kuz might be used to test if Kuz is involved in WT Notch trafficking and mediates 9B2's shift from GPI- to GPI+ endosomes.

5.6.4 Wg and Fz2 antagonise Notch signalling

It was proposed that Ax domain might be involved in the interaction of Notch and Wg (Brennan *et al.,* 1999; Hurlbut *et al.,* 2007), thus it is possible *Ax* mutants affect the interaction of the two pathways. In this chapter, it was shown that Wg and Fz2 expression reduces the basal signalling of Notch and Wg also decreases Dx-dependent signalling. This is in agreement with what was previously proposed, that Wg can antagonise Notch signalling (Hayward *et al.,* 2005; Munoz-descalzo *et*

al., 2010; Collu *et al.,* 2012). This is the first time that it has been shown also to be the case in S2 cells. It is possible this effect is because of a direct interaction between Wg or Fz2 and Notch, which might happen at the cell membrane or in endosomes. Indeed, the effect observed on Notch upon Wg expression cannot be due to downstream events, since S2 cells do not express Fz2 (Gelbart and Emmert, 2013; Flybase FB2018_04), and this might favour a model of direct interaction between Wg and the Notch receptor. Indeed, Wg was found among the Notch binding proteins in *Drosophila* and it was proposed that Wg could be a non-canonical ligand for Notch (Wesley *et al.,* 1999; D'Souza *et al.,* 2010). Wg and Fz2 had the same effect on E2, 9B2 and WT, indicating that the mutants do not disrupt the interaction of Notch with Wg and Fz2 in S2 cells.

5.6.5 *Ax* mutants can dimerise with WT Notch

Notch has been found to dimerise *in vitro* and a number of papers have proposed that the Ax domain might mediate this dimerisation (Luca *et al.,* 2015, Xu *et al.,* 2005; Pei and Baker, 2008). This suggests that the dimerization of Notch might be involved in *Ax* mutants' regulation. In this chapter, it was shown that WT, E2 and 9B2 form dimers with WT Notch in S2 cells and these can be detected by co-immunoprecipitation. It seems that *Ax* mutants have a similar affinity for the binding with WT Notch receptors. However, this means *Ax* mutants do dimerise with other Notch molecules and it is possible this interaction might lead to a different trafficking of WT Notch. For instance, E2 might drag WT Notch in GPI- endosomes and 9B2 might retain more WT Notch on the cell membrane. This hypothesis could be tested through trafficking assays in S2 cells and could explain why *Ax* mutations are dominant over WT Notch *in vivo* (de Celis *et al.,* 1993; de Celis and Garcia-Bellido, 1994). Another possibility is that *Ax* mutants might affect the dimerisation of Notch receptor of the same *Ax* allele, such as E2-E2 or 9B2-9B2 dimers. For instance, it is possible 9B2-9B2 dimers might have an increased affinity since 9B2 showed a tendency toward an increased binding with

WT Notch. Also, the dimerization of *Ax* alleles belonging from different classes, such as E2-9B2 dimers, might have consequences on the localization and activation of Notch. To explore these possibilities, co-immunoprecipitation might be used to detect E2-E2 or 9B2-9B2 and E2-9B2 dimers, followed by trafficking assays to detect differences in the localization of the dimers.

In conclusion, the effect of different regulators on Notch was described and interesting differences were identified which could be of help for the description of *Ax* mutants and WT Notch regulation. Interestingly, some regulators have a specific effect on distinct routes in Notch regulation and this is shown for the first time. Crb and Fz2 affected only the basal and not Dx-mediated signalling, whereas KuzDn and Wg affected both basal and Dx-mediated signalling, suggesting these regulators might perturb different mechanisms. It is also interesting that the Dx-mediated signalling of 9B2 seemed generally more sensitive to the inhibitory effect of different regulators, especially Dl and Crb. It is tempting to speculate that these regulators might either enhance the accumulation of Notch at the cell membrane or the shift into GPI+ endosomes and 9B2 might make Notch more sensitive to these effects. This study represents a promising start for the identification of new regulators of Notch pathway and, more specifically, Ax domain. In chapter 4 it was shown that other mutants around the Ax region have a similar effect on Notch, suggesting some of the regulators might interact with other EGFs in this region. Using the assays developed in this study together with deletion Notch mutants and mass spectrometry, it might be possible to test where different regulators bind and identify functional subdomains in the Ax region. A similar approach was used for the identification of the ligand-binding domain of *Drosophila* Notch (Rebay *et al.,* 1991; Rebay *et al.,* 1993) and human Notch1 (Cordle *et al.,* 2008; Whiteman *et al.,* 2013)

6. Discussion

Chapter 6: Discussion

Drosophila melanogaster has historically been a very powerful tool for the discovery of genes and their functions. Not only was Notch first discovered in the fly over a century ago, but mutations in the Abruptex region were isolated over 80 years ago using the power of mutational genetic screens in this genetically tractable organism. This thesis shows that the fly is still an extremely important model organism for further understanding the molecular mechanism through which these *Ax* mutations affect the function of the Notch molecule. Also, that it is most valuable in further characterising the mode of action of similar mutations in human Notch molecules linked to cancer.

Mutations in the Ax domain of the Notch receptor in *Drosophila* were described as gain-of-function mutations, but their molecular mechanism had never been fully understood. Our analysis described that *Drosophila Ax* mutants alter Notch flux in different endocytic routes, ultimately affecting Notch signalling and leading to Notch gain-of-function. *Ax* mutants showed allele-specific characteristics, suggesting different *Ax* mutants might perturb different mechanisms, although all mutants lead to the same gain-of-function outcome. Our work also showed that *Drosophila Ax* mutants and *Ax* mutants found in human cancers can be functionally classified depending on their position in the Ax domain and this might be relevant in Notch-associated tumours. Finally, it was proposed that the Ax domain might mediate the interaction of Notch with other proteins and that *Ax* mutants might alter these interactions. A number of protein candidates were found to affect Notch ligand-independent pathway and might represent potential binding partners of the Ax domain. In conclusion, our work provides new insights into the molecular mechanism of *Ax* mutants, the function of the Ax domain and the relevance of *Ax* mutants in cancer.

6.1 The regulatory mechanisms of *Ax* mutants

6.1.2 Mechanisms of *Ax* mutant endosomal regulation

The results presented in this thesis indicate that *Ax* mutants, including *E2* and *9B2*, promote the trafficking of Notch in specific endosomal routes and destinations. Our data suggest that the gain-of-function of *E2* and *9B2* arises from two distinct mechanisms which both depend on trafficking and Dx function.

One possibility is that *E2* might upregulate Notch by increasing its endocytosis and endosomal activation, since it was shown that *E2* increases Notch endocytic uptake. However, the mechanism by which E2 could be activated and in which endosomal compartments are still open questions. It was shown that a hypomorphic mutation in the HOPS gene Carnation, only mildly reduces the *E2* phenotype.

E2 might be activated by increasing the retention of Notch on the limiting membrane of an endosomal compartment and its activation might occur prior to lysosomal-fusion in a Carnation independent manner. In this view Dx function might still be to promote E2 retention on the limiting membrane of this endosomal compartment. Another way in which E2 could be activated in the endosomes is through Kuzbanian in the Su(dx) route, however, blocking this signalling route using KuzDN and KuzRNAi reduced but did not strongly suppress E2's ability to signal. These results indicate that the endosomal activation of E2 might not depend exclusively on one of these mechanisms. Indeed, both ligand-independent signalling routes might contribute or perhaps E2 is activated in the endosomes through yet another different mechanism.

In tissues, there was always more endocytic Notch in the *E2, dx*^{152} mutant clone as compared to the wild-type twin spot although the relative increase diminished with rising temperature. This agreed with S2 cell culture work which showed that E2 was readily endocytosed in the absence of Dx. Internalising Notch into interluminal vesicles of an

endosomal compartment, which is promoted by Su(dx), is likely to be sufficient to switch off all ligand-independent routes if Dx is not present to counteract it. If lysosomal degradation is slow Notch may be still lingering but it is already deactivated and this is what we may be seeing in the clones of *E2, dx*^{152} at 29°C where there is a very strong loss-of-function phenotype (Fig. 6.1C-D). If one copy of Su(dx) is depleted in *E2, dx* flies, E2 degradation in the Su(dx) route will be reduced and E2 might be retained on the endosomes and become activated to a certain extent, thus rescuing the *Ax, dx*^{152} phenotype.

Endocytic trafficking and Dx might play a different role in the *9B2* mutant phenotype. As discussed in chapter3, Dx seems to be required for 9B2 endocytosis and degradation. This might be important to maintain the right amount of 9B2 at the cell membrane, which might favour its ligand-dependent activation, and avoid excessive accumulation of 9B2 at the cell membrane. A negative function of Dx was already reported in other contexts, for example Dx was also found to lead to Notch degradation when in complex with Kurtz, a non-visual β-arrestin in *Drosophila* (Mukherjee *et al.,* 2005; Hori *et al.,* 2011) or in other cases in mammalian cells (Sestan *et al.,* 1999; Izon *et al.,* 2002). It is also possible that the Dx-mediated degradation might represent a physiological mechanism triggered by excess of Notch at the cell membrane.

An alternative hypothesis, which could be tested, is that 9B2 might be recycled back to the cell membrane once endocytosed. In this context, 9B2 entry into the recycling route might be antagonised by Dx, leading to a decrease in 9B2 accumulation at the cell membrane (Fig. 6.1E). On the contrary, without Dx, 9B2 might favour Notch entry in the recycling pathway and strongly accumulate at the cell membrane (Fig. 6.1F). According to this hypothesis, the trafficking shift of 9B2 might mark a recycling route which could be antagonised by Dx. A similar idea was proposed in the paper Yamada *et al.,* 2011, in which it was suggested that the accumulation of Notch at the cell membrane in *dx* mutant

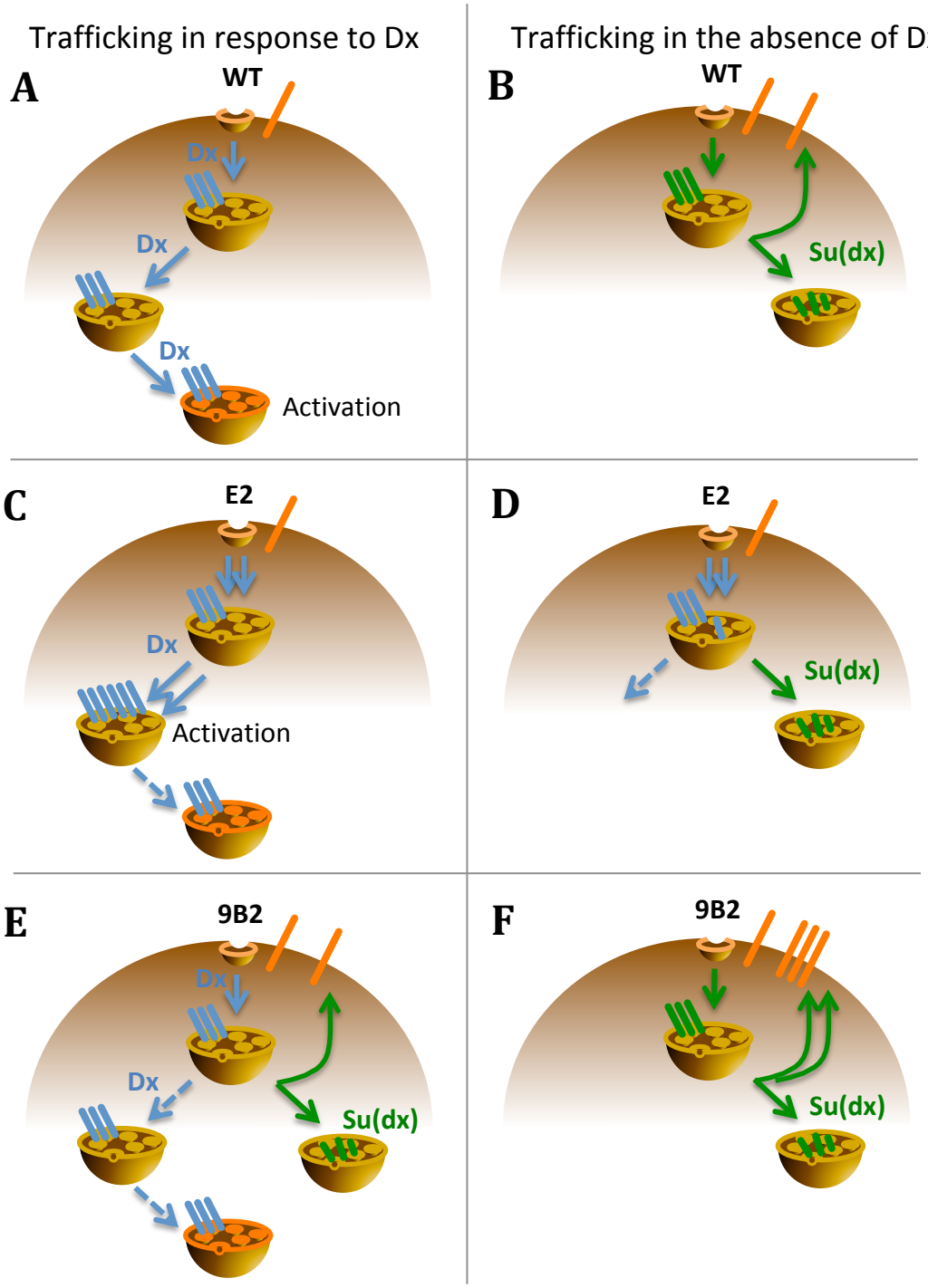


Figure 6.1. Models for the endocytic regulation of Ax mutants. Endocytic trafficking of WT Notch (A, B) and Ax mutants (C-F) in response to Dx (A, C, E) or absence of Dx (B, D, F). Blue and green arrows indicate the GPI- and GPI+ route, respectively. Notch is indicated in orange, blue or green when it is at the cell membrane, in the GPI- route or GPI+ route, respectively. Endo-lysosomes are indicated in orange. In response to Dx: WT Notch is trafficked in the Dx route and activated in endo-lysosomes (A); E2 increases the trafficking in the Dx route and is activated prior to lysosomal-fusion (C); 9B2 is endocytosed by Dx and part of it is trafficked in the Dx route, part of it is shifted to the Su(dx) route or recycled to the cell membrane (E). In the absence of Dx: WT Notch is endocytosed in the Su(dx) route and degraded or recycled to the cell membrane (B); E2 is endocytosed in GPI- vesicles and accumulates endosomally or is degraded by Su(dx) (D); 9B2 is endocytosed in the Su(dx) route and degraded or recycled back to the cell membrane (F).

clones might be the result of Notch recycling in the absence of Dx and not due to reduced endocytosis (Fig. 6.1B). In line with this model, E2 might instead not enter the recycling route and this might favour its trafficking in endosomal compartments in the presence of Dx and its degradation in the Su(dx) route in the absence of Dx. What is very interesting about 9B2 is that it is internalised in GPI- vesicles but then shifted to GPI+ and this might lead to degradation according to the first hypothesis or to the entry into the recycling route in the second hypothesis. In both cases, the shift might be mediated by 9B2 interaction with other regulators that might chaperone Notch from one route to the other as it was proposed in chapter5. Interestingly, preliminary data from our lab showed that 9B2 is not tyrosine-phosphorylated in the presence of Dx, whereas WT is (Z. Huang personal communication), and this might represent a signal for the shift of Notch from one route to the other.

These observations indicate that E2 and 9B2 regulation might represent different mechanisms by which Notch can be upregulated through its endocytic trafficking and this is shown for the first time in *Drosophila* Notch mutants. The altered trafficking might directly contribute to E2 gain-of-function by increasing its endosomal activation and indirectly contribute to 9B2 gain-of-function by increasing its potential for ligand-dependent activation.

6.1.3 The role of ligands in *Ax* mutant regulation

The contribution of ligands to the gain-of-function of *Ax* mutants has been subject to debate for a long time. It was proposed that *Ax* mutants might be more sensitive to ligand-dependent activation or be less sensitive to cis-inhibition and this might contribute to their gain-of-function (de Celis and Garcia-Bellido, 1994; de Celis and Bray, 2000; Perez *et al.,* 2005). The data presented in this thesis, supports a model in which *Ax* mutants might have an effect on cis-inhibition and ligand-dependent activation as a consequence of the different localisation and

trafficking of *Ax* mutants and not by intrinsic properties of the *Ax* receptors. Indeed, our data showed that *Ax* mutants do not affect the affinity for Dl and Ser binding and neither seem to modify the potential for ligand-dependent signalling per se.

A growing body of evidence indicates that the cis-interaction of Notch and ligands in the same cell can determine not only the amount of receptors available for trans-activation, but also the amount of ligands available for trans-activation of Notch in neighbouring cells (Sprinzak *et al.,* 2010; Del Alamo *et al.,* 2011). Since *Ax* mutants affect the localisation of Notch at the cell membrane, they might alter the balance between available ligands and Notch receptors and have an effect on neighbouring cells in a multicellular context, like in a tissue. A similar model was already suggested when *Ax* mutant cells were found to modify the fate of neighbouring cells and their own fate in *Drosophila* tissues (Heitzler and Simpson, 1993; Brennan *et al.,* 1999), presumably depending on the amount of ligands and receptors at the cell membrane of the mutant cells. In E2 mutant cells, a lower number of ligands might be engaged by E2 in cis-interactions since E2 is less localised at the cell membrane, thus increasing the availability of ligands at the cell membrane which can serve to trigger the ligand-dependent activation in neighbouring cells (Fig. 6.2B). An excess of ligands for trans-activation might allow E2 to sustain a normal or increased ligand-dependent signalling even if it is less localised at the cell membrane. Indeed, E2 showed a normal ligand-dependent activation in S2 cells. Interestingly, if the fold ligand-dependent activation of E2 is considered, then E2 ligand-dependent activation might be stronger than in WT (see Fig. 3.7B). Moreover, the fold increase in 16 ligand-dependent signalling would be even more noteworthy. In this view, the effect of ligands might contribute to produce E2 gain-of-function.

On the other hand, since more Notch receptor is at the cell membrane of *9B2* mutant cells, it is possible that 9B2 can give rise to more potent ligand-dependent signalling or less ligands are needed for 9B2 to be activated (Fig. 6.2C), since it was found that 9B2 normally responds to ligand trans-activation in S2 cells and has a higher fold change activation. This effect might be responsible for *9B2* gain of function *in vivo*. On the contrary, the removal of Dx from 9B2 cells leads to an excessive accumulation of 9B2 receptors at the cell membrane, which might sequester all the ligands available for trans-activation of Notch in neighbouring cells and abolish Notch ligand-dependent signalling (Fig. 6.2D).

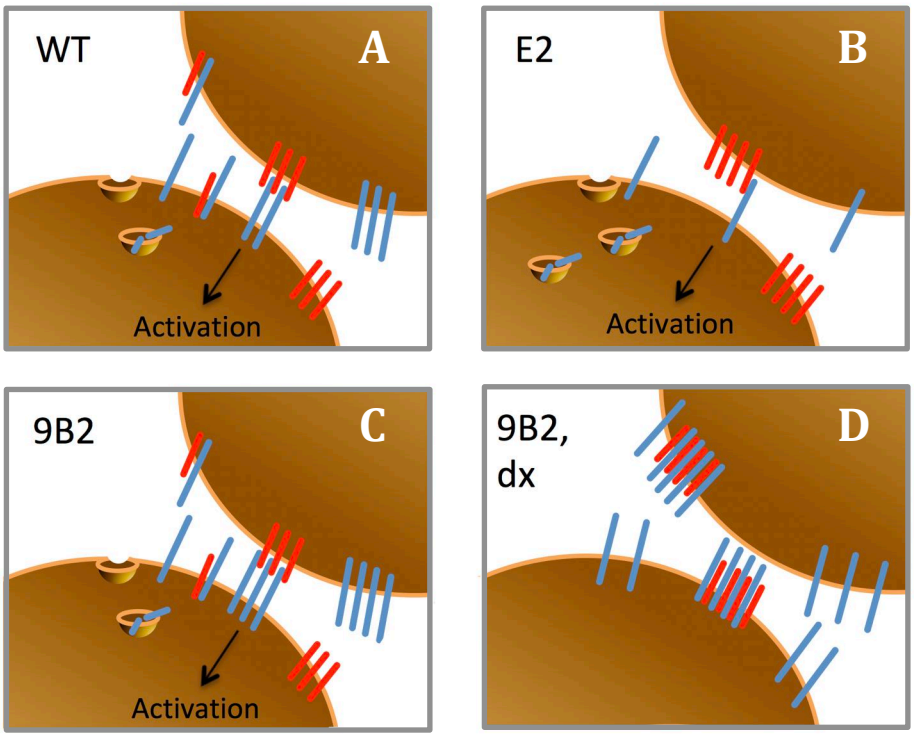


Figure 6.2. Model of ligand-receptor balance in Ax mutants. The ligands (red bars) and Notch receptors (blue bars) at the cell membrane of WT (A), E2 (B), 9B2 (C) or 9B2,dx (D) cells. Endocytosis is indicated by the vesicles. E2 increases the number of ligands available for trans-activation at the cell membrane (B). 9B2 increases the number of receptors available for trans-activation at the cell membrane (C). In the absence of Dx, 9B2 receptors accumulate at the cell membrane and sequester all the ligands (D).

Therefore, ligands might contribute to the gain-of-function of *Ax* mutants, but their contribution depends on the localisation of the mutants at the cell membrane and in turn on their endocytic trafficking. Importantly, according to this model *Ax* mutants might have an effect that goes beyond the single cell level and might impair cell-cell interactions. However, interpreting the ligand-receptor balance is complicated because it has several dependencies and some of them are not well understood. A better understanding could be reach by testing the amount of signal sent and received by *Ax* mutant cells.

6.1.4 The gain of function of *Ax* mutants

In summary, our study indicates that the gain-of-function of *Ax* mutants results from their altered trafficking which might increase their endosomal activation or increase their potential for ligand-dependent activation and these effects are not mutually exclusive. However, in the signalling assays in S2 cells the *Ax* mutants did not show a higher activation compared to WT when normalised to WT. However, maybe this is not the biologically relevant control. If the data is instead normalised to the basal signalling of the mutant concerned, then all *Ax* mutants have increased ligand-dependent signalling, especially marked in 16 (see Fig. 3.7B). Further using the fold induction, E2, 16 and 28 show increased Dx-mediated signalling as well (see Fig. 3.8B).

However, there are other possible scenarios. *Ax* mutant signalling might result from the combination of ligand-independent and dependent pathways, thus cannot be detected when the signalling is individually assessed. Similarly, the gain-of-function of the mutants might ultimately result from their effect at the multicellular level and this could only be achieved *in vivo*. Alternatively, the mutants might give rise to a signalling that is faster or more stable in time and this cannot be detected using standard luciferase assays but would require a live luciferase assay. Finally, the signalling of *Ax* mutants might have a different function. For example, it is not known whether Notch

signalling arising from endosomes is different from the ligand-dependent signalling, thus it might activate a different set of genes or allow Notch to cross-talk with other pathways. Similarly, Met RTK receptor mutants, that increase Met endosomal signalling, were found to promote tumorigenesis as a result of the stability and specificity of their endosomal activation (Joffre *et al.,* 2011).

The cause of the lethality associated with the negative complementation of *E2* and *9B2* had never been elucidated. Our work indicates that the lethality might be caused by a strong gain-of-function because the combination of the mutants caused strong vein loss wing phenotype. This might result from the additive effect of *E2* and *9B2* gain of function mechanisms, such as activation from the endosomes and activation at the cell membrane. In addition, if E2 increases the number of ligands available for trans-activation at the cell surface and 9B2 the number of receptors, these features might give rise to a more potent ligand-dependent activation when combined.

6.2 The function of Ax domain

Ax domain has often been described as a functional domain of Notch receptor, however what its function is, is still unknown. In earlier studies it was proposed that Ax domain might be involved in cis-inhibition (de Celis and Garcia-Bellido, 1994; de Celis and Bray, 2000), however more recent evidence showed that EGF 11-12 are instead required for cis-interactions (Cordle *et al.,* 2008; Becam *et al.,* 2010), excluding the involvement of Ax domain. In addition, it was shown that glycosylation of Notch EGF repeats by Fringe might alter the affinity for the receptor binding with its ligands, but the glycosylation in Ax domain did not seem to modify the affinity for the ligands (Kakuda and Haltiwanger, 2017). Our data support a new view in which the Ax domain might be involved in the endocytosis and trafficking of Notch. Since other EGFs are required for specific functions of Notch we cannot

exclude that Ax domain might be required or necessary for Notch endocytosis and this is a hypothesis to be tested. Interestingly, the domain might also determine Notch sorting into different endosomal compartments since it was shown that *Ax* mutants divert Notch trafficking to different destinations.

The function of the Ax domain might be achieved through its interaction with other regulators. The proteins screened in chapter 5 were shown to modify the ligand-independent activation of WT Notch, further suggesting that the interaction of Notch extracellular domain with other regulators might have an effect on its endocytic trafficking. This model is supported by the study of Contactins, which are neural cell adhesion molecules (F3/contactin1 and NB3/contactin6), that were found to activate Notch1 through Deltex1 and induce glial maturation (Hu *et al.,* 2003; Lu *et al.,* 2008; D'Suoza *et al.,* 2010). Interestingly, Contactins are GPI-linked and are believed to interact with Notch EGF repeats 22-34 (Hu *et al.,* 2003). How the interaction with Contactins has an effect on Dx-dependent activation has not been established yet, but it is very tempting to speculate that this is linked to Ax domain function. Another idea that is worth to explore is whether the Ax domain might mediate Notch receptor-receptor dimerization. Preliminary data from our lab showed that Notch receptors lacking the extracellular domain reduce the formation of dimers in pull-down assays (E. Foteinou personal communication). Further, previous studies have also suggested that the Ax domain might be involved in this interaction (Xu *et al.,* 2005; Pei and Baker, 2008). However, the meaning of the dimerization of Notch is not clear, thus it is difficult to predict its consequences on Notch signalling. If the Ax domain is involved in dimerization, perhaps this process might have an impact on Notch trafficking. A similar example was reported in EGF receptor studies, where it was found that dimerization of the EGF receptor controls its endocytosis (Wang *et al.,* 2005; Wang *et al.,* 2015). Finally, it is possible that different EGFs in the Ax domain might interact with different

proteins, since in chapter 4 it was shown that mutations in different EGFs have different features. For example, some of the candidates screened in chapter 5, which were not affected by *E2* and *9B2,* might interact with the Ax domain but in EGFs other than 24 and 29 where *9B2* and *E2* are located. In order to test this hypothesis, these candidates could be re-screened with mutations made in other EGFs as cancer mutant models (see chapter 4), as well as a Notch construct containing a deletion of the entire Ax domain. If the interaction of the Ax domain with other regulators determines intracellular effects, this might represent a new mechanism of outside-in communication. Additionally, this may further describe non-canonical ligands involved in endosomal activation pathways.

6.3 The significance of *Ax* mutants in cancer

6.3.1 Cancer *Ax* mutations

Notch is highly conserved from *Drosophila* to humans, therefore the functional analysis of *Drosophila Ax* mutants might be relevant for those pathologies in which Notch is misregulated. It is well established that Notch has a high mutational rate in a number of cancer types and its misregulation is associated with different aspects of the disease (Ranganathan *at al.,* 2011; Ntziachristos *et al.,* 2014; Aster *et al.,* 2016). An interesting case is the one of Head and Neck Squamous Cell Carcinoma (HNSCC) patients where Notch1 was found to be highly mutated, especially in the Chinese population. In these tumours the majority of the mutations were found in the Ax domain of Notch1 and associated with a poor prognosis (Song *et al.,* 2013; Zhao *et al.,* 2016). A cluster of mutations can be spotted in the last EGFs of the Ax domain and EGF31. In this cluster the majority of mutations were in EGF29, including a hotspot mutation, C1133Y (Fig. 6.3; Song *et al.,* 2013; Zheng *et al.,* 2018). In the study of C1133Y, it was shown that the mutant localises less at the cell membrane because the mutant protein reaches

the membrane less efficiently, although the endocytic trafficking of the mutant was not examined. It was proposed that the mutant does not activate the ligand-dependent Notch signalling, but activates EGFR-PI3K/AKT pathway leading to increased cell proliferation and invasion (Zheng *et al.,* 2018). Our analysis of EGF29 mutants including E2, 16, G1136V, is particularly relevant to this because it shows that mutations in EGF29 can change the localisation of Notch at the cell membrane and might increase a non-canonical Notch activation route. It is likely that the mechanism of some of the mutations found in HNSCC in EGF29 are similar to *Drosophila Ax* mutants. However, our analysis also pointed out that mutants affecting Cysteine residues might be functionally different from others, thus mechanistic differences might be expected in C1133Y.

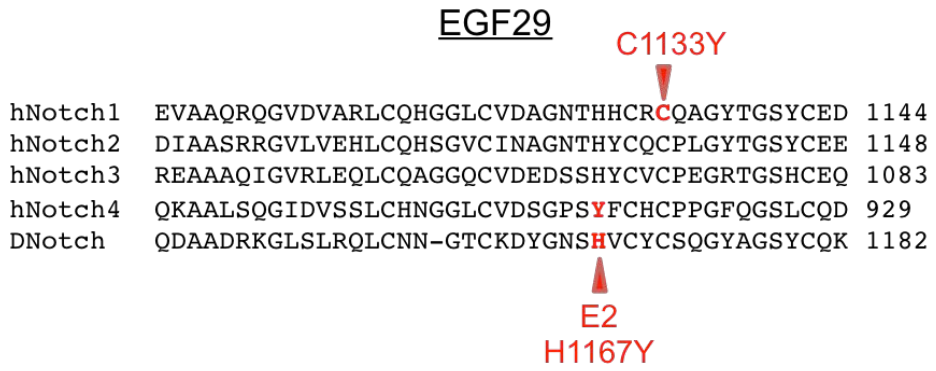


Figure 6.3. Ax mutations in EGF29. This multiple alignment shows *Drosophila* EGF29 and the conserved corresponding EGFs in human Notch receptors. E2 changes the Histidine residue in position 1167 into Tyrosine. A Tyrosine residue is already present in the endogenous human Notch4 in the same position as E2. C1133Y is a mutational hotspot in EGF29 of human Notch1 in Head and Neck carcinoma.

Ax mutations can also be found in other Notch-associated tumours, such as lung and breast cancer, although Ax domain is not defined as a mutational hotspot (COSMIC; Kan *et al.,* 2010; Hammerman *et al.,* 2012). A better record of *Ax* mutations and a comprehensive analysis might reveal that these mutations are also relevant in other tumours.

In chapter 4 it was shown that *Ax* mutants can be functionally classified depending on their position in the domain and make predictions on their outcome. Upon validation in the mammalian system, this kind of classification could be of great help for the treatment and diagnosis of *Ax* mutations found in cancer. For example, if it is possible to predict whether an *Ax* cancer mutation affects Notch trafficking, then it would be possible to treat the tumour accordingly. Also, in the case of *Ax* mutants one of the main features is the different localisation at the cell membrane, a feature that could be easily detected in patient-derived cells in order to make a functional prediction. This would be particularly meaningful in the field of precision medicine, which aims to identify the right treatment for the right patient. As reviewed in Friedman *et al.,* 2015, a limiting factor of today's precision medicine is the lack of understanding of how cancer-associated mutations contribute to the disease, in other words linking the cancer phenotype to its genotype. Our analysis indicates that the use of *Drosophila* for the functional screening of mutations might represent a successful tool to fill up this gap.

6.3.2 E2-like Notch4

Another interesting parallel between *Drosophila Ax* mutant Notch and human Notch, is that E2 amino acid change, H1167Y, is present in wild type human Notch4 (Fig. 6.3) (Dr. M. Baron personal communication). It is tempting to speculate that Notch4 might have a preference of being activated through its endocytic trafficking, like E2. This would be very interesting in the context of cancer, since upregulation of Notch4 signalling was found in Cancer Stem Cells (CSCs) in breast cancer, which are responsible for initiation and recurrence of the tumour, and linked to the maintenance of these cell populations (Harrison *et al.,* 2010; Simoes *et al.,* 2015). It is currently being tested in the Baron group if the up-regulation of Notch4 in CSCs mimics the effect of E2 gain of function. It is also interesting that Notch4 seems to maintain the stem-like features of CSCs, suggesting its activation might trigger the

activation of stem-related genes and this is in line with the idea that signalling from endosomes might have a different signalling function (Joffre *et al.,* 2011). Alternatively, other signalling pathways may be co-regulated by this Notch4 endosomal trafficking mechanism and it is this that defines the transcriptional profile in CSCs. Notch4 signalling in CSCs was found to be less sensitive to the traditional Notch inhibitor drugs (Harrison *et al.,* 2010; Simoes *et al.,* 2015). The reason for the lower sensitivity is unknown and it was proposed to be due to an intrinsic property of CSCs, however it is possible this is because Notch4 regulation and activation are different from other human Notch receptors.

6.3.3 Targeting *Ax* mutants in cancer

It has been proposed that Notch could be a promising target for cancer therapies, however targeting Notch remains a challenge and no FDA-approved anti-Notch therapies are currently available. This is in part because Notch is involved in many key cellular functions and interfering with Notch can cause severe side effects, like intestinal toxicity and increased incidence of skin cancer, unless the treatment is specific for Notch in the cancer cells (van Es *et al.,* 2005; Ryeom, 2011; Yuan *et al.,* 2015). If *Ax* mutations found in human cancers also heavily rely on the endocytic trafficking of Notch, targeting the trafficking might be a specific way to reduce mutant Notch signalling in cancer cells. In this respect, it is very intriguing that preliminary data showed that the area of *E2* mutant clones was increased compared to WT clones, but reduced in *E2, dx*^{152} clones and this was not seen in WT or *dx*^{152} clones (Fig. 6.4). This observation suggests that *E2* might provide the cells with a proliferative advantage, which is suppressed by depletion of Dx. This effect can also be seen in the wing phenotype of *Ax, dx*^{152} mutants in which the loss of function is much more severe than in WT, *dx* flies. This might be because in physiological conditions, the Notch pathway is finely tuned by the different regulatory routes (Shimizu *et al.,* 2014) and so a lack of Dx could be balanced to a certain extent by the other

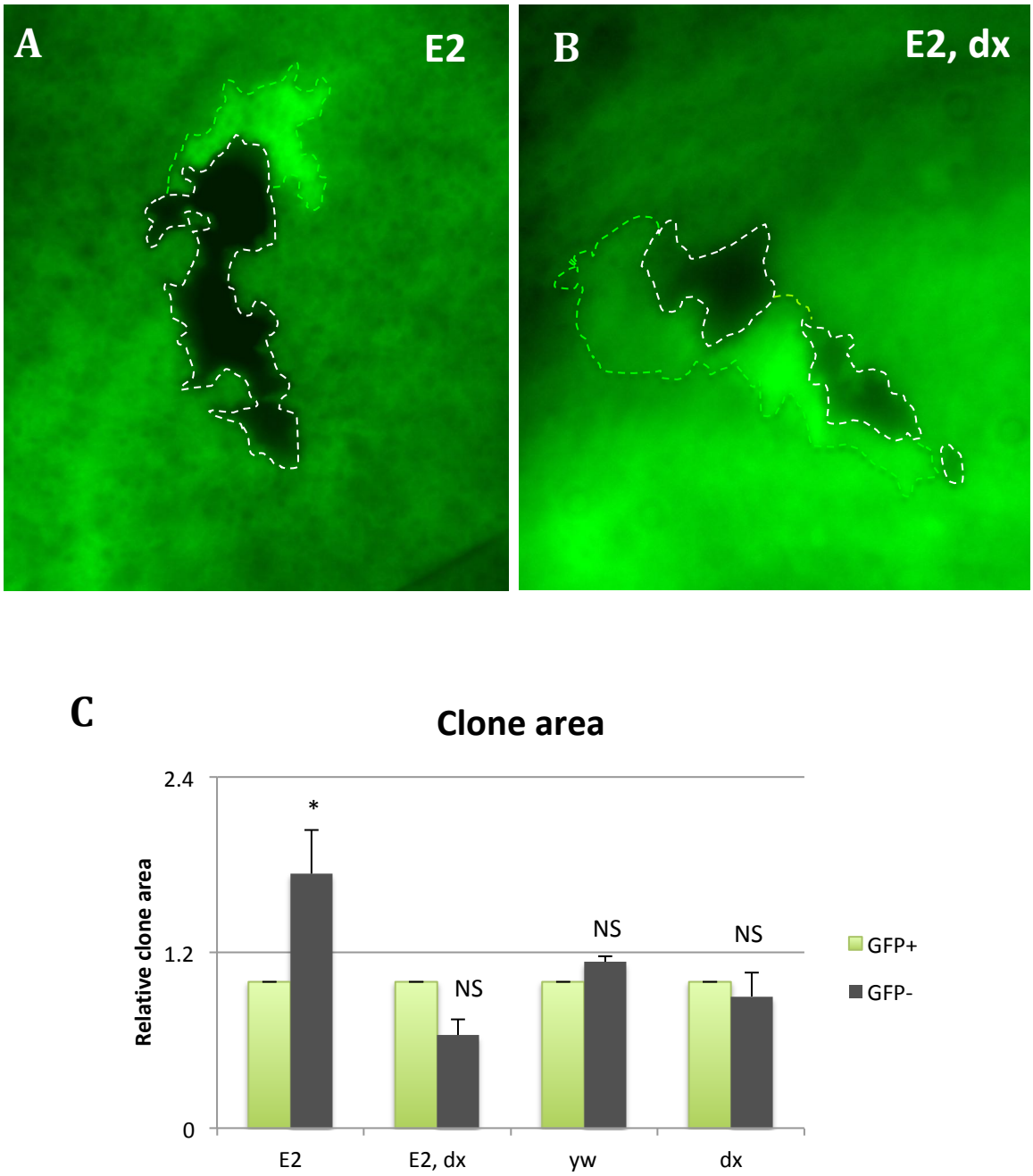


Figure 6.4. Cell proliferation in wing disc clones comparing twin spots. The area of the GFP- and GFP+ twin spots was measured using ImageJ in E2 (A), E2,dx (B), yw and dx wing disc clones. (C) shows the area mean value of GFP- (black columns) and GFP+ (green columns) normalised by the GFP+ mean values of each sample. The statistical significance was determined by Student T test and is relative to the GFP+ values of each sample (NS=non significant; *p<0.05). Error bars indicate SEM (n=5).

routes. However, this is not the case when the balance among the routes is already altered like in *Ax* mutants. These observations suggest that targeting the endocytic trafficking, and more specifically the Dx route, might be a way to switch off the *Ax* mutant Notch signalling and this would be quite specific. A way to increase the specificity of this strategy could be to target the endosomal activation of E2-like mutants or more generally the endocytic uptake of the mutants, both of which might have unique requirements that are not necessary for WT Notch.

Similar strategies have been recently tested to block the signalling of RTK receptors in cancer models. For example, inhibiting endocytosis by different means was reported to reduce the tumorigenic properties of Met receptor mutants that were found to induce neoplastic transformation by increasing endocytosis (Joffre *et al.,* 2011). Importantly, these treatments did not affect WT cells. Another study showed that inhibition of clathrin-dependent endocytosis induces the downregulation of EGFR in non-small cell lung cancer through the activation of a macropinocytosis-lysosomal dependent route where EGFR is degraded and this can overcome the resistance of certain EGFR mutants to tyrosine kinase inhibitors (Menard *et al.,* 2018). Another strategy could be to promote the degradation of the mutants. For example, the induction of degradation of RTK receptors using small molecules showed advantages in the downregulation of RTK mutant signalling compared to the traditional inhibition strategy (Burslem *et al.,* 2018). Therefore, our data fits into the emerging field of endocytosis in cancer and might provide a rational for the use of endocytosis as a strategy to manipulate Notch pathway when misregulated.

6.4 Future directions

The work presented in this thesis shows that *Ax* mutations misregulate Notch by affecting its endocytic trafficking in *Drosophila* and opens new questions and directions in which this study could be brought forward.

The first direction in which the project could be expanded is to further describe the molecular mechanism by which *Ax* mutants are regulated. It would be interesting to determine how E2 is activated prior to fusion with lysosomes since this is clearly an important result that would cast light on new mechanisms of Notch activation in the endosomes. This can be achieved by testing E2 signalling activation upon inhibition of different endosomal components in S2 cells and the use of endosomal markers to determine in which endosomes E2 is localised. Live imaging could also be used to confirm whether E2 is activated at a different stage of endosomal maturation. It would also be important to test if endocytosis or recycling is responsible for 9B2 accumulation at the cell membrane by using biochemical assays in S2 cells or immunostaining of surface Notch after surface stripping in S2 cells and *in vivo* in the wing discs. It was proposed that the localisation of *Ax* mutants at the cell surface could affect the interactions between Notch receptor and its ligands. This could be tested by measuring how much signal an *Ax* mutant cell can send and receive in S2 cells and *in vivo* in wing disc clones. Since the balance between receptor and ligand interaction is complicated, the use of a mathematical model, together with the experimental assays, might be helpful. A mathematical model for ligand-receptor interactions was already designed in Sprinzak *et al.,* 2010 and a similar model could be applied for the study of *Ax* mutants by changing some parameters to account for more or less Notch available at the cell membrane. Finally, another aspect to be tested could be the dynamics of the signalling activation of *Ax* mutants and this could be assessed by live luciferase assay.

Secondly, further study could focus on the function of the Ax domain and which proteins might bind with this region. It would be interesting to generate deletion mutants of Notch extracellular domain and test which is the minimal requirement for Notch endocytosis and sorting and whether the Ax domain is required for these functions. A systematic way of screening interacting candidates in S2 cells was presented in chapter 5 and this can be used to screen other candidates. In addition, a larger scale screening might involve the use of *Ax* mutants and deletion Notch mutants, which lack different domains of the receptor, in combination with mass spectroscopy. The comparison of the binding profile of *Ax* mutants with WT Notch will be essential, not only for the identification of interacting proteins with *Ax* mutants, but also to cast light on the function of the Ax domain.

Finally, it would be very exciting to move the analysis of *Ax* mutants to the mammalian system. The first aim would be to validate what was found in *Drosophila* in mammalian cells, for example the different localisation at the cell membrane and endocytosis of the mutants. At the same time, a comprehensive record of the *Ax* mutations from databases could be done to identify tumours in which *Ax* mutants might have significance. If the mechanism of *Ax* mutants is validated in mammalian cells, a functional analysis could be carried out in *Drosophila* S2 cells as detailed in chapter 4. Moreover, it would be interesting to try to target the *Ax* cancer mutants by inhibiting endocytosis or trigger their degradation as it was done for other receptors. If successful, this strategy could be refined and tailored depending on the mutation and the tumour.

A number of correlations were already found between what was observed in our analysis in *Drosophila* and what has been previously reported in the mammalian system and cancer. Therefore, *Drosophila melanogaster* is an ideal model organism in which to analyse *Ax* mutants and the findings will hopefully ultimately translate into therapies for human conditions.

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