University of South bohemia, Faculty of Science

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RNAi screen of selected NAD(H) binding proteins in Drosophila wing

Bachelor thesis

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Bachelor Thesis

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Annotation

The aim of the thesis was to identify the NAD(H) metabolic sensors involved in Notch. For the detection of these metabolic sensors, we tested the phenotype of an RNAi mediated knock down of forty different NAD(H) binding proteins using the en-Gal4 driver.

Declaration

I hereby declare that I have worked on my [bachelor's – diploma – doctoral dissertation] thesis independently and used only the sources listed in the bibliography. 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 / master / dissertation thesis, in full / in shortened form resulting from deletion of indicated parts 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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Nora Hagleitner

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1.Notch pathway

1.1 Mechanism and components of the Notch signalling pathway-

The Notch receptor, which is encoded by the Notch gene, is a single-pass I transmembrane surface protein. After translation, the newly synthesized receptor protein gets modified through glycosylation by the enzymes O-fut and Rumi. The completely mature Notch receptor is subsequently produced by the first cleavage event (S1 cleavage) that occurs in the trans-Golgi. This cleavage event is mediated by the protein Furin and leads to the formation of a heterodimer, which is composed of a ligand-binding Notch extracellular domain (NECD) and a single-pass transmembrane signalling domain, which is also known as Notch intracellular domain (NICD). These parts of the heterodimer are held together by non-covalent interactions. The heterodimer is then targeted to the membrane surface of the receptor expressing cell. The pathway is ultimately activated when the ligand-binding Notch extracellular domain (NECD) of the heterodimer binds to one of the Delta-Serrate-LAG2 (DSL) ligands, which is generated on the membrane surface of a neighbouring cell. The availability of Notch receptors and its ligands on cell surfaces is regulated by endocytosis and membrane trafficking, which consequently have diverse impact on the overall rate of canonical Notch signalling. Ligand endocytosis induces a mechanical force that leads to conformational changes in the Notch receptor, which ultimately results in the exposure of the second proteolytic cleavage site mediated by TACE (also known as ADAM 17) metalloprotease (S2 cleavage). Thereby NECD is almost released from the outer portion of the membrane and subsequently it is internalized through endocytosis by the ligand expressing cell, where it undergoes lysosomal degradation. Afterwards, the rest of the receptor tethered near the inner leaflet of the membrane of the Notch expressing cell is cleaved by γ-secretase (S3 cleavage), producing the transcriptionally active NICD, which then translocate to the nucleus. In the nucleus, NICD initiates the transcription of target genes, by forming a core transcriptional complex with the DNA-binding protein CLS (CBF1/RBPjκ/Su(H)/Lag-1). The binding of NICD to CSL initiates allosteric changes which ultimately results in the removal of transcriptional repressors that in the absence of Notch signalling repress the transcription of target genes.

Subsequently the transcriptional activator Mastermind (MAM) recognises the NICD/CSL complex and binds to its interface. This triprotein complex then recruits additional coactivators which ultimately results in target gene expression. Afterwards, the nuclear NICD is then phosphorylated and degraded by ubiquitination so that the cell becomes ligand-competent again and a further round of Notch signalling can be initiated. (1, 2).
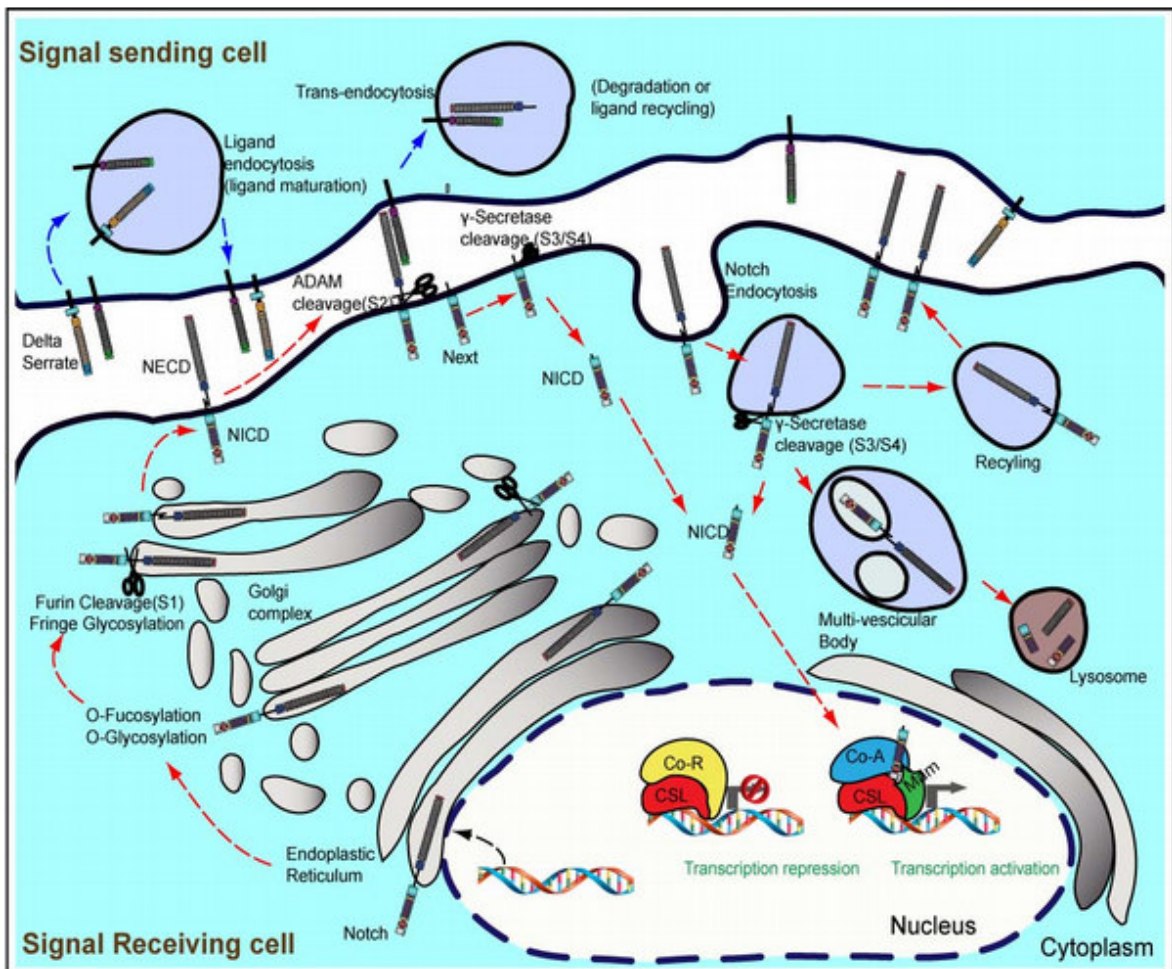
Fig. 1: Illustration of Notch receptor activation and Notch signalling mechanism
(http://www.bsse.ethz.ch/egg/research/epigenetic-pathways-in-cancer/systems-biology-of-notchsignaling/_jcr_content/par/textimage_0/image.imageformat.lightbox.1486509121.png
)

1.2 Processes influenced by the Notch pathway

The Notch signalling pathway plays a role in cell fate determination, lateral inhibition and boundary formation. Furthermore it is essential for stem cell renewal and it controls proliferation and apoptosis in multiple metazoan tissues. Misregulations of the Notch pathway are linked to several human diseases (3).

1.2.1 Lateral inhibition and pattern differentiation

Lateral inhibition is a term that describes a process where the activity in a restricted area prevents similar activity in adjacent areas. These neighbouring areas are consequently forced to adopt an alternative fate.

When two cells express an activator **a,** then both of them have the ability to adopt cell fate **A**. If the expression of A is not inhibited, the concentration of the activator increases until it reaches a certain threshold that determines cell for fate A. However, lateral inhibition allows only one cell to adopt this fate. The Notch pathway is switched on in the neighbours of A (by A expressing Notch ligands on its surface) and promotes the expression of an inhibitor B that reduces the expression of the activator A in these cell. As a consequence, asymmetry between the two cells has been created and the neighbouring cell is ultimately forced to adopt an alternative cell fate **B.** Furthermore if two cells have different ability to adopt a specific fate, then lateral inhibition would operate in a context so that it amplifies some pre-existing bias between cells that have similar, but not equal potential do adopt a fate. The ability of a cell to inhibit the cells in its surrounding depends on the relative concentration of activator in both cells as well as the bias created by earlier pattern mechanisms and the strength of lateral inhibition.

A classic example of lateral inhibition concerning the Notch pathway is neural-epidermal choice in *Drosophila melanogaster*. Some cells of pronerural clusters express both Notch and its ligands, and therefore have the ability to develop into either neuronal precursor cells or epidermal cells. Due to a so far not well understood mechanism, the concentration of Notch and its ligands slightly changes until the cells either express Notch or its ligands. The ability of ligand expressing cells to differentiate into the neuronal linage is inhibited by the Notch expressing neighbouring cell and as a consequence these cells have to adopt an epidermal fate instead (3, 4).

1.2.2 Boundary formation

The formation and maintenance of boundaries between neighbouring groups of embryonic cells is essential because if specific cells should adopt a certain fate, they should be physically separated from other cell populations, to ensure their proper development. Moreover, boundary cells often mediate important signalling

events by acting as organizing centres and thus boundary integrity and shape influence or control the final outcome of downstream patterning events.A classic example for the participation of the Notch pathway in the formation of boundary cells can be shown by the Drosophila wing. The veins in the Drosophila wing have a characteristic width, which is regulated by the activity of the Notch pathway. The Notch-ligand Delta is expressed in the developing veins, whereas the Notch transmembrane receptor is transcribed in broad domains that correspond to the interveins. This asymmetric distribution of Notch receptor and its ligand lead to the activation of Notch in vein/intervein boundaries. As a consequence, boundary cells are formed, which separate veins from adjacent interveins. The ligand is activated by the vein-promoting gene veinless (*ve)*, which transcription is restricted to the domain where Notch is not activated as its transcription is generally supressed by Notch. In boundary cells, the activation of Notch leads to the accumulation of *E(sp)mß* mRNA that maintains the polarity of signalling and preserves the separation of veins from interveins during development. For example, vein-thickening phenotypes and extra vein material are caused by Notch loss-of-function alleles, whereas thinner and incomplete veins are result from Notch gain-of-function alleles. Furthermore the Notch pathway is also involved in the establishment of the wing margin. The cells along the dorsal-ventral boundary of the wing imaginal disc have different properties and activation of Notch is required to initiate a sequence of events leading to the ultimate specification of those cells.Another example of Notch dependent boundary formation is the establishment of dorso/ventral boundary in the wing disc. Notch is persistently activated in the cells of the dorsal-ventral boundary due to the asymmetric distribution of the two Notch-ligands Delta and Serrate (Fig.2). (5, 6, 7).
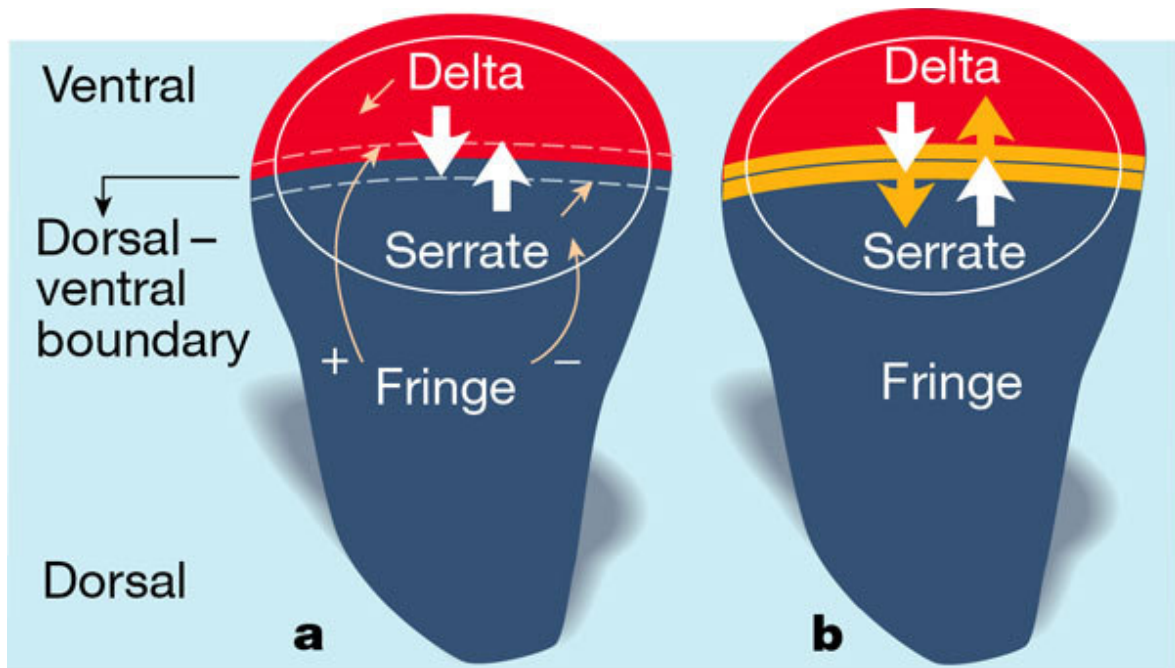
Fig. 2: Establishment of the dorsal-ventral boundary via activation of Notch due to the asymmetric distribution of the two Notch ligands Delta and Serrate and the activity of Fringe protein.

(http://www.nature.com/nature/journal/v406/n6794/images/406357aa.2.jpg)

1.2.3. Stem cells

Stem cells are characterized by their capacity of self-renewal and due to their ability to differentiate into the full spectrum of cells to form a specific tissue or organ. Stem cells can be subdivided into three major groups: embryonic, germinal and somatic stem cells. Embryonic stem cells can be received from the inner cell mass of the blastocyst. They are able to replicate endlessly and furthermore they are able to form all cell types of a mature organism. Germinal stem cells can be isolated from the germinal layer of the embryo. However, this type of stem cells can only form specific organs. Finally, somatic cells have the ability to self-renew and develop into cells that are required or that are characteristic for specific organs or tissues. Stem cells can be found at specific locations, which are called stem cell niches. Three different types of stem cell niches can be differentiated: simple niches, complex niches, and storage niches. Simple niches are specific locations within an organ or tissue, in which stem cells have the opportunity to produce daughter cells and-self renew for an indefinite period of time. In complex niches, one or two different stem cells are supported by the activity of partner

cells. Stem cells that are located in storage niches start to divide and maturate only if they are activated by external signals.

The differentiation and specification of stem cell is influenced by many factors, including Notch signalling pathway and its components. It has been observed the function of a wide variety mammalian stems cells such as hematopoietic, intestinal, and skin stem cells, as well as intestinal stem cells of *Drosophila melanogaster* and germ cells in *C. elegans* are regulated by interplay between the Notch pathway and its components.

For example, a high concentration of Delta in the intestinal stem cells (ICSs) of adult Drosophila midgut leads to the activation of the Notch canonical pathway and a simultaneous down regulation of Delta in the daughter cells. As a result, these daughter cells now differentiate into enterocytes. On the other hand, if the concentration of Delta-vesicles in the intestinal stem cells is relatively low or they are impaired in Notch signalling, their daughter cells will be programmed to develop into enteroendocrine cells. Therefore, in the case of ICSs, the Notch signalling pathway influences the cell fate of the daughter stem cells. Similarly, in mammals, the Notch pathway is essential for the maintenance of stem cells and progenitor cell populations in the intestinal crypt as well as for the differentiation of mature enterocytes (8).

In Drosophila germline stem cells (GSCs), the Notch pathway has a slightly different effect. The germline stem cells are located in a somatic niche and an overexpression of Delta in the germ cells or the activation of Notch in the somatic niche causes the production of extra niche cells.

1.2.4. Notch and disease

Notch signalling participates in the determination of cell fate. Furthermore it is essential for stem cell renewal and it controls cell proliferation and apoptosis in multiple metazoan tissues. As a consequence, it is not surprising that misregulations of the Notch pathway are linked to several human diseases and disorders. In mammals, mutations or variations in the gene dosage of the components of the Notch pathway may result in too weak or too strong signal transduction. Several human disorders are caused by this phenomenon. For example, the Alagille's syndrome is either caused by haploinsufficiency of the Jagged1 or the Notch2 gene. Moreover, haploinsuffuciency of the Notch1 gene is

associated with a subtype of an inheritable aortic disease also known as CADASIL. Moreover, spondylocostal dysostosis is ultimately caused by a missense mutation of the DLL3 protein.

Notch is also linked to various types of cancers. However, it has been discovered that the Notch pathway either promotes or suppresses cancer development or proliferation, depending on the cellular context. In human malignancies such as cervical, lung, colon, head and neck, renal carcinoma, acute myeloid, Hodgkin and Large-cell lymphomas and pancreatic cancer, it has been recorded that Notch is frequently deregulated, accompanied by the up-regulation of Notch receptors and its ligands and thus Notch promotes formation and proliferation of these cancer types. On the contrary, Notch signalling has found to be anti-proliferative in tumour types such as hepatocellular carcinoma, skin and small lung cancer (1, 8, 9, 10).

1.3 Context dependent response of the Notch pathway

The cell context dependant outcome of the Notch pathway may be the result of pathway-cross talk, post translational modifications of Notch components or different receptor and ligand pairs (3).

1.3.1. Chromatin structure and other transcription factors

In general, all cells of multicellular organisms contain the same genetic information and are able to respond to environmental changes. Responses to environmental changes are often dependent on the combination of transcription factors present in the cell and on the regulation of their activity. The activity of transcription factors can be regulated by their association with co-activators, which either stimulate transcription or increase the specificity for the target DNA sequence; or by controlling their transport in or out of the nucleus through post translational modifications; or through their proteasomal degradation by ubiquitination.

Transcription factors bind to specific DNA sequences and interact with chromatin regulators to initiate a change in the overall transcriptional program. Chromatin based mechanisms are also of significant importance for transcriptional control and include post translational modifications like methylation, ubiquitination, propionylation, butyryrlation, carbonylation, and ADP ribosylation of histones and DNA by specific 'eraser and writer' proteins. Subsequently these modifications are

interpreted by so called 'reader' proteins. Furthermore the crosstalk between different histone modifications can influence the ability of reader proteins to recognize an adjacent mark, thereby preventing or promoting the recruitment of enzymes that modify additional sites.

One of the most important posttranslational modifications, which directly affects chromatin structure, is acetylation. The introduction of acetyl groups at protruding histone tails neutralizes the positive charge of histone lysines and thereby decreases the electrostatic attraction between histones and the negatively charged DNA. Subsequently, the chromatin structure unfolds and thereby increases its accessibility towards additional transcription factors and the transcription activator complex. Therefore, acetylation is associated with the activation of gene expression. Acetylation is catalysed by enzymes called histone acetyl transferases, HATs. Deacetylation is regulated by histone deacetylases, HDACs, which repress gene transcription by either condensing chromatin structure or by acting as components of large multiprotein complexes, which recruit inhibitory factors to regulatory DNA elements within gene promotor regions.

Concerning the Notch signalling pathway, it has been shown that the histone acetyl transferases like GCN5/PCAF and p300 are part of the Notch-ICD activator complex, whereas histone deacetylases are part of the RBP-J repressor complex and thereby control inhibition of Notch target genes.

Furthermore, previous findings suggest that many Notch target genes are occupied by various transcription factors that might regulate the transcription of those genes in a manner that is independent of the Notch pathway.

Some of these transcription factors are capable of cooperation with the RBP-J transcription factor (CSL), which actively regulates target gene expression. For example, Ets transcription factors are found at many RBP-J binding sites, suggesting that Ets are positively enhancing recruitment of the RBP-J.

On the other hand, the transcription factors Ikaros and ZNF143 are considered to compete with the RBP-j transcription factor for the same DNA-binding sites (11).

1.3.2 Crosstalk

Signalling crosstalk is defined as the interaction between the proteins of multiple different signalling pathways. Crosstalk is of significant importance due to the fact that the number of different signalling pathways is limited but the amount of cellular decisions is vast. Due to crosstalk, novel input and output combinations are created. As a consequence, the number of transduceable signals is greatly increased, leading to a higher diversity of possible phenotypes. EGFR/MAPK, insulin, TGF-β, Notch, WNT, Hedgehog and JAK/STAT pathways belong to the most important signalling pathways mediating the crosstalk with Notch. Depending on the cellular context, the interactions between these pathways can either be antagonistically or synergistically. For example, during vertebrate osteoblastogenesis, Notch/Wnt cross-talk in ST-2 stromal cells causes those cells to form osteoblasts, whereas in the presence of ectopic Notch1, these cells differentiate into adipocytes. In this case, Notch affects the Wnt signalling antagonistically. On the other hand Notch and Wnt/β – catenin act synergistically to maintain the postnatal hair growth in the mouse epidermis. If both signals are blocked, then a conversion of the hair follicles into cysts of interfollicular epidermis occurs (12, 13).
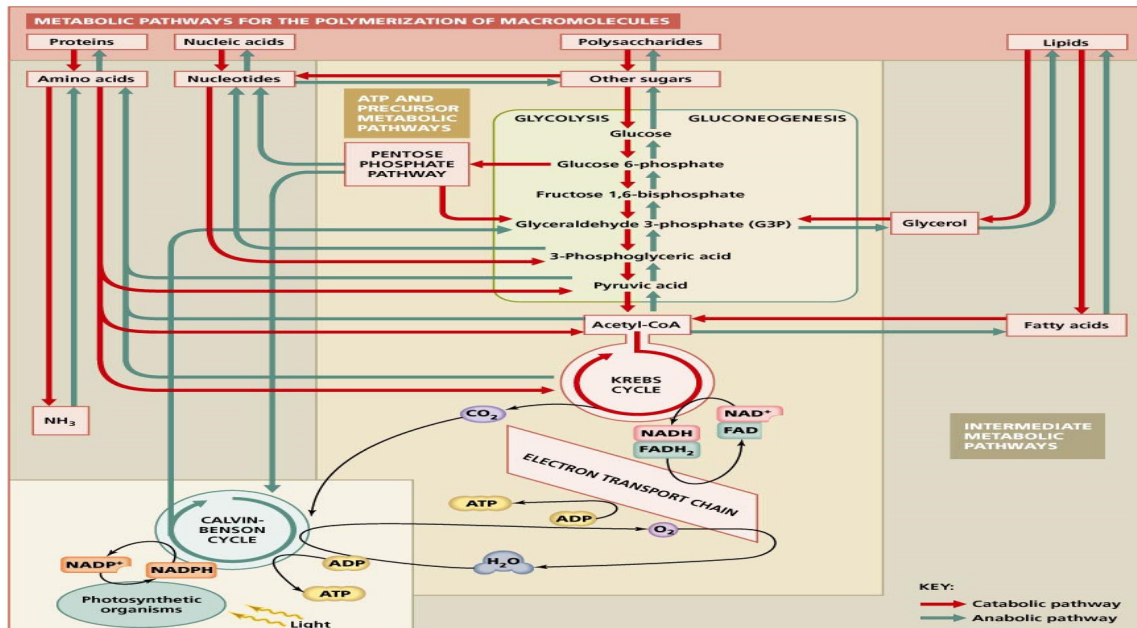
1.3.3 Metabolism

Metabolism is the sum of all enzyme-catalysed reactions taking place inside cells, which are resumed as metabolic pathways. In catabolism, organic nutrients (glucose, lipids and proteins) are degraded and finally converted into smaller and simpler products (lactic acid, CO_2, NH_3), which are useful to the cell. During the course of degradation, chemical energy is released, which is conserved in the form of high energy phosphates, for example ATP (adenosine triphosphate), and reduced electron carriers (NADH, NADPH, $FADH_2$). In anabolism, which is also called biosynthesis; small precursors are utilized for the formation of more complex and larger molecules like lipids, polysaccharides, proteins and nucleic acids. However, these biosynthetic reactions require an energy input, which is usually provided by the ability of ATP to transfer one of its phosphoryl groups or by the reducing power of NADH, NADPH, and $FADH_2$.

Glycolysis, glutaminolysis and the β-oxidation of lipids are important catabolic pathways and thus will be explained in detail. During the course of glycolysis, one molecule of glucose is converted to one molecule of pyruvate. Furthermore two molecules of ATP and NADH are formed as by-products. The next step includes the transport of pyruvate to the mitochondrion, where it passes through the mitochondrial membrane and finally enters the mitochondrial matrix. In the mitochondrial matrix, it is converted to Acetyl COA by the activity of pyruvate dehydrogenase. Acetyl CoA is then oxidized in the citric acid cycle and further molecules of ATP, NADH and $FADH_2$ are formed.

Glutamine is one of the most important available and abundant amino acids in the blood plasma of mammals. During its metabolism it converted to CO_2, pyruvate, lactate, alanine and citrate. Some of these metabolites contribute to the citric acid cycle, whereas other metabolites are alternatively used as building blocks for the synthesis of large biomolecules including, nucleic acids, polysaccharides and glutathione.

Lipids are the major energy source in liver and heart. During β-oxidation, lipids are degraded to Acetyl CoA which is the main substrate of the citric acid cycle and for the further production of NADH and $FADH_2$.

In the mitochondrial respiratory chain, electrons are removed from NADH and subsequently transferred through multiple complexes by the activity of ubiquinone. Due to this transfer, electrons are pumped across the inner mitochondrial membrane, thereby making the intermembrane space more alkaline and establishing a proton gradient, which provides the energy that is necessary to synthesize ATP. Therefore it can be concluded that metabolic pathways are tightly linked to ensure ATP production and cell survival (14, 15).

Fig. 3: Illustration of metabolic pathways and their intersections
(http://academic.pgcc.edu/~kroberts/Lecture/Chapter%205/05-34_CelluarMetabolism_L.jpg)

1.4 NAD$^+$/NADH

NADH is a molecule that is produced NAD+ oxidation in glycolysis, TCA cycle and during b-oxidation and consumed in the mitochondrial respiratory chain. This way it is a key metabolic effector that connects all major metabolic pathways. From this respect it is not surprising that the level of NAD: NADH is constantly monitored by the cells because it can be considered as a good indicator of its overall metabolic status.

The common step in NAD$^+$ biosynthesis is the formation of the dinucleotide from AMP (the adenylate moiety of ATP) and a pyridine mononucleotide. The generation of the pyridine mononucleotide from extracellular precursors is the primary concern of NAD$^+$ biosynthesis, as long as the availability of ATP is sufficient. These exogenous precursors are provided by the diet and encompass nucleobases like nicotinamide and nicotinic acid as well as nucleosides (Nam riboside and NA riboside) which are collectively known as vitamin B$_3$ or niacin. These nucleobases and nucleosides are subsequently converted to the corresponding mononucleotides NMN and NAMN and finally, the dinucleotide is formed by adenylation of the pyridine mononucleotide. This step is catalysed by NMN adenylyl transferases (NMNATs). However, most human NAD$^+$ is formed

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from NAM. This conversion of NAM to NAD^+ is successfully performed through Nam phospho-ribosyltransferase (NamPRT), which is the most crucial NAD^+ biosynthetic enzyme in mammalian physiology and therefore inhibitors of NamPRT can severely affect cellular NAD^+ levels. NAD is also generated endogenously from NAD^+-dependent signalling pathways and NADH has to be recycled immediately to maintain the cellular NAD^+ concentration at sufficient levels (through the NAD salvage pathway).

NAD^+ is an essential molecule in all living organisms. First of all, it is an essential redox carrier and thus it is vital for cellular energy metabolism. Moreover, it`s degradation is a key element of various signalling pathways. Thereby NAD^+ is continuously and irreversibly degraded to molecules that are of key relevance to cellular homeostasis. From this it follows that the cellular NAD^+ levels should be maintained to ensure proper energy transduction and the activity of NAD-dependent signalling pathways. Furthermore NAD^+ is an essential for multiple post translational protein modifications (deacetylation, ADP-ribosylation), which have diverse impact on diverse biological process including gene expression, cell cycle progression, insulin secretion, DNA repair, apoptosis and aging. For example, deacetylation of various protein substrates is catalysed by the activity of members of the highly conserved family of silent information regulator-2 (Sir2) like proteins (sirtuins), which require NAD^+ as cofactor to carry out their enzymatic function. Thus the activity of sirtuins strongly depends on the bioenergetics status of a cell, which is partly reflected by the cellular NAD^+ concentration and the NAD^+/NADH ratio. Deacetylation involves the elimination of nicotinamide and the transfer of the acetyl group to ADP-ribose moiety, leading to the formation of O-acetyl-ADP ribose (OAADPR), whose function is still unknown. Another NAD-dependent protein modification is ADP-rybosilation, which is carried out by two families of enzymes. In the course of the ADP-rybosilation, a single (mono ADP-rybosilation) or multiple (poly ADP-rybosilation) ADP-ribose units are removed from NAD^+ and subsequently transferred to specific amino acid residues of acceptor proteins. Afterwards, polymers of ADP are attached to these acceptor proteins. Furthermore NAD^+ is the precursor of cyclic ADP ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP), which are key regulatory elements of intramolecular calcium signalling. Both metabolites mediate the release of calcium ions from endogenous storage localities. NAADP triggers calcium release via

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activation of two pore channels which have been found in endolysosomes, whereas cADPR activates ryanodine receptors, which ultimately results in the release of calcium ions from the endoplasmic reticulum. On the other cADPR triggers the entry of extracellular cations via activation of plasma membrane cation channel TRPM2. All this processes may be interesting in the development of cancer therapies and are currently evaluated (17, 18).

2 Interplay between signalling pathways and metabolism

In general, cellular signalling and metabolism are tightly linked to each other.

For example, signalling pathways activated by growth factors and hormones initiate a course of events, including the stimulation of cell's metabolic activity, to ultimately promote cell proliferation or differentiation. A good illustration for this mechanism is the binding of insulin to receptors of muscle and fat cells to initiate the uptake of glucose and the formation of metabolic storage localities for triglyceride and glycogen. On the other hand signalling pathways can be regulated by metabolism through intracellular nutrient-sensitive signalling molecules like AMP-activated protein kinase (AMPK), which functions to activate or promote ATP-producing pathways and lower the activity of ATP-consuming pathways, and as a consequence also controls the activity of another intracellular nutrient-sensitive signalling molecule mammalian target of rapamycin complex 1 (mTORC1). Another possibility to modify the activity of signalling pathways is through the modifications of signalling proteins. This can be achieved either through the direct binding of metabolites to metabolic enzymes or signalling proteins or by post translational modifications. Protein modifications have immense impact on transcriptional regulation and gene expression as well as the metabolism of the cell itself and it has been shown that the extent and outcome of proteins modifications significantly depends on the cellular and nuclear availability of particular metabolites such as acetyl-CoA. Thus protein modifications are also nutrient sensitive and depend on the metabolic status of a cell. A well-known example of such a protein modification is acetylation. Acetylation is the transfer of an acetyl group and occurs through the activity of enzymes called histone acetyltransferases. The acetyl group is obtained from Acetyl-CoA, which is synthesised from citrate by the activity of an enzyme called ATP citrate lyase.

Acetylation is a dynamic process that affects non-histone proteins in the nucleus, cytoplasm and mitochondria. Furthermore nearly all enzymes of the central carbon metabolism are acetylated at their lysine residues. The acetylation status of a protein affects its activity. As a consequence it can be suggested that the cellular and subcellular concentration of Acetyl-CoA has a significant impact on metabolic activity as well as on the regulation of gene expression and other physiological processes in the cell.

On the other hand sirtuin deacetylases, which cause the removal of an acetyl group, depend on NAD^+ as a cofactor and therefore respond to the redox state of a cell. A further example of a protein modification that depends on the availability of a particular metabolite is protein methylation, which is carried out by enzymes called methyltransferases. The methyl group is synthesised by the employment and the conversion of S-adenosyl-methionine (SAM) to S-adenosyl-homocysteine, which is regenerated in a one-step carbon metabolism via the conversion of homocysteine into methionine. All this types of modifications are classical examples of how signalling pathways are controlled by metabolism (19, 20, 21).

2.1 Interplay between Notch signalling and metabolism

In tumour cells, a switch from oxidative phosphorylation to glycolysis uncoupled from the TCA cycle is frequently observed (so called Warburg effect). In breast tumour cells, a glycolytic phenotype is caused by the hypo- or hyperactivation of Notch signalling. Both, hypo- and hyperactivated Notch signalling provoke a higher rate of glycolysis by using different mechanisms. Hypoactivated Notch signalling induces a glycolytic phenotype by attenuating the activity of the mitochondrial respiratory chain and decreasing the concentration of p53, whereas hyperactivated Notch signalling modulates the PI3K/AKT signalling pathway, which regulates the expression of glycolytic genes as well as the activity and localization of several rate limiting glycolytic enzymes. However, aggressive tumour growth is only observed in Notch hyperactivated cells, which may be explained by the ability of those cells to switch back to oxidative phosphorylation.

On the other hand, it has been shown by our lab that the mRNA level of Notch target genes after activation of the Notch signalling pathway was significantly lowered in the presence of 2-deoxyglucose, a molecule that blocks glycolysis.

Thus it can be considered that Notch response is sensitive to the level of cellular metabolism. These observations are an example of the coherence and the reciprocal regulation between Notch pathway and metabolism (22).

## 2.2 Interplay between other pathways and metabolism

Transcript profiling studies have revealed that deregulated Wnt signalling alters the mRNA level of metabolic genes. These experiments emphasize that
Wnt-signals control metabolic flux and simultaneously promote cell growth. Therefore β-catenin can be consedered as an oncogene of the Wnt network. The liver-specific and transgenic expression of either wild type or oncogenic β-catenin modifies genes which are involved in gluconeogenesis and glutamine metabolism, thereby ultimately altering the synthetic and metabolic function of hepatocytes. Furthermore, proteomic analyses of livers from liver specific APC knockout mice have identified 56 dysregulated genes. Plenty of these genes are involved in mitochondrial dysfunction and carbohydrate metabolism. Therefore it can be suggested that defects in Wnt signalling lead to a switch of fuel oxidation away from fatty acid oxidation towards glycolysis. On the other hand, ligands of many signalling pathways including members of the Wnt and Hedgehog (Hh) are lipid modified through acylation or prenylation and thereby controlled by metabolism. Furthermore palmitoylation of well conserved cysteine residues in secreted Wnt proteins are responsible for proper signalling and secretion (23, 24).

3. Aim of the thesis

The aim of the thesis was to identify NAD(H) metabolic sensors involved in Notch signalling. For this purpose, we tested the phenotype of an RNAi mediated knock down of various NAD(P)(H) binding proteins, using the engrailed GAL4 driver that is expressed in the posterior part of adult wing. These genes have been selected by my supervisor, after they have already shown promising results in previous test when used in combination with another driver line. As we are primarily interested in metabolic sensors involved in Notch signalling I was specifically looking for phenotypes that would resemble the phenotype of the Notch pathway, like wing notches, thick or thin veins, extra veins or missing veins and deltas at the end of

veins. Fig. 4 shows such phenotypes on the wings where Notch signalling components have been misregulated.

Score Phenotypes:
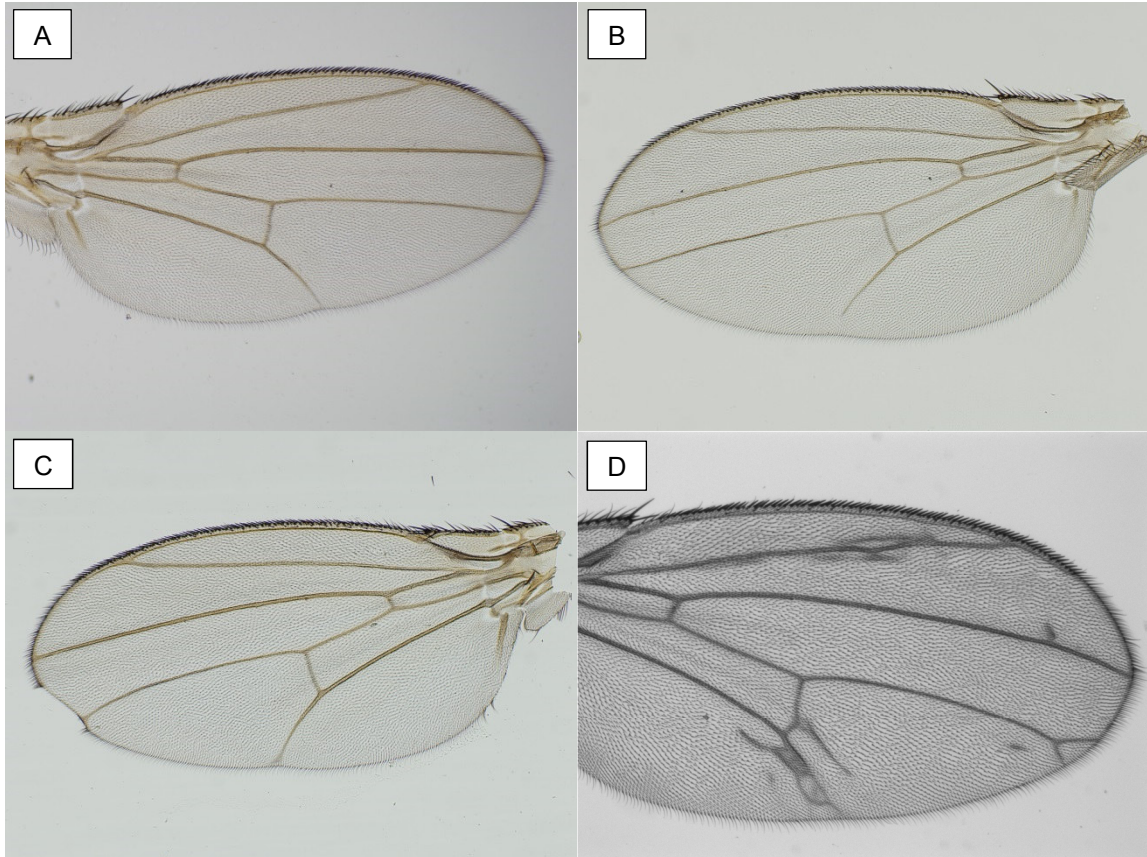
Fig.4: Examples of a wild type wing and of Notch-related phenotypes. (A) Wild type wing. (B) Mutant in Hairless (H^2, gain of Notch). (C) Notch mutant N^{55e11} (partial loss of Notch). (D). Delta mutant (partial loss of Notch).

4. Methods

4.1 UAS/GAL4-system

The UAS-Gal4 system allows the selective and controlled expression of any cloned gene in a wide variety of Drosophila cells and tissue types. On the other hand, it can also be used to reduce or completely switch off the expression of a particular gene by the use of RNAi transgenes.

In the Gal4 system, a promoter (or enhancer) drives the expression of the yeast transcriptional activator Gal4, which subsequently directs the transcription of the Gal4-responsive (UAS) target gene. The key feature of the Gal4 system is the

initial separation of the Gal4 and the UAS-target gene into two distinct transgenic lines. In the Gal4 line, the yeast transcriptional activator Gal4 is placed under the control of a tissue specific promoter. However, the target gene is non-existent in the Gal4 line and thus activation of the target gene is impossible. In the UAS-line, the reguatory regions of target gene (Upstream Activating Sequences, UAS), contain DNA-binding site for GAL4 but is silent because the Gal4 is absent. If the Gal4 line and the UAS-line are crossed, the target gene is expressed in a tissue specific manner in the progeny of this cross (as long as both transgenic lines are homozygous for either the Gal4 or the UAS-target gene).

In our experiment the expression of the UAS-RNAi transgene resulted in the knockdown of a particular NADH-binding protein, which was considered to interact with or be part of the Notch pathway. The possible consequences of this gene knockdown on the Notch pathway activity were examined on the adult wing. The Drosophila wing is subdivided horizontally into two parts, an upper anterior, and a lower posterior part, For my experiments I used *engrailed-Gal4* driver that is only expressed in the posterior region of the wing disc. Therefore we could observe any RNAi mediated phenotype only in the posterior part of the wing (25, 26).
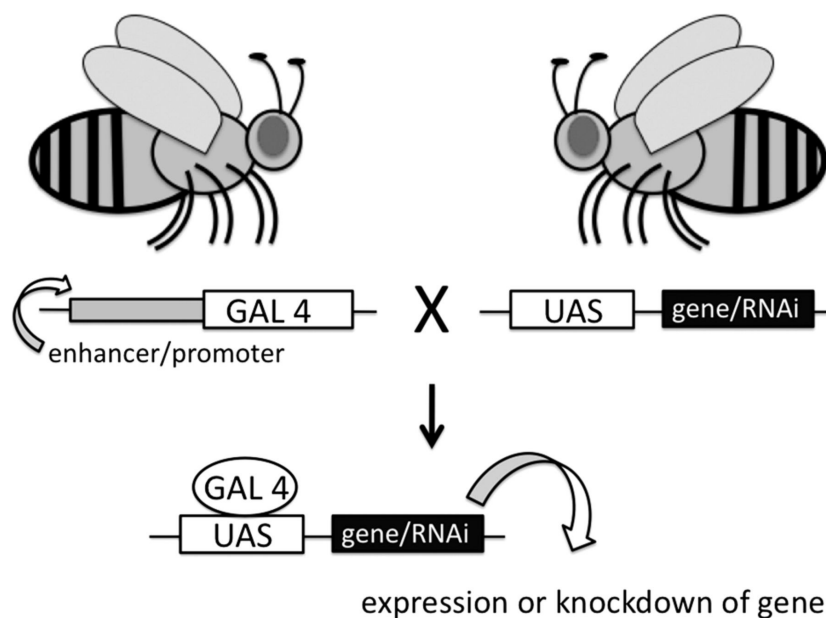
**Fig. 6: Working principle of gene knockdown using the UAS/Gal4-system**
(http://dkqlgfb5sk2dk.cloudfront.net/content/ajpregu/304/3/R177/F2.large.jpg)

4.2 RNA Interference

RNA interference is a technique that leads to the degradation of specific mRNA transcripts and therefore prevents the synthesis of the corresponding protein.

As such it is a rapid and simple method of gene silencing. When dsRNA is present in the cell it is recognized by a protein called dicer that cuts it into 21 to 25 nucleotide fragments called siRNAs. The siRNAs then associate with a complex of proteins to form an RNA-induced silencing complex (RISC). The RNA-induced silencing complex then associates with a cellular messenger RNA that has a sequence of homology with the siRNA fragment in the RISC, leading to rapid degradation of the mRNA and gene silencing. RNAi mediated gene silencing can be evoked experimentally, by the introduction of siRNA into the cells via transfection methods (including liposomes or viral vectors). However, in Drosophila there is no need to synthetize and transfect siRNA but long fragments of dsRNA (several hundred base pairs) can be transfected instead. Similarly, UAS constructs driving the expression of dsRNA can be used in vivo, and in combination with tissue specific Gal4 system in can be used to silence expression of specific transcripts in a tissue specific manner.

As the UAS/Gal4 system comes from yeast its expression at 18C is relatively low but it is much more expressed at 29C (close to the yeast optimal growing temperature). All the RNAi lines I used come from the Vienna Drosophila Research Centre (VDRC) and are so called 'KK' lines that have been designed to give high expression and low off target effects of the RNAi.
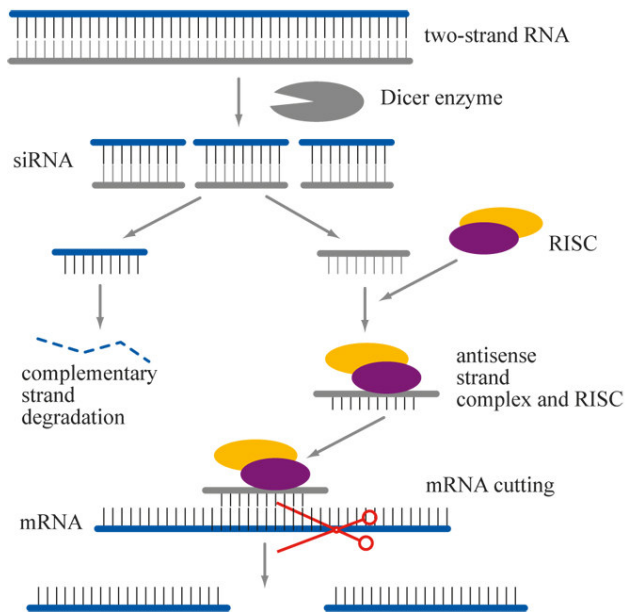
Fig. 7: Process of gene silencing by RNA interference

(http://eng.thesaurus.rusnano.com/upload/iblock/775/RNA-interference_1.jpg)

4.3 Fruit fly metamorphosis

The metamorphosis of fruit flies can be subdivided into four major states: embryonic, larvae, pupal and adult.

Eggs hatch after 24-36 hours. The emerging worm-like lava is called the first instar larva. It feeds from the medium and after another 24 hours, it develops into the second instar larva. It feeds as well and after 24 hours, it matures to the third instar larva. This is the last and the biggest larvae state. It feeds from the substrate and after a certain time it climbs upward away from its food and pupates. After 304 days of pupation the larva matures into the adult fly. The male flies are sexually a few hours after they have emerged. Female flies do not possess ripe eggs until two days after eclosion and therefore need longer time to get sexually active. The whole development time also depends at the temperature of incubation.
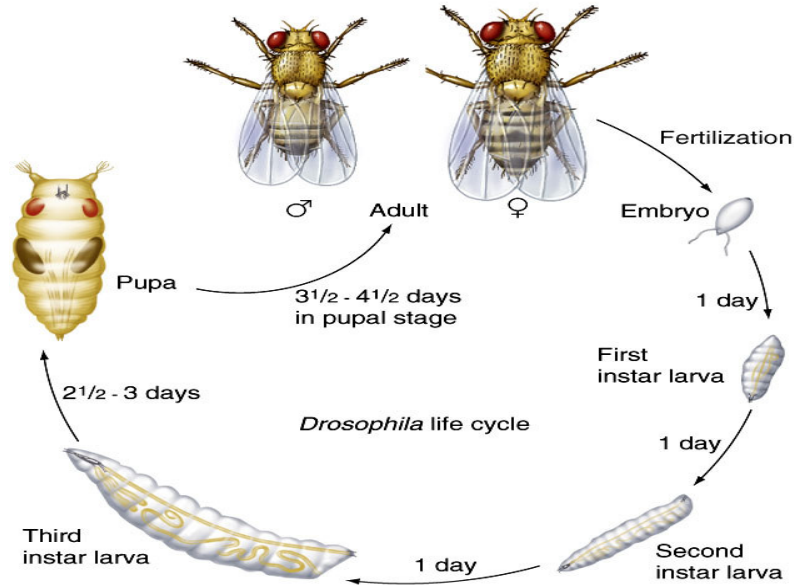
Fig. 5: Fruit fly metamorphosis

(http://skenderianscience.weebly.com/uploads/2/6/1/4/26143564/7822724_orig.jpg)

4.4 Experimental setup

The purpose of my experiments was to downregulate the expression fo 40 candidate genes in the posterior part of the wing using the en-Gal4 line crossed to UAS-RNAi lines, and assessing the resulting phenotype in the adult wings.

Frist of all, I collected virgins of the en-Gal4 flies as well as males of the UAS-RNAi lines. Afterwards five female virgins and three males were transferred to one glass tube and allow to breed at the temperature of 25°C. After two to three days, the first embryos have been embedded in the fly food. Subsequently, the adult flies have been flipped to a new glass tube and incubated at the temperature of 29°C. This way I could compare relatively small knock down of specific genes at 18C with much stronger knock down at 29C. Fly metamorphosis was completed after 8 to 10 days and the progeny was ready for further investigations. We were most interested in the strong knock down of given gene that happens at 29C. However, this was too strong and resulted in adult lethality. If the progeny died at 29°C, the progeny of the same cross at 25°C was analysed instead. If the flies were not capable of surviving even though the incubation temperature has been already reduced to 25°C, the same cross was repeated at an even milder incubation temperature of 18°C.

The wings of approximately 30 flies of every cross were mounted on a slide, pictures were taken on a camera at a microscope and the phenotype of the posterior part of the wing was carefully analysed. Wing with the most interesting phenotypes are shown in the presentation below.

5. Results

Forty different lines were crossed to the en-Gal4 driver. The Table 1 below I summarize the known information about the function of these 40 genes and biological process they are involved (based on Flybase), I state their mammalian orthologues, the RNAi line used and briefly describe the phenotype with the en-Gal4 driver. If the crosses were lethal at 29C or 25C I made a note into the column 'lethality'. Pictures of the most interesting phenotypes follow.
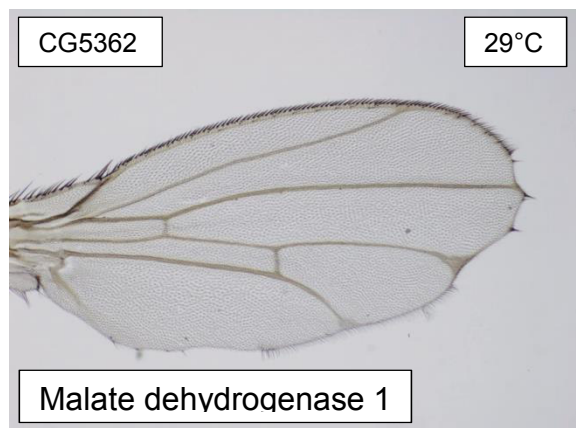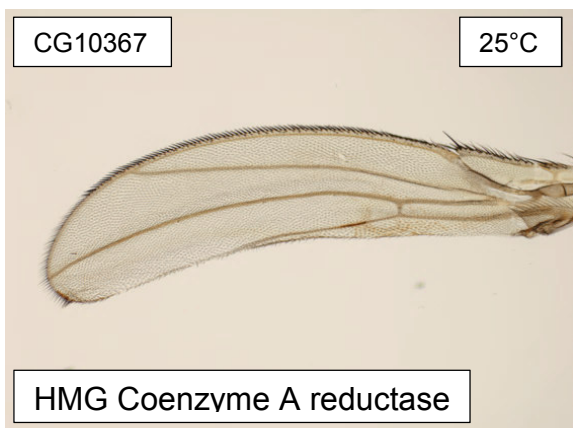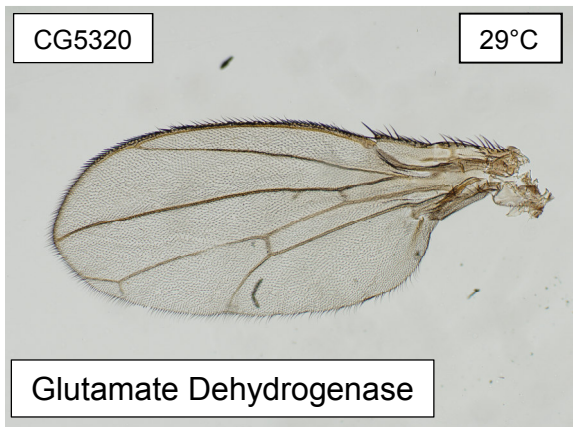
CG	Name	Human orthologue	Molecular function	Biological processes	RNAi line	Lethality	Phenotype
GC5320	Glutamate dehydrogenase	GLUD1(glutamate dehydrogenase 1), GLUD2 (glutamate dehydrogenase 2)	Glutamate dehydrogenase (NAD+) activity; identical protein binding; glutamate dehydrogenase [NAD(P)+] activity	Aerobic respiration, glutamate catabolic process to 2-oxaglutarate, glutamate catabolic process; NADH oxidation	109499	-	Notches, abnormal wing shape; extra vein material; missing vein material
CG3609	-	DHDH (dihydrodiol dehydrogenase)	Oxireductase activity	Oxidation-reduction processes	108742	-	Abnormal wing shape, extra vein material, missing vein material
CG9242	burgundy	GMPS (guanine monophosphate synthase)	GMP synthase activity; ATP binding; pyrophosphatase activity; ubiquitin-specific protease activator activity; GMP synthase (glutamine-hydrolyzing) activity	Histone deubiquitination; regulation of ubiquitin-specific protease activity; axon guidance; GMP biosynthetic process	106467	-	Abnormal wing shape, bubbles, missing vein material
CG33156	-	NADK (NAD kinase)	NAD+ kinase activity	Metabolic process; NADP biosynthetic process	103902		Extra vein material
CG2974	-	APOA1BP (apolipoprotein A-I binding protein), YJEFN3 (YjeF N-terminal domain containing 3)	-	-	110202	-	Extra vein material
CG10749	-	MDH2 (malate dehydrogenase 2, NAD (mitochondrial))	L-malate dehydrogenase activity	Tricarboxylic acid cycle; malate metabolic process; cellular carbohydrate metabolic process	109469	-	Extra vein material
CG12117	Sepiapterin reductase	SPR (sepiapterin reductase (7,8-dihydrobiopterin: NADP+ oxidoreductase))	Sepiapterin reductase activity	Oxidation-reduction process; tetrahydrobiopterin biosynthetic process	108243		Extra vein material
CG6012	-	HSDL1 (hydroxysteroid dehydrogenase like 1), HSD17B3 (hydroxysteroid (17-beta) dehydrogenase 3), HSD17B12 (hydroxysteroid (17-beta) dehydrogenase 12)	Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	Metabolic process	106631	-	-

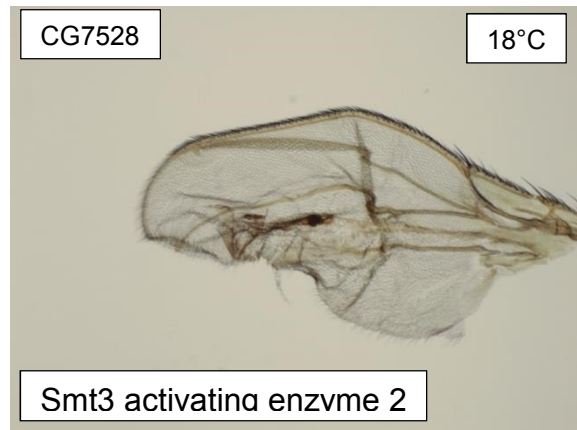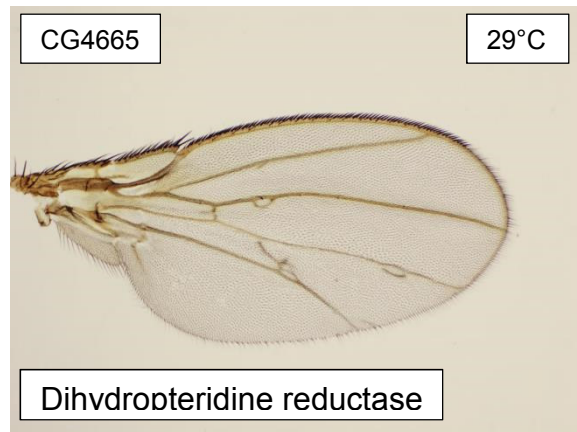| CG number			Molecular function	Biological process			Phenotype
CG2064	-	RDH11 (retinol dehydrogenase 11 (all-trans/9-cis/11-cis)), WWOX; (WW domain containing oxidoreductase), RDH12 (retinol dehydrogenase 12 (all-trans/9-cis/11-cis)), PTPDC1 (protein tyrosine phosphatase domain containing 1), RDH14 (retinol dehydrogenase 14 (all-trans/9-cis/11-cis)),DHRS13 (dehydrogenase/reductase (SDR family) member 13), DHRSX (dehydrogenase/reductase (SDR family) X-linked)	Oxidoreductase activity	Metabolic process	-	-	Extra vein mterial
CG16935	-	MECR (mitochondrial trans-2-enoyl-CoA reductase)	Zinc ion binding; trans-2-enoyl-CoA reductase (NADPH) activity	Oxidation-reduction process; fatty acid metabolic process	100741		Extra vein material
CG14882	-	-	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NAD(P)H as one donor, and incorporation of one atom of oxygen; oxidoreductase activity; [methionine synthase] reductase activity	Oxidation-reduction process	103984	-	Extra vein material, missing vein material
CG11143	Inos	ISYNA1 (inositol-3-phosphate synthase)	Inositol-3-phosphate synthase activity	Phospholipid biosynthetic process; inositol biosynthetic process	100763	-	Extra vein material, missing vein material
CG17560	-	FAR1 (fatty acyl-CoA reductase 1)	Fatty-acyl-CoA reductase (alcohol-forming) activity	Not known	104718	-	Extra vein material, missing vein material, bubbles
CG14893	-	FAR1 (fatty acyl CoA reductase 1)	Fatty-acyl-CoA reductase (alcohol-forming) activity	Not known	105915	-	Extra vein material
CG8665	-	ALDH1L1 (aldehyde dehydrogenase 1 family, member L1), ALDH1L2 (aldehyde dehydrogenase 1 family, member L2)	Hydroxymethyl-, formyl- and related transferase activity; methyltransferase activity; formyltetrahydrofolate dehydrogenase activity; oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	Oxidation-reduction process; one-carbon metabolic process; biosynthetic process; 10-formyltetrahydrofolate catabolic process	101493	-	Extra vein material, missing vein material
CG2070	-	RDH11 (retinol dehydrogenase 11 (all-trans/9-cis/11-cis)), WWOX (WW domain containing oxidoreductase), RDH12 (retinol dehydrogenase 12 (all-trans/9-cis/11-cis)), PTPDC1 (protein tyrosine phosphatase domain containing 1), RDH14 (retinol dehydrogenase 14 (all-trans/9-cis/11-cis)), DHRS13 (dehydrogenase/reductase (SDR family) member 13), DHRSX (dehydrogenase/reductase (SDR family) X-linked)	Oxidoreductase activity	Metabolic process	10691	-	Missing vein material
CG12233	lethal (1) G0156	IDH3G (isocitrate dehydrogenase 3 (NAD+) gamma), IDH3B (isocitrate dehydrogenase 3 (NAD+) beta), IDH3A (isocitrate dehydrogenase 3 (NAD+) alpha)	Magnesium ion binding; NAD binding; isocitrate dehydrogenase (NAD+) activity	Tricarboxylic acid cycle	10792	-	Abnormal wing shape
CG3495	GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase/4-reductase	TSTA3 (tissue specific transplantation antigen P35B)	GDP-L-fucose synthase activity; coenzyme binding; GDP-4-dehydro-D-rhamnose reductase activity	dsRNA transport; GDP-L-fucose biosynthetic process; 'de novo' GDP-L-fucose biosynthetic process	108926	-	Extra vein material
CG7979		UXS1 (UDP-glucuronate decarboxylase 1)	dTDP-glucose 4,6-dehydratase activity; coenzyme binding	Cellular metabolic process	11020	-	Extra vein material
CG7144	Lysine ketoglutarate reductase	AASS (aminoadipate-semialdehyde synthase)	Saccharopine dehydrogenase (NAD+, L-lysine-forming) activity	Oxidation-reduction process; regulation of histone methylation; histone arginine methylation	10960	-	Extra vein material, missing vein material
CG5840	-	PYCR1 (pyrroline-5-	Pyrroline-5-carboxylate	Oxidation-reduction process;	10998	-	-

		carboxylate reductase 1), PYCRL (pyrroline-5-carboxylate reductase-like), PYCR2 (pyrroline-5-carboxylate reductase family, member 2)	reductase activity	proline biosynthetic process			
CG10367	HMG Coenzyme A reductase	HMGCR (3-hydroxy-3-methylglutaryl-CoA reductase)	NADP binding; hydroxymethylglutaryl-CoA reductase (NADPH) activity	Soprenoid biosynthetic process; oxidation-reduction process; locomotory behavior; pole cell migration; germ cell migration; ecdysis, chitin-based cuticle; embryonic heart tube development; coenzyme A metabolic process; germ cell attraction; gonad development	108617	Lethal at 29°C	Posterior wing region missing
CG5362	Malate dehydrogenase 1	MDH1 (malate dehydrogenase 1, NAD (soluble))	L-malate dehydrogenase activity	Oxidation-reduction process; lateral inhibition; malate metabolic process; cellular carbohydrate metabolic process	110604	-	Extra vein material
CG31235	-	-	Oxidoreductase activity, acting on CH-OH group of donors	Metabolic process	107974	-	Extra vein material
CG18031	-	FAR1 (fatty acyl CoA reductase 1)	Fatty-acyl-CoA reductase (alcohol-forming) activity	-	107564	-	Extra vein material, missing vein material
CG10721	-	PYROXD1 (pyridine nucleotide-disulphide oxidoreductase domain 1)	Oxidoreductase activity	Oxidation-reduction process	110731	-	Abnormal wing shape, extra vein material, missing vein material
CG3603	-	DHRS2; (dehydrogenase/reductase (SDR family) member 2), DHRS4; (dehydrogenase/reductase (SDR family) member 4), DHRS4; (dehydrogenase/reductase (SDR family) member 4), HSD17B8; (hydroxysteroid (17-beta) dehydrogenase 8)	Oxidoreductase activity, acting on CH-OH group of donors	Metabolic process	101954	-	Abnormal wing shape, missing vine material, extra vein material, bubbles
CG4199 (II)	-	AIFM3 (apoptosis-inducing factor, mitochondrion-associated, 3)	flavin adenine dinucleotide binding; 2 iron, 2 sulfur cluster binding; oxidoreductase activity	Oxidation-reduction process; cell redox homeostasis	106170	-	Extra vein material, missing vein material
CG4199 (I)	-	AIFM3 (apoptosis-inducing factor, mitochondrion-associated, 39	flavin adenine dinucleotide binding; 2 iron, 2 sulfur cluster binding; oxidoreductase activity	Oxidation-reduction process; cell redox homeostasis	106170	-	Extra vein material
CG31216	Nicotinamide amidase	-	Nicotinamidase activity; calcium ion binding	Response to oxidative stress; determination of adult lifespan; negative regulation of neuron apoptotic process; metabolic process	112461	-	Extra vein material
CG4899	Photoreceptor dehydrogenase	HPGD; (hydroxyprostaglandin dehydrogenase 15-(NAD))	Alcohol dehydrogenase (NAD) activity; retinol dehydrogenase activity	Phagocytosis; retinal metabolic process; oxidation-reduction process; retinol metabolic process	108724	-	Extra vein material
CG1444	-	HSDL1 (hydroxysteroid dehydrogenase like 1), HSD17B3 (hydroxysteroid (17-beta) dehydrogenase 3), HSD17B12 (hydroxysteroid (17-beta) dehydrogenase 12)	Steroid dehydrogenase activity	Metabolic process	100977	-	Extra vein material, bubbles
CG11654	Adenosylhomocyst einase at 13	AHCYL1; (adenosylhomocysteinase-like 1); AHCYL2; (adenosylhomocysteinase-like 2), AHCY; (adenosylhomocysteinase)	Adenosylhomocysteinase activity	One-carbon metabolic process	109393	-	Notches, abnormal wing shape, extra vein material, missing vein material
CG4665	Dihydropteridine reductase	QDPR (quinoid dihydropteridine reductase)	6,7-dihydropteridine reductase activity	Metabolic process	108477	-	Extra vein material
CG7675	-	RDH11 (retinol dehydrogenase 11 (all-trans/9-cis/11-cis)), WWOX (WW domain containing oxidoreductase), RDH12 (retinol dehydrogenase 12 (all-trans/9-cis/11-cis)), PTPDC1	Oxidoreductase activity, acting on CH-OH group of donors	Metabolic process	106051	-	Extra vein material, missing vein material

		(protein tyrosine phosphatase domain containing 1), RDH14 (retinol dehydrogenase 14 (all-trans/9-cis/11-cis)), DHRS13 (dehydrogenase/reductase (SDR family) member 13), DHRSX (dehydrogenase/reductase (SDR family) X-linked)					
CG9914	-	CRYL1 (crystallin, lambda 1)	NAD+ binding; 3-hydroxyacyl-CoA dehydrogenase activity	Oxidation-reduction process; fatty acid metabolic process	106649	-	Extravein material
CG6287		PHGDH (phosphoglycerate dehydrogenase)	NAD binding; phosphoglycerate dehydrogenase activity	Oxidation-reduction process; L-serine biosynthetic process	107505		Bubbles ,extra vein material
CG7113	Scully also known as HL-X	HSD17B10 (hydroxysteroid (17-beta) dehydrogenase 10)	Steroid dehydrogenase activity; estradiol 17-beta-dehydrogenase activity; testosterone dehydrogenase (NAD+) activity; 7-beta-hydroxysteroid dehydrogenase (NADP+) activity; acetoacetyl-CoA reductase activity; 3-hydroxyacyl-CoA dehydrogenase activity	Ecdysone metabolic process; steroid metabolic process; androgen metabolic process; estrogen metabolic process; fatty acid metabolic process; acyl-CoA metabolic process	108042	-	Abnormal wing shape, extra vein material,
CG8303	-	FAR1 (fatty acyl CoA reductase 1)	Fatty-acyl-CoA reductase (alcohol-forming) activity	Not known	103744	-	Extra vein material, missing vein material
CG7528	Smt3 activating enzyme 2	UBA2 (ubiquitin-like modifier activating enzyme 2)	SUMO activating enzyme activity; ATP binding; protein binding; small protein activating enzyme activity; ubiquitin activating enzyme activity	Humoral immune response; positive regulation of NF-kappaB transcription factor activity; protein sumoylation	110173	Lethal at 29°C and 25°C	Abnormal wing shape, bubbles, extra vein material, missing vein material

CG5320 — 29°C

Glutamate Dehydrogenase

CG3609 — 29°C

CG10367 — 25°C

HMG Coenzyme A reductase

CG5362 — 29°C

Malate dehydrogenase 1

CG10721   29°C

CG3603   29°C

CG11654   29°C

Adenosylhomocysteinase at 13

CG4665   29°C

Dihydropteridine reductase

CG9914   29°C

CG7528   18°C

Smt3 activating enzyme 2

6. Discussion

In my thesis I used RNAi lines for fourty genes with predicted NAD binding domains to test their phenotype when downregulating with the engrailed-Gal4 driver. I found several genes that gave an interesting phenotype in the posterior part of the adult wing. I was particularly interested in phenotypes that would resemble Notch gain or loss of function. From this point of view, these were the most promising candidates:

1. CG5320 - glutamate dehydrogenase. There were notches in the wing margin and extra vein material in the posterior domain, consistent with Notch loss of function phenotype. This caught our interest because from our data generated in the lab we know that Notch pathway is sensitive to the level of cellular amino acids, so as less amino acids leads to less Notch signalling. Glutamate is one of the main sources of nutrients for cells and glutamate dehydrogenase is needed for its conversion to 2-oxo-glutarate and its subsequent breakdown in the TCA cycle. Knocking down this gene by RNAi should lead to less glutamate being processed by the cells, leading to slower TCA cycle. Thus the phenotype we see with RNAi line is consistent with our above mentioned observation that low level of amino acids lead to less Notch signalling.

2. CG10367 - HMG Coenzyme A reductase. RNAi knockdown of this gene by en-Gal4 lead to completely missing engrailed domain of the adult wing. This can be caused by various signalling events that cause cell death in this compartment. However, from the data that other members of the lab generated after I finished my thesis, we know that the third instar wing disc is developing normally and that Notch signalling is strongly reduced after the knockdown of this gene. According to Flybase this gene is located to the membrane of endoplasmatic reticulum so we could speculate that the posttranslational processing of the Notch receptor is affectd in these conditions, leading to defects in Notch signalling. Moreover, CG10367 has been shown to positively regulate EGFR signalling that is known to antagonize Notch in many developmental contexts, providing another explanation for the phenotype we observe.

3. CG5362 - malate dehydrogenase 1. This is a cytoplasmic version of malate dehydrogenase 2 that is found in mitochondria and takes part in the TCA cycle. CG5320 is part of the malate-aspartate shuttle that transfers the NADH generated in cytoplasm to mitochondria (not directly shuttling NADH across the membrane). In case this shuttle is not working properly we should expect accumulation of NADH in cytoplasm, slower TCA cycle and slower respiration. From this point of view the loss of Notch function phenotype is consistent with the phenotype we observe with the glutamate dehydrogenase (CG5320) described above.

4. CG10721 - this is a protein with completely unknown function but it was the only one that gave us a phenotype similar to Notch gain of function (similar to Hairless mutant in fig. 1B).

4. CG7528 - ubiquitin activating enzyme 2. This protein is an E1 ligase, activating SUMO and/or ubiquitin pools in the cells so as they can be used by E2 and E3 ligases for specific posttranslational modifications of target proteins. This can lead to protein degradation (in case of polyubiquitination) or it can regulate protein function (in case of monoubiquitination or sumoylation). E1 ligases are not specific for certain proteins but affect all the sumo/ubiquitin events in the cells. It is therefore not surprising that the adult wing with CG7528 downregulation is seriously affected in development. However, we know from the results of other people in our laboratory that the third instar wing discs develop normally and there is a massive upregulation of Notch signalling. Considering the widespread use of sumo.ubiquitin signals other signalling pathways and cellular processes will probably be also affected. In either case, it is interesting that E1 ubiquitin ligases are sensitive to NAD(H) levels and that cellular metabolic status can globally regulate these posttranslational modifications.

My results with RNAi screens in the wing were generated two years ago and meanwhile, other members of the Krejci laboratory took several genes to characterize them further. In fact, my mini-screen was part of a large screen that involved all the proteins with predicted NAD(P)(H) domain. Various phenotypes were identified this way that affects various pathways, not just Notch that we are mostly interested. To link the observed phenotypes to the Notch pathway it would be good to monitore the expression of several Notch target genes in the larval wing disc, while downregulating the NAD(H) binding proteins. Several such reporters exist (like reporter for classical Notch targets enhancer of split genes, or synthetis NRE reporter) or specific antibodies can be used to visualize the endogenous protein signal of certain Notch targets (like cut or wingless). Another way to see if these genes are involved in Notch signalling would be to test their genetic interactions with Notch signalling. In this case the N^{55e11} partial loss of Notch function allele or the Hairless mutant allele (H^2) would be combined with heterozygous mutants of our candidate genes and we would observe whether the N^{55e11} or H^2 phenotype would be modified in such a combination.

Another challenge would be to prove that the phenotypes we observe are really dependent on NAD:NADH levels. To test this we could downregulate or enhance the de novo expression of NAD, using the genes involved in this pathway (like

NAAM and NMNAT), and downregulate (or overexpress) our gene of interest at the same time. We would expect that the RNAi phenotype would be better or worse in these conditions. Another option would be to clone our genes of interest and inactivate their predicted NAD(H) binding domain, creating a dominant negative form of the protein. Again, we would expect differences in the phenotype when expressed in the wing compared to unmutated protein.

Taken together, my results revealed several interesting genes that are good candidates for metabolic sensor proteins involved in Notch signalling. The exact mechanism of their action related to Notch is still not clear but my results help to add one piece into this large puzzle

7. Literature

1. Guruharsha, K. G., Kankel, M. W., & Artavanis-Tsakonas, S. (2012). The Notch signalling system: recent insights into the complexity of a conserved pathway. *Nature Reviews Genetics*, *13*(9), 654-666.

2. Kopan, R., & Ilagan, M. X. G. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. Cell, 137(2), 216-233.

3. Schwanbeck, R., Martini, S., Bernoth, K., & Just, U. (2011). The Notch signaling pathway: molecular basis of cell context dependency. *European journal of cell biology*, *90*(6), 572-581.de

4. Chitnis, A. B. (1995). The role of Notch in lateral inhibition and cell fate specification. Molecular and Cellular Neuroscience, 6(4), 311-321.

5. de Celis, J. F., Bray, S., & Garcia-Bellido, A. (1997). Notch signalling regulates veinlet expression and establishes boundaries between veins and interveins in the Drosophila wing. *Development*, *124*(10), 1919-1928.

6. de Celis, J. F., Garcia-Bellido, A., & Bray, S. J. (1996). Activation and function of Notch at the dorsal-ventral boundary of the wing imaginal disc. Development, 122(1), 359-369.

7. Dahmann, C., Oates, A. C., & Brand, M. (2011). Boundary formation and maintenance in tissue development. Nature Reviews Genetics, 12(1), 43-55.

8. Wang, Z., Li, Y., Banerjee, S., & Sarkar, F. H. (2009). Emerging role of Notch in stem cells and cancer. *Cancer letters*, *279*(1), 8-12.

9. Turnpenny, P. D. (2014). Syndromes and Diseases Associated with the Notch Signalling Pathway. eLS.

10. Gridley, T. (2003). Notch signaling and inherited disease syndromes. Human molecular genetics, 12(suppl 1), R9-R13.

11. Borggrefe, T., & Liefke, R. (2012). Fine-tuning of the intracellular canonical Notch signaling pathway. Cell Cycle, 11(2), 264-276.

12. Lai, E. C. (2004). Notch signaling: control of cell communication and cell fate.*Development*, *131*(5), 965-973.

13. Pálfy, M., Reményi, A., & Korcsmáros, T. (2012). Endosomal crosstalk: meeting points for signaling pathways. Trends in cell biology, 22(9), 447-456.

14. Nelson, D., Cox, M. (2013). Lehninger Principles of Biochemistry. New York. W.H. Freeman and Company.

15. Slaninová, B. V. Regulation of cellular metabolism by the Notch receptor signalling pathway.

16. Diaz-Ruiz, R., Rigoulet, M., & Devin, A. (2011). The Warburg and Crabtree effects: On the origin of cancer cell energy metabolism and of yeast glucose repression. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1807*(6), 568-576.

17. Nikiforov, A., Kulikova, V., & Ziegler, M. (2015). The human NAD metabolome: Functions, metabolism and compartmentalization. *Critical reviews in biochemistry and molecular biology*, (ahead-of-print), 1-14.

18. Chiarugi, A., Dölle, C., Felici, R., & Ziegler, M. (2012). The NAD metabolome—a key determinant of cancer cell biology. *Nature Reviews Cancer*, *12*(11), 741-752.

19. Wellen, K. E., & Thompson, C. B. (2012). A two-way street: reciprocal regulation of metabolism and signalling. Nature reviews Molecular cell biology, 13(4), 270-276.

20. Metallo, C. M., & Vander Heiden, M. G. (2010). Metabolism strikes back: metabolic flux regulates cell signaling. Genes & development, 24(24), 2717-2722.

21. Bi, P., & Kuang, S. (2015). Notch signaling as a novel regulator of metabolism. Trends in Endocrinology & Metabolism, 26(5), 248-255.

22. Landor, S. K. J., Mutvei, A. P., Mamaeva, V., Jin, S., Busk, M., Borra, R., ... & Sahlgren, C. M. (2011). Hypo-and hyperactivated Notch signaling induce a glycolytic switch through distinct mechanisms. *Proceedings of the National Academy of Sciences*, *108*(46), 18814-18819.

23. Sethi, J., & Vidal-Puig, A. (2010). Wnt signalling and the control of cellular metabolism. *Biochem. J*, *427*, 1-17.

24. Teperino, R., Aberger, F., Esterbauer, H., Riobo, N., & Pospisilik, J. A. (2014, September). Canonical and non-canonical Hedgehog signalling and the control of metabolism. In *Seminars in cell & developmental biology* (Vol. 33, pp. 81-92). Academic Press.

25. Phelps, C. B., & Brand, A. H. (1998). Ectopic Gene Expression inDrosophilaUsing GAL4 System. *Methods*, *14*(4), 367-379.

26. Neckameyer, W. S., & Argue, K. J. (2013). Comparative approaches to the study of physiology: Drosophila as a physiological tool. *American Journal of*

Physiology-Regulatory, Integrative and Comparative Physiology, 304(3), R177-R188.