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Investigating the Regulation of Notch Signalling in Drosophila by Trxr-1 gene

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BACHELOR THESIS

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Annotation

In this work, regulation of Notch signalling by *Trxr-1* gene in the *Drosophila* wing disc was monitored using overexpression constructs or inducing mutant clones in the wing discs. The effect on Notch target gene expression was assessed by immunostaining.

Affirmation

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagerism detection system.

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Abstract

In this thesis we investigated the relationship between Notch signalling and cellular redox state, mediated by the Drosophila *Trxr-1* gene. As the enhancer trap line CPTI-000915 showed significant similarity in the expression profile to the Notch target gene *cut*, we decided to test if *Trxr-1* might be involved in the regulation of the Notch pathway activity. We looked at the effects of an overexpression of the mitochondrial and cytoplasmic isoforms of *Trxr-1* on the Notch target genes *cut* and *wingless* in the wing discs by immunostaining. Further, we recombined *Trxr-1* alleles on an FRT chromosome and created mutant clones of cells in the wing discs to again assess the expression of the Notch target genes in these clones. We did not see any effect of *Trxr-1* on the Notch target genes *cut* and *wingless* in either of the approaches. We discuss possible explanation for these observations as well as suggestions for future experiments to investigate the relationship between the *Trxr* genes and the Notch pathway.

List of Abbreviations:

ADAM metalloprotease= A Disintegrin And Metalloprotease

BSA = Bovine serum albumin

CSL transcription factors = CBF1/RBPJ-kappa – Suppressor of hairless – Lag1

DAPI = 4',6-diamidino-2-phenylindole

DV = dorsal-ventral

en = engrailed promoter

FAD = flavin adenine dinucleotide

FLP = Flippase recombination enzyme

FRT = Flippase Recognition Target

GDP = Guanosine diphosphate

GFP = green fluorescence protein

GRX = glutaredoxin

GTP = Guanosine-5'-triphosphate

iPAC = interactome parallel affinity capture

MAM = Mastermind

MIPEP = mitochondrial intermediate peptidase

NADH/NAD+ = Nicotinamide adenine dinucleotide

NADPH/NADP+= Nicotinamide adenine dinucleotide phosphate

NEXT = Notch extracellular truncation

N^{ICD} = Notch intracellular domain

NO = nitric oxide

PBS = Phosphate buffered saline

PCR = Polymerase Chain Reaction

RNAi = RNA interference

ROS = reactive oxygen species

SDS = Sodium dodecyl sulphate

Trx = thioredoxin

TrxR = thioredoxin reductase

UAS = Upstream Activation Sequence

YFP = Yellow fluorescent protein

1. Introduction

1.1. Notch signalling

Notch signalling plays an important role in cell development and adult tissue homeostasis in metazoan organisms. The misregulation of Notch signalling can cause several human disorders including developmental syndromes, adult onset diseases and cancer (Kopan and Ilagan, 2009).

The start of the signalling is when the transmembrane ligands bind to the Notch receptors of the neighbouring cells. Then the process of regulated intramembrane proteolysis begins. A metalloprotease called ADAM cleaves the Notch receptor at the juxtamembrane extracellular site (S2) and generates the membrane-anchored Notch extracellular truncation (NEXT) fragment. Then the NEXT fragment is cleaved by γ-secretase at two other places (S3 and S4) and the Notch intracellular domain (N^{ICD}) is released(Kopan and Ilagan, 2009). A cleavage at an additional, S5, site was recently described that can happen in mitochondria by the mitochondrial intermediate peptidase (MIPEP) and which regulates cell apoptosis by an unknown mechanism (Lee et. al., 2011). Subsequently, the N^{ICD} generated in the cytoplasm translocates into the nucleus and associates into a transcriptional activation complex that is recruited by the CSL transcription factors (CBF1/RBPJ-kappa – Suppressor of hairless – Lag1, Gordon et al., 2008). According to the textbook model, in the absence of signalling CSL binds to the enhancers of Notch target genes but recruits corepressor complexes that prevent gene transcription. N^{ICD} works as a switch for CSL to release the repression and allow gene activation. The molecular surroundings which are present before N^{ICD} arrives determine the available targets for CSL (Kopan and Ilagan, 2009).

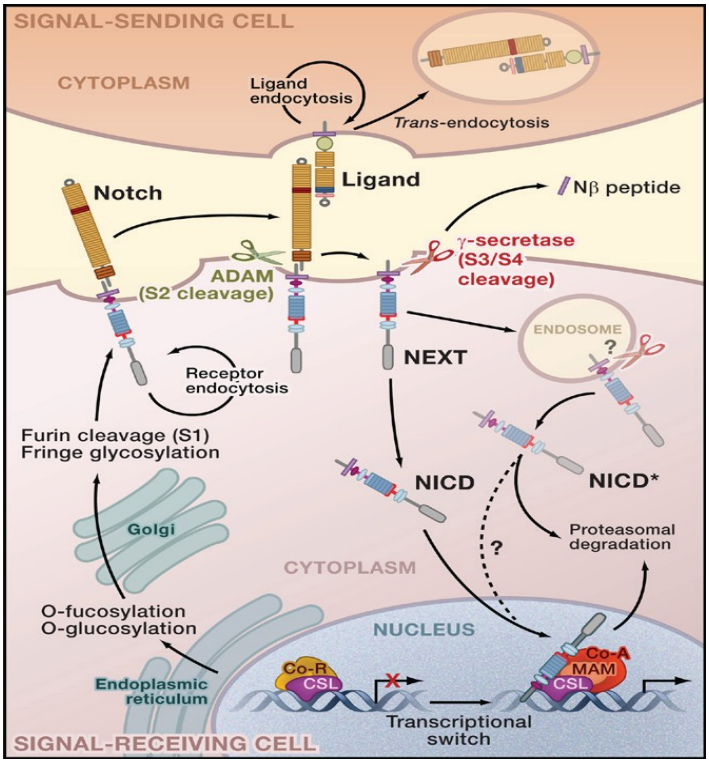


Figure 1: Scheme of the Notch signalling pathway (from Kopan and Ilagan, 2009). After translation the Notch receptor protein is glycosylated and then at site 1 (S1) proteolytically cleaved by PC5/furin. This heterodimer is then moving to the cell surface. There the availability of the receptors and ligands is influenced by endocytosis. The binding of the receptor to the ligand elicits several steps of cleavage: Firstly at the site 2 (S2) by ADAM metalloproteases and then the membrane-anchored Notch extracellular truncation (NEXT) fragment is formed and cleaved at S3 to S4 to release the Notch intracellular domain (N^{ICD}) by γ-secretase. N^{ICD} is then targeted to the nucleus and forms a ternary activation complex with CSL and Mastermind (MAM).

Notch signalling is often used to determine between two alternative cell fates like in lateral inhibition, in the cell lineage specification or during the formation of boundaries (Bray S., 2006). In the case of lateral inhibition, a group of cells has the same developmental potential but only a few of them adopt the potential. By using Notch signalling those 'winning' cells suppress the same fate in the others by enhancing their expression of Notch ligands and over-activating Notch pathway in the surrounding cells. Lateral inhibition happens for example in the selection of a sensory organ precursor in insect neurogenesis. Furthermore, Notch plays a crucial role in the asymmetric cell fate assignation, which is dictated by the asymmetric distribution of negative Notch regulator Numb into the daughter cells (Fiuza and Martinez Arias, 2007).

Another very important function of Notch signalling is the boundary formation. During *Drosophila* wing development the O-fucose on the Notch receptor is extended by the Fringe protein which changes the binding of Notch to its two ligands Delta and Serrate. It increases the binding to Delta but decreases the binding to Serrate. In the larval wing imaginal disc there is a dorsal and a ventral compartment and the border between those two is called dorsal/ventral boundary. In the dorsal compartment Fringe and Serrate are expressed and therefore dorsal cells near the ventral compartment have more Delta-to-Notch signalling from the ventral cells which express Delta. The opposite effect is true for the ventral cells which then experience higher Serrate-to-Notch signalling. This generates a line of strong Notch activation at the dorsal/ventral boundary (Fortini, 2009). Typical Notch target genes expressed at this stage at the DV boundary are the genes *cut* and *wingless*.

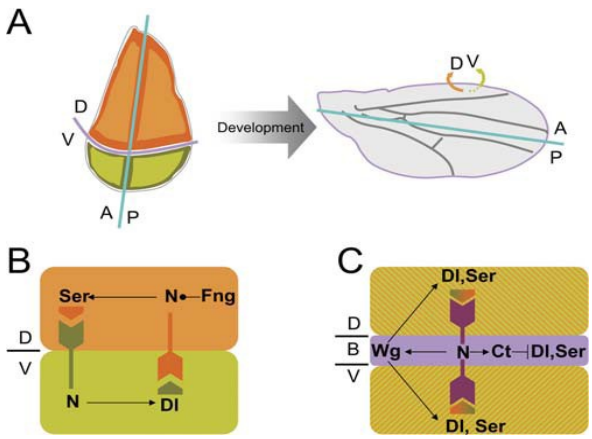


Figure 2: Illustration of the dorsal/ventral boundary formation. A The larval wing imaginal disc (left) is divided horizontally into dorsal (D) and ventral (V) compartments as well as vertically in anterior (A) and posterior (P) compartments which are important for the development of the adult wing (right). B shows the early developmental stage where Serrate (Ser) sends a signal to V cells to activate Notch (N). Simultaneously Delta (Dl) signals to D cells to activate Notch modified by Fringe (Fng) along the dorsal/ventral boundary. C illustrates a later developmental stage where ligand expression is symmetric with respect to the boundary. The positive feedback-loop between Wingless (wg) and Ser/Dl-expressing cells sustains the signalling centre along the dorsal/ventral boundary. The expression of Cut (Ct) inhibits Ser and Dl in boundary cells (Buceta et. al., 2007).

1.2. Cellular redox state and its role in cell signalling

The redox state of a redox couple is defined by the ratio of its reduced and its oxidized form. In cells there are many linked redox couples and therefore it is often referred to as redox environment, which describes the overall reduction potential and reductive capacity of the different couples in the cell (Schafer and Buettner, 2001)

The redox state changes with the amount and type of metabolic fuels and with oxygen supply. Cells have a high reductive capacity in the fed state with abundant fuel and oxygen, and therefore there is also a high ratio of the reduced to oxidized forms of nicotinamide adenine dinucleotides phosphates (NADPH : $NADP^+$) (Agarwal A.K and Auchus R.J., 2005). The pentose phosphate pathway or the tricarboxilic acid cycle are the best known sources for maintaining a high cytosolic NADPH/$NADP^+$ gradient. As NAD^+ being an important electron acceptor for several dehydrogenase reactions cells also need to keep an inverse gradient of NADH/NAD^+. This is usually achieved by the constant removal of electrons from NADH which are used in the mitochondrial electron transport chain (Agarwal A.K. and Auchus R.J., 2005).

However, not only the redox couples which are present at high concentrations like NAD(P)H/NAD(P)+ or malate/oxalacetate influence the redox state of a system. There are also less abundant redox couples that are affected. For example, some transcription factors and cofactors are regulated by NAD(P)H/$NAD(P)^+$ binding. Furthermore, specialized enzymatic functions like synthesis or degradation of biomolecules depend on intermediary metabolism, redox state and the supply of cofactors. Prosthetic groups like iron-sulphur clusters, iron-protoporphyrins (hemes), and flavins (flavin adenine dinucleotide and flavin mononitrate) can serve as sensors, which are most commonly reduced by the electrons from NADPH with a two-electron hydride transfer or with two discreet one-electron steps (Agarwal A.K. and Auchus R.J., 2005).

Imbalances in the cellular redox state can have profound effects on cell signalling also via the production of reactive oxygen species (ROS). ROS covalently modify proteins and other cellular component having adverse effects on cell homeostasis and signalling. Especially the reactions with cysteine residues are of importance in redox mediated signalling as there are many examples of redox sensitive transcription factors or signalling proteins whose activity depends on the formation of disulphide bonds in their structure.

Here the ROS attack the thiol group (-SH) forming a highly reactive sulphenic acid (-SOH). This then forms a disulphide bond upon condensation with a close -SH or a sulphenamide with a close nitrogen. If H_2O_2 is present the sulphenic acid is further oxidized to sulphinic ($-SO_2H$) or sulphonic (SO_3H) acid. Any of these modifications may potentially alter the conformation of the polypeptide and thus influence its function. It should be noted that -SOH is able to be reversibly re-reduced by condensation with a donor -SH where a disulphide bond is formed. Disulphide bonds created either way can be reduced by thioredoxin (Trx) or glutaredoxin (Grx) and thus reverse the effect of ROS. Trx or Grx are then regenerated by their reduction mediated by NADPH-dependent Trx- and glutathione reductases (D'Autréaux and Toledano, 2007).

1.3 Thioredoxin reductases

The thioredoxin system plays an important role in antioxidant defence, cell proliferation and redox-regulated signalling cascades. Both redox active thioredoxins (Trx) and thioredoxin reductases (TrxR) are part of the system. The TrxR are needed to reduce the active site of the disulphide present in the oxidized Trx to a dithiol by using NADPH and therefore the Trx is completely dependent on TrxR (Figure 4, Arnér, 2009). Although thioredoxin reductases are found in all organisms, there are many differences between the enzymes in lower and higher organisms, for example size, substrate specificity and redox active motifs (Arnér and Holmgren, 2000). In mammals both glutathione reductase and thioredoxin reductase are responsible for antioxidant defence whereas in *Drosophila* only the TrxR system is present. Another difference is that the mammalian TrxRs are selenoproteins using NADPH to reduce protein and non-protein substrates in mitochondria and cytoplasm of the cells. (Missirlis et al., 2001)

Drosophila has two TrxR genes, Trxr-1 and Trxr-2. The Trxr-1 encodes three non-selenocysteine-containing isoforms of Trxr, one cytoplasmic (TrxR-$1^{cyto,}$,Trxr-1-RA), one mitochondrial (TrxR-1^{mito},Trxr-1-RB) and one recently described variant with unknown localization (transcript Trxr-1-RC) (http://flybase.org). The isoforms differ in their N-termini and have cysteine residues instead of selenocysteine ones at the C-termini. Although the mitochondrial and cytoplasmic TrxR isoforms have been shown to have similar biochemical properties, their biological functions are not interchangeable (Missirlis et al., 2001).

Based on homology, there is a second TrxR gene in Drosophila, Trxr-2, which has one annotated transcript (http://flybase.org/reports/FBgn0037170.html). There are no alleles available except a weak RNAi line which we tested in our experiments (see the Discussion).

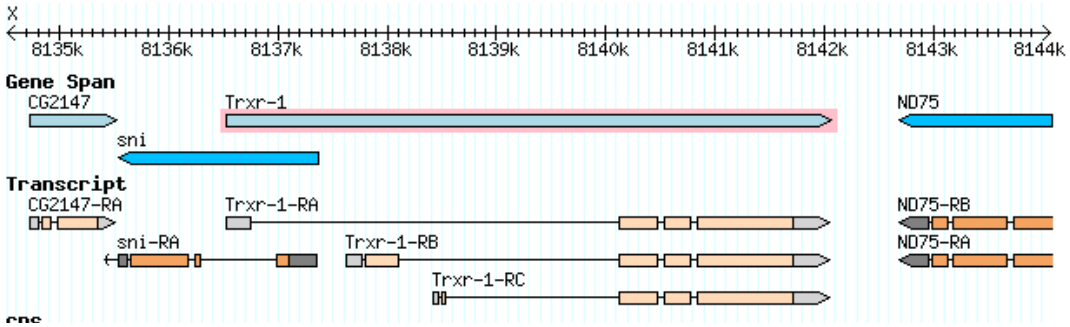


Figure 3: Structure of the *Drosophila* Trxr-1 gene (http://flybase.org/reports/FBgn0020653.html)

The TrxRs belong to the family of the disulphide reductases, which are all homodimers and every monomer is consisting of a flavin binding domain, an interface domain and a pyridine nucleotide binding domain. The enzyme has a N-terminal redox-active disulphide next to FAD and a redox-active C-terminal cysteine pair from the other subunit. In catalysis the reducing equivalents go from NADPH to FAD, which then reduces the N-terminal disulphide, then from the newly formed dithiol pair to the C-terminal redox pair on the other subunit and eventually to the Trx (Bauer et al., 2003).

In Figure 4 the mechanism of the oxidoreductase activities carried out by the thioredoxin system is depicted.

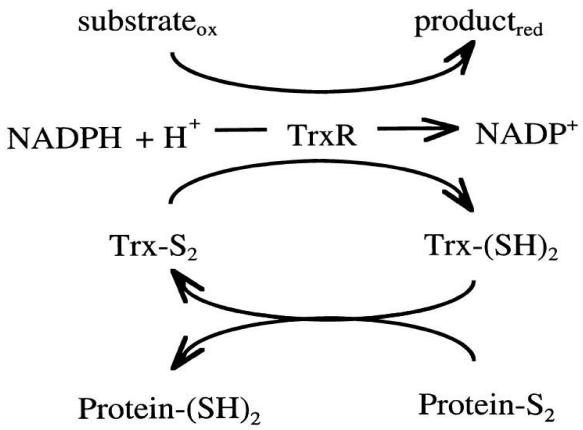


Figure 4: Illustration of oxidoreductase activities of the Trx system. By thioredoxin reductase (TrxR) and NADPH the disulphide in the oxidized thioredoxin (Trx-S_2) is reduced to a dithiol. Subsequently the reduced thioredoxin, Trx-$(SH)_2$ reduces the protein disulphides and is itself

oxidized back to Trx-S_2. Further it is shown that TrxR can also reduce other oxidised substrates than Trx (Arnér and Holmgren, 2000).

There are many different functions of thioredoxins varying in different organisms, which have developed from elementary processes to numerous highly specialised ones. As so far known all organisms need the Trx for DNA synthesis as a hydrogen donor for ribonucleotide reductases (Arnér and Holmgren, 2000).

Furthermore it is also involved in the antioxidant defence system by reducing peroxiredoxins which are essential antioxidant proteins and catalyse the reduction of H_2O_2. Another important Trx-dependent enzyme is the methionine sulphoxide reductase which repairs oxidatively damaged methionine residues of proteins. The mammalian Trx is also supporting some low molecular weight anti-oxidants like ascorbate (Vitamin C), tocopherol (Vitamin E) and ubiquinone (Q10). There are several functions of Trx in the redox-sensitive signalling pathways. Recently a dependence on Trx of potassium channels, cell membrane receptor signalling and protein phosphorylation cascades was observed. Furthermore thioredoxins regulate several transcription factors and are involved in nitric oxide signalling cascades (Arnér, 2009). The eukaryotic cytoskeleton structure is also dependent on Trx, which is balancing the actin and tubulin polymerization. (Arnér, 2009). The Trx system is also related to apoptosis, e. g. Thioredoxin-$(SH)_2$ forms a complex with ASK1 preventing downstream signalling for apoptosis) (Arnér and Holmgren, 2000).

In humans the Trx system may be involved in several diseases as in a number of cancers, where it is highly upregulated. In cell lines and human cancer tumors a strong correlation between the Trx overexpression and the overall tumor aggressiveness was observed. Further it is involved in Alzheimer's disease, hyperoxic lung injury, Cataract, HIV infection but it also plays a role in male infertility, embryonic development as well as ageing (Arnér, 2009).

1.4. Trxr-1 as a possible regulator of the Notch pathway in the wing disc

Johanna S. Rees together with her colleagues from the University of Cambridge in the UK created a collection of several hundreds transgenic flies with the insertion of a construct into the coding region which created fusion proteins marked by Flag, Strep II and Yellow fluorescent protein (YFP) tags (http://xena.gen.cam.ac.uk/flannotator/index.php). Their primary aim was to

express these *in vivo* in the *Drosophila* embryo under the control of their endogenous promoters and use them subsequently for the purification of native protein complexes associated with fusion protein of interest. They called their method interactome parallel affinity capture (iPAC) coupled to mass spectrometry (Rees JS, 2011)

However, these lines can also be viewed as YFP traps to study the expression pattern of certain genes of interests. In other words they can be used as reporters of the endogenous gene expression that can be followed in vivo.

We noticed that one of these YFP trap lines has shown significant similarity in the expression profile to the Notch target gene *cut*. It was the line CPTI-000915, where the YFP is inserted in the first exon of the gene Trxr-1. It was shown to be expressed ubiquitously in the whole organism but remarkably, there seemed to be a region of stronger expression at the dorso/ventral boundary in the wing discs where the Notch pathway is most active. We reasoned that if a gene expression correlates in place and time with the activity of Notch pathway it might be involved in the regulation of the pathway activity. Therefore we decided to either overexpress the mitochondrial and cytoplasmic isoform of Trxr-1 in the wing discs or create clones mutant for Trxr-1 gene and then look on the effect on Notch target genes cut and wingless.

2. Aims

- Verify the expression pattern of Trxr-1 YFP trap line CPTI-000915.

- Look at the effect of Trxr-1 overexpression on selected Notch target genes in the wing disc.

- Recombine Trxr-1 alleles on an FRT chromosome, generate mutant clones of cells in the wing discs and assess the expression of selected Notch target genes in these clones.

3. Materials and Methods

3.1 Trxr-1 reporter line

There are three isoforms of Trxr-1 gene. The RA transcript codes for the cytoplasmic isoform of the protein, the RB codes for a mitochondrial isoform and so far nothing is known about the localization of the protein coded by the recently identified RC transcript. The CPTI-000915 line was obtained from Benedict Sanson from Cambridge where the YFP cassette was inserted in the first intron common to all three transcript and therefore should report the expression of all the three isoforms.

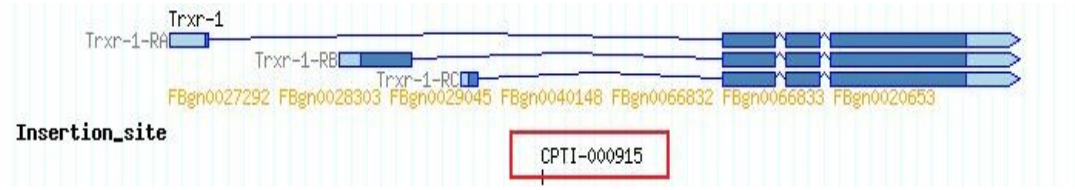


Figure 5: YFP insertion site according to http://www.flyprot.org/stock_report.php

Recently, another YFP trap line has become available from the same source, CPTI-002596. It would be interesting to verify its expression pattern too but this was not possible at the time when I worked on my thesis.

3.2 GAL4/UAS system

The GAL4/UAS system is a very important tool for the study of gene expression in *Drosophila*. When using this system a promoter of the desired driver gene is cloned and inserted upstream of the gene GAL4, which is a yeast transcription factor gene. The promoter then drives the expression of the GAL4 protein in all the cells which express the desired gene. As GAL4 cannot do anything without binding to a UAS region, it has no effect in those cells. In order to activate gene transcription the GAL4 fly strain is crossed with a strain containing the UAS transgene, which is our gene of interest (or its cDNA) cloned after a UAS promoter. Then this gene of interest is expressed only at places where we induce it with the GAL4 driver (Jones, 2009).

For our overexpression studies we obtained stocks from Herbert Jackle from Germany that he used in his JBC paper from 2002. They contain either the cDNA of the cytoplasmic (RA) or the mitochondrial (RB) form of Trxr-1 under the control of the UAS promoter. For the cytoplasmic form we had two constructs, one inserted on the second and one on the third chromosome. For the mitochondrial form we had only an insertion on the third chromosome.

w^-; UAS-Trxr-1 ^{CYTO} (II.)

w^-; UAS-Trxr-1 ^{CYTO} (III.)

w^-; UAS-Trxr-1 ^{MITO} (III.)

Driver stock containing *GAL4* gene under the control of *engrailed* promoter: en-GAL4 (II.)

We set the following crosses and stained the wing discs of third instar larvae by immunostaining for the products of the two Notch targets genes, *cut* and *wingless* (Table 1).

w^-; UAS-Trxr-1 ^{CYTO} (III)	x	en-GAL4
w^-; UAS-Trxr-1 ^{MITO} (III)	x	en-GAL4
w^-; UAS-Trxr-1 ^{CYTO} (II)	x	en-GAL4

Table 1: Crosses set to drive Trxr-1 overexpression in the wing discs.

3.3. FLP – FRT Recombination

With FLP recombinase site-specific recombination occurs between the FLP recombinase target (FRT) sites during replication. In order to obtain mitotic clones, flies with transgenic FRT sites at the same position on homologous chromosomes need to be generated. In case that the site-specific recombination takes place after DNA replication and the two daughter cells segregate correctly, the part of the chromosome arm located distal to the FRT site will be homozygous. Both of its daughter cells will obtain two copies of this region from one of the parental chromosomes (Figure 6; Johnston, 2002).

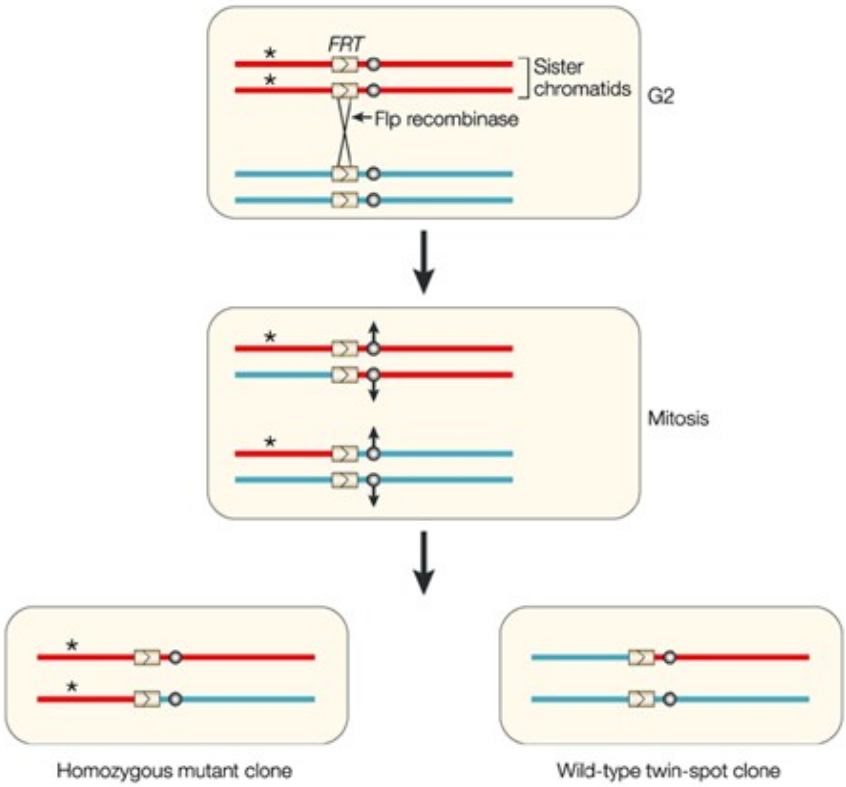


Figure 6: Generating mitotic clones by the FLP/FRT system (Johnston, 2002).

A powerful genetic tool that is often used in Drosophila is the generation of mitotic clones of cells homozygous for a mutation that would be normally lethal if present in the whole organism. Using the FLP/FRT system, mitotic recombination can be induced by the expression of FLP recombinase under a heat shock or tissue specific promoter which then drives recombination between specific FRT sites in the genome. This way clones of homozygous mutant tissue are present next to the wild type homozygous twin spot cells and the rest of the heterozygous cells (Figure 6). The phenotypes of this genetically different cells can then be compared within the same tissue.

To generate the mitotic clones for Trxr alleles we first needed to establish fly stocks where the Trxr-1 alleles would be present next to an FRT site on the same chromosome by meiotic recombination in females. We chose the FRT19A site because Trxr-1 gene lies on chromosome X. We used the following stocks:

a) w-, sn, FRT19A (I.)

Stock containing FRT sites at the location 19A on the X chromosome and the recessive w- and the *sn* alleles as markers. The FRT19A cassette also contains a heat shock inducible gene for neomycin resistance (G418).

b) Stocks from Herbert Jackle from Germany with precise genomic deletion which removed either the mitochondrial and RC isoforms or the cytoplasmic and RC isoforms of Trxr-1 gene (Jackle 2011).

w^-; Trxr-1 $^{\Delta 1}$ / FM6 deletion covering all three Trxr-1 isoforms

w^-; Trxr-1 $^{\Delta 2}$ / FM6 deletion covering only in the mitochondrial and possibly RC isoform

(see Figure 13 for precise localisation of the deleted segments).

Here we rely on the fact that there is a meiotic recombination in *Drosophila* females (and only females, not males) that happens spontaneously during the meiosis without the need for an artificially expressed recombinase (like the above described FLP). We first crossed the w,sn,FRT19A males to the females with Trxr-1 alleles, collected the heterozygous females, crossed it to FM7C balancer males and selected the progeny on neomycin containing food (G418). Because FM7C balancer also contains a *sn* allele, only non *sn*, neomycin resistant flies could potentially be the ones where Trxr alleles were recombined with the FRT site (Figure 8A). We established individual stocks of these flies and tested by real time PCR if the Trxr-1 gene is missing (see methods in section 3.4 and results in section 4.3).

After we confirmed that the stock is correct we crossed the females to males bearing ubx-FLP (a flippase under the control of the ubx promoter which is specifically active only in the wing discs) and an arm-lacZ reporter to generate mitotic clones of Trxr alleles (Figure 8B). Cells homozygous for the Trxr alleles should produce no lac-Z protein and should give no signal after the immunostaining of wing discs with the anti lac-Z antibody.

We selected the third instar females from this cross (as males would not contain the arm-lacZ reporter and FRT19A site for recombination) and analyzed the effect of missing Trxr-1 on the expression of cut and wingless genes by immunostaining (Figure 7).

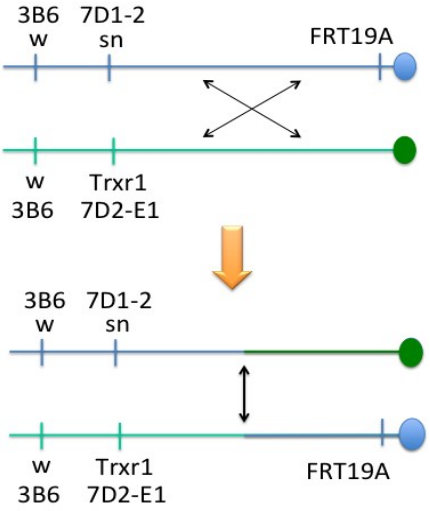


Figure 7: Genomic locations of the individual alleles on the chromosome X we used for recombination and the expected product we select for.

Figure 8A:

$$\frac{w^-,\ sn,\ FRT19A}{y} \quad x \quad \frac{w^-,\ Trxr\text{-}1^{\Delta 1\ (\Delta 2)}}{FM6}$$

$$\frac{w^-,\ sn,\ FRT19A}{w^-,\ Trxr\text{-}1^{\Delta 1\ (\Delta 2)}} \quad x \quad \frac{y}{FM7C}$$

(grown on G418 food, heat shocked)

Possible progeny:

$\frac{w^-,\ sn,\ FRT19A}{FM7C}$ → sn

$\frac{w^-,\ Trxr\text{-}1^{\Delta 1\ (\Delta 2)}}{FM7C}$ → die on G418

$\frac{w^-,\ Trxr\text{-}1^{\Delta 1\ (\Delta 2)},\ FRT19A}{FM7C}$ → not sn, homozygous lethal, establish stock from individual females

Figure 8B:

$$\frac{w^-,\ Trxr\text{-}1^{\Delta 1\ (\Delta 2)},\ FRT19A}{FM7C} \quad x \quad \frac{Y}{FM7\text{-}GFP}$$

$\frac{w^-,\ Trxr\text{-}1^{\Delta 1(\Delta 2)},\ FRT19A}{FM7\text{-}GFP}$ Stock established.

Stain females for cut and wingless in the discs.

Figure 8: Crosses set for obtaining the FRT19A site for recombination.

3.4 Real time PCR

To verify if the Trxr-1 alleles recombined on the FRT19A chromosome we isolated the genomic DNA from individual stocks and quantified the amount of DNA for Trxr-1 gene by the real time quantitative PCR.

We took 10 females flies from the stocks established according to Figure 8B (as males would not contain the arm-lacZ reporter and FRT19A site for recombination and therefore their cells do not lack Trxr-1) and ground them in 150 µl H-buffer which consisted of 160 mM sucrose, 100 mM TrisCl pH 8.0, 80 mM EDTA. Then 250 µl H-buffer, 25 µl proteinase-K (10 mg/ml) and 50 µl 10 % SDS were added. The vial was then kept at 55 °C for at least one hour. Subsequently it was cooled to room temperature and two times extracted with Phenol:Chloroform: Isomylalcohol 25:24:1. The vial was spun for 3 minutes and the aqueous phase was taken. This was extracted with $CHCl_3$. 30 µl 3 M Sodium acetate were added and the DNA was precipitated with equal volume of ethanol. After ten minutes at room temperature the vial was spun for 20 minutes at 4 °C at maximum speed. Then the pellet was washed with 70 % ethanol, dried and redissolved in 40 µl TE with 10 µg/ml RNAse at 68 °C.

Special primers were designed (called ex2 and in2) to verify that the DNA for the specific Trxr-1 alleles are missing in the genome (see Figure 13 in the Result section to see the specific localisation of the primers). Control primers for a gene called wrinkled (W) are also described in Table 2.

Name	Sequence
Trxr ex2 s	CTATGAACTTGACGGGACAGC
Trxr ex2 a	CAATGTGGATGCTGGAAGG
Trxr in 2 s	CAGGAGGCCAAACAGTCTTC
Trxr in 2 a	GATAGGCGGATCGGTAGATG
W real s	GGATCCATAAGTCCGCAGTC
W real a	TTACCACATTGGCCTCCTTG

Table 2: Primers used for the real time PCR

For each primer pair we prepared 14 individual PCR reaction consisted of 4 standards with different dilutions (0.1, 0.01, 0.001, 0.0001 copies/µl), 2 different DNA samples from our stocks of interest and 1 sample with the genomic DNA from yw flies, each of them in duplicate.

Component	Volume (µl)
water	3.9
SYBR green mix	5.0
primer 5'	0.3
primer 3'	0.3
Genomic DNA	0.5

Table 3: Reaction mixture for one vial

Step	Temperature	Time
1: Denaturation	94 °C	15 minutes
2: Denaturation	94 °C	20 seconds
3: Annealing	57 °C	30 seconds
4: Elongation	72 °C	30 seconds

Table 4: Profile of the PCR reaction. Step 2-4 were repeated 40 times. After step 4 every 0.5 °C from 50 °C to 94 °C the melting analysis was performed in order to see if there is only the wanted product.

The fluorescence of SYBR green is measured every cycle and plotted against the cycle number in a logarithmic scale. The amount of genomic DNA in our samples can then be extrapolated from a standard curve.

3.5. Immunostaining

The wing discs from the 3rd instar larvae from the crosses set according the schemes in Table 1 and Figure 8 were stained with antibodies against the *cut* or *wg* proteins (both obtained from the Developmental Studies Hybridoma Bank).

To set the crosses virgin females needed to be collected. Female flies do not mate within the first 8 hours at 25 °C or 16 hours at 18 °C after hatching. So during the day the vials were at 25 °C and overnight at 18 °C and therefore the virgins needed for the crosses were collected

twice a day. The males can be collected at any time. The flies were stored until enough were collected and the cross was started all at once.

Then we collected 3rd instar larvae approximately 5 days after the eggs were laid. The life cycle of *Drosophila* is temperature dependent. At 25 °C it takes approximately 9-10 days from egg to emerged adult. The first three developmental steps from embryo to 1st instar larva, from this to 2nd instar larva and then to 3rd instar larva take each 24 hours. After 48 hours the 3rd instar larva develops into the prepupa, which needs 12 hours to encapsulate into puparium. After 4-5 days at this state the adults hatch.

The 3rd instar larvae were dissected in PBS at room temperature and then fixed for 15 minutes in 4 % formaldehyde in PBS on ice. Afterwards the larvae were washed (2 x 10 minutes) in PBX on ice and then once in PBT for 10 minutes and once in PBT for 1 hour on ice. Subsequently the primary antibody (1:20 for *cut* and *wg* from the Developmental Studies Hybridoma Bank and 1:300 for the Abcam rabbit-GFP antibody) in PBT was added and incubated slightly moving overnight in the cold room. Next day the larvae were washed (3 x 10 minutes) in PBT and then the appropriate secondary antibody in PBT was added (Alexa-444 or Alexa-555 from Molecular Probes, 1:300) and left for two hours slightly moving at room temperature and protected from light. Afterwards they were washed (3 x 10 minutes) in PBT under the same conditions as in the previous step. Then 70 % glycerol in PBS was added and left for at least 20 minutes. Finally the larvae were dissected in 70 % glycerol and the wing discs were mounted with Vectashield on the glass slides and sealed with a cover slip. The glass slides were examined under the GFP microscope as well as under the confocal microscope.

For the double staining with the mutant clones we stained first with the cut antibody and then lacZ.

PBS: 137 mmol NaCl, 2.7 mmol KCl, 10 mmol $Na_2HPO_4 \bullet 2\ H_2O$, 2 mmol KH_2PO_4

PBX (50 ml): 5 ml 10 x PBS, 0.1 ml TX-100

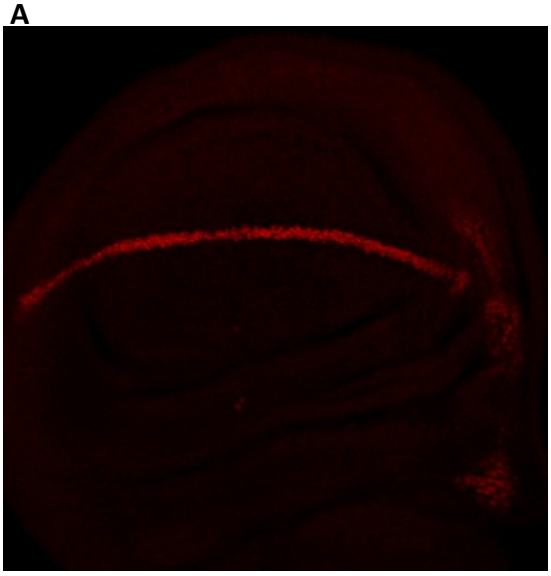
PBT: PBX, 0.05 g BSA for each 10 ml of PBX

4. Results

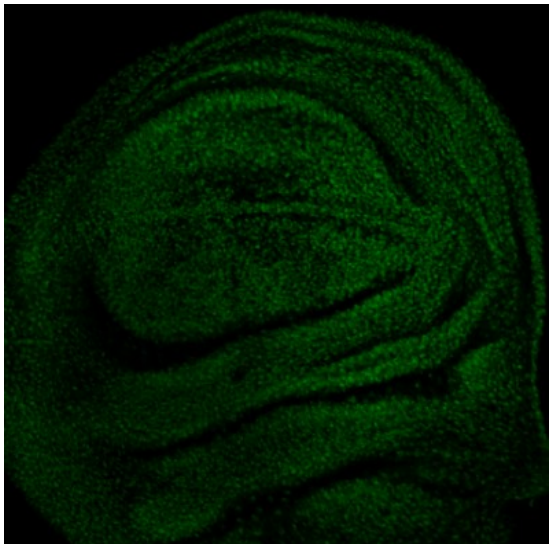
4.1. Verifying the expression pattern of the Trxr-1 YFP trap line CPTI000915

In order to prove the similarity of the Trxr-1 and Notch expression pattern we co-stained the CPTI000915 line for *GFP* (as the GFP antibody recognizes also the YFP protein) and for the Notch target gene *cut* (Figure 9). The CPTI line showed a stripe of enhanced GFP expression at the dorso/ventral boundary of the disc which overlapped with the cut staining. The GFP signal from the D/V boundary cells overlapped with the cut staining (Figure 9B) but as opposed to *cut* it showed both nuclear and cytoplasm localization, suggesting the expression of both the cytoplasmic and mitochondrial forms of Trxr-1 in the wing disc.

A

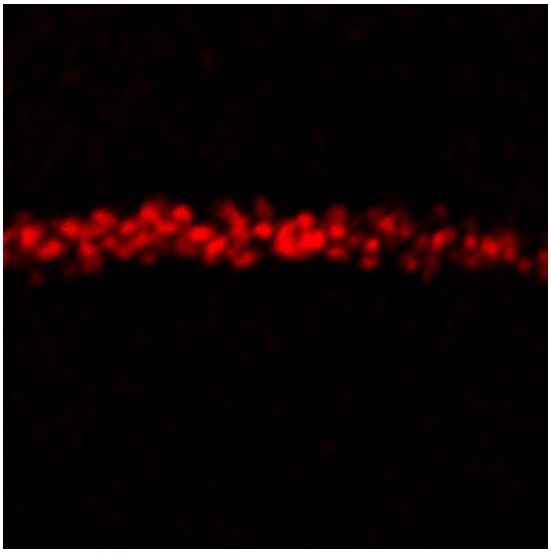


cut staining

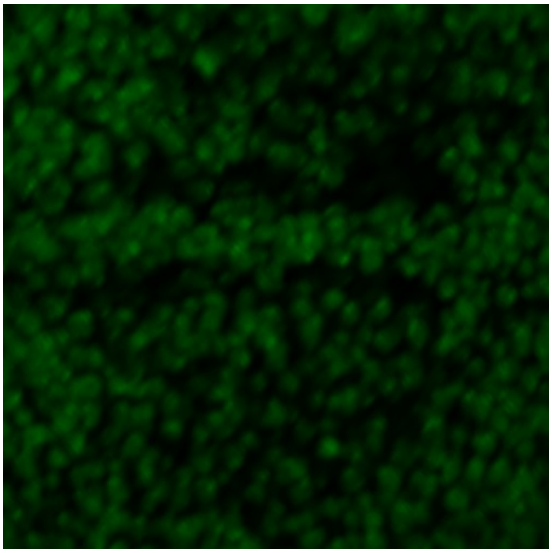


GFP staining

B



cut staining



GFP staining

C

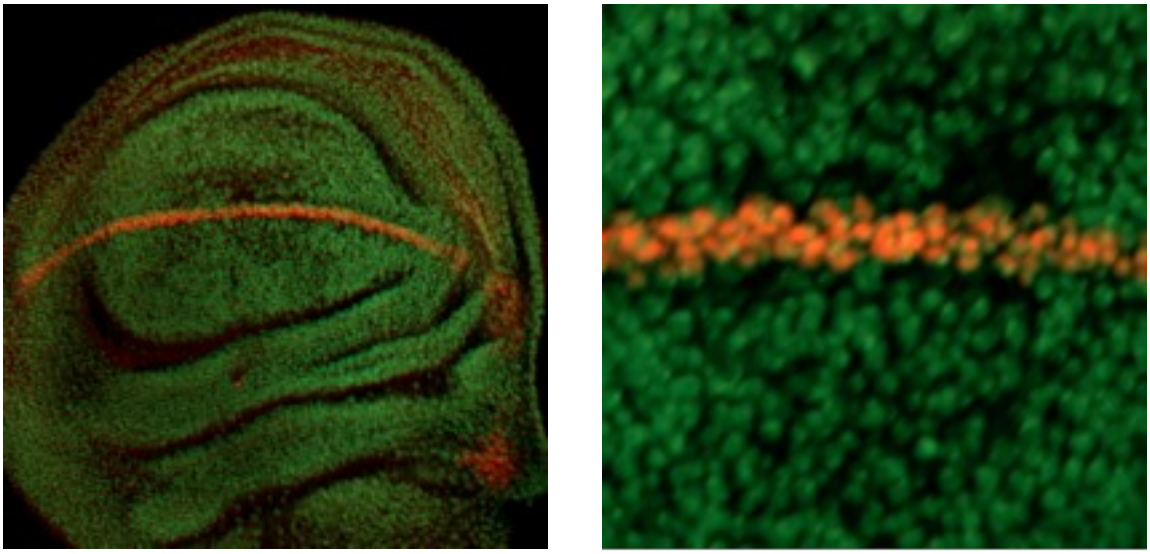


Figure 9: A. Wing discs from CPTI000915 larvae stained against GFP (green) and cut (red). B. High magnification of the green and red signal from the DV boundary. C. Overlay of GFP (green) and cut (red) channel with normal and high magnification.

4.2. Looking at the effect of Trxr-1 overexpression on Notch target genes in the wing disc

First we crossed the eng-gal4 driver to a UAS-GFP line and immunostained the discs for GFP to see the expression pattern of the en-GAL4 driver. The GFP was only expressed on the posterior side of the wing disc as expected (Figure 10).

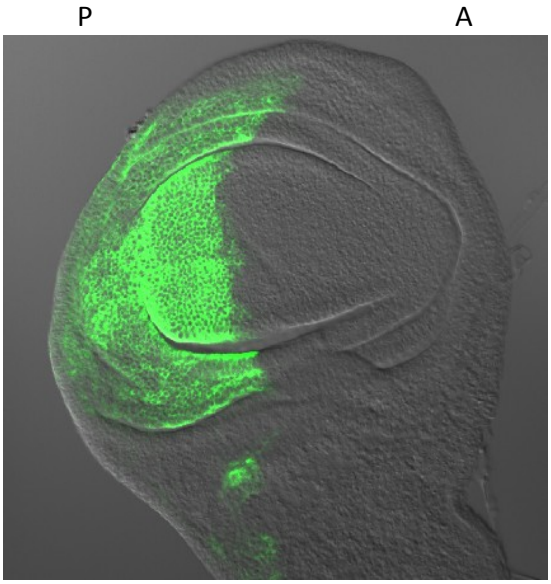


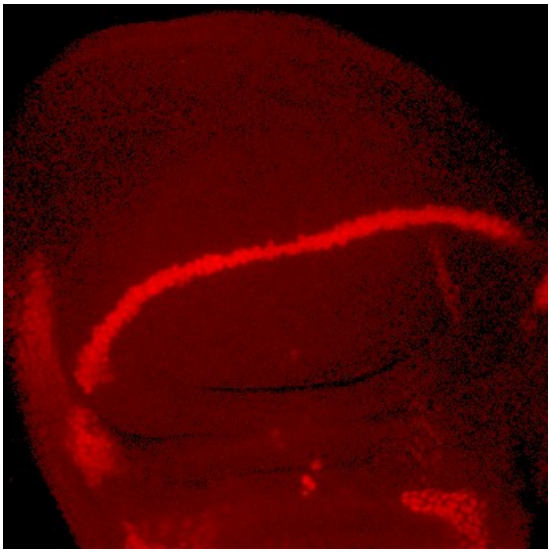
Figure 10: GFP staining of a wing disc where the GFP expression was driven by the engrailed driver.

P – posterior part of the disc, A- anterior part of the disc.

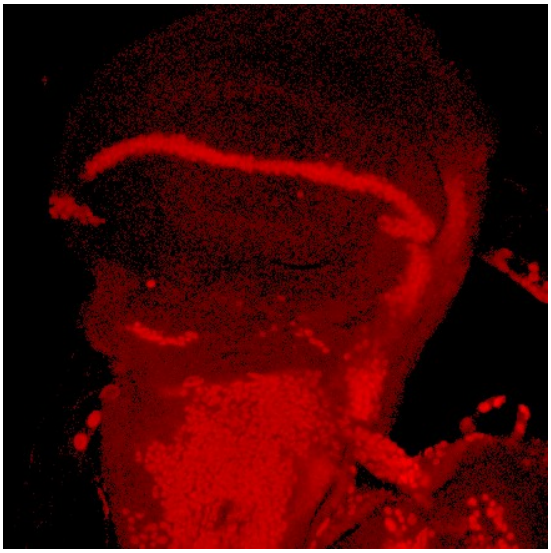
Overexpression of Trxr-1

After verifying the expression of the en-GAL4 driver we set a cross where the Trxr CYTO or MITO forms were expressed in the eng domain of the disc (see Methods). This way we had the wild type cells and the cells that overexpress Trxr-1 isoforms in the same disc and we could simply compare the strength of the cut and wingless staining in the anterior and posterior domains. As shown in the Figures 11 and 12, there was no obvious effect observed on the cut or wingless expression when Trxr-1 CYTO or MITO was overexpressed in the engrailed domain.

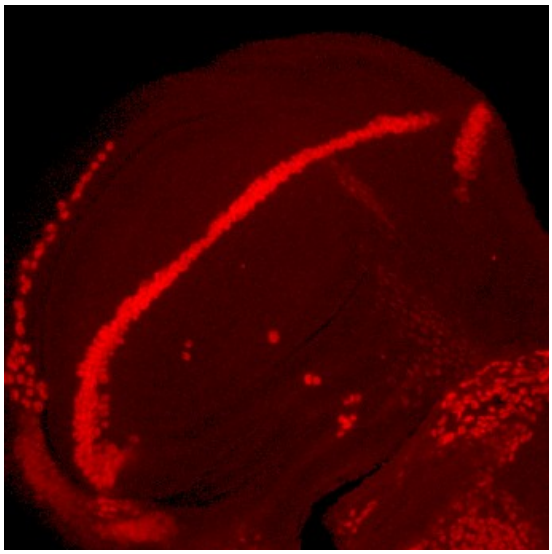
There was once an ectopic cut expression seen on a wing disc of Trxr-1 ^{CYTO} (II) with cut staining but this was not considered to be a phenotype as it did not appear again in any of the four repeats of the experiment.



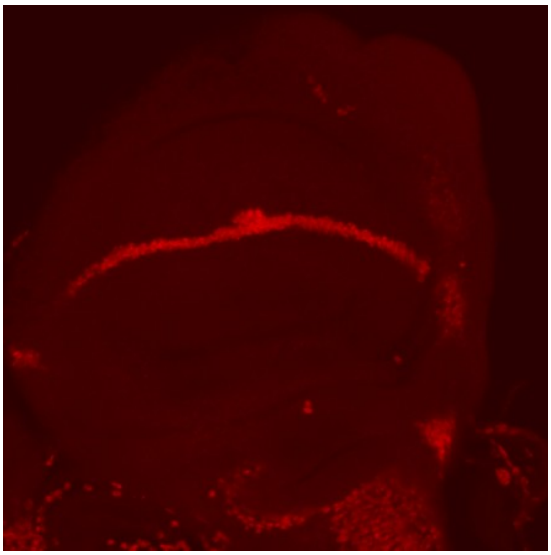
Trxr-1 ^{CYTO} (III)



Trxr-1 ^{CYTO} (II)

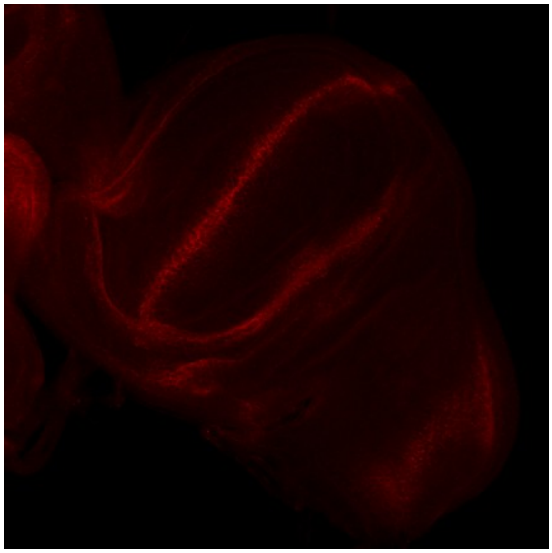


Trxr-1 ^{MITO} (III)

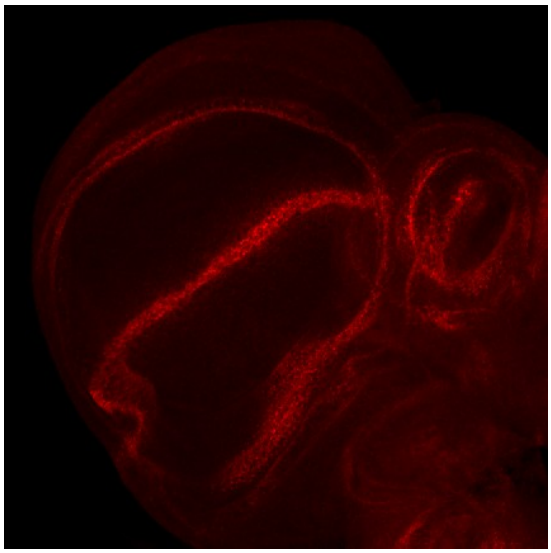


Trxr-1 ^{CYTO} (II) showing an ectopic cut expression

Figure 11: Cut staining of wing discs of larvae where Trxr-1 CYTO or MITO overexpression was driven by the en-GAL4 .



Trxr-1 ^{CYTO} (III)



Trxr-1 ^{MITO} (III)

Figure 12: Wingless staining of wing discs of different larvae. Both wing discs show the normal wingless staining.

4.3 Recombining the Trxr alleles on FRT19A and verification of the stocks

The scheme of fly crosses that we set to recombine the Trxr alleles on FRT19A chromosome are described in the Methods (Figure 8). Here are the results of the real time PCR reactions that verified that certain part of the Trxr-1 genomic DNA was missing (Figure 13)

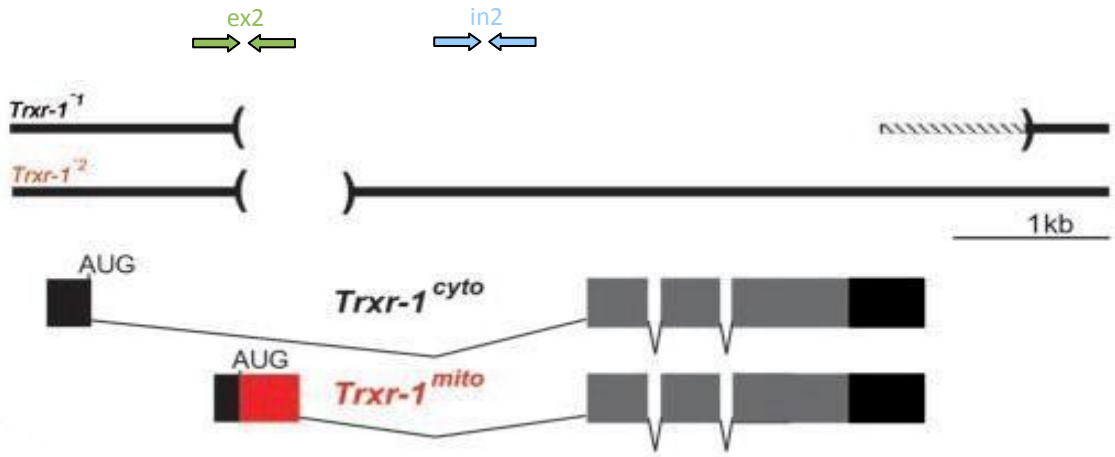


Figure 13: Physical map of Trxr-1$^{\Delta 1}$ and Trxr-1$^{\Delta 2}$ with the arrows showing the positions of the primers and the two transcripts with four exons each.

We first tested the presence/absence of the ex2 region in the Trxr-1 gene, in comparison to a control genomic region of the W gene that should not be missing. If the Trxr-1 DNA was not missing (the recombination in our stocks did not occur), the ratio between the signal from

Trxr-1 and the control W gene should be 1:1 (or we can say 2:2 as there are 2 copies of each in the fly genome). However, if part of the Trxr-1 gene was missing, the Trxr-1 to W ratio should be 1:2 (Table 5, 6 and Figure 14).

To confirm the identity of the delta1 and delta2 stocks we also calculated the ratio of in2 to ex2 signal. In the delta2 allele the in2/ex2 ratio should be close to 2 as there is the first exon missing but not the first intron (so in the balancer stock females there will be two copies of the intron DNA but only one copy of the exon DNA that comes from the balancer). In the delta1 stock both the intron2 and exon2 are missing so the ratio will be 1:1 (Table 5, 6 and Figure 14).

	ex 2	average	in2	average	W	average
Trxr-1 $^{\Delta 2}$	0.4363	0.4088	0.5134	0.7823	0.6676	0.8306
	0.3813		1.0512		0.9935	
Trxr-1 $^{\Delta 1}$	0.0434	0.0468	0.0613	0.0617	0.0723	0.0827
	0.0502		0.0620		0.0931	
yw	0.0092	0.0089	0.0092	0.0101	0.0062	0.0072
	0.0086		0.0110		0.0082	

Table 5: Arbitrary numbers expressed as the dilution factor of standard genomic DNA (from yw flies).

	ex2/W	in2/ex2
yw	1.24	1.13
Trxr-1 $^{\Delta 2}$	0.49	1.91
Trxr-1 $^{\Delta 1}$	0.57	1.32

Table 6: Ratios between the primer ex2 and the control primer and between ex2 and in2

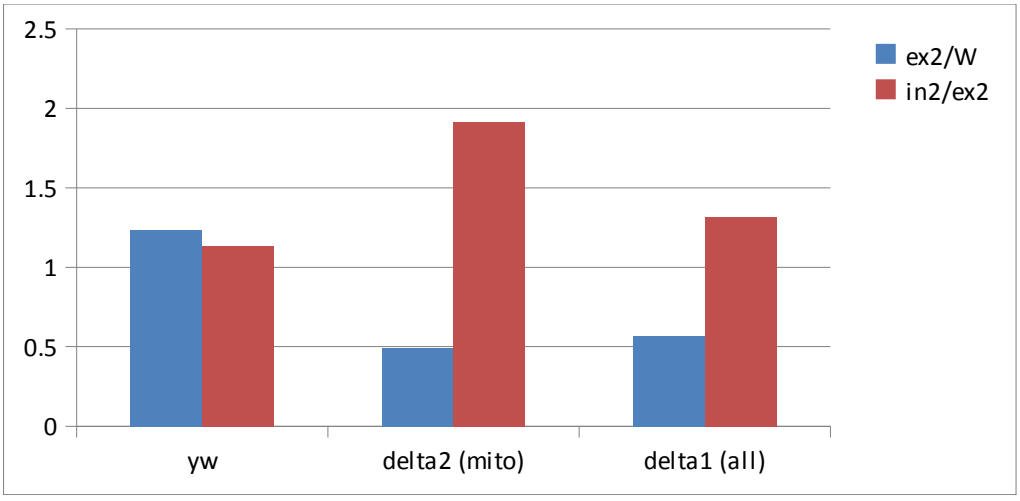
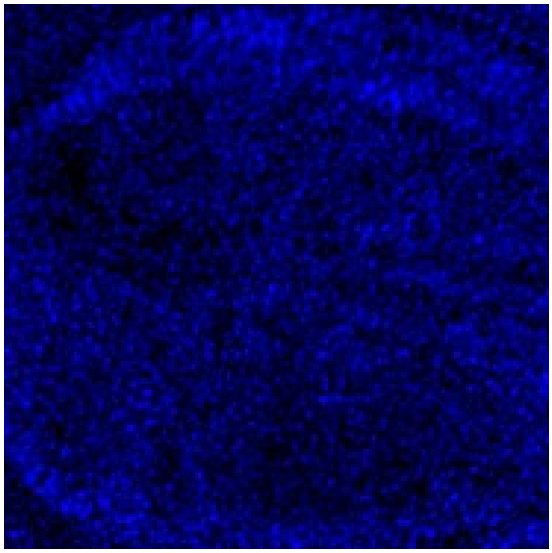


Figure 14: Ratios between the real time PCR signal for ex2 and the control region from the Wrinkled gene (W) and between ex2 and in2 from our stocks with recombined Trxr-1 delta 1 and delta 2 alleles.

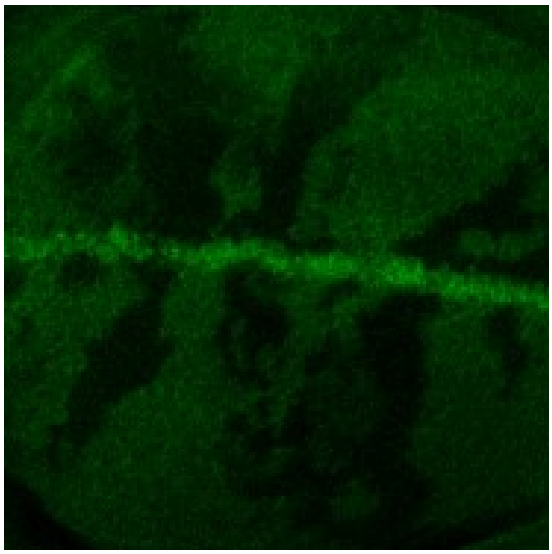
From graph on Figure 14 it can be easily seen that our effort to recombine the Trxr-1 alleles on FRT19A was successful. There was not much difference in the ratio of ex2/W and the ratio of in2/ex2 for the genomic DNA from yw flies as it does not have the Trxr-1 gene missing and therefore is amplified with both primers. However, the ex2/W ratio was lower in delta 1 and delta 2 stocks suggesting that the ex2 was missing in both of them. On the other hand, the in2/ex2 ratio was doubled in the delta2 stock which confirmed that here the in2 was not missing and therefore this is the variant of Trxr-1 that deletes only the mitochondrial (and RC) form. The delta1 stock showed reduced signal from both ex2 and in2 proving that it is the stock where all the three isoforms of Trxr-1 are missing.

Generating the mitotic clones of the Trxr-1 alleles

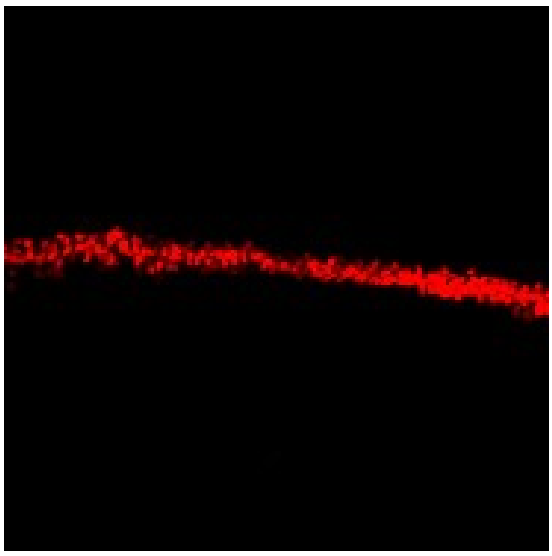
We set the crosses as described in the Methods (Figure 8B). Then we stained the wing discs with the mutant clones of cells lacking Trxr-1 $^{\Delta 1 \text{ (or } \Delta 2 \text{ resp.)}}$ for DAPI (to visualize nuclear DNA), cut and lacZ or DAPI, cut and wg. Clones of cells being homozygous for the Trxr-1 alleles were the ones missing the lacZ staining (in green). The green signal at the DV boundary even in cells presumably lacking the lacZ staining (clones of cells spanning the DV boundary) comes from a cross-reactivity of the mouse secondary antibody with the cut primary antibody (both the cut and lacZ antibodies are mouse, sequential staining was performed first with cut and then with lacZ). As apparent from Figure 15 there was no effect seen on the Notch target genes of both the Trxr-1 $^{\Delta 1}$ and Trxr-1 $^{\Delta 2}$.



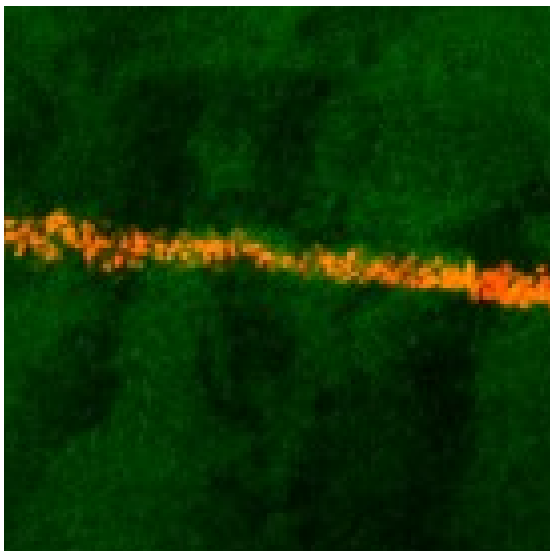
DAPI staining



LacZ staining

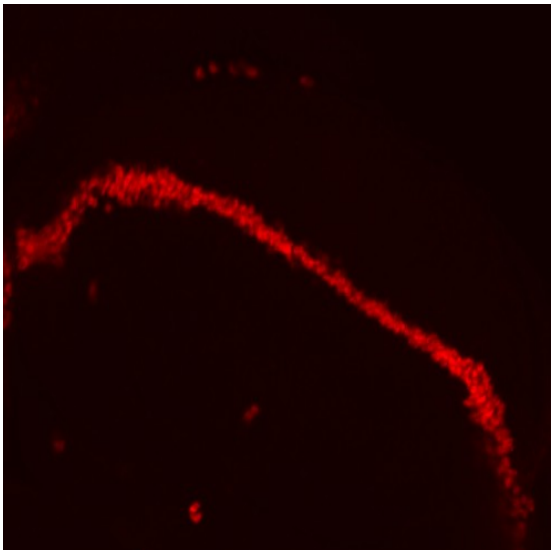


Cut staining

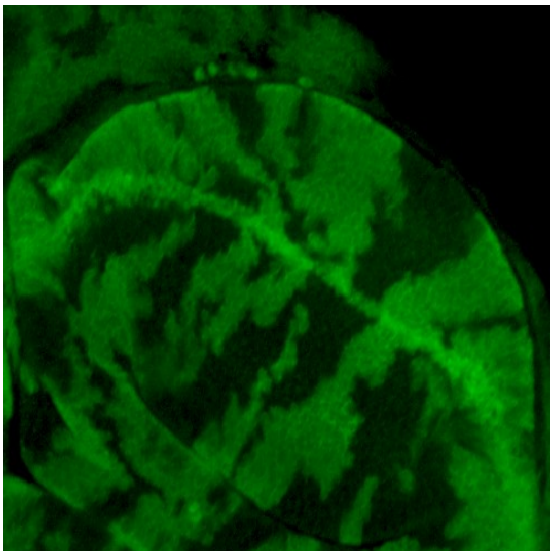


LacZ (green) and cut (yellow) overlaid

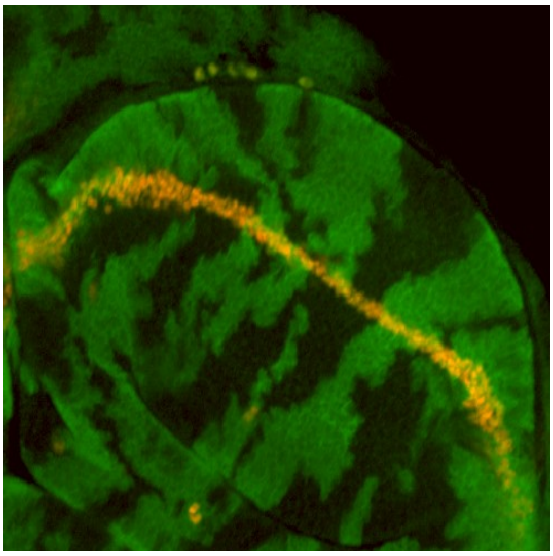
Figure 15: Wing discs of Trxr-1 $^{\Delta 1}$ larvae.



LacZ staining



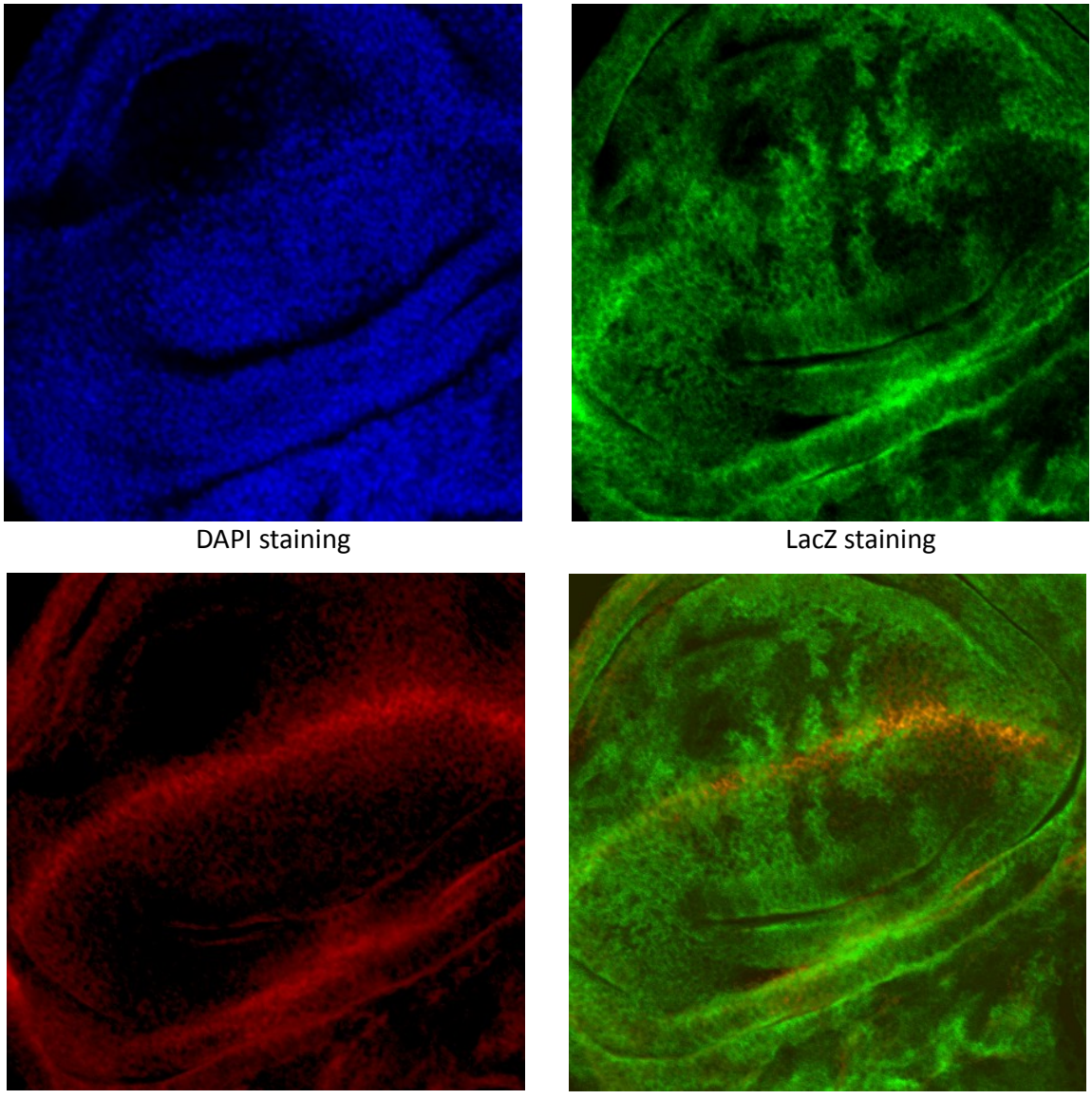
Cut staining



LacZ (green) and Cut (yellow) overlaid

Figure 16: Wing discs of Trxr-1$^{\Delta 2}$ larvae.

The cloned cells have the same Notch activity as the other cells.



DAPI staining | LacZ staining

cut staining | LacZ(green) and cut (yellow) overlaid

Figure 17: Wing discs of Trxr-1 $^{\Delta 1}$ larvae with DAPI, wingless and lacZ staining.

4.4 Additional data to the Trxr project

These are data from other members of the lab (Veronika Caisova and Alena Krejci) which I want to include to make a complete picture of the project.

The UAS lines with Trxr-1 CYTO or MITO were crossed to several other GAL-4 drivers. The overexpression of either form of Trxr-1 using scabrous-GAL4 driver gave extra bristles on the

scutum in comparison to the control flies with the GAL-4 driver only (scabrous GAL-4 driver is specific for cells from which the bristles on the scutum develop).

Furthermore, RNAi against Trxr-1 or Trxr-2 was driven by en-GAL4 (KK line 10648_for Trxr-1 and GD line 16768 for Trxr-2, both from Vienna Drosophila Research Centre) and the phenotype was inspected in the adult wings (Figure 18). Interestingly, while the Trxr-2 RNAi gave only a very mild phenotype (missing intervein at 29C), the whole engrailed domain was missing in the Trxr-1 RNAi flies. The mild phenotype for Trxr-2 might be explained by the fact that the GD lines are generally weaker than KK lines.

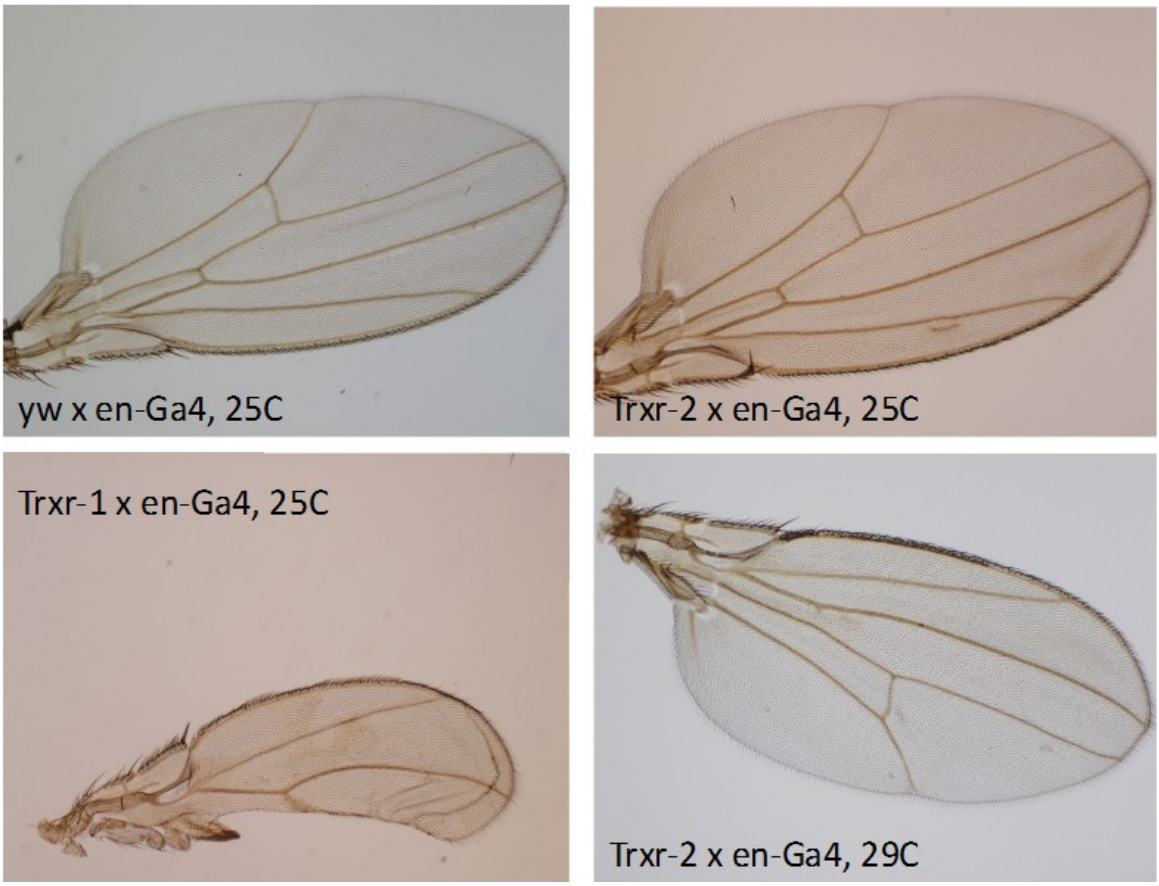
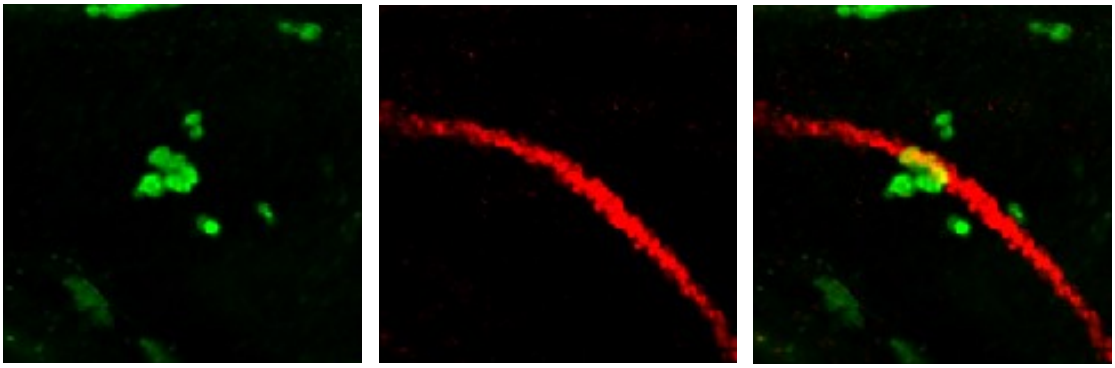


Figure 18: Wings where RNAi against Trxr-1 or Trxr-2 was driven by en-GAL4.

Additionally, flip-out clones of Trxr-1 RNAi line were generated (instead of using the obg-GAL4 driver). Here no effect on cut was observed. However, these clones were rather small, possibly because of a late recombination event, and one can not exclude that the RNAi did not have enough time to knock out the Trxr-1 gene. Alternatively, it is also possible that bigger clones induced earlier die as expected and there is no effect seen in the smaller clones because they were induced later and there is still enough Trxr-1 protein remaining to see a phenotype. Further investigation is needed to resolve these alternatives.



LacZ staining | Cut staining | LacZ (green) and Cut (red) overlaid

Figure 19: Pictures of clones of Trxr-1 RNAi lines.

5. Discussion

With the Trxr-1 overexpression there was no effect observed on Notch target genes in the wing disc. Further we recombined Trxr-1 alleles on FRT chromosome, generated mutant clones of cells in the wing discs and in order to assess the expression of Notch target genes in these clones. Here we found out that the clone cells had the same Notch activity as the others.

There can be several reasons why the overexpression of the Trxr-1 did not show any phenotype. Firstly, the overexpression did not show any effect because there is already a lot of the Trxr-1 protein present in the discs and putting any extra will not make an effect. Secondly, we inspected only the expression of two Notch target genes, cut and wingless. It is still possible that we would see an effect on the other Notch targets in the wing. Due to the lack of specific antibodies specific reporter stocks would have had to be crossed to our fly lines and in the time scale given this was not possible to do.

Our clones of the cells lacking Trxr-1 $^{\Delta 1}$ or Trxr-1 $^{\Delta 2}$ did not show any effect on the Notch signalling pathway. This could be real but there is an alternative explanation. There is the Trxr-2 gene which maybe compensates for the loss of the Trxr-1.

The data from the other lab members where RNAi against Trxr-1 was driven by en-GAL4 and caused a completely missing engrailed part of the adult wing opened a question: Why the Trxr-1$^{\Delta 1}$ and Trxr-1 $^{\Delta 2}$ clones do not show any effect in the larval wing? Knocking down a gene by RNAi should be similarly effective as using its mutant allele.

There are four possible explanations:
a) My Trxr-1 delta 1 and 2 lines on the FRT19A chromosome were not correct (Trxr-1 is not missing). This was verified by PCR and therefore it can be excluded.
b) The Trxr-1 RNAi line has an off-target that gives the phenotype (so the effect we see is not because of missing Trxr-1).
c) Trxr-1 RNAi line actually knocks-down also Trxr-2 because of its close homology. Therefore the RNAi phenotype is stronger than my mutant allele clones missing just Trxr-1.
The sequences that were used to create the RNAi lines are shown in Figure 20. In the alignment there is a region of high homology between the Trxr-1 and Trxr-2 RNAi but as it is less than 19bp

Trxr-1 RNAi should not be very effective in silencing also Trxr-2, at least not by the siRNA machinery. Presumably, the miRNA mechanism could still be applied to silence the protein translation. This is an attractive explanation that can not be excluded and it definitely would be worth testing.

d) The effects of RNAi observed in the adult wing only happens in the pupae stage, the larval discs are not affected. We would need to stain the larval discs of these flies to see if the effect is seen already at this stage.

```
          10         20         30         40         50         60
Trxr-1 GATGTTTACGACACCGTTCTGTGGGCCATCGGCCGCAAGGGTCTGGTGGACGATCTGAAC
       :: :::: ::::::::: :::::::::: :: ::::: :::::  : :: :: :: :::
Trxr-2 GACGTTTTCGACACCGTGCTGTGGGCCATTGGGCGCAAAGGTCTCATCGAGGACCTCAAC
             40         50         60         70         80         90

          70         80         90        100        110        120
Trxr-1 CTGCCCAATGCCGGCGTGACTGTGCAGAAGGACAAGATTCCAGTGGACTCCCAGGAGGCT
       :::  :   ::::: ::::     ::  : :::::::::   :: ::: ::  ::::::
Trxr-2 CTGGACGCCGCCGGAGTGAAGACTCATGACGACAAGATTGTGGTAGACGCCGCGGAGGCC
            100        110        120        130        140        150

         130        140        150        160        170        180
Trxr-1 ACCAATGTGGCAAACATCTACGCTGTCGGCGATATCATCTATGGCAAGCCAGAGCTGACG
       ::::  ::: :  : :: :  :: :: :: :: ::::: :::::    :::::::: :::
Trxr-2 ACCAGCGTGCCTCATATATTTGCAGTTGGGGACATCATATATGGTCGACCAGAGCTAACG
            160        170        180        190        200        210

         190        200        210        220        230        240
Trxr-1 CCCGTCGCCGTTTTGGCTGGCCGTTTGCTGGCCCGCCGCCTGTACGGAGGATCTACCCAG
       :: :: ::: :  :: : :::::  :::: ::: : :: :::: ::  :: :: :: :::
Trxr-2 CCGGTGGCCATCCTGTCGGGCCGCCTGCTTGCCAGGCGTCTGTTCGCCGGCTCCACGCAG
            220        230        240        250        260        270

         250        260        270
Trxr-1 CGCATGGACTACAAGGATGTGGCCACC
       :  ::::::::    :: ::::: :::
Trxr-2 CTGATGGACTATGCCGACGTGGCAACC
            280        290
```

Figure 20: The DNA sequence used for generating the Trxr-1 RNAi line aligned with the Trxr-2 mRNA.

Taken together, solely based on the data from my experiments we would tend to conclude that Trxr-1 does not have any effect on the Notch signalling. However, the fact that overexpression of either form of Trxr-1 using scabrous-Gal4 driver gives extra bristles on the scutum suggests that Trxr-1 might have a negative effect on Notch in the context of the development of sensory organ precursors (more bristles mean less Notch signalling during lateral inhibition). Also, the effect of RNAi lines supports the view that Trxr-1 plays an important role during the development of the wing disc. Further experiments need to address these opened questions.

6. Conclusion

First we verified the expression pattern of the Trxr-1 YFP trap line. As we wanted to prove the similarity of the Trxr-1 and Notch expression pattern we co-stained the CPTI000915 line for *GFP* and for the Notch target gene *cut*. There was an overlap of the enhanced GFP expression at the dorso/ventral boundary and the cut staining.

Further we had a look at the effect of overexpression of the mitochondrial and cytoplasmic isoform of Trxr-1 on the Notch target genes in the wing discs. Therefore we verified the expression pattern of en-GAL4 driver by crossing the en-GAL4 driver to a UAS-GFP line and immunostaining the discs for GFP, which was only expressed on the posterior side of the wing disc. Then we set a cross where the mitochondrial and cytoplasmic isoform of Trxr-1 were expressed in the eng domain of the disc where no effect was observed.

Further we recombined Trxr-1 alleles on FRT chromosome in order to create mutant clones of cells in the wing discs and then assessed the expression of Notch target genes cut and wingless in these clones. Therefore we established fly stocks where the Trxr-1 alleles were present next to an FRT site by meiotic recombination in females and verified the correctness by real time PCR. Afterwards those females were crossed to males bearing ubx-FLP and an arm-lacZ reporter. At the immunostaining of the wing discs with the anti lac-Z antibody the cells homozygous for the Trxr alleles did not show any lac-Z signal.

7. References

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