Optogenetic modulation of Notch signaling during tissue morphogenesis in *Drosophila*

A thesis submitted towards the partial fulfilment of BS-MS dual degree programme

Submitted by

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Certificate

This is to certify that this dissertation entitled “Optogenetic modulation of Notch signaling during tissue morphogenesis in *Drosophila*” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune, represents original research carried out by Mr.Rohit Krishnan.H at the European Molecular Biology Laboratory (EMBL), Heidelberg, under the supervision of Dr.Stefano De Renzis, Group leader, Developmental Biology unit, during the academic year 2015-2016.

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Group leader,

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22nd March, 2016

Declaration

I hereby declare that the matter embodied in the report entitled “Optogenetic modulation of Notch signaling during tissue morphogenesis in *Drosophila*” are the results of the investigations carried out by me at the Developmental Biology unit, EMBL Heidelberg, under the supervision of Dr.Stefano De Renzis and the same has not been submitted elsewhere for any other degree.

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Abstract

Precise activation of signaling pathways is essential for the development of a multicellular organism. Signaling systems display a high range of complexity with quantitative differences in levels of activation often leading to altered cellular responses. A thorough quantitative analysis of these signaling modules would require the ability to modulate individual pathway components with high spatial and temporal precision. Here, I have employed optogenetics to manipulate an endogenous Notch signaling component during *Drosophila* embryogenesis. I have utilised an optogenetically tagged allele of the Notch ligand, Delta (Opto-Delta), which was generated in the host laboratory. I demonstrate that Opto-Delta functions as a light sensitive loss of function allele and characterise the mechanism underlying light mediated inhibition of Notch activation. Combining Opto-Delta with live transcriptional reporters of Notch activity, I further establish a system to regulate Notch signaling with sub-cellular and second scale precision, which can now be used to gain a quantitative understanding of the input-output relationship underlying Notch signaling.

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1. Introduction

The development of a multicellular organism results from the co-ordinated interaction of diverse cell types. Cells are required to adopt a particular fate or modulate their behaviour, in response to their environment. All the key events in development, from embryonic patterning to organogenesis, rely on intercellular communication, mediated by short and long range signaling pathways. Surprisingly, all these years of research on cell differentiation and morphogenesis has yielded the identification of only a few core signaling cascades, which raises the question of how such a limited number of pathways are responsible for mediating a wide range of cellular responses (Perrimon et al., 2012).

There are often instances where one signaling pathway mediates a multitude of responses, depending on the context. For example, in rat neuronal cells, quantitative differences in the duration of activation of Mitogen activated protein kinase (MAP-K) pathway, enable the induction of either proliferation or differentiation. Another example is the tumor suppressor function of Transforming growth factor-β (TGF-β) in the early stages of tumorigenesis and its tumor promoting role in advanced metastatic stages (C.J.Marshall., 1995; Jakowlew., 2006).

A major obstacle towards understanding how such signaling systems function, is the high degree of complexity associated, with the available amounts of individual components varying dynamically over time. Conventional genetic and genomic approaches have been successful in qualitatively characterising complex signaling relationships over longer time scales but do not provide the spatio-temporal precision required for a thorough quantitative analysis. It is therefore essential to develop tools which would allow real time tracking and modulation of pathway components with the appropriate spatial and temporal resolution, so as to dissect out the precise link between variability in information flow and cell fate decisions.

1.1. Optogenetics to modulate signaling systems

Optogenetic systems have been known to provide precise modulation of signaling pathways in living cells through light induced protein-protein interactions. These systems are highly effective compared to the conventional genetic, biochemical or physiological approaches owing to their specificity, rapid reversibility and high spatial

and temporal resolution. A commonly exploited protein in such studies is the Arabidopsis Cryptochrome 2 (Cry2), which is a blue light receptor involved in photoperiodic flowering and light mediated cell elongation (Guo et al., 1998; Wu and Spalding., 2007). It contains a conserved N-terminal photolyase homology region (PHR) which binds flavin and pterin chromophores and mediates light-responsiveness. In its photoexcited state, Cry2 interacts with a basic helix loop helix protein CIB1 and the complex dissociates in darkness (Liu et al., 2008) **[Fig 1.A]**. The Cry2/CIB1 dimerisation module has been used to manipulate protein-protein interactions in various contexts (Kennedy et al., 2010; Boulina et al., 2013). Interestingly, Cry2 is also capable of undergoing N-terminal mediated homodimerisation in response to blue light (Bugaj et al., 2013) **[Fig 1.B]**. This provides us with a much desirable light-sensitive system that facilitates the use of a single protein to modulate signal transduction and various other cellular processes.

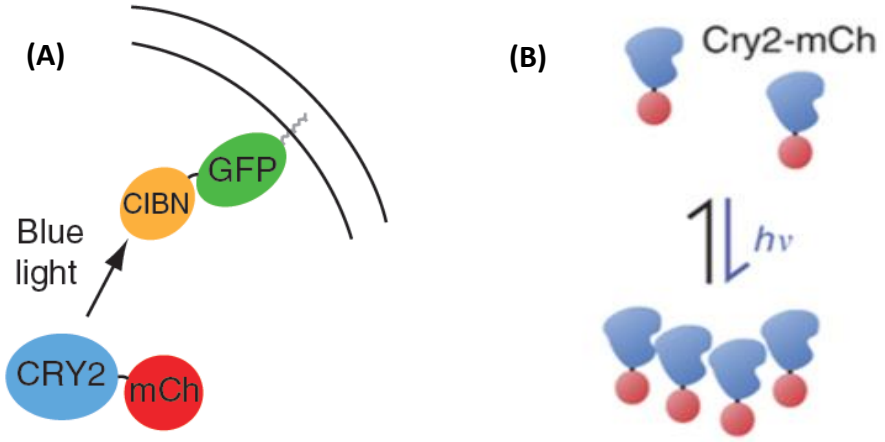


Fig 1: Blue light mediated protein-protein interactions. A) Arabidopsis Cry2 interacts with BHLH protein CIB1 in the presence of blue light (Adapted from Kennedy et al., 2010). **B)** Cry2 also undergoes homodimerisation in its photoexcited state (Adapted from Bugaj et al., 2013).

1.2. Notch signaling

The Notch pathway is a highly conserved short range signaling mechanism, which influences cell fate decisions and tissue homeostasis in multiple aspects of metazoan development (Artavanis-Tsakonas et al., 1995). Dysfunctioning of this pathway has been implicated in a variety of developmental disorders as well as in

cancers, where it promotes tumor growth in some contexts and inhibits it in others. Notch signaling was first discovered in *Drosophila melanogaster*, where its role in intercellular communication was extensively studied during embryonic neurogenesis (Poulson., 1937; Artanavis-Tsakonas et al., 1995). In this context, the upregulation of Notch signaling in all but one cell, in an initial group of equipotent cells, via a mechanism known as lateral inhibition, causes the cell to delaminate and become a neuroblast, while the remaining sheet of cells gives rise to epidermal structures. Lack of zygotic Notch increases the number of cells taking up the neuronal fate, giving rise to a 'neurogenic' phenotype **[Fig 2]**.

Inductive signaling is yet another mode of regulation exhibited by Notch. In this process, the transmission of signals between two non equivalent cell types induces a new fate at their interface. A well known example is the establishment of dorso-ventral boundary in the *Drosophila* wing (Irvine and Vogt., 1997). Apart from lateral inhibition and inductive signaling, there are also instances where asymmetric cell divisions lead to the activation of Notch in one daughter cell and not in the other, giving rise to distinct cell lineages (Bray., 1998) **[Fig 3]**.

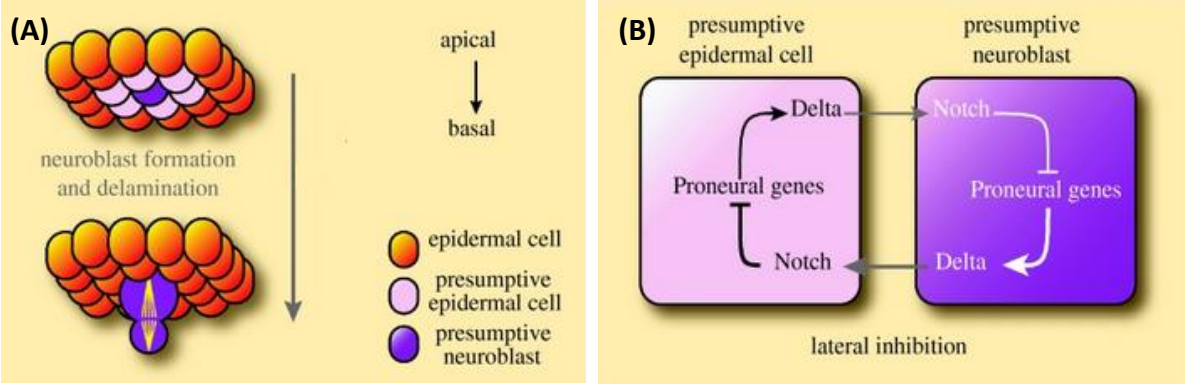


Fig 2: Notch in embryonic neurogenesis. A) Lateral inhibition causes a single cell to delaminate from an equipotent group of cells and become a neuroblast, which now divides along the apico-basal axis in a stem cell-like manner. **B)** Activation of Notch signaling by its ligand Delta leads to suppression of proneural genes in presumptive epidermal cells. Since the expression of Delta is dependent on proneural transcription factors, repression of proneural genes downregulates the activation of Notch signaling in the neighbouring cells. These cells with low levels of Notch activity take up the neuronal fate and delaminate, owing to increased expression of proneural genes (Adapted from Boris et al., 2008).

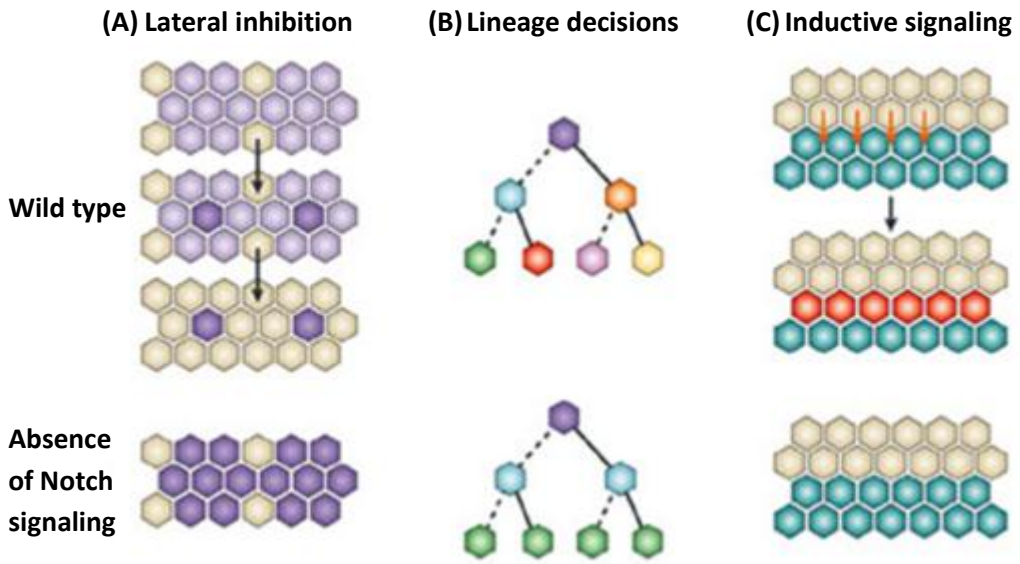


Fig 3: Three distinct modes of Notch mediated cell fate regulation. A) Notch signaling facilitates an equipotent group of cells to inhibit each other's ability to adopt a particular fate. **B)** Asymmetric cell divisions lead to activation of Notch in one daughter cell and not in the other, resulting in distinct cell fates. **C)** Transmission of Notch signals between two different neighboring cell types induces a new fate at the interface (Adapted from Haines and Irvine., 2003).

The Notch gene in the fruit fly codes for a 300kDa single pass type 1 transmembrane receptor. Its extracellular domain (ECD) consists of 36 EGF repeats and 3 Notch/Lin-12 repeats while its intracellular domain (ICD) has 6 cdc-10/ankyrin repeats and a PEST sequence (Wharton et al., 1985; Kidd et al., 1986). Delta and Serrate, which are the two canonical Notch ligands in *Drosophila*, are also type 1 transmembrane proteins that are characterised by three structural motifs- an N terminal DSL domain (Delta/Serrate/Lag-2), a DOS domain (Delta and OSM-11 like) and EGF like repeats (Cordle et al., 2008; Komatsu et al., 2008).

Trans-interaction between Notch from one cell and its ligand from a neighbouring cell is essential for the activation of signaling. The ligand, via its DSL domain, binds EGF 11-12 of Notch ECD (Rebay et al., 1991; Fleming, R.J., 1998). The ligand is then ubiquitinated by the RING finger ligase Neuralised and endocytosed into the signal sending cells. This facilitates a conformational change to the Notch receptor and

provides access to the Notch intramembraneous S2 site for cleavage by ADAM/TACE metalloproteases. This results in the shedding of Notch extracellular domain (Notch ECD), which is trans-endocytosed into the signal sending cells. (Parks et al., 2000; Nichols et al., 2007). The Notch extracellular truncated (NEXT) fragment that remains tethered to the membrane in signal receiving cells is now cleaved at the S3 site by a gamma secretase complex (De Stooper et al., 1999). This releases the Notch intracellular domain (Notch ICD or Notch^{intra}) from the membrane, which now translocates into the nucleus and interacts with the DNA binding protein, CSL (CBF-1, Su(H) and LAG-1) (Schroeter et al., 1998). Transcriptional coactivators like Mastermind (Mam) are now recruited to facilitate the transcription of target genes such as Hairy and enhancer of split (Hes) (Jarriault et al., 1995; Lecourtois and Schweisguth., 1995; Kidd et al., 1998) **[Fig 4]**.

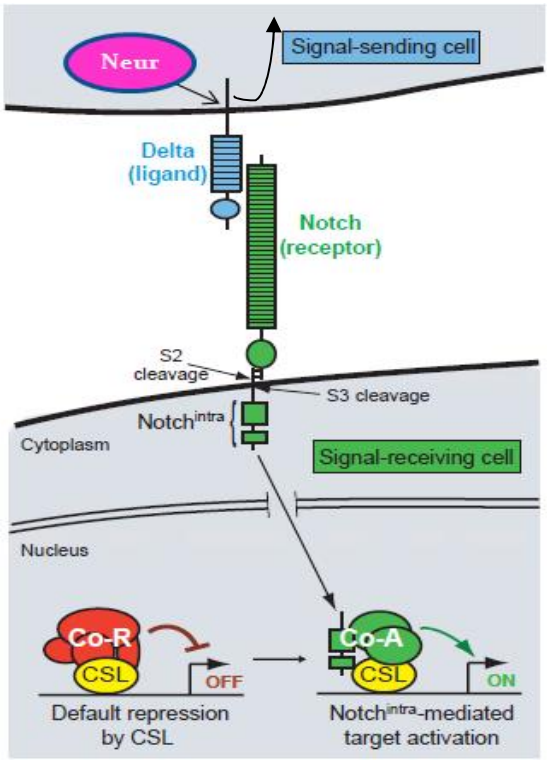


Fig 4: Molecular mechanism of Notch activation. The Notch receptor and the Delta ligand are both transmembrane proteins. Neuralised mediated ubiquitination and endocytosis of Delta results in cleavage and release of Notch^{intra} into the neighboring cell. Notch^{intra} enters the nucleus and forms an activation complex with CSL, which facilitates the transcription of target genes. In the absence of Notch signaling, CSL, along with other co-repressors actively represses transcription (Adapted from Eric.C.Lai., 2004).

1.3. Notch in *Drosophila* embryonic mesectoderm

The development of the fruit fly embryo involves thirteen rapid cycles of nuclear division, resulting in the accumulation of about 6000 nuclei at the unseparated cytoplasm of the oocyte, under the control of maternally deposited proteins. During the interphase of cycle 14, cellularisation transforms the syncytial embryo into 6000 columnar epithelial cells, producing the cellular blastoderm, after which the ventral furrow forms marking the beginning of gastrulation.

The anteroposterior and dorsoventral axes of the embryo are established by the asymmetric distribution of maternal signals in the oocyte. This results in a nuclear localisation gradient of the transcription factor Dorsal along the D-V axis in the pre-blastoderm embryo. High levels of Dorsal in the ventral nuclei mediate the activation of mesoderm determining genes, *twist* and *snail*. Low nuclear levels of Dorsal in the lateral ectoderm induce the neuroectoderm specific genes, *rhomboid* and *sog*, which are repressed ventrally by Snail. Absence of nuclear Dorsal in the dorsal side of the embryo allows the expression of genes such as *decapentaplegic* and *tolloid*, which enable dorsal fates (Perrimon et al., 2012).

The *Drosophila* embryo is an excellent model to study the dynamics of Notch signaling. In pre-cellularising embryos, the Notch receptor and its ligand Delta are both maternally deposited and uniformly distributed at the plasma membrane. Delta in the ventral mesoderm interacts with Notch in the lateral ectoderm. Neuralised mediated ubiquitination and endocytosis of Delta in the mesoderm results in the activation of Notch in a single row of cells abutting the mesoderm, called the mesectoderm. The Notch ICD, which translocates into the nucleus, relieves the repression by Su(H) and, together with Dorsal and Twist, mediates the transcriptional activation of *single minded* (*sim*) at the mesectoderm. Activation of *sim* is inhibited in the ventral mesoderm by the transcriptional repressor, Snail (Morel and Schweisguth., 2000; De Renzis et al., 2006). In the ectoderm, internalisation of Delta and thereby Notch signaling is prevented due to the inhibition of Neuralized by the Bearded family of proteins (Allison and Schweisguth., 2006) **[Fig 5, 6]**.

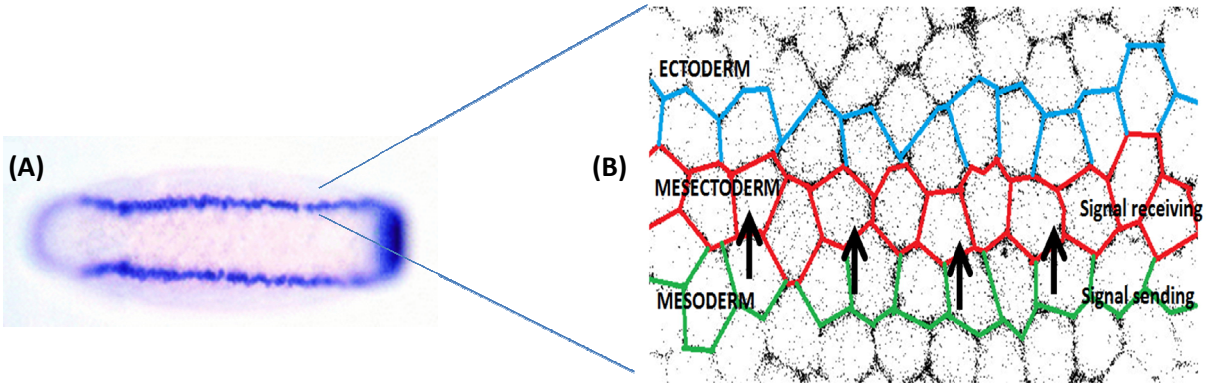


Fig 5: *Drosophila* embryonic mesectoderm. A) *In situ* hybridisation for *single minded*. Shown in figure, ventral view of *Drosophila* embryo at mid-cellularisation; anterior is towards left. **B)** Representation of mesectoderm specification: Delta in the mesoderm interacts with Notch in the lateral ectoderm, resulting in the activation of *sim* at the mesectoderm.

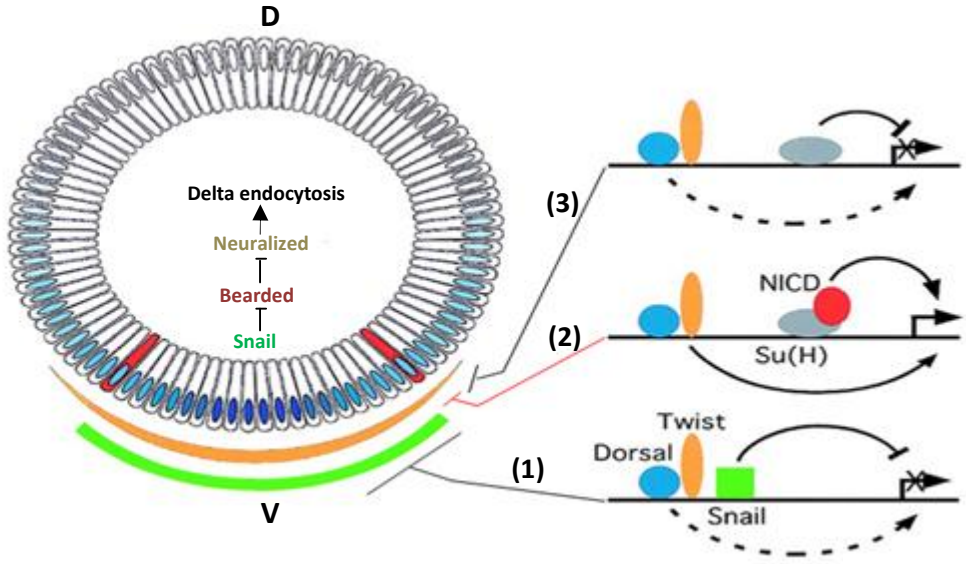


Fig 6: Mechanism of activation of *single minded* at the mesectoderm. D-V nuclear localisation gradient of Dorsal is shown in blue. The boundary of expression of *snail* (green) coincides with the mesoderm-mesectoderm boundary. The accumulation of Twist (orange) fades towards the neuroectoderm. Inhibition of Bearded proteins by Snail results in Neuralized mediated endocytosis of Delta in the mesoderm. **(1)** Transcriptional repression of *sim* by Snail at the ventral mesoderm. **(2)** Notch ICD relieves repression by Su(H), and, together with Dorsal and Twist, activates *sim* at the mesectoderm. **(3)** Su(H) acts as a repressor due to the absence of Notch signaling at the neuroectoderm (Adapted from Morel and Schweisguth., 2000).

1.4. Specific aims of the project

As mentioned earlier, Notch signaling has been implicated in a variety of cellular processes such as proliferation, migration, fate specification, stem cell maintenance, apoptosis and boundary formation. It is indeed surprising that a simple linear pathway like Notch is able to specify such a huge repertoire of developmental outcomes. Quantitative differences in the level of Notch activation have been found to influence the choice of cellular responses in multiple contexts. For instance, a reduction in the relative levels of Notch alters the fates of developing B and T cells in vertebrates (Washburn et al., 1997; Witt et al., 2003). Similarly, halving the gene dosage of wild type Notch disrupts the specification of dorso-ventral wing margins in *Drosophila*, leading to a 'notched' wing phenotype (Fehon et al., 1991).

A thorough understanding of how various functions of Notch correlate with different threshold levels of signaling would require the development of tools to control endogenous pathway components at will with high spatial and temporal accuracy.

Hence, the goal of this study is to:

i) Characterise an optogenetic tool to regulate Notch signaling with high spatial and temporal precision during *Drosophila* embryogenesis.

ii) Combine this tool with live reporters of Notch activity to gain a quantitative understanding of Notch signaling dynamics, by focusing on the activation of *single minded* expression during embryonic mesectoderm specification.

2. Materials and Methods

2.1. Fly stocks

N-; *NotchintraGFP* procured from F.Schweisguth (DOI: 10.1038/ncb2419 stock number 314); *NotchextraYFP* (I) from CPTI, Kyoto stock center (DGRC no: 115544); *MCPGFP* (II) from Thomas Gregor (fly base ID: FBgn0067412); *Sqh: GAP43-mCherry/TM3* from Wieschaus, E (fly base ID: FBtp0073096).

2.2. Embryo collection

Flies of the appropriate genotype are put into cages with agar coated apple juice plates and incubated in the dark at room temperature (RT). The plates are changed after a 4hr day collection or an overnight collection and the embryos are used for further experiments.

2.3. Cuticle preparation

Pre-cellularising embryos are selected using halocarbon oil and photo-activated until the stage they are about to hatch (36-48 hrs). These are bleached and transferred into a drop of Hoyer's-based medium (add 15g of gum arabic to 25mL of water; heat to 65°C and stir overnight; successively dissolve 100g of chloral hydrate and add 10g of glycerol; centrifuge for 30 min at 10000 rpm) in a glass slide. The slide is then heated at 60°C overnight. The inner parts of the embryo get digested, leaving the epidermis behind. Repeated belts of denticles are clearly observed on the ventral surface.

2.4. Dechorination and fixation

The embryos are dechorionated by treatment with bleach (100%) and rinsing with 1X PBS to remove the bleach. They are then fixed by shaking in fixative (4% PFA in 1X PBS, and heptane in 1:1 ratio) for 20 min at room temperature. The lower aqueous phase is removed and 100% methanol is added to the solution so as to get a 1:1 mixture of methanol: heptane. The devitallinized embryos, which sink to the bottom after vortexing for 20 sec, are collected and rinsed thrice with methanol. They can now be stored in methanol at -20°C.

2.5. Immunofluoresence staining

The fixed embryos are rehydrated after storage in methanol by washing them thrice for 20min each with 1ml $PTx_{0.1\%}$ (0.1% Triton-x-100 in 1X PBS). The samples are then blocked using 500µL $PB_{10}Tx_{0.1\%}$ (10% BSA and 0.1% Triton-x-100 in 1X PBS) and rotated for 60 min at RT, followed by overnight incubation at 4°C (or 2 hours at RT) with primary antibody, diluted in 400-500 µL of $PB_5Tx_{0.1\%}$ (5% BSA and 0.1% Triton-x-100 in 1X PBS). The embryos are now washed 4 times with 1ml $PTx_{0.1\%}$ for 10min each and incubated with 1:500 concentration of secondary antibody in $PB_5Tx_{0.1\%}$ for 1.5 hours at RT (or overnight at 4°C). After incubation with secondary antibody, the samples are washed 4X for 10min each with $PTx_{0.1\%}$ and equilibrated by treating with 50% glycerol for an hour. The embryos at the appropriate stage are mounted using Aqua-Poly/Mount, heated for 5min at 37°C and imaged (within 3 days) using the 63X oil immersion objective (NA 1.3) at the Perkin Elmer Ultraview VoX spinning disk confocal microscope.

The following primary antibodies were used in this study:

Mouse anti-extracellular Notch EGF repeats #12-20 (1:20 dilution) (Development Studies Hybridoma Bank, DSHB), Rabbit anti-GFP (1:1000) (Torrey Pines Biolabs, CA), Rat anti-DE Cadherin (1:10) (DSHB).

All secondary antibodies were Alexa-conjugated (1:500 dilution) (Molecular probes, OR).

2.6. Probe synthesis for *in situ* hybridization

Clones for *single minded* were obtained from the Berkeley *Drosophila* gene collection (Sim DGC Clone RE54280). The template was PCR amplified using the following primers:

Forward- TGAGAAGGTGCTCGCAACAGTC

Reverse- <u>TAATACGACTCACTATAGGGAGA</u>AGCGGACCCTGCACTATTTA

The underlined sequence incorporates the T7 RNA polymerase binding site. The amplified fragments were separated out via gel electrophoresis and extracted using the Qiagen PCR purification kit.

The Sim antisense RNA was then transcribed using T7 RNA polymerase, through the following steps:

1-13µL of template is incubated with the reaction mix (2µL of 10X NTP labelling mix (Sigma-Aldrich), 2µL of 10X transcription buffer, 1µL of protector RNase inhibitor and 4µL of T7 RNA polymerase) for 4 hours at 37°C and the solution is covered with an aluminium foil. 2µL of DNase1 is then added to remove the template DNA, followed by treatment with 30µL LiCl and 30µL of RNase free water, which precipitates the RNA. The RNA concentration is quantified using nanodrop.

2.7. *In situ* hybridization

The fixed embryos are transferred from methanol into xylene and washed for 20 min to remove the yolk. These are again rinsed with methanol before a 3X treatment with 1ml $PTw_{0.1\%}$ (0.1% Tween20 in 1X PBS) for 2 min each. The samples are incubated in PBTK (3µg/mL ProteinaseK in $PTw_{0.1\%}$) at room temperature for 30 min, followed by a 2X wash with PBTG (2mg/mL glycine in $PTw_{0.1\%}$) to stop ProteinaseK digestion. They are now rinsed twice with $PTw_{0.1\%}$ prior to post fixation for 20 min at RT in 1ml fixative. They are then washed 5X with $PTw_{0.1\%}$ to remove all traces of fixative.

The samples are rinsed once with 1:1 mixture of hybridization buffer: $PTw_{0.1\%}$, which is then replaced by 100% hybridization buffer. The prehybridisation buffer is prepared by boiling the hybridization buffer for 5 min at 100°C and cooling it on ice for 5 min. The embryos are treated with the prehybridisation buffer for 2 hours at 65°C, followed by incubation for 15 hours in the probe solution (50-100ng of probe in 100µL of hybridization buffer along with 5% dextran sulphate) at 65°C.

Post hybridisation, the samples are washed for 5 times at 65°C with 100% hybridization buffer for 10 min each, followed by sequential washing for 15 min, each with 3:1, 1:1, and 1:3 mixtures of hybridization solution: $PTw_{0.1\%}$, at the same temperature. They are then rotated 5X for 5 min each with $PTw_{0.1\%}$ at 65°C and thereafter cooled to RT. This is followed by blocking with 5% BSA in $PTw_{0.1\%}$ for one hour at RT, after which the samples are incubated with 1:500 dilution of DIG antibody in 1.5% BSA in $PTw_{0.1\%}$ for two hours at RT. They are now rinsed twice with 1.5% BSA in $PTw_{0.1\%}$ for 10 min each and then 7X for 10 min each with $PTw_{0.1\%}$.

For detection, the embryos are rinsed thrice with fresh Alkaline phosphatase (AP)-wash buffer for 5 min each, following which, they are transferred to 24 well plates and incubated with 1:50 concentration of NBT/BCIP: AP-colour buffer, till the colour develops. The reaction is stopped by washing several times with $PTw_{0.1\%}$ and then with an ethanol series to reduce the background. The embryos are rehydrated by rinsing with $PTw_{0.1\%}$ and stored in 50% glycerol. All *in situ* images were procured using the Olympus SZX 16 stereo microscope.

Reagents and buffers:

Hybridization buffer- 1% blocking reagent, 0.1% chaps, 50% formamide, 5X SSC, 1X Denharts, 0.1% yeast tRNA, 0.01% Heparin and 0.1% Tween20 in water

AP Wash buffer- 0.1M Tris HCl (pH-9.5), 0.1M NaCl, 50mM $MgCl_2$, 1mM Tetramisol and 0.1% Tween20 in water

AP Colour buffer- 0.1M Tris HCl (pH-9.5), 0.1M NaCl and 0.1% Tween20 in water

2.8. Photo-activation and imaging

For all live imaging experiments, the embryos were dechorionated and mounted in a dark room using a standard upright microscope, with the light source covered using a yellow filter to prevent unwanted blue light illumination. For global photo-activation, we used the 488nm laser at the Perkin Elmer Ultraview VoX spinning disk confocal microscope (sensitivity=190; exposure=200ms), equipped with a 63X (NA 1.3) oil immersion objective (C-Apochromat). Individual samples were illuminated at the rate of one slice per minute for all experiments, except for the time lapse analysis experiment, in which embryos were photo-activated by illuminating one slice every 3sec for 6min.

Local photo-activation was achieved using a Zeiss LSM 780 NLO confocal microscope with a C-Apochromat 633 water immersion (NA 1.2) objective (Carl Zeiss). The embryos at the start of cellularisation were illuminated locally using a one photon argon laser (λ=488nm; laser power-30%) by acquiring 5μm deep z stacks (each plane separated by 1μm) from the apical surface, every 30sec, with a pixel dwell of ~5μs, for a total time of 10min. This was followed by two photon illumination, till the beginning of ventral furrow formation (~30min), using a pulsed laser (140fs)

(λ=930nm; laser power -12%) by taking 10μm deep z stacks from the apical surface, every 40sec, with the same pixel dwell.

For all immunolabelling and *in situ* hybridisation experiments, embryos were persistently illuminated for an hour using the Olympus SZX 16 stereo microscope, whereas for inducing various developmental phenotypes (eg: cuticle preps), embryos or pupae (raised in dark till this point) were continuously exposed to white light from a standard upright microscope.

2.9. Time lapse analysis of clusters

All images for time lapse analysis were pre-processed using ImageJ. Image segmentation and cluster detection was then carried out in an automated fashion using a pipeline developed in Cell Profiler 2.1.1. The ratio of intensity in clusters to the total intensity of image for each time point was calculated. This was depicted in terms of percentage of the maximum value attained and plotted against the time axis. Statistical significance was tested using a student's t-test.

3. Results and Discussion

3.1. Generation of Opto-Delta constructs

The primary goal of this project was to generate a light-sensitive allele of Delta and thereby achieve precise control over Notch signaling. It was recently demonstrated that the Arabidopsis photoreceptor Cryptochrome 2 (Cry2) undergoes blue light mediated oligomerization. Prior to my arrival in the lab, Aleksandar Necakov, a postdoctoral fellow with whom I was collaborating, inserted a Cry2 tag into the third chromosomal endogenous locus of Delta (Opto-Delta), using ΦC31 integrase mediated homologous recombination (Bateman., 2006). A series of constructs were generated containing either a Cry2 tag alone (DeltaCry2) or Cry2 fused to EGFP (DeltaGFPCry2) or tagRFP (DeltatagRFPCry2). *DeltaCry2* homozygous flies were viable and fertile in the dark while the other two constructs displayed reduced fertility and viability **[Fig 7]**.

Our working hypothesis was that the Cry2 induced Delta oligomerization should interfere with its normal function. Consistent with this hypothesis, we could show that homozygous Opto-Delta embryos and flies grown in the light display typical Notch phenotypes. At the embryonic stage, prolonged exposure to blue light (photo-activation) gave rise to a neurogenic phenotype at the expense of ectodermal cell fate, which was observed by embryonic cuticle preparation. A complete lack of hatching was also observed in photo-activated Opto-Delta embryos. The phenotypes in the adult fly included defective wing vein formation, rough eye morphology and a decrease in sensory bristle density at the notum **[Fig 8]**.

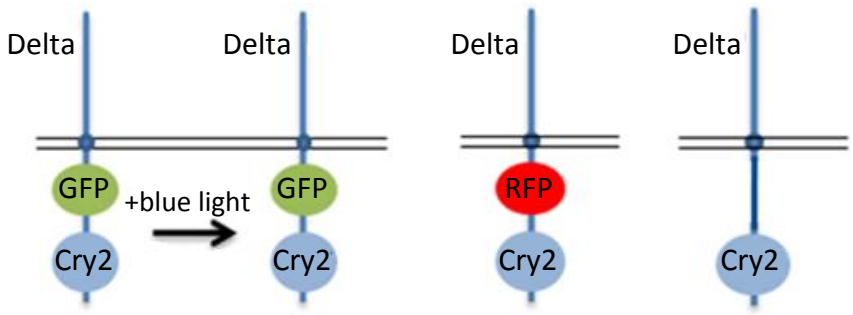


Fig 7: **Schematic of Opto-Delta constructs.** Three Opto-Delta constructs were generated, containing a Cry2 tag in the intracellular domain**.** Opto-Delta undergoes Cry2 mediated oligomerization upon blue light illumination (left).

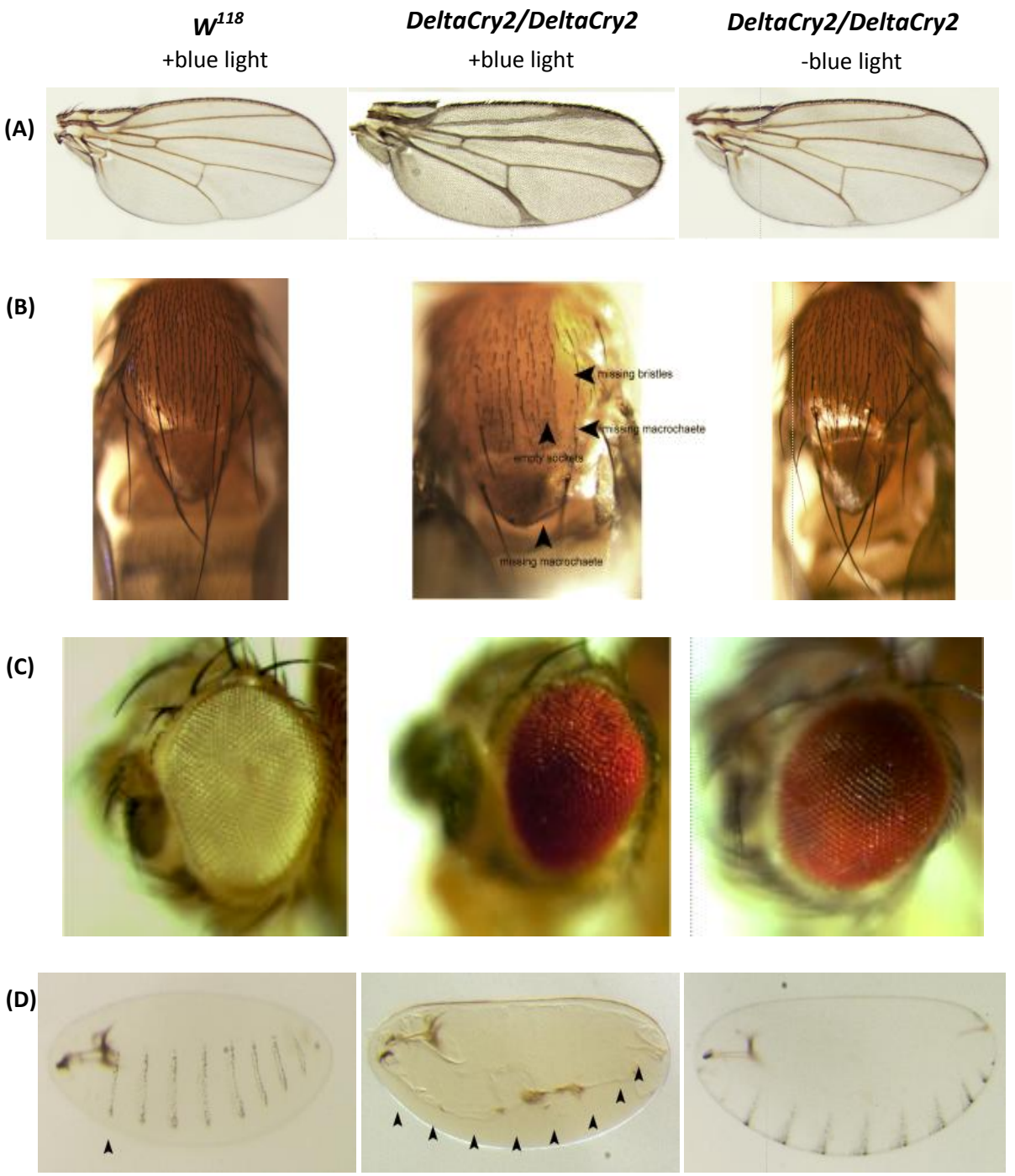


Fig 8: Photo-activation of Opto-Delta induces Notch loss of function phenotypes. Phenotypes exhibited by blue light illuminated homozygous *DeltaCry2* embryos and flies (middle column) have been compared to that of non photo-activated *DeltaCry2* (right) and a photo-activated wildtype (left). **A)** Defective wing margins. **B)** Altered density of sensory microchaetae and macrochaetae (arrowheads) at the notum. **C)** Rough eye morphology. **D)** Loss of ectodermal denticles (arrowheads) observed through embryonic cuticle preparation. This is used as a proxy for the embryonic neurogenic phenotype.

3.2. Optogenetic induction of Delta clustering

To characterize the molecular mechanisms underlying loss of function of Delta activity upon light exposure, I first decided to test whether Cry2 would induce Delta clustering. To this end, I used DeltaGFPCry2 as a proxy for DeltaCry2 and showed that a brief pulse of blue light (photo-activation) with a 488nm laser is sufficient to trigger Delta clustering at the plasma membrane ($t_{1/2}$ ~40s) in *DeltaGFPCry2/+* embryos **[Fig 9.A]**. I observed the same result in a few homozygous *DeltaGFPCry2* embryos obtained, and upon constant photo-activation under blue light for one hour, I could show using *in situ* hybridisation that the Notch target gene *single minded* (*sim*) was not expressed **[Fig 9.B]**. In contrast, *DeltaGFPCry2/+* embryos treated in a similar manner showed normal *sim* expression, which I attribute to the presence of a wild type copy of Delta. Taken together, these results strongly argue that DeltaCry2 is a blue light-sensitive loss of function allele in the homozygous state.

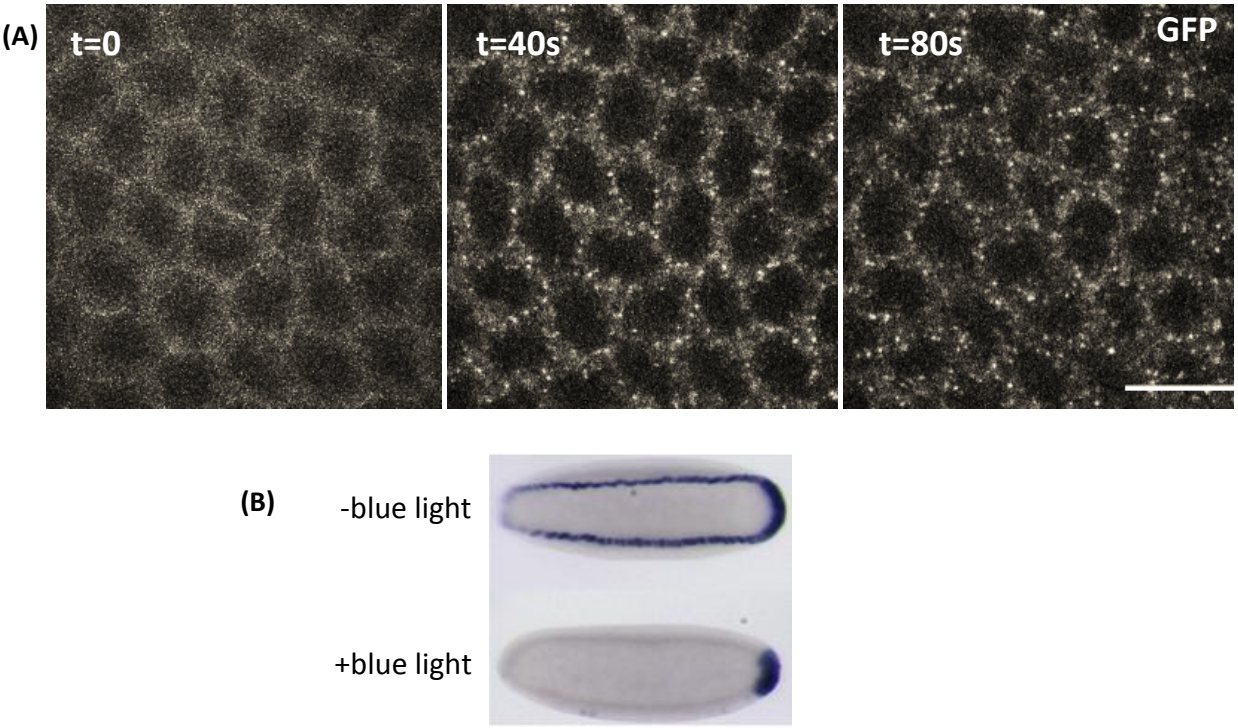


Fig 9: Characterisation of Opto-Delta constructs. A) Blue light illumination triggers Delta clustering in *DeltaGFPCry2/+* embryos. Shown in figure, mesoderm of an early cellularising embryo at 0s, 40s and 80s into photo-activation. Scale bar =10um **B)** Lack of *sim* expression in 1hr photo-activated homozygous *DeltaGFPCry2* embryos (below) in cycle 14, demonstrating that blue light illumination blocks Notch signaling.

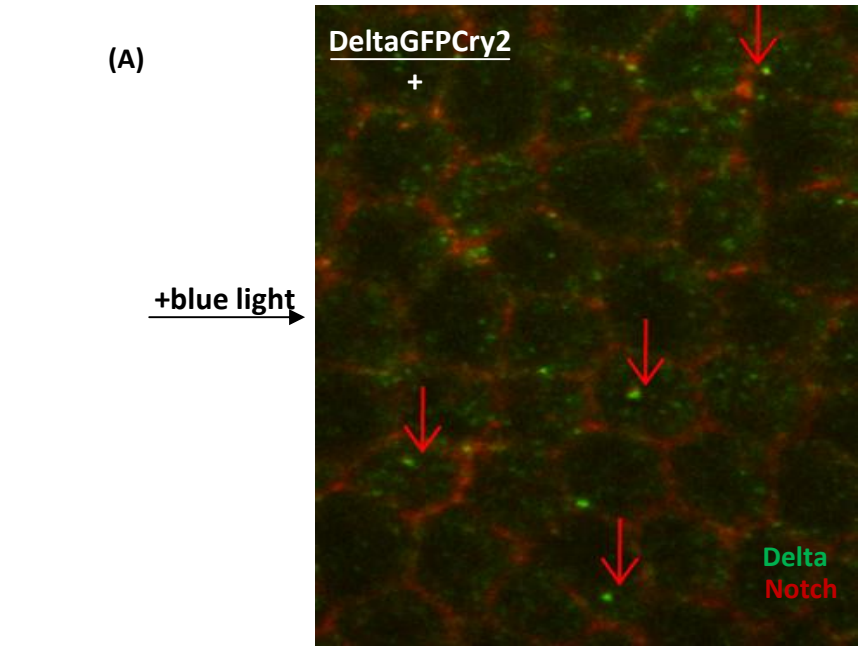
3.3. Molecular mechanism of Notch inhibition

It is well-established that the endocytosis of Delta in the mesoderm is essential for the activation of Notch signaling (Chitnis., 2012). In order to test whether optogenetically induced Delta clusters undergo endocytosis, antibody staining against GFP was carried out in 1hr photo-activated *DeltaGFPCry2/+* embryos in cellularisation. The presence of GFP positive internal clusters hinted that the clustered Delta molecules are normally endocytosed at the mesoderm **[Fig 10.A].** This was validated by co-expressing a plasma membrane marker (*sqh::GAP43 mCherry*) in *DeltaGFPCry2/+* embryos, followed by blue light illumination and imaging for six minutes using the confocal microscope **[Fig 10.B]**. Using this approach, I could successfully monitor blue light induced clusters of Delta and their subsequent internalisation in the mesoderm. I also observed that optogenetic Delta clusters got internalised more rapidly compared to the *DeltaGFP/+* control, suggesting that clustering makes Delta a potent substrate for Neuralised mediated ubiquitination and subsequent endocytosis.

The observation that signaling was inactive in homozygous *DeltaGFPCry2* embryos even when both clustering and endocytosis took place raised the question of whether Delta molecules in clusters were able to bind Notch. To address this question, I generated a fly line expressing Notch tagged with GFP and DeltaCry2-tagged with RFP *(N-; NotchintraGFP; DeltatagRFPCry2/+).* This line carries a loss of function mutation in the endogenous Notch locus and a second chromosomal insertion carrying Notch tagged with GFP in its intracellular domain. *DeltatagRFPCry2* homozygous embryos showed loss of *sim* expression, similar to homozygous *DeltaGFPCry2*, upon light exposure. However, since the maturation time for tag-RFP is very long, it was not possible to visualise RFP positive clusters at the required developmental time point but only at later developmental stages.

Using the *N-; NotchintraGFP; DeltatagRFPCry2/+* fly line, I could induce Delta clustering and simultaneously observe the behavior of NotchintraGFP. The result of this experiment demonstrated that Notch clusters in the ectoderm but not in the early mesoderm. This difference is in contrast to the presence of Delta clusters throughout the embryo **[Fig 11, 12]**.The lack of Notch clusters in the early mesoderm indicates

that the Delta clusters in the mesoderm are unable to bind Notch at the developmental stage required for *sim* activation.



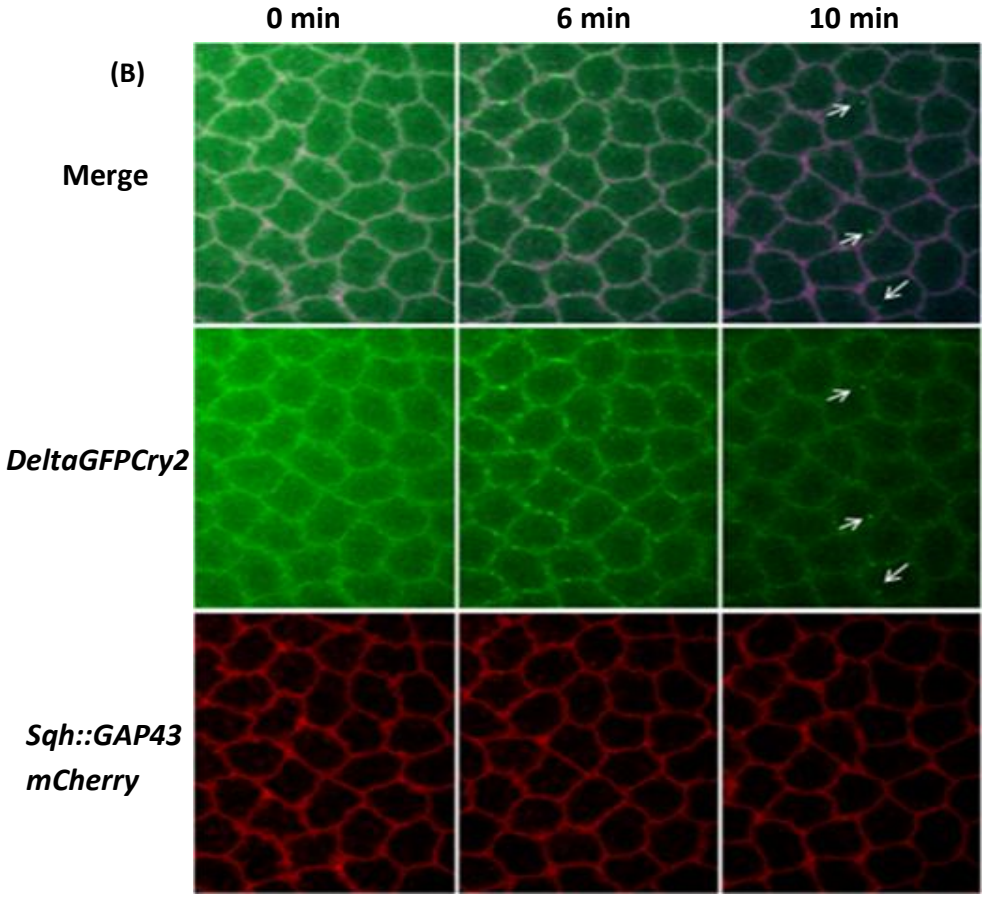


Fig 10: Endocytosis of Opto-Delta in the mesoderm. A) GFP immunolabelling (green) shows internal Delta clusters in the early mesoderm of 1hr photo-activated *DeltaGFPCry2/+* embryos. Notch ECD staining (red) shows accumulation of Notch at the membrane during this developmental time point. **B)** Live imaging of *DeltaGFPCry2/ Sqh::GAP43 mCherry* embryos at cellularisation. Blue light mediated Delta clustering and subsequent internalisation visualised at 6min and 10min into photo-activation, respectively. Arrows depict internalised Delta clusters.

To address the reason behind the inability of Delta clusters to bind Notch in the early mesoderm, I carried out a time lapse analysis (every 3 sec for 6 min) of both Notch and Delta clustering in the ectoderm of late cellularising embryos **[Fig 11.C]**. This analysis revealed that the rate of Notch clustering is delayed in comparison to Delta clustering, indicating that optogenetically induced Delta clusters have some latency in binding Notch. This, along with the earlier observation that optogenetic induction of Delta clustering makes it prone to rapid endocytosis, led us to hypothesise that Opto-Delta clusters in the mesoderm do not get sufficient time to bind Notch, owing to their rapid internalisation. In the late ectoderm, endocytosis of Delta clusters is prevented by the inhibition of Neuralized by the Bearded family of proteins. This results in the persistence of Delta clusters at the plasma membrane ensuring sufficient time for their interaction with Notch **[Fig 16]**.

We also carried out live imaging of *NotchextraYFP; DeltatagRFPCry2/+* embryos. In this line, the ECD of endogenous Notch is tagged with YFP. The embryos were photo-activated and imaged after 5 minutes. Time lapse analysis could not be carried out, owing to high rate of bleaching of YFP. Nevertheless, the pattern of Notch clustering in response to optogenetic clustering of Delta was consistent with that of *N-*; *NotchintraGFP; DeltatagRFPCry2/+* embryos **[Fig 13]**.

To further validate the live imaging data, antibody staining against Notch ECD was carried out in photo-activated *DeltaGFPCry2* homozygous embryos. As expected, Notch clusters were detected in the early ectoderm and were absent in the mesoderm **[Fig 14]**. Also, Notch ECD was found to be retained at the plasma membrane in the late mesoderm of *DeltaCry2* homozygous embryos, rather than getting removed through trans-endocytosis by Delta **[Fig 15]**.

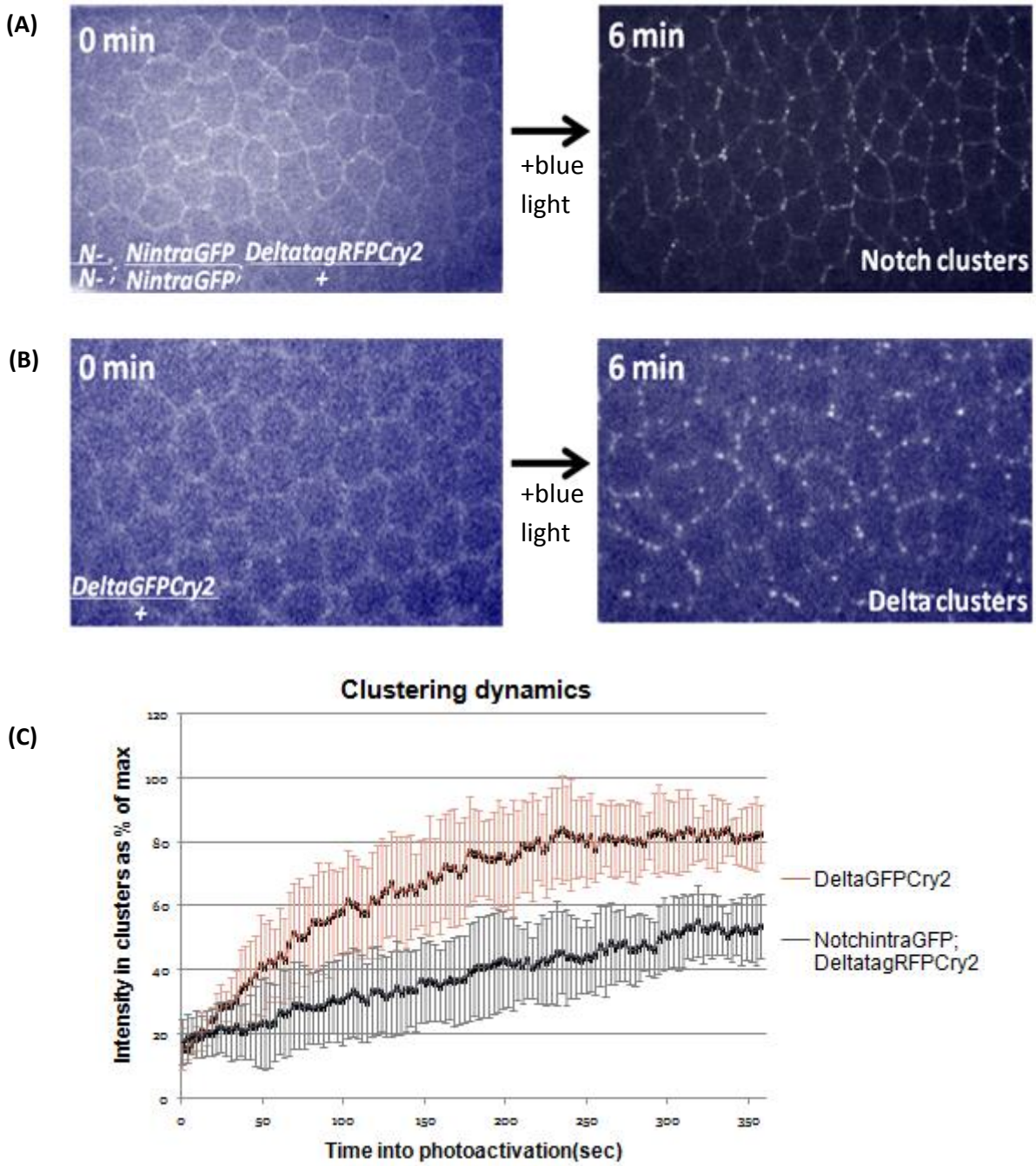


Fig 11: Dynamics of Notch clustering in the ectoderm. A) Notch clustering in response to blue light mediated Delta clustering in the ectoderm, in *N-*; *NotchintraGFP; DeltatagRFPCry2/+* embryos visualised using the 488nm laser. **B)** Blue light mediated Delta clustering in the ectoderm of *DeltaGFPCry2/+* embryos. **C)** Time lapse analysis of Notch-Delta clustering in the ectoderm. Note the difference in kinetics of Notch clustering (black) compared to Delta (red) (P value<0.05).

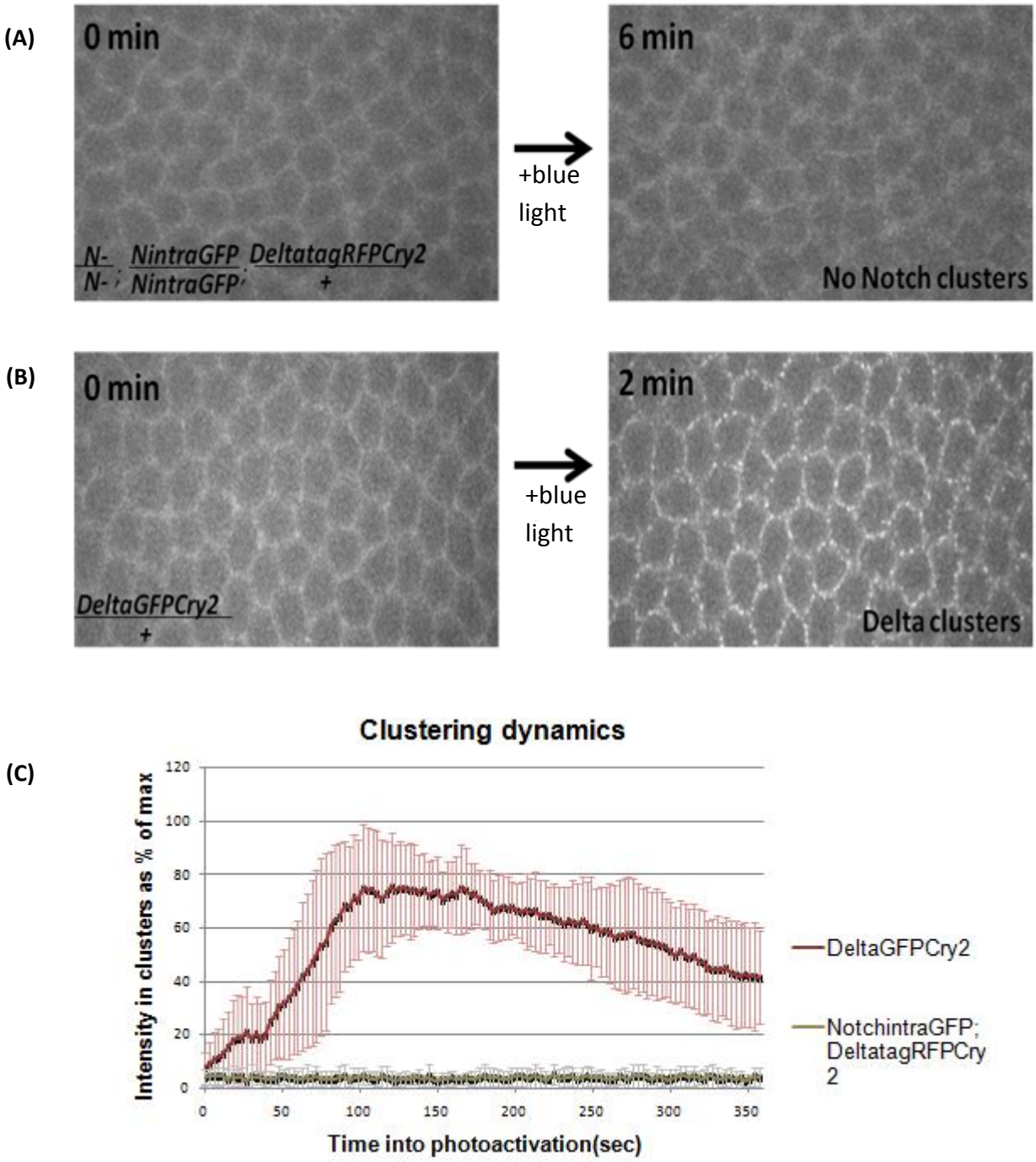


Fig 12: Absence of Notch clustering in the mesoderm. A) Lack of Notch clustering in response to blue light mediated oligomerization of Delta in the early mesoderm of *N-* ; *NotchintraGFP; DeltatagRFPCry2/+* embryos. **B)** Delta clustering in the mesoderm of *DeltaGFPCry2/+* embryos in response to photo-activation. **C)** Time lapse analysis of Notch-Delta clustering in the mesoderm. Delta graph (red) shows a decrease in the number of detectable clusters after 2 min, which we attribute to endocytosis and cluster disassembly.

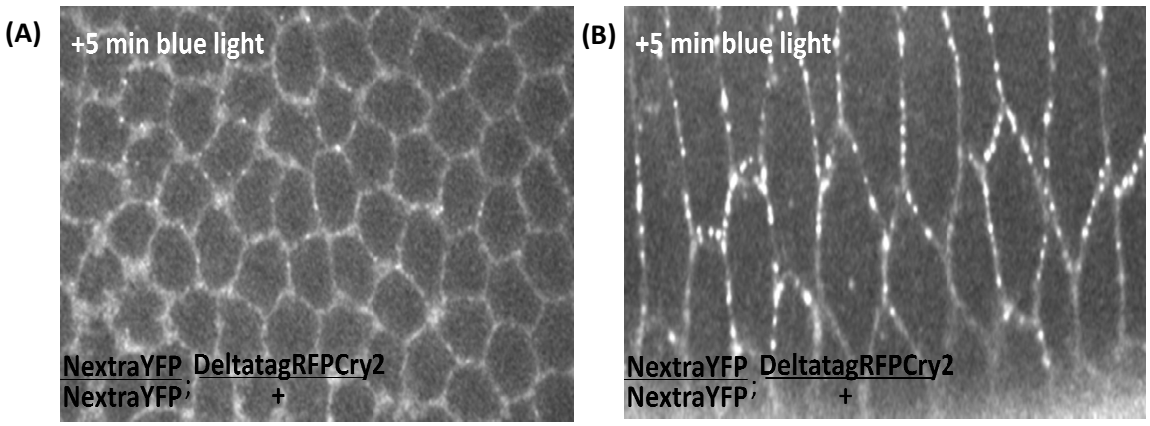


Fig 13: Validation of Notch clustering responses. A) Absence of Notch clustering in the mesoderm of *NotchextraYFP; DeltatagRFPCry2/+* embryos even after 5min of photo-activation using the 488nm laser. **B)** Notch clusters are however formed in the lateral ectoderm.

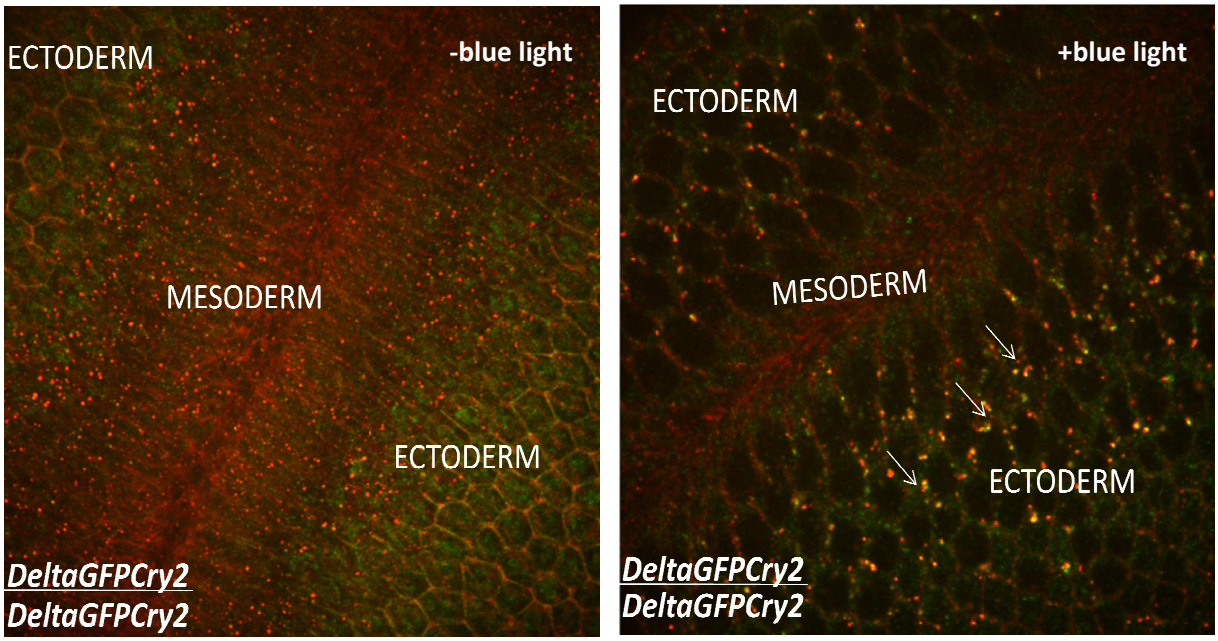


Fig 14: Notch ECD immunolabelling validates live imaging results. Presence of Notch clustering (yellow) in the lateral ectoderm of 1hr photo-activated *DeltaGFPCry2* homozygous embryos (right) at late cellularisation. A non photo-activated embryo of the same genotype has also been shown (left). DeltaGFPCry2 is stained in green (anti-GFP)

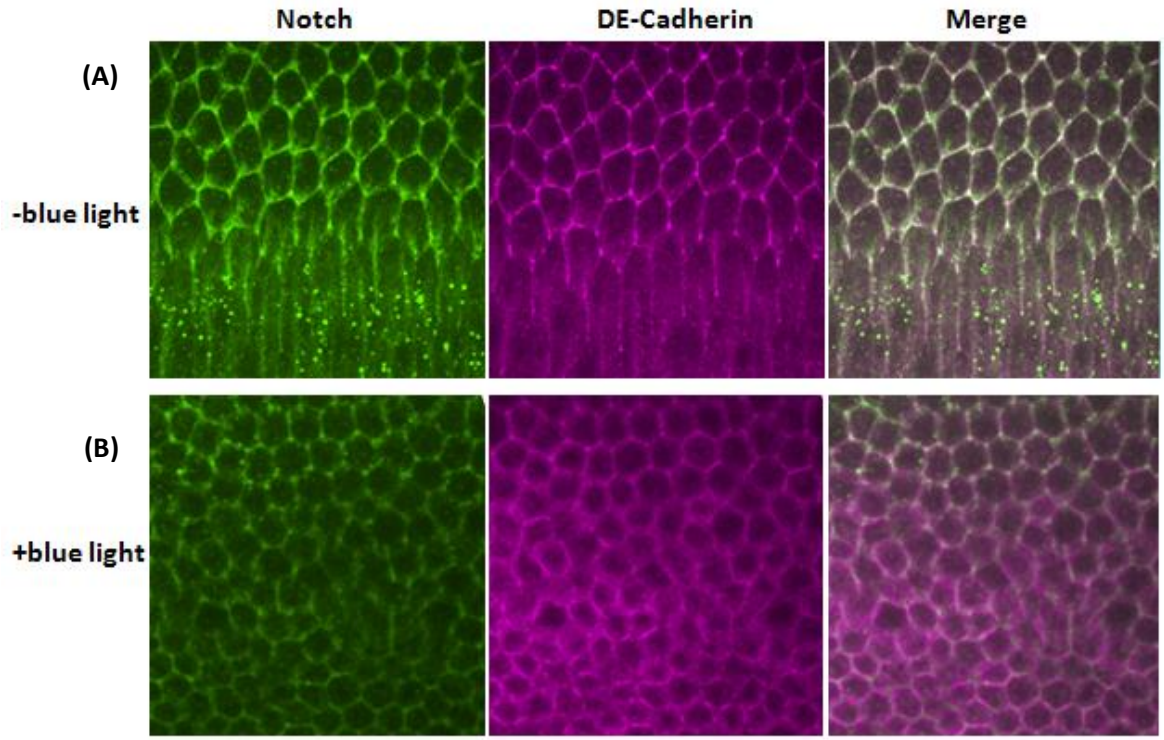


Fig 15: Retention of Notch ECD at the mesoderm membrane upon photo-activation. A) The late mesoderm view of a non photo-activated *DeltaCry2* homozygous embryo, stained for Notch ECD (green). Note the presence of internal Notch staining. These are Notch ECD being removed from the membrane through trans-endocytosis by Delta into the neighboring cells. DE-Cadherin (purple) serves as a membrane marker. **B)** The late mesoderm view of a 1hr photo-activated *DeltaCry2* homozygous embryo. Notch ECD is retained at the membrane instead of being trans-endocytosed, demonstrating the inability of Delta clusters in binding Notch.

The live imaging and immunostaining results are thus consistent with my hypothesis for mechanism of light mediated inhibition of Notch activity using Opto-Delta **[Fig 16]**. A definitive proof to this hypothesis would be to monitor Notch clustering in a *neuralized* mutant background. If my hypothesis is correct, Delta clusters should persist at the plasma membrane of mesodermal cells in the absence of Neuralized activity. This in turn should provide sufficient time for Notch to interact with Delta and therefore, one would expect to see Notch clusters in the mesoderm, similar to what has now been observed in the lateral ectoderm.

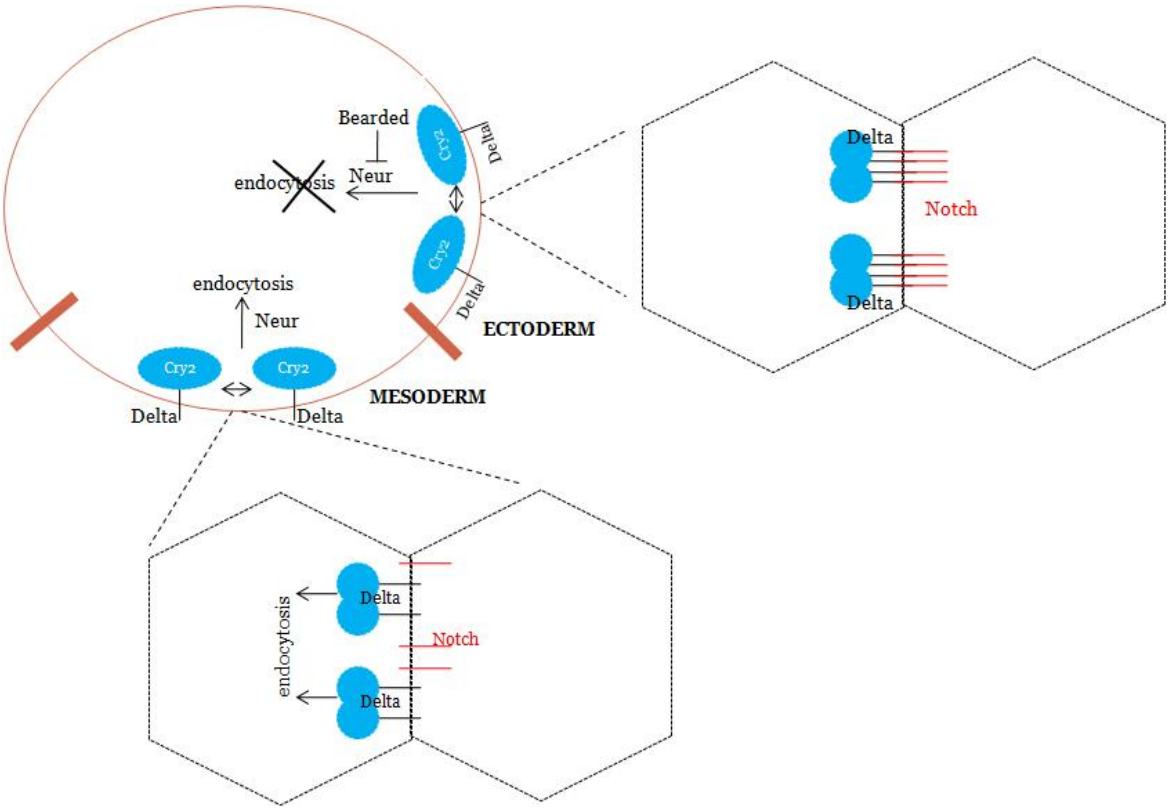
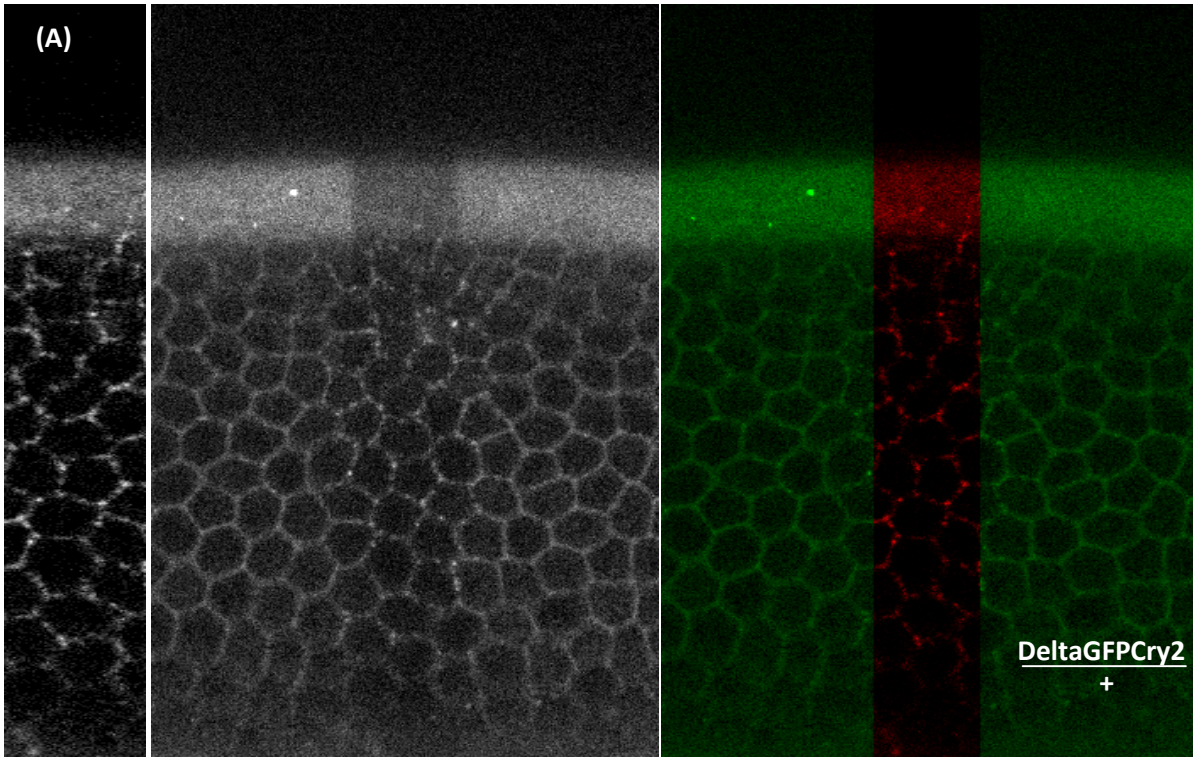


Fig 16: Schematics for mechanism of Notch inhibition. Opto-Delta clusters are potent substrates for Neuralized mediated ubiquitination and endocytosis. Rapid internalisation prevents these clusters from binding Notch in the mesoderm. Hence, Notch clusters are not observed in the mesoderm. Inability of Delta in binding Notch at the mesoderm results in the inhibition of *sim* expression during mesectoderm specification. However, in the ectoderm, inhibition of Neuralized by Bearded proteins facilitates retention of Delta clusters at the membrane, which are now able to bind and cluster Notch.

3.4. Tools to understand Notch signaling dynamics

Having thus understood how Cry2 mediated Delta clustering disrupts Notch activation, the next step was to use this tool to investigate the spatio-temporal dynamics of Notch signaling. In this regard, I first demonstrated the use of two photon lasers in inducing Opto-Delta clustering within a narrow stripe across *DeltaGFPCry2/+* embryos **[Fig 17.A]**. Thereafter, I developed a two photon activation protocol (see Materials and Methods) to locally photo-activate homozygous *DeltaCry2* embryos throughout cellularisation without inducing toxicity. *In situ* against

single minded in these embryos showed inhibition of *sim* expression in the narrow region, thereby demonstrating that it is possible to inhibit Notch signaling with even sub-cellular precision **[Fig 17.B]**.



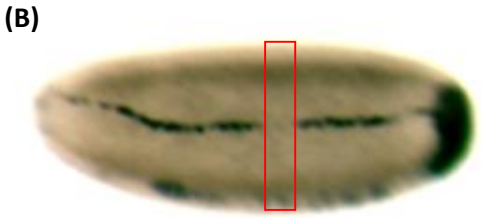


Fig 17: Inhibition of Notch signaling with sub-cellular precision. A) Photo-activation of *DeltaGFPCry2/+* embryos in a thin stripe using the two-photon laser. Image after 6 minutes of local photo-activation (left) and a subsequent global image (middle) have been shown in figure. The overlay (right) demonstrates absence of unwanted photo-activation through scattering and thus restriction of Delta clustering with high spatial precision. **B)** *In situ* hybridisation against *single minded* in locally photo-activated (throughout cellularisation ~40min) *DeltaCry2* homozygous embryos shows inhibition of Notch activity in the illuminated stripe (red box).

A key step towards understanding the spatio-temporal dynamics of Notch signaling was to monitor Notch activity in real-time. In this regard, I utilised the MCP-MS2 system to visualize live sim mRNA expression. This system allows the fluorescent labelling of nascent mRNA transcripts *in vivo*. It relies on the interaction between a GFP tagged MS2 coat protein (MCP) and the RNA of interest, modified by the introduction of 18-24 bacteriophage MS2 stem loops in the 5' or 3' UTR (Bertrand et al., 1998).

For my purposes, I used the *sim MS2* construct, which expressed sim mRNA modified by 24 stem loops in its 5'UTR. Activation of Notch signaling induces sim mRNA production from mid cellularisation in the mesectoderm. In *MCPGFP/simMS2* embryos, maternally deposited MCPGFP interacts with the sim MS2 stem loops and produces a bright punctuate nuclear signal in all the cells at this boundary **[Fig 18]**.

I then generated a fly line of the genotype: *MCPGFP; DeltaCry2* and crossed the females to *simMS2* males. The embryos from this cross were photo-activated throughout cellularisation using the 488nm confocal laser. A 25µm deep z stack at the beginning of gastrulation demonstrated the absence of MCPGFP coated sim mRNA, in contrast to the presence of punctuate GFP signals in persistently photo-activated *MCPGFP/simMS2* controls **[Fig 19]**. This indicates that continuous blue light illumination disrupts the activation of Notch signaling in *MCPGFP; DeltaCry2* embryos. This result was further validated by *in situ* hybridisation against *single minded* in 1hr photo-activated embryos of the same genotype. The embryos from this line also developed a strong neurogenic phenotype upon continuous photo-activation.

I have thus generated a system which can be used to investigate the transcriptional dynamics of *sim* activation. In this regard, an immediate step will be to demonstrate in real-time, the local inhibition of Notch signaling in *MCPGFP; DeltaCry2/simMS2* embryos using the two-photon microscope, followed by an experiment in which Delta clustering is induced locally from different time points during cellularisation. This will allow us to determine whether the Notch signaling module is an on/off switch and if so, the precise time point at which Notch mediated transcriptional switch occurs. Spatial regulation will enable the comparison of sim transcript levels inside the stripe with the region outside, thereby providing an ideal control.

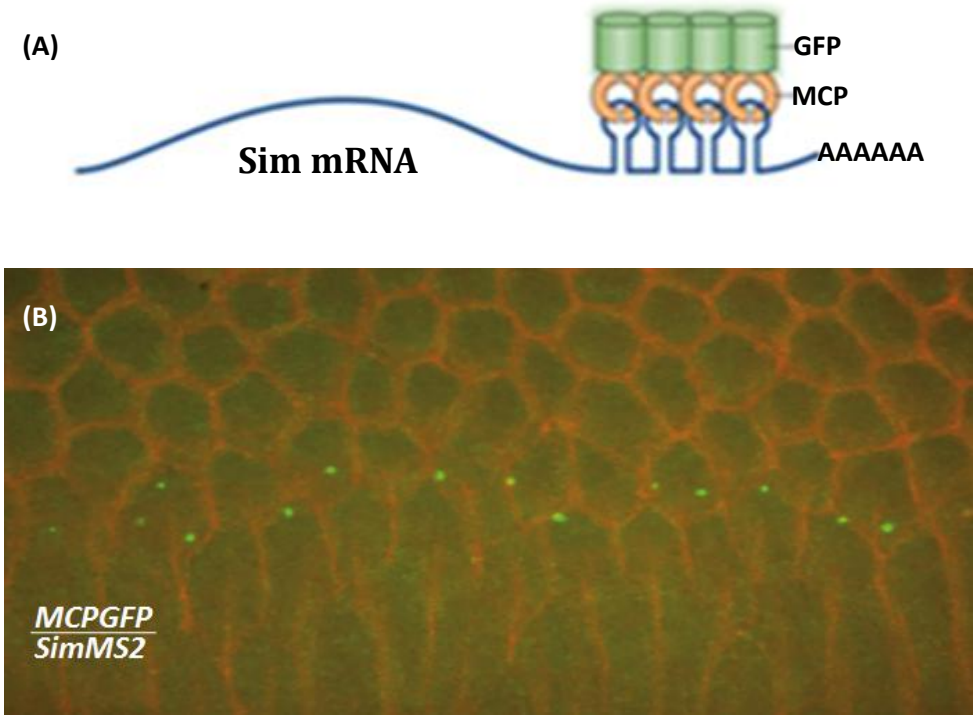


Fig 18: Real-time reporters of Notch activity. **A)** Schematics of the MCP-MS2 system (adapted from Adina et al., 2015). **B)** Live imaging of sim mRNA (green) in *MCPGFP/simMS2* late cellularising embryos. Sqh: GAP43-mCherry (red) serves as a membrane marker.

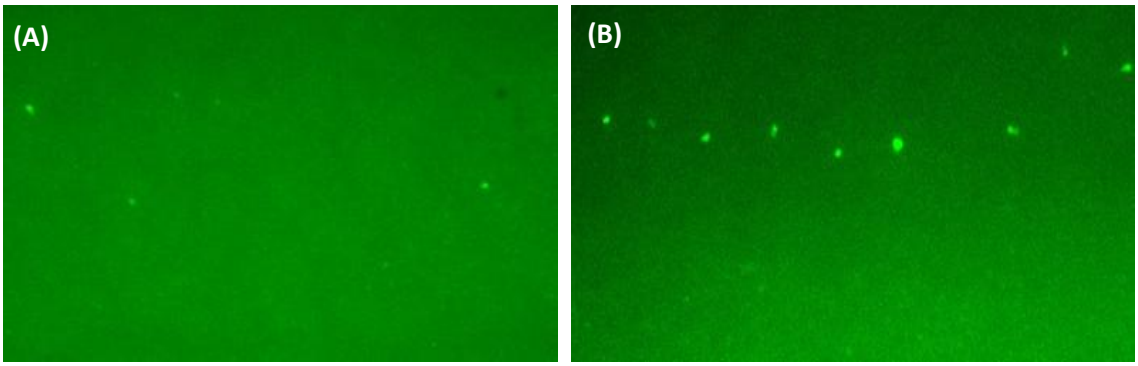


Fig 19: Live demonstration of Notch activity inhibition. A) Persistent blue light illumination inhibits expression in *MCPGFP; DeltaCry2/simMS2* embryos during cellularisation. **B)** Notch activity remains unaffected in similarly treated *MCPGFP/SimMS2* controls.

Since the strength of the signal is proportional to the number of sim transcripts produced, implementing a temporal control over Delta clustering would also allow us to examine how Notch levels correlate with the dynamics of *sim* expression, in case signaling is found to be active throughout cellularisation. Furthermore, the spatio-temporal precision provided by this system can be exploited for involved experiments such as to understand the relationship between Notch signaling and cellular architecture.

Taken together, my results demonstrate the use of a Cry2 tagged light sensitive allele of Delta to modulate endogenous Notch signaling with high spatial and temporal precision. Opto-Delta can now be used in combination with live transcriptional reporters of Notch activity to gain a quantitative understanding of the input-output relationship underlying Notch signaling. The effectiveness of Cry2 mediated clustering in downregulating ligand activity *in vivo* can also be exploited to modulate other signaling pathways and thus greatly enhance our understanding of animal development and cancers.

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