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The Interplay Between the Notch Signaling Pathway and Cellular Metabolism

Ph.D. thesis

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Annotation

We identified four metabolic genes as direct targets of Notch signaling pathway both *in vitro* and *in vivo* and investigated the hypothesis that Notch directed metabolic changes support the growth of the imaginal wing dics. Vice versa, we observed the influence of metabolic changes on the activity of Notch signaling pathway and we identified Sirt1 as a metabolic sensor for the Notch pathway that helps to elicit an efficient response to Notch signal, in a metabolism sensitive manner.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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České Budějovice, dne ..... Věra Slaninová

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

Slaninova V, Krafcikova M, Perez-Gomez R, Steffal P, Trantirek L, Bray SJ, Krejci A. **2016** Notch stimulates growth by direct regulation of genes involved in the control of glycolysis and the tricarboxylic acid cycle. Open Biol. **6**: 150155. http://dx.doi.org/10.1098/rsob.150155

Vera Slaninová searched for candidate genes, cloned their enhancers into the luciferase vectors and measured the activity of the reporters in the presence of N^{icd} *in a luciferase assay. She also performed all time course in Fig. 14, measured metabolite levels by Seahorse Xfe flux analyser and helped to prepare the cells for NMR metabolite measurements. She did the* hairy *and* Hairless *RNAi in the S2N cells, dissections of all wing discs samples which were subjected to Q-RT-PCR analysis, immunostainings of all wing discs with* Hex-A LacZ *reporter expression,* in situ *hybridizations in the imaginal wing discs, growth restriction analysis of the wing discs with double RNAi against metabolic genes and also experiment to rescrue* N^{icd} *induced hyperplasia by downregulation of metabolic genes.*

Horvath M, Mihajlovic Z, **Slaninova** V, Perez-Gomez R, Moshkin Y, Krejci A. **2016** The silent information regulator 1 (Sirt1) is a positive regulator of the Notch pathway in Drosophila. Biochemical journal, 473, 4129-4143 (2016).http://dx.doi.org/10.1042/BCJ20160563

Věra Slaninová contributed by measuring the activity of NRE *reporter in the presence of Sirt1 in a luciferase assay and also by expressing the Su(H) protein using baculovirus system, its purification and analysis of its acetylation status via western blots*

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1. Introduction

In multicellular organism each cell needs to stay in touch with its surroundings and cooperate in order to preserve integrity of the individual. Multiple signaling pathways has developed to secure communication of the cells within the whole organism as well as to mediate the information about the environmental state to the nucleus.

One of the signaling pathways involved in the regulation of multiple cellular processes is an ancient pathway leading through the Notch receptor that is highly conserved amongst all metazoan species. Notch pathway is active mainly during the development where it influences cell fate decisions, cell proliferation, differentiation and apoptosis in many organs in either a positive or a negative manner. In adult organism it participates in the regulation of proliferation of regularly renewing tissues such as gut epithelium (Kopan & Ilagan 2009) and in the regulation of the immune system. If disrupted, Notch signaling can contribute to development of disseases such as multiple sclerosis (Brosnan & John 2009), cerebral autosomal dominant artriopathy with subcortical infarcts and leuko-encephalopathy (CADASIL) (Spinner 2000), Alagille syndrome (McDaniell et al. 2006) or Diabetes (Ahn & Susztak 2010). It is also long known that Notch signaling can function as both a tumour suppressor and tumour-promoting factor in several types of cancers such as lymphoid neoplasm, breast cancer, lung cancer, skin cancer or colorectal cancer (Ntziachristos et al. 2014).

Notch signaling pathway is dependent on the contact of two neighboring cells and does not require any second messengers or other mediators to spread the signal. After a ligand on the surface of one cell is linked to the receptor represented on the plasma membrane of the other cell, intracellular domain of the receptor is cleaved off and travels to the nucleus where it directly regulates gene expression by binding to its transcription factor from the CSL family (Kopan & Ilagan 2009). Nevertheless, high level of regulation is required to secure the vast range of actions driven by Notch signaling.

In this part of my thesis I will introduce the Notch signaling pathway including the ways of its activation, its role in development and the mechanisms of Notch signaling regulation. I will also summarize recent knowledge about the regulation of metabolism and growth by Notch signaling, both in mammals and *Drosophila*.

1. 1. Notch signaling pathway

The number of the Notch receptors and its ligands differs within different organisms, nevertheless the mechanism of Notch signaling are very similar from *C. Elegans* to human. Notch signaling can be triggered either canonically through a ligand binding to the receptor on the surface of the cell or non-canonically in a ligand independent manner (Fig. 1).

The Notch signaling pathway is one of the very few pathways dependent on a proteolytic cleavage. In fact, several proteolytic cleavages are employed to activate Notch signaling. First cleavage (S1) takes place in Golgi apparatus. In here, a furin like protease cleaves the newly emerged Notch polypeptide straight after its translation, creating the future extracellular and intracellular domain which are connected by dissulfidic bonds in the transmembrane region. Second cleavage (S2) is specific for the canonical signaling and occures after a ligand is bound to the Notch receptor. Metaloproteases from the ADAM family cleaves off the extracellular domain creating Notch extracellular truncation (NEXT) which is substrate for the third and fourth cleavages (S3 and S4) mediated byγ-secretase. After that the Notch intracellular domain (N^{icd}) is released from the membrane and goes to the nucleus (Kopan & Ilagan 2009). During the non-canonical Notch signaling NEXT is created in dfferent mechanism but it serves as a substrate for S3 and S4 cleavage as well (Palmer & Deng 2015). There is another cleavage site within the N^{icd} (S5) that was discovered rather recently. It was identified as the site for the mitochondrial intermediary peptidase (MIPET). The cleavage of N^{icd} in the mitochondria results in a decreased cell viability and mitochondrial membrane potential (Lee et al. 2011).

Another property of the Notch signaling is its dependency on endocytosis. In the canonical Notch signaling, endocytosis and recycling of Notch ligands in the signal sending cells is needed for proper signal transduction. It was hypothesized that endocytic recycling enables ligand clustering which is necessary for DSL ligand activity. Recycling can also drive ligand to a specific part of the plasma membrane. It is also known that DSL ligands are endocytosed into the signal sending cells together with bound Notch extracellular domain (NECD). It is possible that during endocytosis NECD is removed from DSL ligand which can then be recycled back to the plasma membrane securing effective levels of ligand at the cell surface (Nichols & Miyamoto 2007).

Moreover, endocytosis of the Notch receptor is required for not only signal regulation and degradation but also for the signal activation. When ligand is not present on the surface of the neighbouring cell, Notch receptor will be marked for internalization by ubiquitin ligase called Deltex (Dx). Once internalized, Notch receptor can either return to the plasma membrane to be activated by the ligand, be forwarded for degradation or activated in ligand-

independent manner. Whether internalized Notch receptor will be degraded or activated by non-canonical Notch pathway is determined by its ubiquitination state. While polyubiquitination forwards receptor for degradation, monoubiquitination leads to its activation in Dx-dependent manner or its recycling to the plasma membrane (Palmer & Deng 2015; Hori et al. 2004; Barth & Köhler 2014). Vaccari et al. showed that the endocytosis of NEXT is necessary for the efficient activation of Notch signal via the cannonical Notch pathway (Vaccari et al. 2008). It is not clear why NEXT needs to be internalized to be cleaved effectively but it is suspected that γ-secretase cleaves more efficiently in acidic conditions present in endosomes. Alternatively, endocytosis can lead to changes in the conformation of NEXT making it more susceptible for γ-secretase. This event occures in early endosomes before their integration into the multivesicular bodies (MVB´s) (Palmer & Deng 2015). In case of the ligand independent branch of Notch signaling internalization of the Notch receptor is vital for the generation of NEXT. Internalized Notch receptor is forwarded from early endosomes into the MVB´s and further to the lysosomal limiting membrane in the way that N^{ecd} is enclosed in the lysosome while N^{icd} is displayed in the cytoplasm. The higly acidic environment of the lysosome is then responsible for the degradation of N^{ecd} creating NEXT that can be cleaved by γ-secretase (Andersen et al. 2012; Yamada et al. 2011).

Notch pathway possesses only one known transcription factor called CSL (CBF1/RBPjκ in mammals, Su(H) in *Drosophila*, Lag-1 in *C. elegans*). In the absence of Notch signal CSL recruits a repressor complex which represses the expression of Notch target genes. Once N^{icd} is cleaved off by γ-secretase it multimerize in the cytoplasm and creates the preactivation complex with SKIP and MAM. This complex then travels into the nucleus where it interacts with the CSL-repressor complex. While one N^{icd} and MAM is attached to CSL, unicorporated N^{icd} monomers and SKIP are released alongside with corepressor proteins (Vasquez-Del Carpio et al. 2011). CSL/N^{icd}/MAM complex then recruits other coactivators creating an activation complex that triggers the expression of Notch target genes (Kopan & Ilagan 2009). It should be noted that not all notch target genes must be occupied by CSL prior to Notch pathway activation (Krejčí & Bray 2007). Moreover, Hori et al. showed that non-canonical branch of Notch signaling pathway is sufficient to trigger not only the expression of the CSL-dependent Notch pathway target genes but also the expression of the Notch target gene *vestigial* in a CSL-independent manner. (Hori et al. 2004). It was proposed that there is another yet unknown transcription factor subjected to Notch signaling pathway. Nevertheless, the option that Notch regulates *vestigial* secondarily through activation of another transcription factor wich expression is regulated by CSL cannot be excluded.

While canonical Notch signaling and its functions are rather well understood, our knowledge about non-canonical Notch pathway remain poor. Mukherjee et al. showed that in *Drosophila* crystal cells ligand independent Notch signaling is necessary for their survival (Mukherjee et al. 2011). Notch deficiency leads to crystal cell destruction but inhibition of Notch ligands Dl and Ser after crystal cell fate determination does not affect cell survival rate suggesting that crystal cells induce Notch signaling self-autonomously. It was hypothesized that circulating blood cells such as crystal cells have no guarantee to meet a ligand source. In such case ligand independent induction would be highly beneficial.

Also, Notch signaling is remarkably stable to temperature variation. Shimizu et al. discovered that this thermal robustness is secured via Suppressor of Deltex (Su(Dx)) which is under normal conditions considered as a negative regulator of Notch signaling. Both Su(Dx) and Dx are E3 ubiquitin ligases responsible for internalization of Notch receptor. However, meanwhile endocytosis by Dx leads to Notch signal activation, internalization by Su(Dx) drives Notch receptor to lysosome and degradation. It was shown that canonical branch of Notch pathway is more effectively activated in high temperature. In these conditions Su(Dx) competes with Dx for Notch receptor, even overrides instructions of Dx, and drive its degradation and therefore decrease level of ligand independent Notch signaling. On the other hand, when temperature is low, the canonical Notch signaling is not effective enough. In that case Su(Dx) cooperates with Dx and helps to trigger ligand independent Notch pathway. In this manner, non-canonical Notch signaling helps to maintain desired level of activation (Shimizu et al. 2014).

Hayashi et al. showed that non-canonical notch signaling regulates the expression of synaptic vesicle proteins in excitatory neurons in a ligand-dependent but γ-secretase independent manner (Hayashi et al. 2016). Non-canonical Notch pathway also modulate Wnt signaling. In *Drosophila*, Membrane-bound form of Notch physically interacts with β-catenin and consequently negatively regulates its activity (Andersen et al. 2012). Taken together, it is clear that canonical and non-canonical Notch signaling pathway are highly orchestrated to secure all Notch related functions.

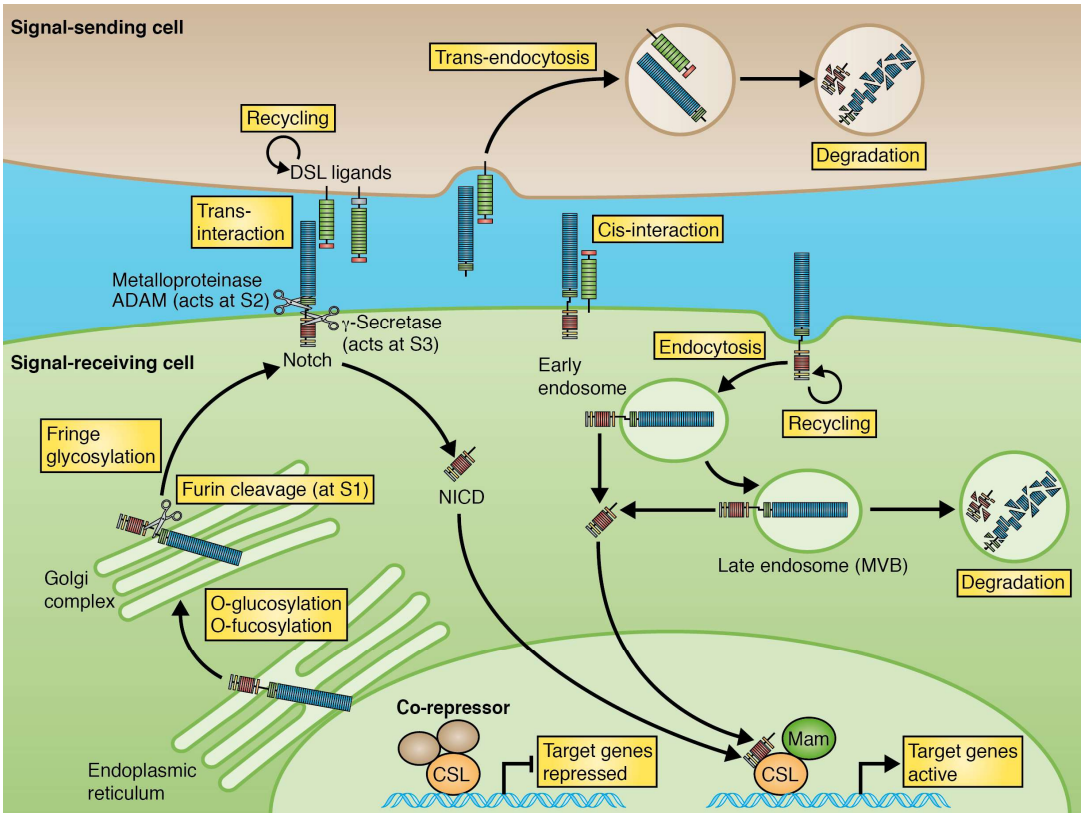


Fig. 1 Scheme of activation of Notch receptor and Notch receptor signaling pathway. Image courtesy of **Journal of Cell Science.** (Hori et al. 2013)

1. 2. Regulation of Notch signaling

The range of actions mediated by Notch signaling is rather broad and mechanisms of activation are very complex. It is obvious that high degree of regulation is necessary to secure such a versatility of Notch effects in the context dependent manner. The machinery regulating Notch signaling is still poorly understood. Nevertheless, some mechanisms explaining the context dependency of Notch signaling were proposed.

The first layer of Notch regulation is determined by the posttranslational modifications of both the ligand and the receptor and their cleavages. For example, ubiquitination by the E3 ligases like mindbomb or neuralized is needed for the activation of ligands as well as receptors and it sets the context to the Notch signaling (Bras & Loyer 2011). Spatial regulation of Notch signaling is secured by fucosylation (Shi & Stanley 2003). Glycosylation by specifically expressed glycosyl transferase Fringe regulates distinct interactions of the receptor with its ligands (Bray 2006). Moreover, Notch receptors are synthesized as single precursor proteins which need to be cleaved and assembled again in order to be activated by its ligands (Kopan & Ilagan 2009). Furthermore, Delta and Jagged ligands undergo proteolytic cleavage as well. While both the mechanism and purpose of the receptor cleavage is well understood, the role of ligand proteolysis is still not properly uncovered. Nevertheless, it was proposed that

cleavage of both Delta and Jagged serves to negatively influence Notch signaling. Mishra-Gorur with colleagues suggested that Delta cleavage serves to limit the amount of ligand available at the cell surface in nonideal conditions and therefore prevent Notch activation in signal receiving cell (Mishra-Gorur et al. 2002). Alternatively, LaVoie and Selkoe shown that not only Notch receptor but also both of its ligands undergo cleavage by γ-secretase and therefore limiting the enzyme pool available for Notch receptor. They also found that Jagged intracellular domain travels into the nucleus and stimulates expression of several genes via transcription factor AP1, the effect which is abrogated by N^{icd} (Lavoie & Selkoe 2003).

Second step of regulation involves the selection of target genes which are to be activated in the cell. The pool of activated genes is highly tissue and stage specific. According to a textbook model, transcription factor CSL binds in absence of Notch signal to either a low (RTGRAR) or high (YGTGRGAA) affinity binding site (Nellesen et al. 1999). Together with protein SKIP (Ski-interacting protein, Bx42 in *Drosophila*) it initiates assembly of corepressor complex (fig. 2) composed of such proteins as ETO (Eight twenty-one), CtBP (C terminal binding protein), Gro (Groucho), SMRT (Silencing Mediator for Retinoid and Thyroid hormone receptor), SHARP (SMRT and HDAC associated repressor protein, Hairless in *Drosophila*), NCoR (Nuclear receptor corepressor) or CIR (CBF1 interacting corepressor) (Schwanbeck et al. 2011). Corepressor complex further recruit histone deacetylases (HDACs, SAP30 (Sin3A associated protein 30 kDa), NAP1 (Nucleosome assembly protein 1), Sirt1) or demethylases (LSD1, ASF1, NAP1) (Bray 2006; Borggrefe & Oswald 2009). Presumably, the composition of repressor complexes on different Notch target promoters is highly variable and therefore the degree of target gene repression also differs. In many cases CSL is bound to the promoter only after Notch pathway activation suggesting that Notch signaling pathway is highly dynamic process (Krejčí, Bernard, Housden, et al. 2009).

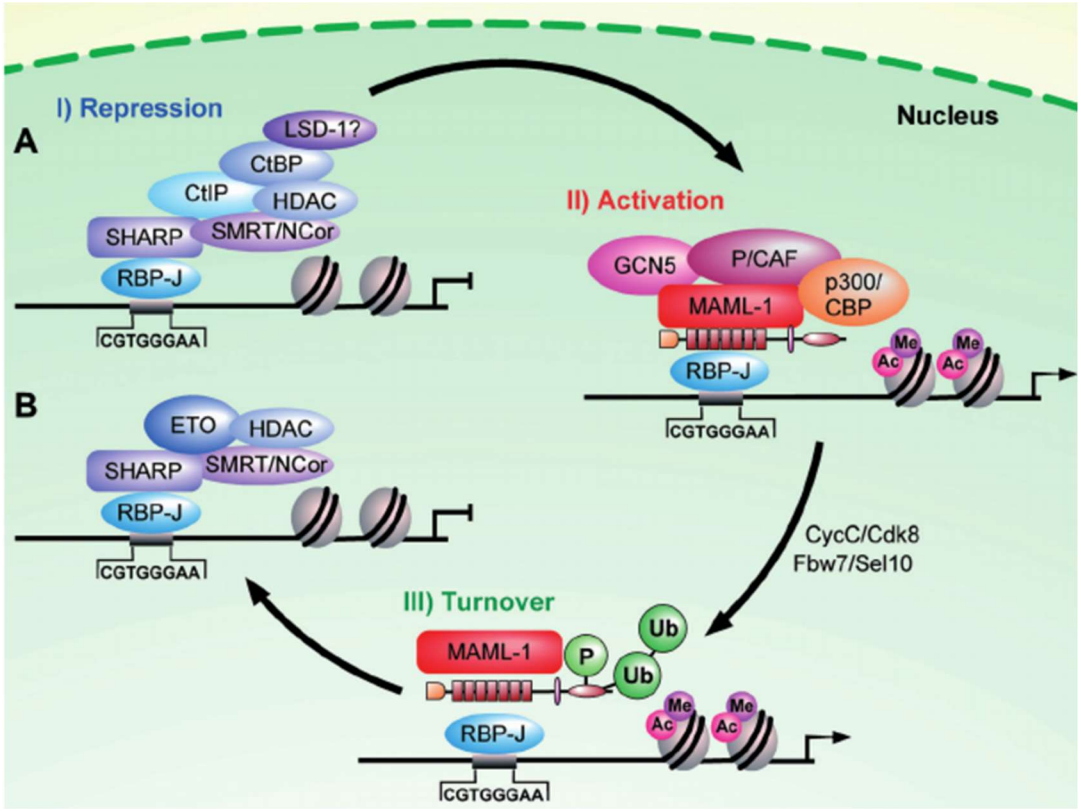


Fig. 2: Composition of the mammalian corepressor and coactivator Notch complexes recruited by CSL. (Borggrefe & Oswald 2009). RBP-J is different name for CBF-1, the mammalian homologue of CSL.

Upon Notch pathway activation N^{icd} translocates to the nucleus and together with coactivator mastermind (MAM) binds to CSL which leads to release of the corepressor complex (Fig. 3). The complex then recruit the general transcription factors and acetyltransferases such as p300, CREB-binding protein (CBP), K(lysine) acetyltransferase 2B (KAT2B), GCN5 acetyltransferase or H2B ubiquitinase Bre1 (Fryer et al. 2002). SKIP is also part of the activation complex. It was shown that at least in mammals multimerization of N^{icd} in the cytoplasm and binding to the SKIP is necessary for its transfer to the nucleus (Vasquez-Del Carpio et al. 2011). The Notch coactivator complex is rather unstable and its turnover is mediated by phosphorylation of N^{icd} by cyclin dependent kinase 8 (CDK8). Phosphorylated N^{icd} is tagged for ubiquitination and decomposition (Fryer et al. 2004). The composition of coactivator complexes is as versatile as in case of corepressor complexes. Moreover, if two CSL binding sites are close proximity N^{icd} can dimerize directly on DNA (Arnett et al. 2010).

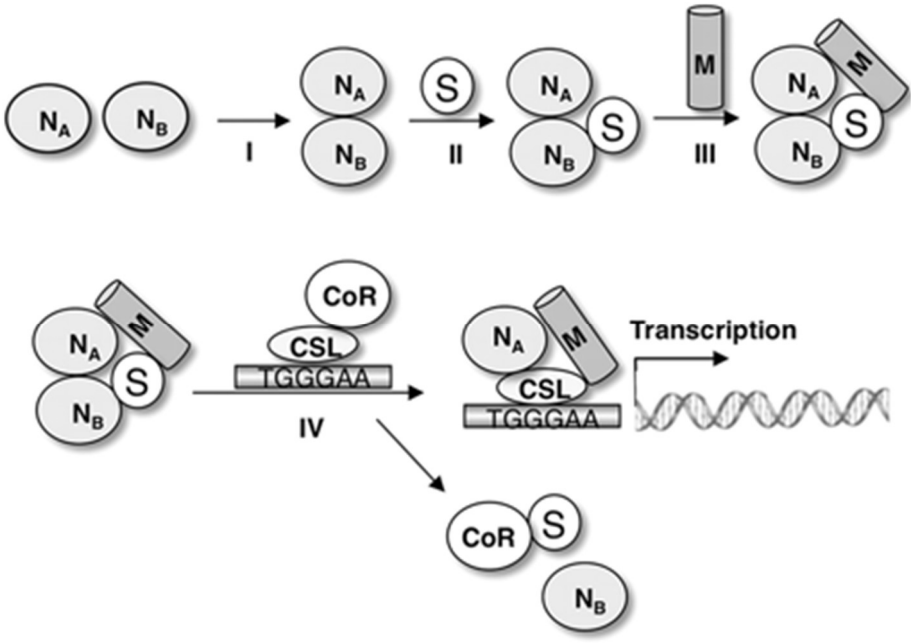


Fig. 3: Assembly of the Notch coactivator complex involves multimerization of N^{icd} in the cytoplasm. (Vasquez-Del Carpio et al. 2011).

Nevertheless, the majority of Notch pathway regulation is secured by a cross-talk with other signaling pathways and transcription factors. For example activation of NF-κB signaling leads to increased expression of both Notch receptor and its ligands and vice versa during the development of marginal zone of B lymphocytes (Moran et al. 2007). Interaction of Notch and NF-κB was also uncovered in several types of cancer (Vilimas et al. 2007; Maniati et al. 2011). Notch signaling cooperates with growth factor / cytokine signaling during self-renewal and differentiation of neural stem cells (Nagao et al. 2007). In hepatic stellate cells Notch pathway cooperates with Hedgehog signaling to drive their differentiation into the myofibroblasts (Xie et al. 2013). Notch signaling also cooperates with PI3K/AKT signaling during megakaryopoiesis (Cornejo et al. 2016), with estrogen receptor in breast cancer or Wnt signaling during intestinal epithelial cell fate decision (Rizzo et al. 2008; Nakamura et al. 2007). Moreover, cooperation of Notch signaling with transcription factors such as grainyhead (Furriols & Bray 2001), daughterless (Cave et al. 2005) or Twist (Bernard et al. 2010) helps to define target gene selection in specific tissues. Last but not least, Notch cooperates with HIF-1 for example in *Drosophila* crystal cells or glioma stem cells. In *Drosophila* crystal cells, Sima (an orthologue of mammalian Hif-1α) interact with full-length Notch in early endosomes and activates it through ligand-independent mechanism to promote hemocyte survival both in normoxic conditions and during hypoxic stress (Mukherjee et al. 2011). In mammals, it was shown that under hypoxic conditions Hif-1α interacts with N^{icd} and is recruited to Notch target genes promoters to enhance their expression. This interaction is necessary to maintain the cells

in undifferentiated state (Gustafsson et al. 2005). Lastly, Hu et al. showed that in glioma stem cells Hif-1α and Hif-2α competitively bind to N^{icd} to either promote the Notch activation or repress it in order to drive or suppress cell differentiation depending on oxygen tensions (Hu et al. 2016).

1. 3. Notch target genes

The Notch pathway target genes are studied for decades. The best known targets of Notch signaling is Enhancer of split (Hairy/Enhancer of split in mammals) complex which comprise a cluster of 10 highly homologous genes. They are transcriptional repressors at least some of them are expressed in all tissues where Notch is active and in many cases the Notch mediated phenotypes can be explained by the action of E(Spl) genes (Oswald et al. 2005; Kageyama et al. 2007).

Several studies revealed novel Notch target genes in different tissues using genome wide approaches. Krejčí et al. searched for the changes in mRNA expression and for sites occupied by Su(H) after activation of Notch in *Drosophila* muscle progenitor cells. They uncovered that Notch cooperates with the tissue specific transcription factor Twist to switch on the expression of genes encoding core components of different signaling pathways such as RTK, TGF-β, Wnt and also Notch receptor itself as primary targets of Notch signaling. Moreover they discovered that in many cases Notch activates the expression of certain genes and at the same time the expression of their repressors which creates a negative feed-forward loops (Krejčí, Bernard, Housden, et al. 2009).

Djiane revealed vast pool of Notch target genes which are activated during Notch induced hyperplasia in the *Drosophila* imaginal wing discs. Amongst them there were genes involved in proliferation, growth, cell death, metabolism or cell signaling. They also identified two novel targets of the Notch pathway, BTK Tec kinase homologue Btk29A and CG6191 which *Drosophila* homologue of CABLES1/2 (a substrate of Cdk5 and ABL). Moreover, many genes identified to be upregulated in hyperplastic wing discs are homologous to the ones which are regulated by Notch in human cancer cells (Djiane et al. 2012).

Meier-Stiegen with colleagues found vast pool of Notch1 target genes in murine stem cells including transcription factors, lineage determinants, cell cycle regulators, intracellular signaling mediators, receptors and ligands of different signaling pathways. Amongst them e.g. Pax6, Sox9, Runx1 or inhibitors of the Id family (Meier-Stiegen et al. 2010). Notch also directly activates cell cycle regulatory genes such as Cyclin D1 and therefore deregulate cell cycle progression (Koch & Radtke 2007). Furthermore, activated Notch can in certain tumor

cells cause cell cycle arrest by upregulating p21/Waf1 (Rangarajan et al. 2001). Taken together, the pool of Notch target genes is highly specific both spatially and temporally.

For example, in the developing brain the Notch pathway is responsible for the maintenance of neural precursor cells (NPCs) pool. On the other hand, Notch actively promotes differentiation into the astrocytes from NPCs. It is clear that the subset of activated genes must be completely different in these processes (Namihira et al. 2009). Moreover, there are several genes such as *cut* which is activated by Notch in dorso-ventral boundary of the wing or *scalloped* and *strawberry Notch* that are known to be target of Notch pathway in the wing anlagen but not in other cell types (Nagel et al. 2001). This complexity makes searching for Notch target genes even more challenging.

One of the problems while identifying new targets of Notch signaling pathway is how to distinguish between the primary and secondary or even tertiary targets. For example, it is difficult to truly separate responses caused by the direct signaling of Notch to CSL and a secondary or tertiary responses caused by a mediator in between when comparing transcriptional profile of normal tissue and a tissue with over activation of N^{icd}. Nevertheless, there are several methods which helps to identify primary and secondary targets. For example, inhibition of protein synthesis by cycloheximide (inhibits elongation by interfering with translocation step of protein synthesis) can be useful when using cell lines (but not tissues) (Kannan et al. 2001). Nevertheless, secondary responses still might be mediated by a protein mediator which was already synthesized before protein synthesis inhibition. Also, there are several commercially available γ-secretase inhibitors (prevent Notch signaling pathway activation by inhibiting N^{icd} cleavage) which can be powerful tool while identifying Notch targets genes. But again, possible secondary response cannot be excluded. The best way how to identify novel targets is to combine expression studies with bioinformatics/computational approach (*in silico* prediction of CSL binding sites within the genome) and chromatin immunoprecipitation (ChIP) that identifies elements occupied by a transcription factor *in vivo* (Wang 2005). Only genes that have Su(H) or N^{icd} bound in their enhancers and at the same time their mRNA level is upregulated after Notch activation can truly be considered as primary targets of Notch signaling (Krejčí, Bernard, Housden, et al. 2009).

1. 4. The role of Notch signaling in development

During development the Notch pathway plays a role in multiple processes, namely lateral inhibition and lateral induction, linage specification and boundary formation.

Both in vertebrates and *Drosophila* lateral inhibition is typically used during neurogenesis. It drives the differentiation of initially equal progenitor cells which express more or less the same amount of both the Delta ligand and Notch receptor. In a time one of the cells start to express more Delta and becomes a neuron. It sends an activation signal to neighboring cells instructing them to undergo different developmental path (Bolós et al. 2007). It is suggested that lateral inhibition creates a negative feedback loop because Notch signaling cannot be activated in the signal sending cell and also level of Delta ligand expression is decreases in the signal receiving cells. Other than neurogenesis, lateral inhibition is further involved in bristle patterning in *Drosophila* (Hartenstein & Posakony 1989), inner ear patterning in vertebrates (Eddison et al. 2000) or development of pancreas (Bolós et al. 2007).

Lateral induction is to some extend similar to lateral inhibition. In the cluster of progenitor cells one starts to differentiate but instead of instructing surrounding cells to take different path it guides them to share the same fate. Notch activation drives production of the Notch ligand, Serrate (Jagged) in the signal receiving cells by a positive feedback loop and these cells send the signal further to the neighboring cells. Nevertheless, Notch activity in the middle cell is still blocked by the regulator Numb which is similar to the situation occurring during lateral inhibition. Lateral induction takes place in cardiovascular system development (Lewis 1998) or differentiation of lens fibers (Saravanamuthu et al. 2009).

Notch signaling pathway is also involved in the regulation of lineage specification. During the asymmetric cell division Notch regulators such as Numb are inherited unevenly by the daughter cells which causes Notch signaling being active only in certain proportion of the cells. This leads to specification of divergent cell subtypes. Notch pathway is involved in lineage specification of sensory organ precursors (SOP) (Culi & Modolell 1998) in *Drosophila*, specification of projection neuron precursors (PN) (Lin et al. 2010) or specification of hematopoietic progenitors (Ikawa et al. 2006; Duvic et al. 2002).

Notch pathway is also involved in processes required to form a boundary between two distinct populations of cells. To some extend boundary formation is similar to lateral inhibition in a way that cells which express more of Delta ligand trans-activates Notch signaling in adjacent cells while cis-inhibits Notch activation within Delta expressing cells employing once again negative feedback loop. Nevertheless, in the process of boundary formation this happens in the stripe of cells making a boundary between two groups of cells rather than within one

cell only as it does during lateral inhibition (Matsuno et al. 2003). Notch controlled boundary formation happens in the *Drosophila* wings (Parks et al. 2000) or intestine as well as midbrain organizer formation in chicken embryo (Kopan et al. 2002).

1. 5. Notch signaling in disease

The relationship between Notch signaling and cancer is known for a long time. It is a known player in the carcinomas of T-cells, breast tissue, colon, etc. However, the connection with other diseases such as CADASIL, Allagile syndrome or diabetes was also discovered. Here are several example how aberrant Notch signaling can promote development of disease.

Joutel et al. discovered that certain mutations in Notch3 genes can lead to the development of CADASIL disease (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy). Most of the CADASIL patients display missense mutations which leads to addition or loss of a cysteine residue within the EGF-like repeats in Notch3 protein. That leads to synthesis of Notch3 protein with odd number of cysteine residues which is predicted to interfere with dissulfidic pairing and therefore changed stability of Notch3 protein and abnormal extracellular domain accumulation. These mutations are present in 90% of CADASIL patients. Nevertheless, mechanisms in which Notch3 extracellular domain accumulation causes CADASIL are still not clear (Spinner 2000).

In multiple sclerosis, dysfunction of non-canonical Notch signaling pathway leads to the demyelination of the axons. In a normal brain the canonical Notch signaling is involved in the maintenance of oligodendrocyte precursor cell (OPC) pool. Binding of canonical ligands such as Delta or Jagged leads to transduction of the Notch signal through the CSL/N^{icd}/MAM inducing transcriptional activation of the inhibitory genes of hairy/Enhancer of split family and subsequent inhibition of the OPC maturation. On the other hand, ligands of the non-canonical Notch signaling such as Contactin activates Notch pathway through N^{icd}/Deltex signaling cascade which leads to elevated expression of such genes as myelin-associated glycoprotein and therefore promoted OPC differentiation and myelination. Nakahara and colleagues showed that despite the fact that Contactin is abundantly expressed on demyelinated axons in human chronic MS lesion and that Notch1 is activated in OPC, N^{icd} intracellular domain fails to translocate to the nucleus. They found that although N^{icd} succeed to create a complex with nuclear transporter Importin β in cytoplasm this complex also aggregate with TAT-interacting protein 30kDa which is a proapoptotic factor inhibiting transport of the N^{icd} into the nucleus and consequently prevents oligodendrocyte differentiation and demyelination (Brosnan & John 2009).

The Notch signaling is vital for glomerular and proximal tubule development of kidneys and when the developmental processes are finished Notch activity is dramatically decreased. Nevertheless, growing evidence suggest that activation of the Notch signaling in podocytes may play crucial role in the pathogenesis of podocyte injury. In mouse model of diabetes expression of Notch1 in podocytes leads to albuminuria and glomerulosclerosis which are the signs of diabetic nephropathy. In HEK293 cells (human embryonic kidney cell line) and rat kidneys hyperglycemia leads to activation of Notch signaling. In podocytes, Notch pathway cooperates with the TGF - β pathway creating positive feed-back loop in which TGF – β transcriptionally upregulates expression of Jagged1 ligand and in turn Notch activation increases TGF – β expression. TGF – β is a potent profibrotic activator in glomerular disease suggesting that Notch can be one of the regulators during glomerulosclerosis. Moreover, Notch1 activation induces VEGF (Vascular endothelial growth factor) expression, causing decreased expression of nephrin and induction of podocyte apoptosis and subsequent albuminuria. This effect can be reversed by γ-secretase inhibitor DAPT which opens new therapeutic strategy for the cure of diabetic nephropathy (Ahn & Susztak 2010).

Allagile syndrome (AGS) is defined by hepatic bile duct insufficiency and cholestasis in combination with cardiac, skeletal, and ophthalmologic symptoms. AGS is known to be caused by mutations in the Notch pathway ligand Jagged1. These mutations are found in 94% of patients. Moreover, McDaniell et al. hypothesized that AGS is actually heterogeneous disorder and implicated that Notch2 mutations can be involved as well. Spatial and temporal expression pattern of Notch2 in tissues involved in AGS implicates that it can be the receptor interacting with Jagged1. Furthermore, they identified Notch2 mutations in two probands with AGS diagnosis which lacked mutations in Jagged1 adding new pavestone into the understanding how Notch can trigger Alagille syndrome (McDaniell et al. 2006).

Alzheimer's disease (AD) is one of the most common cause of dementia related with neurodegeneration. There are two neuropathological lesions in the brain of Alzheimer´s disease patients. They are composed of extracellular amyloid plaques consisting of 4-kDa amyloid-β peptide and intracellular neurofibrillary tangle consisting of hyper phosphorylated forms of tau protein. Neurotoxic amyloid β-peptide is generated by cleavage of the amyloid precursor protein (APP) by γ-secretase (presenilin) which also cleaves Notch receptor, Notch ligands Delta and Jagged and several other type I membrane proteins. Mutations in both APP and presenilins are connected with early onset of familial Alzheimer´s disease (FAD). FAD patients account for less than 5% of all AD patients but out of those over 90% display mutations in presenilins genes. Moreover, it was implied that Notch signaling plays a role in learning and long term spatial memory and also that misregulation of Notch can also contribute

to neuronal degeneration (Woo et al. 2009). Nevertheless, despite of the fact that both APP and Notch uses γ-secretase the involvement of the Notch signaling in Alzheimer´s disease was not validated.

Finally, out of all diseases related with Notch, relationship between Notch signaling pathway and cancer is the one the best studied. Depending on the context it can serve as both oncogene and tumor suppressor. For example, translocation t(7;9) leads to non-regulated expression of 5´deleted transcripts of Notch1. It was shown that this translocation leads to changes in proliferation as well as survival of improperly differentiated T-lymphocytes and is a cause of lymphoid neoplasm of T-all (Allenspach et al. 2002). Another example of Notch working as an oncogene comes from the breast cancer model. In here, Mouse Mammary Tumor Virus (MMTV) insert itself into the Int-3 region inside of the Notch4 gene which leads to misregulation and higher expression of the active form of Notch 4 and therefore higher proliferation of these cells (Bolós et al. 2007). In guts, Notch pathway supports maintenance of proliferative cells which are necessary for renewal of Lieberkühn´s crypts. Nevertheless, constitutive activation of Notch in gut epithelium leads to accumulation and subsequent proliferation of non-differentiated epithelial cells (Koch & Radtke 2007). But in some cases it is inactivation rather than over activation of Notch pathway which leads to tumor progression. In these contexts, Notch pathway plays a role as a tumor suppressor. In neuroendocrine cells of lungs, Achaete-scute gomolog-1 (ASH-1) is crucial for their differentiation. Nevertheless, its expression in epithelial cells leads to unguarded proliferation of epithelial tissue. This is prevented by expression of Hes triggered by activated Notch signal. Hes protein binds to ASH-1 promoter and inhibits its expression. Nonetheless, in lung tumors Notch signaling is inhibited which leads to overexpression of ASH-1 and subsequently to over proliferation of epithelial cells (Bolós et al. 2007).

Notch pathway can contribute to cancerous growth by various mechanisms. It is known that Notch pathway affects many oncogenes and tumor suppressors. For example it was found that gene *Myc*, which is major regulator of cell growth, proliferation, metabolism, differentiation, apoptosis and also one of the best known proto-oncogene, is a direct target of Notch signaling (Efstratiadis & Klinakis 2007). There is also connection between Notch and tumor suppressor p53 which can be downregulated by Notch signaling (Beverly et al. 2005) or in other cases p53 can downregulate Notch signaling (Lefort et al. 2007). Notch signaling also influence expression of PTEN (phosphatase and tensin homologue) which is a tumor suppressor involved in the regulation of the cell cycle and also AKT. It prevents cells to grow and divide too quickly. In prostate adenocarcinoma, Notch1 signaling is lost and PTEN expression is decreased (Whelan et al. 2009). Finally, activation of Notch signaling pathway

can influence cellular metabolism and eventually trigger the Warburg effect. This happens not only indirectly by modulating Myc or PI3K/AKT signaling but also directly by triggering expression of several metabolic genes such as Glucose transporter 1 or Hexokinase-A (Landor et al. 2011).

From vast range of action which Notch signaling pathway orchestrate it is obvious that misregulation of Notch signaling can cause or at least participate in the development of diseases. Indeed, many diseases have already been connected with aberrant Notch activation and their amount is still growing. Therefore, development of drugs targeting Notch pathway may be good strategy for treatment of these diseases.

1. 6. Metabolism of the cell

Each cell accepts nutrients in form of sugars, lipids and proteins. These nutrients are further decomposed by catabolic reactions to smaller units. During decomposition processes, energy is gained mostly in form of ATP (adenosine triphosphate), NADH (reduced nicotinamide adenine dinucleotide) or $FADH_2$ (flavine adenine dinucleotide). Both building blocks and energy created during catabolism are later consumed to create new biomolecules such as proteins, lipids, sugars or nucleic acids (Fig. 4).

Most important source of energy as well as carbonic building blocks for a cell is glucose. This molecule is metabolized in the cytoplasm in the process called glycolysis, followed by pyruvate decarboxylation, citric acid cycle and finally oxidative phosphorylation by electron transport chain (ETC) in mitochondria. This way, up to 36 molecules of ATP can be formed from one molecule of glucose. Another important sources of nutrients are lipids and amino acid glutamine.

In the first step of glycolysis, glucose is transformed to glucose-6-phosphate which is either further decomposed to two molecules of pyruvate or it is redirected into pentose phosphate pathway. In here, ribose-5-phosphate (precursor vital for creation of nucleotides) and also huge amount of NADPH (reduced nicotinamide adenine dinucleotide phosphate, co-factor for fatty acid synthesis) are produced. During glycolysis itself, two molecules of ATP, two molecules of NADH and also glyceraldehyde-3-phopshate (precursor for triaclyglyceride synthesis) are created. In the last step of glycolysis glucose is remodeled to two molecules of pyruvate which is further transported through mitochondrial membranes to mitochondrial matrix. In here, pyruvate is transformed to acetyl coenzyme A (Acetyl CoA) by pyruvate dehydrogenase.

Mitochondrial matrix contains enzymes of Citric acid cycle which uses Acetyl CoA as the main substrate and produces not only precursors for synthesis of nucleic acid, lipids and amino acids but also reduced energy molecules in form of NADH and $FADH_2$. Co_2 is also created as a waste product of this process.

In the ETC, on mitochondrial inner membrane, electrons are extracted from either NADH or succinate and further disseminated through different ETC subunits by coenzyme Q (ubiquinone). As electrons are transported from one subunit to another, large amount of protons flows through ETC complexes (except complex II) from mitochondrial matrix to intermembrane space creating proton gradient. At the end of the ETC electrons are passed on the oxygen to create water. Protons are pumped back from intermembrane space to the mitochondrial matrix through the ATP synthase (also called complex V of ETC) which produce ATP.

Another important source of energy for the cells, especially the ones which are rapidly dividing, is glutamine. Glutamine is the most abundant amino acid in the mammalian plasma and provides nitrogen for synthesis of amino acids and proteins. When consumed by the cell, glutamine is imported into the mitochondria where it is decomposed in the process of glutaminolysis to glutamate, aspartate, CO_2, pyruvate, lactate, alanine and citrate. These metabolites are then used as building blocks for the synthesis of nucleic acids, polysaccharides or glutathione. Some of them can also fuel Citric acid cycle and therefore create energy in form of NADH or $FADH_2$.

Last nutrient and source of energy to mention are lipids. They are main source of energy for both liver and heart. During process of β-oxidation they are decomposed to acetyl CoA which serves as substrate for Citric acid cycle. During decomposition of the lipids, further molecules of NADH or $FADH_2$ are produced as well. It should be noted that all of the above mentioned metabolic pathways are dependent on each other and they cooperate to produce both energy and building blocks for synthetic pathways (Murray et al. 2012).

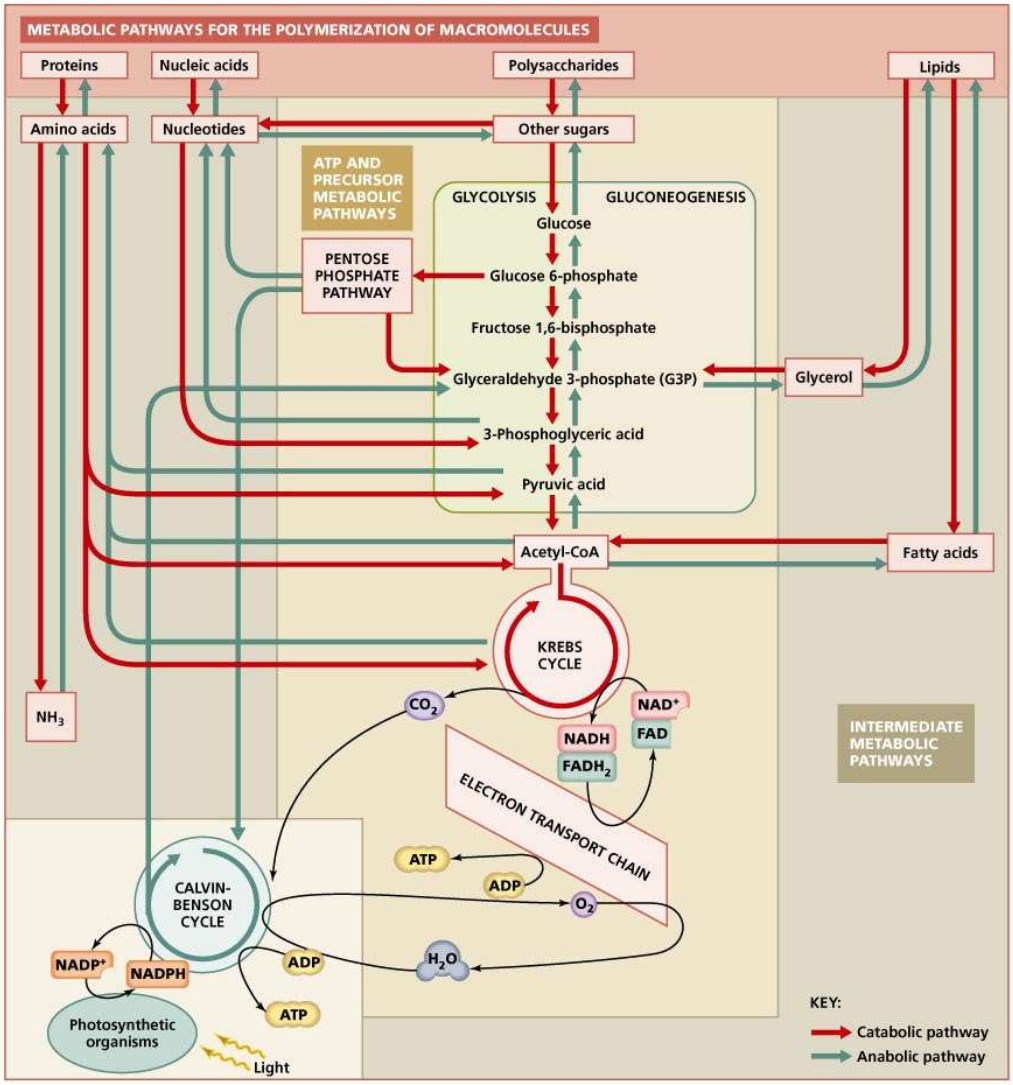


Fig. 4: Connection of metabolic pathways. (http://biology-pictures. Blogspot.com/2011/11/cellular-metabolism-summary.html). Metabolism of glucose, amino acids, lipids, Krebs cycle and other metabolic pathways provides building blocks for respiratory chain.

1. 7. Warburg effect

The metabolism of rapidly dividing cells such as cells during development, tissue renewal, immune response or even cancer cells differs fundamentally from the one present in adult differentiated healthy tissue. Rapidly dividing cells produce energy and building blocks almost exclusively through glycolysis while Citric acid cycle and electron transport chain are downregulated, even in the presence of oxygen (Fig. 5). This phenomenon is called Warburg effect and it was observed for the first time in 2nd decade of the 20th century by chemist Otto Warburg who was studying metabolism of the cancer cells. Until then, it was considered that cells gain energy via glycolysis only when they suffer from hypoxia (so called Pasteur´s effect). Warburg hypothesized that mitochondrias and therefore also respiration are either damaged in

cancerous cells or the amount of mitochondrias is reduced in these cells forcing them to gain energy through glycolysis. He believed that mitochondrial damage is necessary to trigger all biochemical processes leading to cancerous growth. Moreover, Warburg and Pasteur´s effects can go hand-in-hand because there is lack of oxygen inside of large solid tumors (Warburg 1956).

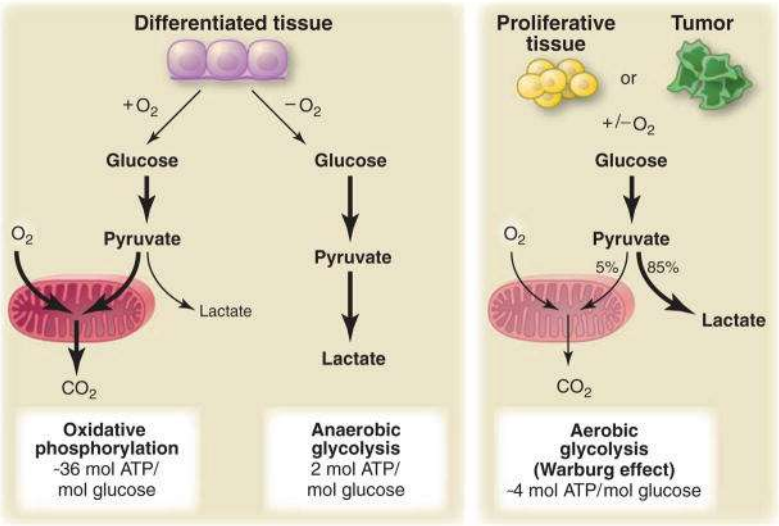


Fig. 5: Warburg effect scheme (Vander Heiden et al. 2009)**.** Normal tissues gain energy from electron transport chain, cancerous and proliferating tissues use glycolysis as a source of energy

Nevertheless, in following years Weinhouse showed that cancer cells are able to oxidize both glucose and fatty acids to carbon dioxide in rates comparable to those in healthy tissue (Weinhouse et al. 1951). He postulated that downregulation of mitochondrial activity is due to upregulation of glycolytic flux which inhibits mitochondria as discovered by Crabtree (Crabtree 1929). Moreover, Shell discovered that mitochondrial functions in colon cancer cells are downregulated rather due to lack of pyruvate directed into mitochondria. This effect can be rescued when pyruvate supply into the mitochondria is restored which exclude the possibility that the mitochondrias in these cells are irreversibly damaged (Schell et al. 2014). But why do cancer cells gain energy through glycolysis which is much less efficient in this matter? Why to waist both carbon and energy source by converting pyruvate to lactate which is secreted out of the cell?

Warburg effect might seem as wasteful strategy but the opposite is true. Firstly, glycolytic rate is 10 – 100x higher when impaired from the mitochondrial metabolism and it was shown that amount of synthesized ATP per time unit is comparable regardless of whether it was synthesized by glycolysis or oxidative phosphorylation (Shestov et al. 2014). Moreover, glycolysis provides also sufficient amount of the building blocks for synthetic pathways. For example, phosphoenolpyruvate, pyruvate, 3-phosphoglycerate and glucose-6-phosphate,

metabolites derived from the glycolysis, are necessary for synthesis of amino acids. Cytosolic Acetyl-CoA derived from pyruvate is fundamental for generation of fatty acids. And ribose-5-phosphate and NADPH produced in pentose phosphate pathway, a „side branch "of glycolysis, are necessary for synthesis of nucleic acids and fatty acids respectively. (Murray et al. 2012).

Furthermore, rapidly dividing cells produce more reactive oxygen species than differentiated non-dividing ones. And altered glycolytic metabolism provides weapons to fight it. Superoxide radicals are oxidants toxic for merely all living organisms by damaging DNA, proteins and lipids. They are produced in large quantities by NADPH oxidase as a reaction to microbial or fungal infection (Segal et al. 2012). Nevertheless, they are also created as a byproduct of mitochondrial respiration, mostly by Complex I and Complex III of ETC. In that case they represent a threat for cell survival and need to be defused. Superoxide dismutase (SOD) hunts for superoxides and transforms them to peroxide and water (Miller 2004). Peroxides, which are the main reactive oxygen species in the cell are further disposed by glutathione peroxidases and catalases. Catalase transforms peroxides to oxygen and water and does not need any cofactor to do so (Weydert & Cullen 2010). On the other hand, glutathione peroxidases needs glucose-6-phosphate derived from glycolysis and NADPH produced in pentose phosphate pathway to transform peroxide to water (Kelner et al. 1995). Also, in SK-N-SH neuroblastoma cells, peroxides are scavenged by pyruvate, terminal metabolite produced in glycolysis, which converts them to water. Moreover, Wang et al. discovered, that pyruvate is in these cells able to reduce superoxide production caused by inhibition of Complex I or Complex III of ETC (Wang et al. 2007).

Also, key enzyme of glycolysis, Hexokinase, is known to interact with voltage dependent anion channels (VDACs) on outer mitochondrial membrane of rapidly proliferating cells. This interaction block release of cytochrome C from mitochondria and therefore prevents cell to undergo apoptosis (Azoulay-Zohar et al. 2004). Another glycolytic enzyme, glucose-6-phosphate isomerase (PGI), was shown to induce cell migration which implies its role in metastasis (Watanabe et al. 1991). Furthermore, Tsutsumi showed that PGI gain of function leads to proliferation of 3T3 fibroblast. Therefore, PGI can be considered as oncogene (Tsutsumi et al. 2003).

Finally, even production and secretion of lactate, a valuable source of carbon, is not as spendthrift as it seems to be. During glycolysis, when glyceraldehyde 3-phosphate is decomposed to 1, 3-bisphosphoglycerate, NAD^+ is reduced to NADH. Under normal condition, NAD^+ is recovered during oxidative phosphorylation in mitochondria. But in rapidly dividing cells, mitochondrial metabolism is impaired from glycolysis. Instead, NAD^+ level is restored by converting pyruvate to lactate which is subsequently secreted out of the cell. Secreted

lactate is then transported into the liver where it is recovered back to glucose in Cori cycle (Murray et al. 2012). Furthermore, it was shown that lactate can serve as a source of energy for myocardial cells (Bergman et al. 2009) and for brain (van Hall et al. 2009).

In addition, in cancer cells lactate has yet another functions. Inside large solid tumors, there is lack of oxygen. These cells have no choice but to gain energy by glycolysis (Pasteur´s effect). But it was discovered that lactate secreted from these cells can serve as a source of energy for oxygenated cells at the edge of the tumor which saves all the glucose for the inside hypoxic cells (Sonveaux et al. 2008). Lactate production is also one of the escape mechanisms of the tumor cells from immune system. Extracellular lactate inhibits differentiation of monocytes to dendritic cells and inhibits their ability to release cytokines (Gottfried et al. 2006). Lactate also has inhibitory effect on cytotoxic T-cells. Activated T-cells uses glycolysis as a main source of energy themselves. But high level of lactate in the surroundings of the tumor cells prevents T-cells from secreting their lactate as well and therefore decreases their function (Fischer et al. 2007). Furthermore, it was shown that secretion of lactate can induce angiogenesis (Porporato et al. 2012) and enhances migratory properties of cancer cells and therefore influences metastatic tumor development (Goetze et al. 2011).

Nevertheless, mitochondrial metabolism is not completely attenuated even in the cells with glycolytic phenotype such as cancer cells. Even though suppressed, mitochondrial metabolism is still running to maintain mitochondrial membrane potential and integrity. Mitochondrial function is partially secured by pyruvate which is still in smaller quantities transported into the mitochondria to fuel citric acid cycle but mainly by glutamine which is at least by cancer cells consumed in high amount despite of the fact that it is not essential amino acid. Glutamine in these cells serves not only as a source of nitrogen for elevated synthesis of amino acids but also as substrate for citric acid cycle and subsequently electron transport chain (Vander Heiden et al. 2009). In fact, some cancer cells develop addiction to glutamine and its deprivation can induce apoptosis in these cells (Wise & Thompson 2010). In total, all of the above described points to the fact that Warburg effect is not only beneficial but also necessary for rapidly dividing cells.

1. 8. Regulation of metabolism

Cells of multicellular organism are living in environment with unlimited amount of sugars, proteins, lipids and amino acids. Nevertheless, nutrient uptake in these cells is strictly

regulated. Nutrient uptake dysregulation may lead to unguarded cell proliferation and subsequently to cancer or some other metabolic disease. Main regulators of the cellular metabolism during normoxia are signaling pathway leading through PI3K/AKT, Myc, Src or H-Ras. During hypoxic conditions, when cells gain energy solemnly through glycolysis, metabolism is regulated by transcription factor Hif-1α. Moreover, feedback loop regulation by intermediate metabolites is involved as well (Levine & Puzio-Kuter 2010).

As mentioned above, main source of energy and carbons for the cells is glucose which is imported via glucose transporters (GLUTs). GLUTs are positively regulated in response to insulin by PI3K/AKT. It mediates translocation of GLUT4 to plasma membrane and therefore enhanced glucose consumption (Hajduch et al. 2001). GLUTs expression is also upregulated in hypoxic conditions by Hypoxia inducible factor (HIF). Under normoxic conditions Hif-1α is hydroxylated on proline residues by proline hydroxylases (PHDs) and subsequently ubiquitinated by von Hippel-Lindau (VHL) E3 ubiquitin ligase and targeted for degradation. PHDs enzymatic activity is dependent on α-ketoglutarate, an intermediate metabolite of citric acid cycle. During hypoxia, mitochondrial respiration is disabled and citric acid cycle rate is lowered. As a consequence, production of α-ketoglutarate drops to minimum and is not available as a cofactor for PHDs anymore. In that case hydroxylation of Hif-1α is lost and therefore cannot be distinguished by VHL. Hif-1α is stabilized, travels to the nucleus and create a heterodimer with Hif-1β. This complex then trigger expression of its target genes (Hayashi et al. 2004). Finally, expression of GLUT1 and GLUT4 is downregulated by p53 which directly binds to GLUTs promoters and prevents their transcription (Schwartzenberg-bar-yoseph et al. 2004).

Glucose imported into the cell needs to be processed and the first step is driven by enzyme hexokinase (HEX). Osawa et al. showed that transcription of hexokinase in L6 cells is regulated by cAMP and also through PI3K/$p70^{S6K}$ branch of insulin pathway (Osawa et al. 1995; Osawa et al. 1996). Transcription of hexokinase is also regulated in HIF-1α dependent manner (Riddle et al. 2000). Hif-1α and also hypoxia inducible vascular endothelial growth factor (VEGF) signaling regulate transcription of another glycolytic enzyme, glucose-6-phosphate isomerase (Funasaka et al. 2005). On the contrary, phosphofructokinase and phosphoglycerate mutase are negatively regulated by tumor suppressor p53 (Kondoh et al. 2005; Bensaad et al. 2006). Hexokinase is also a direct target of Notch signaling (Slaninova, 2016).

Last step of glycolysis, conversion of phosphoenol pyruvate (PEP) to pyruvate is driven by pyruvate kinase (PK). PK has two isoforms produced by alternative splicing: PKM1 which is expressed in adult organism and PKM2 which is specific for embryonic tissue and tumors.

It was discovered that PKM2 has lower enzymatic activity. This is one of the critical rate limiting steps. Since transformation of PEP to pyruvate is slower, glycolysis intermediate metabolites accumulates in cytoplasm in higher quantities which pushes them to enter pentose phosphate pathway (Gupta & Bamezai 2010). Furthermore, accumulated PEP can serve as phosphate donor for phosphoglycerate mutase and therefore increase its activity (Vander Heiden et al. 2010). Moreover, as mentioned above, PKM2 isoform favors cell proliferation and it is characteristic for cancer cells. Nevertheless, it was shown that when PKM2 is depleted and PKM1 isoform is reconstituted, it inhibits Warburg effect in cancer cells and prevents their proliferation (Christofk, Vander Heiden, Harris, et al. 2008). Expression of M2 isoform is negatively regulated by tyrosine kinase signaling which is often deregulated in cancers (Christofk, Vander Heiden, Wu, et al. 2008) .

There are two ways how pyruvate created in glycolysis can be utilized. Firstly, it can be converted to lactate and secreted out of the cell. This process is typical for cells with Warburg effect and also during hypoxia. Reaction is driven by enzyme lactate dehydrogenase which expression is induced by Hif-1α (Semenza et al. 1996) and also c-myc (Shim et al. 1997). Secondly, pyruvate can enter mitochondria where it is converted to acetyl CoA by pyruvate dehydrogenase (PDH). This enzyme is negatively regulated by phosphorylation by pyruvate dehydrogenase kinase which is directly upregulated by Hif-1α (Holness & Sugden 2003; Kim et al. 2006).

Important source of energy and nitrogen for the cell is glutamin which is processed in glutaminolysis. Decomposition of the glutamin is vital especially for cells with Warburg effect in which puruvate is diverted out of the mitochondria. In here, glutamin is responsible for maintaining activity of mitochondrial metabolism as well as maintaining mitochondrial membrane potential. Glutaminolysis rate is increased by Myc which activates expression of both glutamine transporter ASCT2 (ASC amino acid transporter 2) and glutaminase which converts glutamine to glutamate (Wise et al. 2008).

Acces of the pyruvate is the first step of regulation of mitochondrial metabolism in response to changing environment. Nevertheless several other factors are involved in different conditions. For example in *Drosophila* transcription factor hairy was shown to directly downregulate transcription of several metabolic enzymes involved in citric acid cycle and therefore promoting glycolytic phenotype during hypoxia (Zhou et al. 2008).

Also, there are several enzymes of citric acid cycle whose mutations lead to disregulation of mitochondrial metabolism, Warburg effect and cancerogenesis. One of them is isocitrate dehydrogenase (IDH) which mutations are rather typical for several types of cancer (Reitman & Yan 2010; Murugan et al. 2010; Zou et al. 2010). Moreover, mutated IDH does not convert

isocitrate to α-ketoglutarate anymore. Instead, it transform α-ketoglutarate to oncometabolite 2-hydroxyglutarate which alteres cellular methylation of DNA by inhibiting Jumonji C family of histone demethylases. Furthermore, 2-hydroxyglutarate inhibits prolylhydroxylases and therefore stabilizes Hif-1α promotes glycolytic phenotype (Gross et al. 2010).

Moreover, mutations in succinate dehydrogenase (SDH) were connected with hereditary paraganglioma (Baysal 2003) and there is also relationship between mutations of fumarate hydratase (FH) and development of renal cell cancer or inherited leiomyomatosis (Toro et al. 2003). Moreover, mutations of SDH or FH leads to accumulation of succinate and fumarate in cytoplasm which inhibits activity of PHD and therefore promotes stabilization of Hif-1α even in the presence of oxygen (Morin et al. 2014).

Alongside all of the rather straight forward regulations described above there are some other mechanisms which are more tangled. For example Hif-1α is directly activated by AKT, Myc, Src, Tor or Ras (Agani & Jiang 2013; Lim et al. 2004; Yeung et al. 2008; Carrero et al. 2000; Land & Tee 2007). On the other hand AKT, Myc and Tor can be activated by loss of PTEN (Kim et al. 2014; Bonnet et al. 2011; Mulholland et al. 2012). P53 regulates PTEN, Insulin receptor pathway and also NfκB (Webster et al. 1996; Kawauchi et al. 2008; Stambolic et al. 2001) and vice versa it is regulated by NfκB and PTEN (Freeman et al. 2003; Webster & Perkins 1999).

It is obvious that regulatory network between cellular metabolism and signaling pathway is very complex and needs to be properly tuned. Moreover, most of the signaling pathways involved in regulation of cellular metabolism are known to be disregulated in cancers. Maybe deeper understanding of both signaling and metabolic pathways and their connection would allow us to develop effective treatment against cancer and other diseases connected with metabolism.

1. 9. Mechanisms involved in the regulation of growth

Size of the adult organism is not fixed in most animal species. In fact, it is known that the final body size is determined not only by genetical background but it is also influenced by environmental conditions. There are several regulatory mechanisms which drives tissue growth and they have to be in perfect concordance.

Growth regulation in *Drosophila* and mammals differes in many ways. For example, since the body of adult *Drosophila* is enclosed in chitinose exoskeleton and cannot grow

anymore, final body size is determined during larval development (Blair 1999). On the other hand growth of the mammals continues even after they reach sexual maturity and it was implied that for example ears are growing during the whole lifetime (Niemitz et al. 2007). Nevertheless, many molecular mechanisms involved in growth regulation are very similar both in mammals and *Drosophila*.

Regulatory mechanism specific for *Drosophila* is mediated by molting hormone ecdyson. After hatching, larva feasts on the food, grows and depending on the developmental stage undergoes three sets of molting and subsequent pupariation and metamorphosis into the adult. These events are initiated by prothoracicotropic hormone (PTTH) which stimulates synthesis and release of ecdysone, probably through Ras-Raf-MAPK pathway (Huang et al. 2008). Ecdyson together with the eclosion hormon then drives larva towards molting or pupariation. Whether larva molts into another instar or if it rather undergo metamorphosis is regulated by presence or absence of juvenile hormone (JH) during ecdysone secretion. If JH is present, larva will undergo another set of molting. When level of JH drops, PTTH is released, stimulates ecdysone production and process of metamorphosis is initiated (Nijhout 2003).

The timing of molting hormones release and therefore duration of larval growth is one of the regulatory mechanisms which influence final body size. Ecdyson is not released until larva reaches critical size. Nevertheless, there is a delay, so called terminal growth period, betwen acquisition of critical size and ecdyson secretion. During this time, larva still grows and can actually even triple its size (Stieper et al. 2008). Final size of the animal is then given by both the critical size of the larva and the growth achieved during terminal growth period. Critical size of larva is controlled primarily by the insulin/insulin-like signaling (IIS) pathway that regulates the growth of the prothoracic gland (Mirth et al. 2005). Mass gain during terminal growth period is then mainly determined by nutrient acces. Even more important than reaching the critical larval size is to reach critical size of imaginal tissue though. Organs which still did not reach their critical size secrete insulin-like peptide (Dilp) 8 which inhibits ecdysone production and therefore prevent premature molting and pupariation (Colombani et al. 2012). In *Drosophila* there are eight insulin-like peptides (Dilp) most of which activates growth of the tissues (Brogiolo et al. 2001). Dilps originate from different organs such as brain, gut, fat body, muscles or ovaries. Such versatility of origin suggest different functions both spatially and temporary for individual Dilps. They bind to InR and activate a downstream pathway which is similar to insulin/insuline-like growth factor signaling in vertebrates (Géminard et al. 2006).

During starvation, Dilps accumulate in insulin-producing cells (IPC) in brain which suggest that their secretion is modulated by nutrient availability (Géminard et al. 2009).

Secretion of Dilps from IPCs is regulated by fat body. This organ serves to accumulate glycogen and triacylglycerol which are used as energy source during starvation and also pupal stage (Rusten et al. 2004). Accept its function as a nutriment warehouse, fat body also collects informations about larval metabolic status and changes in nutrient availability. These information are forwarded to IPCs through fat body-derived signals (FDSs). FDSs are released from the fat body in response to high-fat, sugar or aminoacid diet, binds to receptors on the IPCs and drives secretion of Dilps. Dilps then activates insulin-like growth factor signaling (IIS) which induces growth of the imaginal tissues. Activation of IIS in fat body also enhances TOR signaling which is already elevated by acces of amino acids and therefore promotes biosynthesis of proteins, lipids, ribosomes and nucleotiedes (Koyama et al. 2013).

In mammals, similar mechanisms are employed with growth hormone (GH) and the insulin growth factor (IGF) signaling pathway as master regulators of growth (Lupu et al. 2001). During embryogenesis the growth of budding limb is driven by fibroblast growth factors (FGF) secreted from apical limb bud cells (Cohn et al. 1995). A critical role in the regulation of mammalian growth is also implied for the AKT signaling pathway. It was shown that the malfunction of the AKT pathway can result in dwarfism or Proteus syndrome that causes overgrowth of bones, muscles, skin and connective tissue (Peng et al. 2003; Lindhurst et al. 2011). Moreover, mutations in AKT signaling are observed in most of the tumors (Altomare & Testa 2005).

Similar sensitivity to nutrient acces driven by similar processes is observed also in mammals. In here, AKT/mTOR pathway can be regulated by amino acid availability (Tato et al. 2011). Moreover, it was shown that during strarvation IGF-1 levels are lowered. To maintain apropriate circulating IGF-1 levels, both energy and protein needs to be available in sufficient amount. Synthesis and activity of IGF-1 and IGF binding proteins are regulated by nutrition in multiple levels. During starvation, binding of growth hormone in liver is decreased which can lead to lowered IGF-1 production. Lack of proteins in the diet leads to malfunction of GH signaling as well even though GH receptor function is not affected. And amino acid availability is vital for IGF-1 expression (Estívariz & Ziegler 1997).

Nonetheless, various tissues present different sensitivity to nutrient acces. In *Drosophila,* one of the mechanisms involved leads through forkhead transcription factor O (FoxO) which negatively regulates protein translation in response to starvation. In the presence of the nutrients, FoxO is inhibited by IIS downstream effector AKT. One of the tissue in *Drosophila* which shows lowered sensitivity to nutrient acces are male genitalia imaginal discs. Tang et al. showed that there is lowered production of FoxO in this tissue which makes them less sensitive to nutrient shortage (Tang et al. 2011). Similarly, Reptor, a downstream effector of

the TORC1 pathway, was shown to play a critical role in energy homeostasis and promoting animal survival upon nutrient restriction. Active TORC1 phosphorylates REPTOR on two serine residues (Ser527 and Ser530) which leads to retention of REPTOR in the cytoplasm. Nevertheless, when TORC1 is inhibited, REPTOR becomes dephosporylated, trevels into the nucleus where it binds to its partner REPTOR binding protein (REPTOR-BP) and together they activates the transcription of the target genes to induce stress response (Tiebe et al. 2015)

Moreover, it was shown that critical organs, such as brain, keeps growing even during starvation at the expense of other tissues in order to preserve the live of the whole organism. In neuroblasts, anaplastic lymphoma kinase (Alk) rather than IIS activates PI3-kinase signaling and therefore promotes growth. Both Alk and its ligand Jelly belly (Jeb), which is constitutively produced by the surrounding glial cells, are highly expressed regardless of the nutritional status of the larva which can be one of the mechanims impairing neuroblast gorwth from overall nutrient availability (Cheng et al. 2011). Such brain-sparing mechanism was also observed in mammals. Even though the mechanism in which the mammalian brain is protected from nutrient deprivation is still not uncoverd, it was shown that Alk plays role also during neurigenesis in zebrafish. It is tempting to speculate that similar mechanism can play a role in mammals as well (Lanet & Maurange 2014).

Another factor which influences both growth and final body size is oxygen acces. It was shown that size of the flies which suffer from hypoxia during larval development is reduced (Shingleton et al. 2009). In Drosophila, the transcriptional response to hypoxia is mediated by two hypoxia inducible factors (HIF), Similar and Tango (homologues of mammalian Hif-1α and Hif-1β). During hypoxia, HIF induces transcription of Scylla which in turn suppress TOR signaling and therefore reduces all biosynthetic processes. Moreover, Sima can be activated by PI3K/AKT signaling creating yet another feedback loop of growht regulation (Dekanty et al. 2005). Hypoxia induces fetal growth restriction also in mammals presumably by mechanisms similar to the ones present in *Drosophila* (Orgeig et al. 2011).

Finally, growth of Drosophila is also influenced by temperature. It was shown that organ size of larvaes kept in higher temperatures is smaller (Shingleton et al. 2009). Nevertheless, mechanisms by which temerature regulates body and organ size are still unknown. Temperature changes also influences growth rate of cultured mammalian cells by affecting its progression through G1 and S phase. Nonetheless, regulatory mechanisms are unknown as well (Watanabe & Okada 1967).

However, in healthy organisms, even when conditions are optimal, organs do not grow infinitely. Moreover, shape of singular organs is still more or less the same regardless of the final size. How is this possible? The shape is regulated by patterning genes which organize

spatial arrangement of differentiated tissues (Edgar 2006). Patterning signals consist of morphogenes and signaling pathways which react to them. Morphogenes, such as Decapentaplagic (Dpp), Hedgehog (Hh), Notch or Wingless (Wg) in *Drosophila* or bone morphogenic proteins (BMPs) in mammals are secreted out of the cells, diffuse to the surroundings to create morphogenes gradient, regulate cell proliferation and defines which part of the body will be created from the particular tissue (Tabata & Takei 2004; Wang et al. 2014).

The evolutionarily conserved Hippo/Wts pathway assures that organs do not grow more then they shoud. Hippo can sense variations in morphogen levels such as bone morphogenic proteins through Fat and Dachsous cadherins and regulate the growth proportionaly (Zecca & Struhl 2010; Ishiuchi et al. 2009).

Hippo also follows apicobasal polarity and adhesion of the cells. When the tissue architecture is disrupted, Hippo is activated to promote organ growth (Tapon & Harvey 2012). Regulation of Lats, the downstream effector of Hippo pathway, by G protein-coupled receptor is also involved suggesting rather systemic control of Hippo signaling (Yu et al. 2012; Zeng & Hong 2008). Overall, regulation of growth of both *Drosophila* and mammals is very complex but despite of all differencies also conserved. Moreover, signaling pathways wich regulates growth are the ones which are the most often disrupted in human diseases including cancer. Therefore, understanding of growth regulatory mechanisms is necessary for well established treatment of these diseases.

1. 10. Mechanisms involved in the regulation of signaling pathways by cellular metabolism

In chapter 1. 8. I have described mechanisms in which signaling pathways regulates metabolism to fulfill the needs of the cell. But this is not a one way street. Signaling pathways respond to metabolic status and they are regulated by availability of nutrients and energy. Nutrient sensing pathway drives cells to anabolism and storing processes when the food is abundant. On the contrary, when nutrient levels drops, signaling pathways shuts down all synthetic processes and drives cells to metabolize internal stores. Informations about cellular metabolic status is forwarded to signaling pathways by so called metabolic sensors. They are proteins within signaling pathways which are capable to follow changes in energy status or levels of certain metabolites derived from metabolic processes.

The main nutrient sensing signaling pathway that regulates the overal metabolic homeostasis is the Mechanistic target or rapamycin (mTOR) pathway. The mTOR is a protein kinase highly conserved amongst eucaryotes that regulates cell growth, proliferation, motility, survival, protein synthesis, autophagy and transcription. As the name suggest, mTOR is sensitive to rapamycin treatment which inhibits its function. mTOR protein creates two different complexes, mTOR complex 1 and mTOR complex 2 (mTORC1 and mTORC2) with differential regulation, function and even sensitivity to rapamycin. It was suggested that defects in mTOR signaling can contribute to several human diseases including type II diabetes or cancer (Dazert & Hall 2011). There is not much known about the mTORC2 mostly due to the fact that are no specific inhibitors available. Nevertheless, recent evidence from yeasts show that TORC2 play a role in the maintenance of genome stability under both oxidative and replicative stress (Schonbrun et al. 2009; Shimada et al. 2013). mTORC1 is the primary nutrient sensing complex within mTOR pathway that is able to follow levels of multiple essential nutrients such as amino acids, glucose or energy levels and modulate homeostasis of the whole organism accordingly. Not only it mediates one of the cell autonomous mechanisms to sense levels of amino acids through its interaction with Ras-related GTPase (Rag, see below) but it also collect signals from other nutrient-sensing signaling pathways. For example activation of insulin signaling pathway increase mTORC1 activity by inhibiting its suppressors, tuberose sclerosis complex 2 (TSC2) and proline-rich AKT substrate of 40 kDa (PRAS40) (Dan et al. 2014). During hypoxia or glucose deprivation, AMPK phosphorylates TSC2 on sites distinct from AKT to activate it which leads to suppression of mTORC1 (Mihaylova & Shaw 2012). AMPK can also directly downregulate mTORC1 activity by phosphorylating one of its subunit, Raptor (Gwinn et al. 2008). The action of mTORC1 are driven mostly by its two direct substrates, ribosomal S6 kinase (S6K) and the eucaryotic initiation factor 4E (eIF4E)-binding protein (4EBP), both promoting protein translation (Yang et al. 2014). Moreover, when active, mTORC1 phosphorylates its transcription factor REPTOR to secure its cytoplasmic localization. In case of mTORC1 inactivation, REPTOR is dephosphorylated, travels into the nucleus to bind to its partner, REPTOR binding protein, and activates the transcription of target genes in order to maintain cellular energy homeostasis (Tiebe et al. 2015).

AMP-dependent protein kinase is a metabolic sensor which is able to follow changes in cellular energy levels. AMPK is activated when energy levels drops and AMP/ATP ratio is high. In this situation, AMP is bound to AMPK γ-subunit which allows activation of catalytic α-subunit. Active AMPK then inhibits TORC1 which blocks all anabolic processes and cellular growth (Mihaylova & Shaw 2012). Moreover, Chopra et al. showed that at least in

rodent muscles, AMPK is responsible for phosphorylation of insulin receptor and therefore ligand-independent activation of insulin pathway during glucose deprivation. (Chopra et al. 2012). This would suggest rather complex mechanism in which AMPK regulate cellular energy homeostasis. Activation of insulin pathway initiates translocation of GLUT4 to plasma membrane to increase glucose uptake which, together with inhibition of TORC1, might help to normalize energy status of the cell. Nevertheless, effect of high AMP/ATP ratio can be overwritten by glycogen that binds to AMPK β-subunit when energy reserves are high and prevents AMP dependent AMPK activation (McBride et al. 2009).

The second index of the cellular energy status is NAD^+/NADH ratio. Its changes are sensed by proteins that use NAD(H) as their cofactors or substrates. There are several proteins known to bind either NAD^+, NADH or both. One of them is C terminal binding protein (CtBP) that serves as general transcriptional cofactor. It usually serves to recruit histone deacetylases (HDACs), histone lysine methyltrasferases and other players which modifies chromatin status of target genes to repress their expression. Repressive function of CtBP was observed for example in case Notch, Wnt and Dpp signaling pathways (Aihara et al. n.d.). Nevertheless, there are cases when CtBP can act also as activator. Bhambhani showed, that in case of Wnt signaling it is rather oligomeric state of CtBP which determines its activating or repressive function (Bhambhani et al. 2011). Moreover, Itoh discovered, that CtBP and its binding to NAD^+ is necessary for expression activation of circadian clock activator genes *Clock* and *Cycle* (Itoh et al. 2013). Furthermore, CtBP is one of the sensors which can bind both NAD^+ and NADH as a cofactor in context dependent manner. It was shown that whereas NADH binding promotes CtBP recruitment to promoters of E-cadherin CDH1 or Breast cancer early-onset 1 (BRCA1), it inhibits it binding to neuron-restrictive silencig factor/repressor element 1-silencing transcription factor (NRSF/REST) (Garriga-Canut et al. 2006).

Another known metabolic sensor which is influenced by NAD^+/NADH ratio are the lysine specific protein deacetylases from the sirtuin family. In mammals, there are seven sirtuins with localization in nucleus (Sirt1, Sirt2, Sirt6, Sirt7), cytoplasm (Sirt1, Sirt2) and mitochondria (Sirt3, Sirt4, Sirt5). Nevertheless, despite their localization, all of them need NAD^+ as a cofactor which means that during nutrient deprivation activity of sirtuins is low. Sirtuins were already shown to influence processes such as energy metabolism, senescence, DNA repair, circadian rhythm or cellular stress (Satoh et al. 2011). There are several pathways which were shown to be influenced by sirtuins. For example in liver during fasting, Sirt1 deacetylate transcription factor FoxO and simlutaneously inhibit sterol-ragulatory-element-binding protein (SREBP) which leads to upregulation oxidation rate of fatty acids and gluconeogenesis while inhibiting lipogenesis (Liu et al. 2008). Sirt1 was also shown to induce

transcriptional activity of Hif-2α under hypoxia. In kidney, Hif-2 is responsible for induciton of vascular endothelial growthh factor, erythropoietin and other genes which are regulated by oxygen levels (Hao & Haase 2010). Moreover, it was discovered that sirtuins (namely Sirt1, Sirt3 and Sirt6) are essential for modualtion of IGF-AKT activation on different levels. Sirt1 deacetylates AKT in order to increase PIP_3 binding, Sirt3 is involved in regulation of ROS-mediated AKT activation and Sirt6 is involved in suppression of IGF-AKT target genes by deacetylating histone 3 at their promoters (Pillai et al. 2014).

Nevertheless, cells do not track only thier energetic status but they also directly sense nutrients and their derivates. For example there are purinergic membrane receptors from the G-protein-coupled family that sense levels of adenosine, nucleotides or ATP (Burnstock & Ulrich 2011). Cytoplasmic acetyl CoA is nececcary as an acetyl donor group donor for acetylases (Takahashi et al. 2006). Cells can also sense levels of lipids; G-protein-coupled receptors GPR40 and GPR120 binds free faty acid and modulate insulin pathway by distinct mechanisms (Itoh et al. 2003; Moran et al. 2014).

Levels of glucose are monitored by glucokinase (GCK) and Glucose transporter 2 (GLUT2) respectively. While GLUT2 is responsible for glucose intake into the cells, GCK mediates its primary decomposition in glycolysis. Unlike other glucose transporters and hexokinases, both GLUT2 and GCK are activated only when glucose levels are relatively high due to their lower affinity to glucose. For example, GCK is the most abundant hexokinase in livers and it is responsible for regulation of gluconeogenesis and glycogen decomposition during starvation (Nordlie et al. 1999). In hepatocytes, GLUT2 multimerize with InR to regulate hepatic glucose homeostasis (Eisenberg et al. 2005). And in pancreas, Glut2 is vital for insulin release from β-cells (Thorens 2014).

Amino acids are sensed cell-autonomously through the activation of mTOR pathway, via TORC1 which is sensitive to nutrient levels. It consist of several proteins, namely Raptor, mLST8, PRAS40, FKBP38 and mTOR itself. mLST8 and Raptor are activators of mTOR whereas PRAS40 and FKBP38 regulate mTOR function negatively. Activation of mTORC1 leads to phosphorylation of its substrates S6K and 4EBP1 that mediate downstream effects of mTOR signaling. mTORC1 is modulated by upstream regulators such as Rheb which is vital for mTOR activation or TSC which inactivates Rheb and therefore the whole mTOR complex1 in response to signals from other nutrient sensing pathways. Nevertheless, direct amino acid sensing by mTORC1 is TSC independent (Kim 2009). It was implied that amino acid sensing properties of mTORC1 are mediated by Ras-related GTPase (RAG). In mammals, there are four RAGs (A-D). Highly homologous RAG-A or RAG-B create a heterodimer with RAG-C and RAG-D respectively. Amino acid binding converts the RAG heterodimer to its active

conformation. Active RAG heterodimer binds RAPTOR and recruits the whole mTORC1 complex to the lysosome surface where it is activated by Rheb. RAG – mTORC1 complex is anchored to lysosomal membrane by heteropentameric complex called RAGULATOR which also works as guanine nucleotide exchange factor for RAG-A/B. On the other hand, when amino acids are scarce in the presence of growth factors, inactive RAGs bind TSC and recruit it to the lysosome to inhibit Rheb and followingly TORC1. There are other amino acid sensing mechanisms influencing activity of mTORC1. Nevertheless, mechanism of their function is rather elusive (Kim & Guan 2009; Sancak et al. 2010).

Accept the cell-autonomous mechanism of amino acid sensing that is mediated by mTORC, there are other mechanisms regulating metabolic processes across the whole body. It was shown that adipose tissue is sensitive to the changes in amino acid availability and that it release long-range factors in order to modulate metabolism, reproduction and other processes accordingly (Rosen & Spiegelman 2014). Moreover, dysfunction in adipocytes has been linked to diseases such as diabetes or cancer (Vucenik & Stains 2012) In *Drosophila,* fat body, an organ that stores energy reserves in the form of glycogen and triglycerides and is therefore homologous to vertebrate livers, was shown to be involved in amino acid sensing in both mTORC dependent and independent manner (Rusten et al. 2004). Armstrong showed that partial inhibition of amino acid transport into adult adipocytes results in the reduction of ovarian germ stem cells number independently of mTOR signaling (Armstrong et al. 2014). The amino acid sensing properties of the fat body is mediated by multiple amino acid transporters. Amongst them, the highest importance was implicated for Slimfast (Slif). It is cationic amino acid transporter which inhibits tissue growth during amino acid deprivation. Exact mechanism is not fully understood but Colombani et al. proposed that this regulation leads through mTOR pathway. They found, that Slif mediated mTOR inhibition prevents expression of Acid labile substrate in fat body and subsequent suppression of PI3K/AKT signaling in other larval tissues (Colombani et al. 2003). Moreover, another amino acid transporter, minidisc, is responsible for regulation of the growth of imaginal tissue (Martin et al. 2000)

One more organ responsible for amino acid sensing is the gut. It is responsible not only for food digestion but also for regulation of nutrient intake. Guts collect information about amino acids in the diet via G-protein coupled receptors and several amino acid transporters. For example, T1R receptors of G-protein coupled receptors family expressed in enteric endocrine cells and intestinal brush cells are able to sense most of the L-amino acids accept tryptophan (Shirazi-Beechey et al. 2014). Another class of G-protein coupled receptors, CaSR, is activated by L-aromatic amino acids such as L-phenylalanine or L-tryptophan (Conigrave

et al. 2000). And G-protein coupled receptor 6A is known to follow several basic and small neutral amino acids such as L-lysine, L-arginine, L-serine, L-glycine or L-alanine (Wellendorph et al. 2005). Finally, there are multiple amino acid transporters in the gut. Out of them, Sodium dependent neutral amino acid transporter 2 (SNAT2) is the one identified as the main amino acid transporter and sensor in the gut which modulate homeostasis dependent on glutamine levels (Young et al. 2010). Presence of amino acids in the gut sensed by both G-protein coupled receptors and amino acid transporters then triggers release of peptide hormones such as glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK) or peptide tyrosine-tyrosine (PYY) from enteric endocrine cells into the veins. In here, these hormones activate both extrinsic and intrinsic afferent neurons or other target cells. They also trigger response of afferent vagal nerve which forward the signal to the brain to regulate food intake and digestion and this gut-brain regulatory axis is of high importance to maintain homeostasis of the organism (Sam et al. 2012; Kondoh et al. 2009; Tsurugizawa et al. 2014).

1. 11. The interplay between Notch signaling, metabolism and growth

Cell signaling and metabolism has only recently been recognized as two tightly coupled cellular processes. The cell constantly monitors the availability of nutrients and energy and vice versa, its metabolic status influences the activity of signaling pathways. How the Notch signaling fits in this scheme remains to large extend elusive.

As mentioned above, the Notch signaling pathway is involved in the regulation of several processes in the cell including modulation of metabolism. As we recently published and as I will describe in my thesis Notch regulates directly several metabolic genes and directs cellular metabolism towards the Warburg effect (Slaninova et al. 2016) (see Chapter 3.1). The Notch pathway regulates growth in several developmental contexts (such as the growth of the wing pouch) and it is active in tissues that display Warburg effect (such as immune cells, stem cells or cancer cells). It is therefore plausible that Notch helps to metabolically reprogram certain cells or tissues by the direct regulation of metabolic genes.

At the same time, Notch signaling is regulated by the metabolic status of the cell. Saj et al. were the first ones to discover by their large RNAi screen in *Drosophila* that genes involved in pyruvate metabolism do influence the strength of Notch signaling in the wing disc (Saj et al. 2010). This is in agreement with our data where we show that the strength of the Notch phenotype *in vivo* is dependent on the composition of diet the larvae are feeding during their development and amino acids are critical in this process. What could be the metabolic sensors involved in Notch signaling? I will discuss possible role of four of the candidates - Hif-1, Sirt1

and CtBP and TOR. This way, the Notch pathway integrates with the sensing of oxygen, NAD^+: NADH ratio, amino acid availability and nutrient/energetic status of the cell.

The first known factor influencing Notch signaling according to cellular metabolism is the hypoxia inducible factor 1 (HIF-1). Canonically, HIF-1α is stabilized in hypoxic conditions and activates the expression of its target genes. Nevertheless, it also modulates the output of several signaling pathways and one of them is Notch signaling. In myogenic and neural precursor cell line, Notch activation is necessary to maintain pool of undifferentiated cells. Gustafsson et al. discovered that during hypoxia HIF-1α binds to N^{icd} and promotes Notch activation to further suppress differentiation (Gustafsson et al. 2005). Stabilization of N^{icd} by HIF-1α was also shown by Qiang and col. (Qiang et al. 2011). On the contrary, negative effect of hypoxia on N^{icd} is mediated by HIF-2α (Hu et al. 2016).

The NAD^+ dependent protein deacetylase called Silent information regulator 1 (Sirt1) also influences Notch signaling. Guarani with colleagues showed that Sirt1 dependent deacetylation of Notch intracellular domain leads to N^{icd} destabilization and therefore negative regulation of Notch signal (Guarani et al. 2011). Similar mechanism of N^{icd}-Sirt1 interaction was also implied in regulation of self-renewal of neural stem cells and neural progenitor cells (Ma et al. 2014). Moreover, Sirt1 was isolated in a complex with the histone demethylase LSD1 and with CtBP and it was proposed that it may be part of the Notch corepressor complex (Mulligan 2011). Moreover, it was shown that Sirt1 can modulate angiogenic activity to promote lung tumor growth by downregulating Notch signaling in lung endothelial cells (Xie et al. 2012). As we showed in our recent publication where I am also an author, Sirt1 positively influences the activation of Notch targets in the context of the development of sensoric organ precursors and wing disc (Horvath et al. 2016).

The C-terminal binding protein (CtBP) binds NADH and it is a known metabolic regulator of several signaling pathways. It is also part of the Notch corepressor complex. The role of CtBP in Notch regulation is not clear but there is a possibility that it might serve as yet another metabolic sensor for Notch signaling (Byun & Gardner 2013). There are over 200 proteins in the *Drosophila* genome that are predicted to bind NAD(H) and hundreds of others identified / predicted to bind other metabolites. Many of them have been implicated in the regulation of the Notch pathway but how they connect the cellular metabolic status to Notch signaling remains unknown.

In this thesis I will describe the fascinating interplay between Notch signaling pathway and cellular metabolic status in which Notch pathway influence metabolism and growth of the cells by direct regulation of metabolic gene expression and vice versa that it is regulated by cellular metabolic status via Sirt1.

2. Aims

During the course of my PhD I set three goals to follow:

I. To identify Notch transcriptional targets involved in the regulation of metabolism and to dissect the role of these gene in Notch driven tissue growth.

II. To test whether changes in NAD:NADH ratio affect Notch response in vivo

III. To investigate the role of Sirt1 as a metabolic sensor for the Notch pathway

3. Methods

3. 1. Methods related to published article Slaninova V, Krafcikova M, Perez-Gomez R, Steffal P, Trantirek L, Bray SJ, Krejci A. Notch stimulates growth by direct regulation of genes involved in the control of glycolysis and the tricarboxylic acid cycle. Open Biol. 6: 150155 (2016).

3. 1. 1. Selection of potential Notch target genes involved in metabolism

The genes potentially regulated by the Notch pathway were selected based on both published (Krejčí, Bernard, Krejčí, et al. 2009) and unpublished data of Krejčí and colleagues. They searched for the regions with Suppressor of Hairless (Su(H)) binding sites throughout the genome using ChIP-chip experiments in different tissues and cell lines. Experiments were performed with three Drosophila cell lines (BG2 – neural cells progenitors, Kc167 – hemolymph progenitors, DmD8 – muscle progenitors) and three types of the Drosophila imaginal wing discs (yw-control, "Giant Su(H)" – overexpression of Su(H) in the patched domain, "Giant N^{icd}" – overexpression of N^{icd} in large clones of cells throughout the discs). Using integrated genome browser (Nicol et al. 2009) we searched for peaks of Su(H) binding in the neighborhood of several metabolic genes in any of the cell lines or imaginal discs. In the best case scenario, we the peaks that overlapped with computationally predicted Su(H) binding sites (predicted by a weight matrix or using a set of experimentally verified Su(H) binding sequences which we call a "dictionary").

We found several genes with Su(H) peaks in their vicinity. Out of these, we chose seven to test further (see Tab. 1). Selected genes were either those with the most profound peaks in their potential regulatory regions or genes whose peaks were less prominent but the genes play a crucial role in the glycolysis or citric acid cycle. These genes were then used for both *in vitro* and *in vivo* studies with *Drosophila* cell lines and third instar larvae imaginal wing discs.

Responsiveness of their enhancers to the Notch pathway was tested in luciferase assays, upregulation of their mRNA after the Notch pathway stimulation in cells and wing discs was quantified using real time PCR and *in situ* hybridizations, their protein expression pattern in the imaginal wing discs was tested by immunostainings and metabolic parameters of imaginal wing discs with Notch pathway overactivation were tested by the Seahorse FX analyzer and NMR.

Tab. 1: List of selected metabolic genes potentially regulated by the Notch signaling

pathway

Name of the gene	Symbol	Function
Glucose transporter 1	Glut1	Transfers glucose through plasma membrane
Hexokinase A	Hex-A	Phosphorylates glucose to Glucose-6- phosphate
Trehalase	Treh	Convets trehalose to glucose
CG13334	-	Predicted L-lactate dehydrogenase activity, converts pyruvate to lactate
Ecdyson-inducible gene L3	Impl3	Predicted L-lactate dehydrogenase activity, converts pyruvate to lactate
Isocitrate dehydrogenase	IDH	Decarboxylates isocitrate to α-ketoglutarate
hairy	h	Transcription factor, regulator of cellular metabolism during hypoxia

3. 1. 2. Analysis in cell lines

3. 1. 2. 1. PCR amplification and cloning of enhancers

Some of the selected genes contained more than one "good" Su(H) binding peak within their enhancer. In that case all of those regions were amplified and cloned into the vector. Out of seven originally selected genes we have amplified and cloned eleven enhancer regions. The lengths of the amplified sequences differed from 152 bp to 641 bp (for cloned sequences see Fig. S1).

Enhancer regions were amplified by polymerase chain reaction from genomic DNA extracted from yw stock of Drosophila melanogaster using BioTaq polymerase (Bioline) and oligonucleotides (Sigma-Aldrich) listed in table S1. Primers were designed by Primer3 online software (Koressaar & Remm 2007) and unique 5 bp sequence was added upstream to the restriction site (RE I) to enable proper binding of the restriction enzyme (for list of used oligonucleotides see Tab. S2). PCR was performed according to the protocol in tab. 2. Final products were checked by 1% agarose gel electrophoresis.

PCR products were cleaned by phenol: chlorophorm and precipitated in ethanol by sodium acetate. Pellet was resuspended in 20 µl of DEPC H2O. PCR product and 5 µg of cloning vector were then cleaved by restriction enzyme according the table 2 overnight at 37 °C. Cleaved vectors were treated with alkaline phosphatase (Roche) to prevent religation. The ligation reaction was performed overnight at 16 °C with T4 DNA ligase (Roche) and transfected into the DH 5α *E. coli* using 45 second heat shock at 42 °C. Colonies after

transformation were verified by PCR using a forward primer from the enhancer region and a reverse primer RV3 from the vector (see tab 3). Plasmids from the positively verified colonies were purified by High-speed plasmid mini kit (Geneaid) and sequenced.

Table 2: Polymerase chain reaction protocol

Reaction mix protocol		Three-step cycling protocol	
Components	Volume		
10x NH4 reaction buffer	5 µl	94°C	90 s
50mM MgCl2 solution	1,5 µl	**94°C**	**40 s**
100mM dNTP mix	1 µl	**56°C**	**40 s**
Primer mix (10mM each)	2,5 µl	**72°C**	**40 s**
Water	up to 50 µl	72°C	5 min
Template	1 µl (150 ng)		
BioTaq polymerase	0,5 µl	30x	

Table 3: Restriction endonucleases (RE I) used for cloning of enhancers

Enhancer	**Fw**	**Rev**
Glut1	Kpn I	Bgl II
Hex-A (1)	Kpn I	Bgl II
Hex-A (2)	Kpn I	Bgl II
Hex-A (3)	Kpn I	Bgl II
Treh	Kpn I	Bgl II
CG13334 (1)	Kpn I	Bgl II
CG13334 (2)	Mlu I	Bgl II
CG13334 (3)	Mlu I	Bgl II
Impl 3	Bgl II	Kpn I
IDH	Mlu I	Kpn I
hairy	Mlu I	Bgl II

Genomic DNA extraction protocol

- Homogenize 50 *Drosophila* flies in 500 µl H-buffer (120 mM Sucrose, 100mM Tris-Cl pH 8, 80 mM EDTA)
- Add 25 µl of 10mg / ml proteinase K and 50 µl 10% SDS, incubate over night at 55 °C
- Extract by 500 µl of phenol: chlorophorm: isoamylalcohol, vortex and spin 5 min at 14000 g, 4 °C (repeat twice)
- Clean with chlorophorm: isoamylalcohol (24:1), vortex and spin 5 min at 14000 g, 4 °C

- Add 1 ml of 100% ethanol and 40 µl of sodium acetate and put to the -80 °C for a few hours, then spin 20 min at 14000 g, 4 °C, discard supernatant
- Wash pellet with 70% ethanol, spin 5 min at 14000 g 4 °C, discard a supernatant
- Dry the pellet and resuspend it in adequate volume of water

3. 1. 2. 2. Mutagenesis

In order to test that the effects we observed in a luciferase assay truly depend on the Notch pathway activity we mutated the Su(H) binding sites within the genomic regions cloned into the reporter vectors and tested them in a luciferase assay. If the cloned wild type genomic regions responded to Notch activation in luciferase assay the signal should be lost in the vectors with mutated Su(H) binding sites. Some of the enhancers contained more than one predicted Su(H) binding site. In that case either the most conserved Su(H) site or the one which overlapped in both of the computationally predicted Su(H) binding peaks was chosen for mutagenesis.

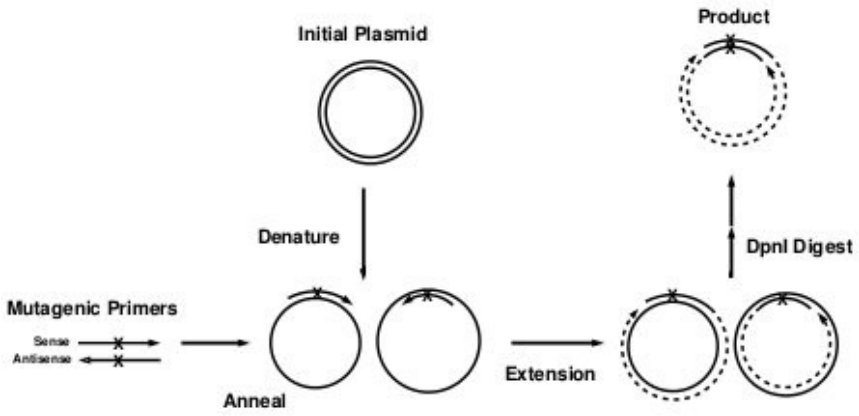


Fig. 6: Principle of the mutagenesis protocol: (https:// web.stanford.edu/~loening/protocols/ Site_Directed_Mutagenesis.pdf). Initially cloned enhancers were mutated within the Su(H) binding site using 47 bp mutagenic primers and a proofreading Pfx polymerase. Non-mutated plasmid was then removed from the PCR product by the DpnI restriction enzyme.

Primers for mutagenesis were 47 bp long, with 7 bp of the Su(H) binding site out of which 3 bp were mutated and 20 bp at each side of the mutated Su(H) binding site to allow proper annealing (see table S2). PCR amplification was performed using proofreading Pfx Platinum polymerase according to a protocol provided by supplier (Invitrogen). Original plasmids containing cloned enhancers were used as a template. The PCR products were treated by the DpnI restriction endonuclease (specifically recognizing only its methylated DNA recognition sequence) in order to remove the non-mutated template plasmids isolated from bacteria and therefore methylated. Vectors were transformed into DH 5α strain of *E. coli*. Plasmids were isolated by High-speed plasmid mini kit (Geneaid) and sequenced. Verified

plasmids were purified using the Plasmid midi kit (Quiagen). Both mutated and non-mutated plasmids were transfected into S2 cells alongside with pMT-N^{icd} to perform luciferase assay and the luciferase activity was measured with the Dual-luciferase reporter assay system (Promega).

3. 1. 2. 3. Testing Su(H) enhancers in the luciferase assay

Genomic fragments of the potential enhancer regions of selected metabolic genes identified above were tested in a luciferase assay in order to assess their responsiveness to the Notch pathway. Each of the selected genomic regions were cloned into a modified pGL3-Basic vector that contains the luciferase reporter gene and a Hsp70 minimal promoter (pGL3—min, fig. 7).

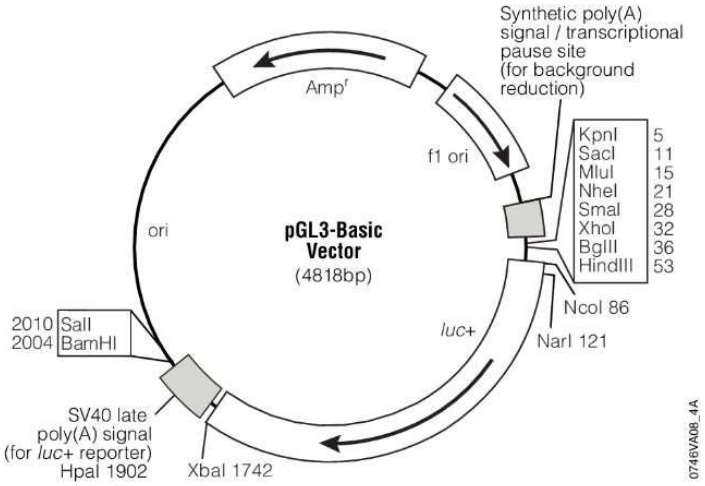


Fig. 7: Map of pGL3-Basic vector used for cloning of selected enhancer regions: https://worldwide.promega.com/products/reporter-assays-and-transfection/reporter-vectors-and-cell-lines/pgl3-luciferase-reporter-vectors/

The vectors were then transfected into the S2 cells together with a vector containing copper inducible Notch intracellular domain (pMT-N^{icd}) and a normalizing pRL-TK-Renilla construct. In case that the cloned enhancers contained functional binding sites for Su(H), enhanced expression of the *luciferase* gene was triggered by N^{icd} in comparison to the basal expression of the reporter alone (Fig. 8). Basal expression was determined in cells transfected with the luciferase reporter plasmid and an empty pMT plasmid instead of pMT-N^{icd}.

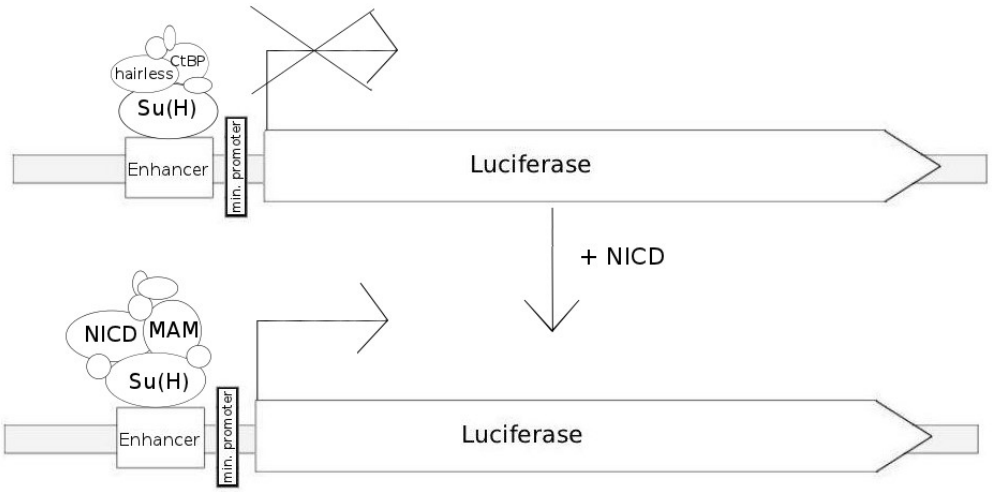


Fig. 8: Principle of the luciferase enhancer assay. If the cloned DNA functions as an enhancer, a coactivator complex is assembled on the DNA after the Notch pathway induction and transcription of the *luciferase* gene starts.

Two individual reporter enzymes (*luciferase* and *Renilla*) were expressed simultaneously within one experiment. Whereas the expression of *luciferase* was controlled by the genomic regions we cloned into the pGL3-basic vector, the pRL-TK-Renilla plasmid served as an internal control of experimental variability such as variations in cells viability, transfection efficiency or pipetting errors.

The luminescent signals from the luciferase and Renilla enzymes were measured consequently from the same sample using the Dual-luciferase reporter assay system (Promega). Briefly, the luminescence of the luciferase was triggered after the addition of the Luciferase assay reagent II, the reaction was subsequently attenuated by Stop & Glo reagent that also induced Renilla luminescence (Fig. 9).

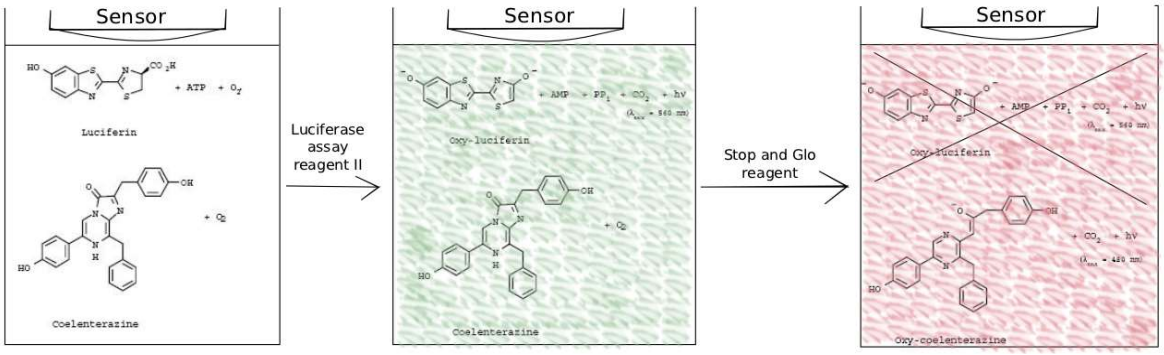


Fig. 9: Bioluminescent reaction catalyzed by firefly and Renilla luciferase. Luciferin and coelenterazin are both present in the sample. After Luciferase assay reagent II is added luciferin is changed to oxyluciferin. Subsequently added Stop & Glo reagent stop luciferin reaction and coelenterazin is converted to oxycoelenterazin.

Drosophila S2 cell line was transfected using the Fugene transfection reagent (Roche). For each well in a 24-well plate total amount of 1200 ng of DNA was used (200 ng pRL-TK-Renilla (Promega), 500 ng reporter plasmid containing cloned enhancer, 200 ng pMT-N^{icd} and 300 ng empty pMT) in the following protocol:

- For each sample mix 40 µl of Opti-MEM (Invitrogen) with 200 ng of pRL-TK-Renilla and 500 ng of the luciferase reporter plasmid containing cloned enhancer of interest
- Add 200 ng of pMT-N^{icd} (coding the copper inducible Notch intracellular domain) and 300 ng of the empty pMT plasmid (or 500 ng of pMT plasmid as a negative control without pMT-N^{icd})
- In another vial mix 25 µl of Opti-MEM with 3 µl of Fugene, vortex lightly and let stand for 5 min on RT
- Combine vials 1 and 2, vortex lightly and incubate on RT for 30 min
- Suck off medium from the cells, keep only 250 µl in the dish and add transfection mix into medium by drops
- After 6 hours replace medium for medium containing 600 µM $CuSO_4$ to activate the N^{icd} expression
- After 24 hours suck off the medium, wash the cells with 1x PBS and lyze them in 50 µl of 1x lysis buffer

In order to measure the luciferase expression, samples were diluted 5x in 1x lysis buffer and 10 µl of diluted sample was measured on the Orion II microplate luminometer (Titertek-Berthold) according to the following protocol:

- Add 50 µl of the Luciferase assay reagent II
- 2,05 s delay
- 10 s measurement of *Luciferase*
- 10 s delay
- Add 50 µl Stop & Glo reagent
- 2 s delay
- 10 s measurement of *Renilla*

If necessary, lysed cells were stored at -20 °C to the next day or at -80 °C for longer time before measurement. All transfections were prepared in duplicates and at least three independent biological replicates were performed on separated days.

3. 1. 2. 4. Analysis of the mRNA expression profile after the activation of the Notch pathway in cell lines using Q-RT-PCR

In order to investigate mRNA expression of selected metabolic genes after Notch pathway activation we performed a time course analysis in S2N and dmD8 cells. The S2N cell line are S2 cells stably transfected by a copper inducible full length Notch construct. Expression of Notch was triggered by 600 μM CuSO4 overnight and the Notch pathway was activated by 2 mM EDTA in PBS for 15 min. This should provide us good induction of Notch activation without starving the cells by the lack of media during the activation (Krejčí, Bernard, Krejčí, et al. 2009). Both S2N and dmD8 cells were kept in Schneider´s insect medium (Gibco). Experiment was performed in 6-well plate and the samples were collected according to the following scheme:

Non-activated control

15 min EDTA in PBS

15 min EDTA in PBS + 15 min Schneider´s medium

15 min EDTA in PBS + 30 min Schneider´s medium

15 min EDTA in PBS + 45 min Schneider´s medium

15 min EDTA in PBS + 105 min Schneider´s medium

Cells were collected by centrifugation at 700 rpm for 1 min and lyzed in TRI reagent (Sigma-Aldrich). RNA was isolated according to the manufacturer´s protocol. Residual traces of genomic DNA were removed with DNase I (DNAfree reagent from Ambion). Reverse transcription reaction was performed using the M-MLV reverse transcriptase (Sigma-Aldrich) with the use of random primers (Promega). The length of the regions amplified by the real time PCR varied between 120 and 136 bp. Q-RT-PCR reaction was performed according to the protocol (tab. 4) using the Bio-Rad CFX96 machine:

Tab. 4: Q-RT-PCR protocol

Reaction mix protocol		Two-step cycling protocol		
Components	Volume			
DEPC H_2O	3,9 µl	95°C	5 min	
GoTaq Q-PCR master mix	5 µl	**95°C**	**60 s**	**40x**
10 mM primer mix	0,6 µl	**65°C**	**30 s**	
cDNA (or genomic DNA)	0,5 µl	**65°C**	**3 min**	

Calibration curves were constructed from the genomic DNA and signal was normalized to the levels of *CG11306* gene and all reactions were performed in duplicates.

We also incubated S2N cells with 5 μM γ-secretase inhibitor Compound E (Abcam) or 100 μM protein synthesis inhibitor cycloheximide (Sigma-Aldrich) respectively for 16h prior to Notch activation and also during the time course experiment in order to confirm that selected metabolic genes are true primary targets of Notch pathway. Similarly, treatment of S2N cells with 50 μM PI3K/AKT inhibitor LY294002 (Abcam) 1 hour before and during the experiment was used to rule out the possibility that increased expression of several metabolic genes is due to PI3K/AKT signaling stimulation after Notch activation, as observed after hyperactivaton of Notch1 in breast cancer cells (Landor et al. 2011). Inhibition of PI3K/AKT signaling by inhibitor LY294002 was verified by testing expression of its downstream target *Thor*. All primers used for Q-RT-PCR analysis are listed in supplementary table S2.

For each experiment at least three biological replicates were performed on separate days and all of the time course experiments were performed according to the above described scheme.

3. 1. 2. 5. RNAi treatment in S2N cells

PCR product (714 bp long) that was used as a template to create double-stranded RNA for *hairy* was amplified from genomic DNA using primers with T7 promoters attached to theirs ends (see table S2). Double stranded RNA was transcribed using RiboMax system (Promega). Cells were seeded into the six-well plate and RNAi was performed according to the following protocol:

- Suck out the medium and replace it with 300 μl Opti-MEM containing 20 μg of dsRNA
- Incubate the cells for 30 min at 25C and rock the plate every 5 min to secure equal distribution of the mixture
- Add fresh Schneider´s medium pre-warmed to 25C
- After 48 h repeat steps 1 – 3
- Harvest the cells by spinning at 700 g / 60 s and resuspend in 500 μl of TRI reagent
- Isolate RNA and process samples as described in chapter 3. 1. 2. 3. (above).

3. 1. 3. Analysis in vivo

To investigate the effect of overexpression or down regulation of the Notch pathway on selected metabolic genes *in vivo* we performed *in situ* hybridizations, fluorescent immunohistochemistry and quantitative real time PCR analysis in the wing discs of 3rd instar *Drosophila* larvae.

3. 1. 3. 1. *In situ* hybridization in imaginal wing discs

For *in situ* hybridizations, the Su(H) – VP16 fusion (a fusion with a strong viral VP16 activation domain) was used to induce the expression of genes that contained Su(H) binding sites in their regulatory regions. These flies were crossed with Ptc-Gal4; Tub-Gal80^{ts} driver line to induce Su(H) overexpression specifically in *patched* domain of the imaginal wing disc (fig. 10). Gal4 is a transcription activator protein binding the upstream activation sequence (UAS) that works as a tetramere. Gal80 protein replaces one or more of Gal4 protein in this tetramere and therefore inhibits its function. In our study, thermal sensitive mutant of Gal80 (Gal80^{ts}) was used that is unstable at temperatures above 29 °C. Larvae were first grown at 25C (with Gal80^{ts} active) and then exposed to elevated temperature of 29 °C for 72 hours before dissection (to make Gal80^{ts} inactive and trigger the expression of Su(H)-VP16 construct).

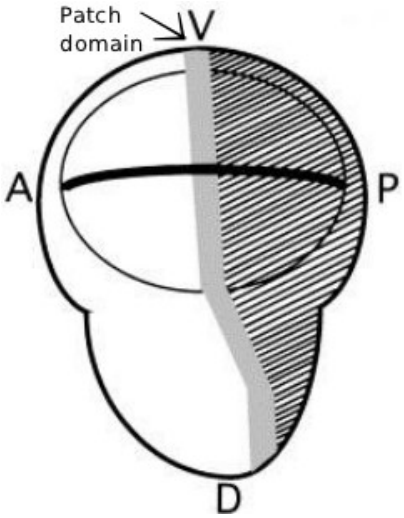


Fig. 10: Drosophila imaginal wing disc. The *patched* domain is marked by an arrow. A – anterior side of the disc (*Cubitus interruptus* domain), P – posterior side of the discs (*engrailed* domain), D – dorsal side of the disc, V – ventral side of the disc.

Digoxigenine labeled sense or antisense probes were synthesized using the sense or the antisense oligonucleotides with T7 promoter attached to their 5´ ends (tab. S2). PCR products (555 bp – 717 bp long) were amplified with BioTaq polymerase (Bioline). Amplified DNA was purified by phenol: chlorophorm extraction and precipitated in ethanol.

RNA probes were prepared with T7 RNA polymerase and DIG RNA labeling mix (both Roche) according to the following protocol:

Preparation of the RNA probes

- Mix: 1 µg of PCR product

  2 µl of DIG RNA labeling mix

  2 µl of 10x transcription buffer

  2 µl of T7 RNA polymerase

  0,5 µl of RNAsin (Promega)

  Water up to 20 µl
- Incubate 4 hours at 37 °C
- Add 2 µl of DNase I (Ambion) and incubate 15 min at 37 °C
- Add 1 µl of 500 mM EDTA (pH 8) to stop the reaction
- Precipitate the probe by adding 1,28 µl of LiCl and 75 µl of 100% ethanol
- Incubate on ice for 60 min, centrifuge at 13000 g at 4 °C for 30 min
- Remove the supernatant, dry the pellet and resuspend in 20 µl of DEPC H2O

For in situ hybridizations 15 larval heads (partially dissected larva containing its anterior part that included brain and wing discs) for antisense and 15 for sense probe were dissected in 1x PBS and fixed immediately in 4% formaldehyde in PBS for 30 min at room temperature. Fixed heads were washed three times 5 min in PBT-Tween 0,1% and fixed again in 4% formaldehyde in PBT-Tween 0,1% for 20 min. Heads were washed three times 5 min once more and another wash step in 50% Hybridization solution (HS) in PBT-Tween 0,1% for 5 min was included. Followingly, heads were put into the pure HS and kept in -20°C until hybridization with the probes.

Before prehybridization, heads were washed with HS for 10 min and incubated in fresh HS at least 3 hours at 62°C. 7ul of probe had been mixed with 100 µl of HS, incubated 10 min at 80°C and chilled on ice to prevent renaturation. HS was removed from the heads, replaced by the probe in HS and incubated at 62°C overnight.

Following morning, probe was removed and the heads were washed according to the following steps:

- 5 min with HS at 62°C

- 15 min with HS at 62°C
- 5 min with 70% HYBE in 30% PBT-Tween 0,1% at 62°C
- 5 min with 50% HYBE in 50% PBT-Tween 0,1% at 62°C
- 5 min with 30% HYBE in 70% PBT-Tween 0,1% at 62°C
- Four times 10 min with PBT-Tween 0,1% at room temperature in agitation

Following of the washing steps, heads were incubated with PBT-BSA for 30 min at RT and then with digoxigenine antibody in PBT-BSA for 2 hours. After that heads were washed by PBT-Tween 0,1% four times 10 min in agitation. Heads were washed once more with staining solution for 15 min in agitation and then incubated with NBT / BCIP (Roche) in staining solution until the staining was either visible or did not shown any progress. Reaction was attenuated by washing the heads with PBT-Tween 0,1% for three times 5 min in agitation. Heads were washed with glycerol (30%, 50%, 70%) and imaginal wing discs were dissected and mounted on a microscope slide.

Solutions:

Saline sodium citrate (SSC) buffer:
3 M sodium chloride
300 mM trisodium citrate
Adjust pH to 7.0 with HCl

HYBE:
50% Formamide
50% 5x SSC buffer

Hybridization solution (HS):
50% Formamide
5x SSC buffer
100 µg/ml DNA salmon sperm
50 µg/ml Heparin
0,1% Tween 20

Staining solution:
100 mM NaCl
50 mM MgCl2
100 mM TrisHCl
adjust pH to 9,5
0,1% Tween 20

3. 1. 3. 2. Q-RT-PCR analysis of the imaginal wing discs

To investigate if the mRNA levels of selected metabolic genes respond to Notch signaling also *in vivo* imaginal wing discs from N^{ts2} (BL3075) *Drosophila* stock were dissected.

N^{ts2} is a thermosensitive allele of Notch that leads to its degradation when shifted to 30 °C therefore mimicking conditional Notch loss of function. Larvae were kept in 18 °C and transferred to 30 °C 16 hour prior to dissection. To test whether Notch can downregulate tricarboxylic acid cycle genes in wing discs and if this could be mediated by transcription factor hairy as seen in S2N cell lines we also dissected imaginal wing discs from the hypomorph mutant of *hairy* (h^1, BL513) which were kept in 25 °C. In both cases, as a control of normal expression of selected metabolic genes in wing discs Oregon R *Drosophila* strain was used.

For all the genotypes, larvae were scraped out 2 hours prior to experiment and only newly emerged larvaes were used. 40 imaginal wing discs were dissected and dissolved in 500 µl of TRI reagents. Discs were dissected into the ice cold 1x PBS in 10 min intervals after which they were spun and dissolved in TRI reagents to prevent RNA degradation. Discs were gradually dissected until the final amount of dissected discs was reached. The extracted RNA was threated and Q-RT-PCR analysis was performed in the same manner as in case of S2N cell lines (see chapter3. 1. 2. 4.). The same primer sets for Q-RT-PCR analysis were used as well (see table S2). All samples were run in duplicates and three biological replicates were prepared for each genotype.

3. 1. 3. 3. Immunohistochemistry in the imaginal wing discs

To test whether changes in mRNA expression correspond to changes in protein translation we performed immunostaining of 3rd instar *Drosophila* larva imaginal wing discs after overexpression or downregulation of Notch signaling.

Stock containing *Hex-A* Gal4 enhancer trap (DGRC 105136) together with UAS-Lac Z reporter (Hex-A Gal4; UAS-Lac Z) was prepared and subsequently crossed with N^{ts2} flies to perform time course analysis of Hex-A protein expression. Oregon R flies were used as control. Larvae were kept in 18 °C prior to heat shock in 30 °C. Discs were dissected in several time points (0h, 2h, 4h, 6h, 8h, 12h, 16h and 20h). To verify the results, Hex-A Gal4, UAS-Lac Z stock was crossed also with UAS-MAM^{DN} (BL26672, dominant negative form of Notch co-activator Mastermind) and UAS-N^{icd}. UAS-GFP stock was used as a control. Larvae were kept in 18 °C ad moved to 30 °C 16 h and 72h prior to dissection respectively.

Lastly, Hex-A Gal4; UAS-Lac Z stock was crossed with Hairless mutant line (H^2 allele of transcriptional corepressor Hairless, BL517) to see if the effect on Hex-A expression can be achieved also by over activation of Notch signaling downstream of the pathway. Larvae were kept at 25 °C. All stainings were performed according to the following protocol:

- Dissect 10 larval heads into the ice cold 1x PBS for maximal period of 10 min
- Fix larval heads in 4% formaldehyde for 30 min at room temperature
- Wash larval heads in PBST (PBS with 0,2% Triton X-100) three times 10 min at room temperature in agitation
- Keep the larvae in fresh PBST at 4 °C until preincubation with 1x PBST - 0,5% BSA
- Wash larval heads in PBST –0,5% BSA for 30 min at room temperature in agitation
- Incubate larval heads with PBST –0,5% BSA containing primary antibody (α-Lac Z, 40-1A, Hybridoma Bank) for 16 hours (9 hours for N^{ts}) at 4 °C in agitation
- Wash larval heads in PBST – 0,5% BSA three times 10 min at room temperature in agitation
- Incubate larval heads with PBST – 0,5% BSA containing secondary antibody (α-mouse Alexa 488, (Jackson immunoresearch, 1: 500) for 9 hours at 4 °C in agitation
- Wash larval heads in PBST three times 10 min at room temperature in agitation
- Incubate heads in 80% glycerol on ice for 30 minutes
- Dissect imaginal wing discs and mount on a slide into the Citifluor AF1 (Citifluor)

The strength of the signal from the Hex-A Gal4 reporter was quantified in ImageJ (Abràmoff et al. 2004) by calculating the integrated density of Hex-A immunostaining form the whole wing disc (sum of pixel values) divided by area of the wing discs. Z-stacks of confocal pictures spanning the whole disc thickness were used. Background was subtracted from the part of the disc with no signal and value plotted as “Hex-A intensity per area”.

To test responsiveness of the *hairy* transcription factor to Notch activity *in vivo* we prepared C-terminal fusion of *hairy* with eGFP in 43 kb FlyFos031638 fosmid clone according to the protocol described by Ejsmont (Ejsmont et al. 2011) using S0062-R6 K-2xTY1-eGFP-FNF3xFLAG (gift from M. Sarov). The fosmid was injected into embryos carrying the attP40 site for stable transgene integration. Flies carrying this construct were crossed to UAS-N^{RNAi}; Ptc-Gal4, Tub-$Gal80^{ts}$ and kept at 29 °C for 72h in order to downregulate Notch signaling in the *Ptc* domain of the wing disc. *hairy* FlyFos crossed to Ptc-Gal4, Tub-$Gal80^{ts}$ alone was used as a control. Immunostaining with monoclonal α-GFP antibody (ab290, Life technologies) was performed according to the above described protocol.

3. 1. 4. Functional analysis of Notch-dependent metabolic changes

3. 1. 4. 1. Measurement of the cellular metabolites by nuclear magnetic resonance in S2N cells

To test whether Notch induced changes in metabolic enzyme expression are sufficient to induce changes in overall metabolism we measured levels of lactate and fumarate and glucose consumption in S2N cells. For the measurements of lactate and fumarate we seeded S2N cells in Schneider´s medium and induced Notch expression by 600 µM $CuSO_4$ overnight. Control plates without Notch induction were included as well. The Notch signaling was then activated by 15 min pulse of EDTA (2 mM) in 1x PBS that was then replaced with Schneider´s medium if longer incubation times were desired. Non-activated cells (0 min) as well as cells 15 min, 25 min, 50 min and 75 min after activation were collected according to the following protocol:

- Scratch down all cells from the plate and transfer them gently into the ice cold 15 ml falcon tube.
- Wash the dish with small amount of ice cold 1x PBS and add it into the falcon tube.
- Spin the cells at 750 g for 2 min at 4 °C and discard the supernatant.
- Resuspend the cells in 1 ml of ice cold 1x PBS and add the 1x PBS up to 10 ml.
- Spin the cells again, discard the supernatant and resuspend cells in 1 ml of fresh ice cold 1x PBS, transfer into ice cold 2 ml Eppendorf tube.
- Spin the cells at 750 g for 1 min at 4 °C and discard the supernatant.
- Resuspend the cells in 250 µl of ice cold MiliQ water and add 250 µl of frozen methanol, Mix gently and transfer into the liquid nitrogen for minimally 30 min
- Thaw the samples in ice bath and spin at 16000 g for 1 min at 4 °C.
- Transfer the supernatant into new ice cold 1,5 ml Eppendorf tube.
- Resuspend pellet in 500 µl of 50% frozen methanol and transfer the sample into liquid nitrogen for at least 30 min again.
- Thaw the samples in ice bath and spin at 16000 g for 1 min at 4 °C, take supernatant and combine with supernatant from the previous elution.
- Resuspend pellet in 500 µl of MiliQ water and snap freeze in liquid nitrogen for subsequent assessment of protein content.
- Lyophilize the samples and send for NMR measurement.

Glucose consumption was determined using isotopically labelled [2-13C] glucose. The S2N cells were treated with 600 µM CuSO4 and activated by EDTA for 15 minutes as

described in the section above. After Notch activation, medium containing [2-13C] glucose was added and cells were collected 75 min after activation as follows (Delgada et al. 2004):

- Wash the dish twice with ice cold PBS and apply 3 ml of ice cold acetonitrile: MiliQ water (1: 1)
- Rock the plate on ice for 10 min
- Scrape the cells into sample tubes and spin at 16000 g for 5 min at 4 °C
- Transfer the supernatant to the ice cold tube, pellet resuspend in 500 µl of MiliQ water and snap freeze in liquid nitrogen
- Lyophilize the sample

Metabolite measurements and quantifications were performed by NMR as described in Sellick (Sellick et al. 2009) and Delgado (Delgada et al. 2004) respectively by L. Trantírek and M. Krafčíková from CEITEK. Lyophilized samples were resuspended in 550 µl D_2O (Sigma-Aldrich) containing 0,005% sodium 3-(trimethylsilyl)propionate-2,2,3,3-d4 (TSP) (Sigma-Aldrich) for chemical shift reference. The samples were measured at 700MHz Bruker Avance spectrometer using standard (Bruker) pulse sequence in order to determine 1D ^1H NMR spectra. Pre-saturation was used to suppress residual water signal. All spectra were acquired at 25°C, processed using TopSpin 3.2 (Bruker, USA) and referenced with respect to TSP. Peaks of selected metabolites were identified by comparison to reference values for chemical shifts (Ulrich et al. 2008) and confirmed by titration of the samples with pure compounds (Sigma-Aldrich). Signals which corresponded to the glucose, lactate and fumarate were integrated manually using built-in routines of TopSpin 3.2 software. Volumes of the signal were normalized to concentration of the TSP employed as an internal standards and subsequently also to total protein concentration. Total protein concentration was determined from the cellular pellet resuspended in MiliQ H_2O. 25 µl of the resuspended pellet was transferred into a 96-well plate in duplicates and mixed with Biquinonic acid reaction mix according to the manufacturer´s protocol (Sigma-Aldrich). BSA of concentration 250 – 1000 ng resuspended in MiliQ H_2O was used as standard, pure MiliQ H_2O was used as blanc. Samples were incubated at 37 °C for 30 min and measured on SpectraMax 340PC384 Absorbance Microplate Reader (Molecular Devices) at 562 nm. Two independent measurements of the sample were performed and average value was taken for protein normalization.

3. 1. 4. 2. Measurement of cellular metabolism by Seahorse flux analyzer *in vivo*

Metabolic status of imaginal wing discs was measured by the XFe24 Extracellular Flux analyzer (Seahorse Bioscience). Two different parameters were measured in the medium surrounding the tissue: oxygen consumption rate (OCR) which represents activity of the respiratory chain, and extracellular acidification rate (ECAR) that is the indicator of the rate of glycolysis.

Flies with N^{icd} induced hyperplasia (AbxUbxFLPase; Act > y > Gal4, UAS-GFP; FRT82B Tub-Gal80x UAS-N^{icd}MH3; FRT82B) were kept in 25 °C until L3 larvae emerged. AbxUbxFLPase; Act > y > Gal4, UAS-GFP; FRT82B Tub-Gal80x UAS-GFP; FRT82B were used as control. In order to keep the discs at the bottom of the measuring well during sample mixing involved in the Seahorse protocol, Seahorse XF24 Islet Capture Microplates were used. Samples were dissected in DMEM medium without sodium bicarbonate (Sigma-Aldrich) and measured in triplicates. Background was subtracted from empty wells and three-step cycle (1 min of mixing, 2 min of waiting and 3 min of measuring) was used. Basal metabolic status was measured for 42 min after which three inhibitors were subsequently added: 5 μM oligomycin (inhibits ATP synthase by blocking its proton channel which should lead to lowered respiration compensated by higher glycolysis), 2 μM H+ ionophor FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, depolarize the mitochondrial H^+ gradient leading to higher oxygen consumption rate), 5 μM antimycin A (binds to the Qi site of cytochrome C reductase and inhibits electron transport chain). Metabolic parameters were recorded for another 24 minutes after each drug was added.

Measured values were subsequently normalized to the amount of the proteins. Discs were lyzed in plate by 60 μl of RIPA lysis buffer, shaken on ice for 15 min and placed into -20 °C. Next day, discs lysates were transferred into pre-cooled Eppendorf tubes and spun for 5 min at 16 000 g in 4 °C. After centrifugation, lysate was diluted 4x in fresh RIPA buffer. 25 μl of lysate was transferred into a 96-well plate in duplicates. For the determination of the amount of protein Biquinonic acid kit (Sigma-Aldrich) was used. After reaction mix was added samples were incubated at 37 °C for 30 min and measured on SpectraMax 340PC384 Absorbance Microplate Reader (Molecular Devices) at 562 nm. Three biological replicates of the experiment were prepared.

3. 1. 4. 3. Fly strains used to investigate the interplay between Notch signaling and metabolism during the regulation of imaginal wing discs growth.

To evaluate the reduction in wing disc growth caused by metabolic perturbations we prepared flies with both the en-Gal4 driver and Hex-A RNAi (VDRC 100831) or Impl3 RNAi

(VDRC102330) transgenes on the second chromosome by recombination. We then crossed these flies with the aim to strongly downregulate glycolysis by having double dose of en-Gal4 and targeting two genes of the glycolytic pathway for degradation. RNAi line for Glut1was also from VDRC (VDRC108683).

To evaluate the reduction in wing disc growth caused by Notch pathway downregulation, en-Gal4, TubGal80ts flies were crossed to UAS-MAMDN (BL26672), UAS-N^{RNAi} (BL7078) and UAS-N^{DN}. As the controls UAS-GFP and *white* RNAi flies (BL35573) were used. Progeny was kept in 25 °C for 40 h and then transferred to 29 °C for 120 h before dissection of the wing discs from L3 larvae.

Further, we tested if hyperplasia induced by Notch overexpression can be rescued by dietary restriction and, more importantly, by knocking down selected metabolic enzymes. Firstly, UAS-N^{icdMH3} flies were crossed to Ptc-Gal4, TubGal80^{ts} driver line with UAS-GFP line used as a control. Secondly, Ptc-Gal4, Tub-Gal80^{ts}; UAS-N^{icd} flies were crossed to RNAi lines of Hex-A, Impl3 and Glut1. Control KK line (VDRC60100, KK stock used for creating all KK lines without inserted transgene) was used as a control. In both cases larvae were kept on two different diets ("high nutrient food" containing 160 g of yeast and 160 g of sugar per 1 liter and "low nutrient food" containing 20 g of yeast and 20 g of sugar pre 1). N^{icd} overexpression was induced by transferring the larvae from 18 °C to 29 °C for 72 h prior to wing discs dissections. N^{icd} induced overgrowth was measured in ImageJ as described in (Djiane et al. 2012).

In all of the above mentioned experiments immunostaining was performed to visualize the *Cubitus interruptus* (*Ci*) domain (α-Ci antibody 2A1-S from Hybridoma bank) according to the protocol described in chapter 3. 1. 3. 3. and the size of *en* domain was determined as part of the wing disc lacking signal the *Ci* staining.

3. 2. Methods related to published article Horvath M, Mihajlovic Z, Slaninova V, Perez-Gomez R, Moshkin Y, Krejci A. The silent information regulator 1 (Sirt1) is a positive regulator of the Notch pathway in *Drosophila*. Biochemical journal, 473, 4129-4143 (2016).

3. 2. 1. Luciferase assays to test the functional interaction of Sirt1 with N^{icd}

In mammalian systems, Sirt1 has been described as a negative regulator of Notch signaling (Mulligan 2011; Xie et al. 2012). However, according to our data Sirt1 influence

Notch pathway in a positive manner. To test the effect of Sirt1 on Notch activation luciferase assay was performed. S2 cells were transfected with 350 ng of pGL-*m3* reporter and 200 ng of pTL – TK – Renilla together with either 350 ng of pMT – *Sirt1* vector or 200 ng of pMT-N^{icd} vector or combination of both. Total amount of the transformed DNA was 1200 ng and empty pMT was used as stuffing vector.

3. 2. 2. Baculovirus expression of Su(H) protein

Bac-to-Bac expression system (Invitrogen), based on site specific transposition, was used to clone and express the protein. Su(H) protein was cloned into the baculovirus vector pFastBac-TOPO that creates a fusion protein tagged with 6x His tag at the C-terminus. The expression from this vector is under the control of a multiple polyhedrin promoter and the whole expression cassete is flanked with Tn7 transposon. Vector was transformed into the DH10Bac *E. Coli* that is carrying a helper plasmid with a transponase and also a bacmid, i.e. a huge plasmid containing mini-attTn7 target site and also coding all genes necessary for the production of functional baculoviruses in insect cells. Recombinant bacmid is produced by a transposition of the gene of interest from the pFastBac TOPO donor. Recombinant bacmid was isolated by PureLinkTM HiPure Plasmid Miniprep Kit (Invitrogen) according to the manual, verified by sequencing using pUC/M13 rev and Su(H) fw and transformed into Sf9 cells (Spodoptera frugiperda) to create baculoviral stock P1 (fig. 11).

Sf9 cells were infected by baculovirus carrying Su(H) twice more to create a P3 stock with appropriate titer (10*8). To determine the baculoviral titer the dilution assay was performed. Sf9 cells were infected with different volume of baculoviral stock and we selected the lowest volume sufficient to infect all cells within 24 hours as MOI (multiplicity of infection; amount of the particles per one cell) equal to 1. The titer was then calculated from the formula $Inoculum\ required\ (ml) = \frac{MOI\ x\ number\ of\ cells}{titer\ or\ viral\ stock}$. Cells were kept in TNM-FH insect medium (Sigma-Aldrich) at 27 °C.

Hi5 cells were seeded into 6 cm tissue culture dish a day before experiment in TNM-FH insect medium and in a manner that they would reach 100% confluency by the following morning. In the day of the experiment, cells were infected by P3 baculoviral stock of a MOI = 1 (multiplicity of infection = amount of baculoviral particle per cell). After 5 hours TNM-FH insect medium was replaced for Express five serum free medium (Invitrogen) specifically designed for protein expression in Hi5 cells and treated with either 20 μM Resveratrol and 20 μM SRT1720 (activators of Sirt1) or 25 μM EX527 and 10 μM Sirtinol (inhibitors of Sirt1).

Treatment with DMSO only was used as control. Cells were left for 48 h at 27 °C and harvested according to the following protocol:

- Scratch the cells off the plate by plastic cell scraper into the precooled falcon tube and spin at 500 g for 3 min at 4 °C
- Discard the supernatant, wash the cells by ice cold 1x PBS and spin again
- Discard the supernatant and resuspend cells with 1500 µl of lysis buffer containing 20mM NaH2PO4, 300mM NaCl, 10mM imidazole, 1% Triton X-100 and 1x protease inhibitor cocktail (Roche), pH 7,5
- Sonicate cells at 20 kHz, 320 W for 10 min and spin 20 min at 14000 g at 4 °C

Protein was purified from the lysate by HisPur Ni-NTA superflow agarose beads (Thermo fisher) according to the following protocol:

- Add 20 µl of the beads into the lyzate and incubate at 4 °C over night
- Spin the cells at 700g for 3 min at 4 °C
- Discard the supernatant and was the beads with 1 ml of cold Washing buffer (20mM NaH2PO4, 300mM NaCl, 50mM imidazole, pH 7,5) on ice
- Spin the cells and repeat the washing step twice more
- Spin the cells at 700g for 3 min at 4 °C, discard the supernatant and resuspend the beads in 200 µl of Elution buffer (20mM NaH2PO4, 300mM NaCl, 300mM imidazole, pH 7,5)
- Incubate the beads with Elution buffer for 10 min at 25 °C in agitation
- Spin the cells at 700g for 3 min at 4 °C and transfer the eluate into the fresh Eppendorf tube

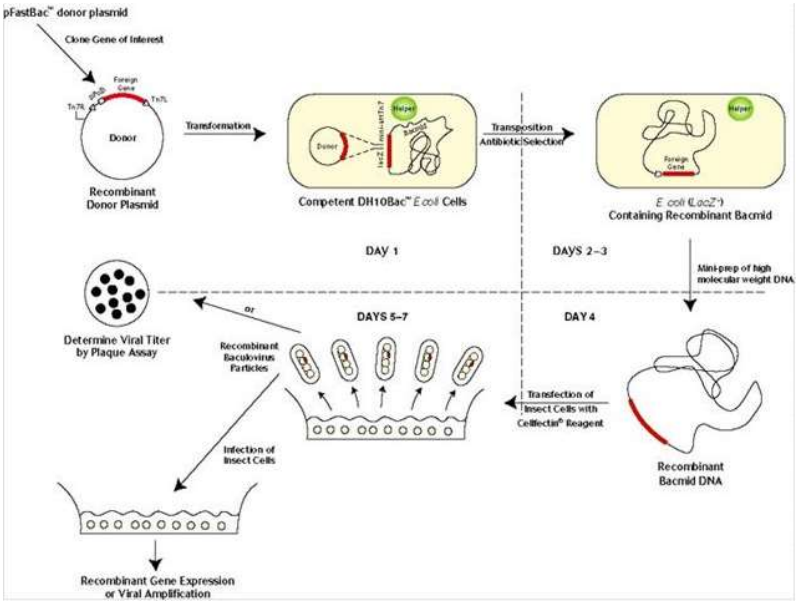


Fig. 11: Bac-To-Bac expression system cloning mechanism
(https://tools.thermofisher.com/content/ sfs/manuals/bactobac_topo_exp_system_man.pdf). In the first step coding region of the protein is cloned into the pFastBac vector that is then recombined into the DH10BAC *E. coli* carrying helper plasmid together with bacmid containing all genes needed for assembly of functional baculovirus. Recombinant bacmid is subsequently transfected into the insect cells to create functional recombinant baculovirus carrying the protein of interest.

3. 2. 3. Western blott analysis

Protein samples were analyzed by western blot analysis. Approximately the same amount of the total Su(H) protein isolated from Sf9 cells with / without Sirt1 inhibitors or activators (estimated by previous Coomassie staining) was loaded to 10% SDS denaturation gel and blotted on PVDF membrane using XCell II™ Blot Module blotting system (Novex). Membranes were washed in 1x PBT 3x 15 min at room temperature in agitation and subsequently blocked in 1x PBT – 5% BSA for 30 min at room temperature in agitation. HRP conjugated α-His antibody (Thermo Fisher, 1:4000, in 1x PBT – 5% BSA) and α-acetyl-K antibody (PTM Bioloabs, 1:1000, in 1x PBT – 5% BSA) were used for incubation overnight at 4C in agitation.

Following morning membranes were washed 3x in 1x PBT and the one incubated with α-acetyl-K antibody was subsequently also incubated with HRP α-rabbit secondary antibody (Jackson immunoresearch, 711-035-152) for 2 hours at room temperature in agitation. To develope the membranes, Clarity™ Western ECL Substrate (Biorad) was used. Signal intensities were collected on ChemiDoc MP system machine (Biorad) using

Chemi-Hi resolution settings for 5s for α-His antibody and 16s for α-acetyl-K antibody respectively. Band intensities were quantified in ImageLab software (Biorad).

3. 3. Methods related to unpublished results

3. 3. 1. Seahorse measurements of the effect of 2-deoxy-glucose treatment on metabolism of S2N cells

According to our data, treatment of S2N cells by 10 mM 2-deoxy-glucose (DOG) leads to changed expression of several Notch target genes from the Enhancer of split complex. To test whether DOG treatment leads to changes in cellular metabolism, respiration and glycolysis rates were measured using Seahorse XFe24 analyzer. S2N cells were seeded into the Seahorse XFe24 Cell Culture Microplate either in complete Schneider´s medium or in medium containing 2 mM DOG for 16 h. Schneider´s medium was exchanged for DMEM medium without sodium bicarbonate containing 2 mM DOG 30 minutes prior to measurement. Cells were measured for 48 minutes for basal after which 110 mM DOG was added and cells were measured for additional 24 min. Signal was normalized to protein content. Measurements and protein normalization were performed as described in chapter 3. 1. 4. 2..

3. 3. 2. Fly stocks used for testing the effect of changes in metabolism on the expression of Notch target genes in vivo

Homozygous *yw* stock as well as *yw* flies crossed to GFP fused reporter of *M6* and Lac Z fused reporter of *mβ*, both targets of Notch pathway belonging to Enhancer of split complex, were set on "high" and "low" nutrient diet (see chapter 3. 1. 4. 3.). Flies with double en-Gal4 and RNAi against ImpL3 and Hex-A, carrying Notch response element fused with RFP (NRE-RPF) to induce metabolic changes by different mechanisms, were prepared as well (for reference of KK lines see chapter 3. 1. 4. 3.). All flies were kept at 25 °C and only L3 larvae were dissected and stained according to the protocol described in chapter 3. 1. 3. 3.. Antibodies against Lac Z, cut and deadpan (all Hybridoma) and against GFP (G10362, life technologies) and RFP (632496, Clontech) were used. Flies containing en-Gal4 driver were stained also with α-Ci antibody (2A1-S, Hybridoma). Signal of Notch target gene expression was quantified according to protocol written in chapter 3. 1. 3. 3.. NRE signal was plotted as a en: Ci ratio.

3. 3. 3. Luciferase assay for testing the effect of changes in NAD^+: NADH ratio on the expression of Notch target gene in S2 cells

To investigate whether changes in NAD^+: NADH could influence response of the cells to the Notch signaling pathway we turned to S2 cells and performed luciferase assay. Cells were seeded into 24-well plate to 80% confluency and transfected with either 500 ng of pGL3-Basic vector with cloned *m3* enhancer region in front of Luciferase gene (pGL-m3, (Bernard et al. 2010)) or empty pGL3 vector as a control. 200 ng of pRL – TK – Renilla was transfected into the cells as well together with either 200 ng of pMT-N^{icd} or 200 ng of pAC-dNAAM (gift from Guri Tzyvion) or combination of both. pMT empty vector was added to make the total DNA used for transfection to 1200 ng.

4. Results

4. 1. Results related to Slaninova V, Krafcikova M, Perez-Gomez R, Steffal P, Trantirek L, Bray SJ, Krejci A. Notch stimulates growth by direct regulation of genes involved in the control of glycolysis and the tricarboxylic acid cycle. Open Biol. 6: 150155, (2016)

In this work we decided to test the hypothesis that some of the metabolic genes are direct transcriptional targets of the Notch pathway and that Notch signaling is iportant for the modulation of cellular metabolism. First, we searched previously published ChIp-chip data of Su(H) to select potential candidate genes, then we characterized their responsivness to Notch in a luciferase assays and *in vivo* in *Drosophila* wing disc. We measured several metabolites in S2N cells and we showed that even a short pulse of Notch activation triggers long lasting metabolic effects. The Notch driven metabolic changes stimulate higher glycolysis and slower TCA cycle, directing a metabolic reprogramming of the target cell towards the Warburg effect. The Notch dependent growth of wing discs requires induction of metabolic genes and Notch is able to support the growth of wing disc even in conditions of low nutrient availability. We proposed a model how the activity of Notch pathway modulates the metabolic parameters of the target cells that may be relevant in other tissues where Notch pathway is active, such as stem cells, immune cells or Notch-dependent cancer cells.

4. 1. 1. Selection of potential Notch target genes involved in metabolism

We took advantage of the published (Krejčí & Bray 2007) and unpublished ChIP-chip data from two cell lines (muscle precursor derived DmD8 cells and neuronal precursor BG2 cells) after 30 minute activation of Notch and from wing disc tissue (wild type yw strain, N^{icd} overexpressing wing discs and Su(H) overexpressin wing discs), looking for peaks of Su(H) binding in the regulatory regions of genes involved in the regulation of cellular metabolism. More than twenty genes contained Su(H) binding site and therefore potential enhancers in their promoter regions. From these we selected seven genes that either had the most profound peaks and/or an important role in basic metabolism (Fig. 12). These were *glucose transporter 1, hexokinase-A*, a key regulatory enzyme of the glycolysis, *trehalase*, two predicted lactate dehydrogenases (*Impl3* and *CG13334*) and *isocitrate dehydrogenase* We also included hairy into our analysis, a transcription factor that has been shown to be a master regulator of cellular metabolism during hypoxia (Zhou et al. 2008).

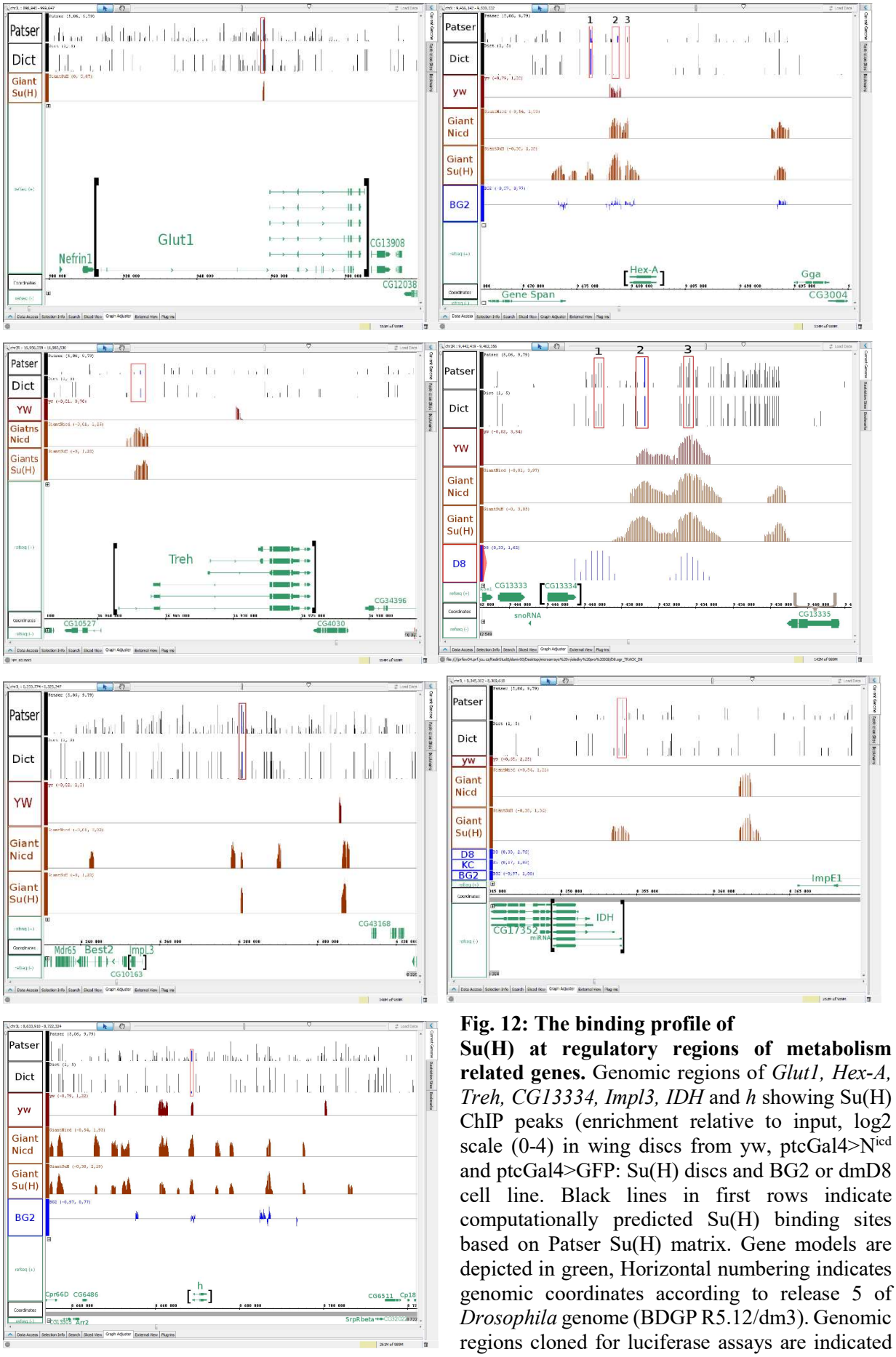


Fig. 12: The binding profile of Su(H) at regulatory regions of metabolism related genes. Genomic regions of *Glut1, Hex-A, Treh, CG13334, Impl3, IDH* and *h* showing Su(H) ChIP peaks (enrichment relative to input, log2 scale (0-4) in wing discs from yw, ptcGal4>N^{icd} and ptcGal4>GFP: Su(H) discs and BG2 or dmD8 cell line. Black lines in first rows indicate computationally predicted Su(H) binding sites based on Patser Su(H) matrix. Gene models are depicted in green, Horizontal numbering indicates genomic coordinates according to release 5 of *Drosophila* genome (BDGP R5.12/dm3). Genomic regions cloned for luciferase assays are indicated by redrectangles.

4. 1. 2. Testing Su(H) enhancers in the luciferase assay

First of all, we decided to test the genomic regions around the Su(H) peaks in a luciferase assay to see if they work as Notch dependent enhancers. For cloning into the luciferase vector we selected regions 152 - 641 bp long around the top of the peaks containing also a high predicted Patser or 'dictionary' site(s). When some genes showed more than one interesting ChIP peak we decided to test more than one region in the luciferase assay. Also, one cloned fragment often contained more than one predicted Su(H) binding site. In total eleven regions were cloned (fig. 12).

Eleven predicted enhancer regions were amplified by PCR (see supplement 1 for sequences) and cloned into the pGL3 vector in front of a luciferase reporter gene and a minimal promoter. Luciferase assays were performed in S2 cells in duplicates for each sample, in at least three independent experiments on separate days. As a positive control a vector containing the Su(H) dependent enhancer from the *m3* gene was used. As a negative control we used a plasmid containing *grainyhead* binding sites and four mutated non-functional Su(H) binding sites (called NME). Out of the eleven cloned regions six showed a signal that was higher when cotransfected with N^{icd} plasmid in comparison to basic expression without N^{icd}. These were the genomic fragments from *Hex-A, Glut1, CG13334* and *hairy* (fig. 13).

To see if the luciferase response we saw is really dependent on Su(H) binding we then decided to mutate the predicted *Su(H)* sites within all the responding enhancers. In case a fragment contained more than one predicted *Su(H)* site we chose to mutate the one that overlapped between the dictionary and Patser or that had the higher score in either of the prediction methods. In H*exokinase-A* fragment1 we mutated two sites, one common for both computationally predicted and experimentally verified and one that was only computationally predicted. The fragments from *Hex-A 1, Glut1* and *hairy* significantly lowered their response to N^{icd} suggesting that we are working with the true Notch dependent enhancers.

We also decided to mutate fragments that did not respond to N^{icd} to serve as controls. As expected, none of the *Hex-A2*, *treh* or *Idh* fragments significantly changed their responses. However, in case of *Impl3* we saw a decrease in the luciferase response suggesting that this may still be a true Notch enhancer. Data showed in the graph in Fig. 13 is the average of all measurements.

Fig. 13. Luciferase assay in S2 cells using genomic regions indicated in fig. 12. NME is negative control (Notch mutant enhancer); m3 contains Notch responsive element from *bHLHm3*. Blue columns indicate responses of the same regions where *Su(H)* sites were mutated. Two different *Su(H)* sites were mutated in Hex-A region 1. Significance according to Krustall-Wallis test comparing groups against NME.

4. 1. 3. Short pulse of Notch activation elicits transcriptional response of Glut1, Hex-A, Impl3 and hairy that is primary and independent of levels of Notch receptor

We decided to investigate whether the *Glut1*, *Hex-A*, *Treh*, *Impl3, CG13334, Idh* and *hairy* regulatory elements could regulate Notch dependent transcription of the endogenous genes in their imminent vicinity. To answer this question we turned to S2N cells in which we can activate the Notch pathway in a precisely controlled manner using a pulse of EDTA treatment (Krejčí, Bernard, Housden, et al. 2009). We activated the Notch pathway for 15 minutes and then assayed relevant gene mRNA expression in a time course up to 120 minutes after the activation (Fig. 14 A-B). Enhanced *Hex-A, Impl3* and *hairy* transcription was observed at 15-30 minutes after Notch activation after which the response was quenched. *Glut1* mRNA was also upregulated but only 45-60 minutes after Notch activation. *Treh, Idh* and *CG13334* did not respond to Notch activation and therefore were excluded form further analysis.

To confirm that responses in S2N cells were specifically dependent on Notch cleavage we incubated cells with the γ-secretase inhibitor *compound E* alongside the EDTA treatment

(Fig. 14 B). All four previously responding genes lost their transcriptional response suggesting that indeed, it is the Notch pathway that is responsible for their upregulation.

To see if Notch dependent activation of metabolic genes is direct and does not require a Notch induced secondary protein intermediate we incubated cells with cycloheximide, to inhibit *de novo* protein synthesis (Fig. 14 C). The response of all genes remained undiminished and in fact, the responses of *Glut1* and *hairy* were even more pronounced suggesting a Notch dependent transcriptional repressor might be responsible for quenching the initial peak of activation.

Another possible mechanism to explain the increased expression of several metabolic genes is that PI3K/Akt signaling is stimulated following Notch activation, as was observed after hyperactivation of Notch1 in breast cancer cells (Landor et al. 2011). To test if the transcriptional upregulation of our candidate genes required PI3K/Akt signaling we incubated S2N cells with the PI3K inhibitor LY294002 (Fig. 14 D). The responses of the *Hex-A, Impl3* and *hairy* were unaffected, implying that these genes do not require additional PI3K/Akt signal to respond to Notch activation. In contrast the *Glut1* response was lowered, although not abolished, upon PI3K/Akt inhibition suggesting that it may be regulated by combined actions of Notch and PI3K/Akt pathways. Nevertheless, taken together, our experiments with cycloheximide and PI3K/Akt inhibitors support the conclusion that *Glut1, Hex-A, Impl3* and *hairy* are direct transcriptional targets of Notch signaling.

To exclude the possibility that this regulation of metabolic genes was only possible due to the relatively strong Notch signals we generated when overexpressing the Notch receptor in our S2N cell model, we assayed the Notch responsiveness of the metabolic genes in *DmD8* muscle precursor cells. In this case only the endogenous Notch is present, resulting in much lower receptor levels (Fig. S3). As shown in Fig. 14 E *Glut1*, *Hex-A* and *hairy* were also transcriptionally upregulated in DmD8. Although *Impl3* did not respond in this cell type, another lactate dehydrogenase that could functionally substitute for *Impl3*, *CG13334*, that was previously also included into our screen but did not respond in S2N cells, was upregulated. CG13334 is proximal to two genomic regions with robust Su(H) binding in ChIP in DmD8 cells and in wing discs that were N^{icd} responsive in our luciferase assays (see Fig. 12 and 13). It is not expressed in S2N cells. These data from *DmD8* cells support the hypothesis that Notch dependent transcriptional upregulation of metabolic genes occurs with endogenous levels of Notch.

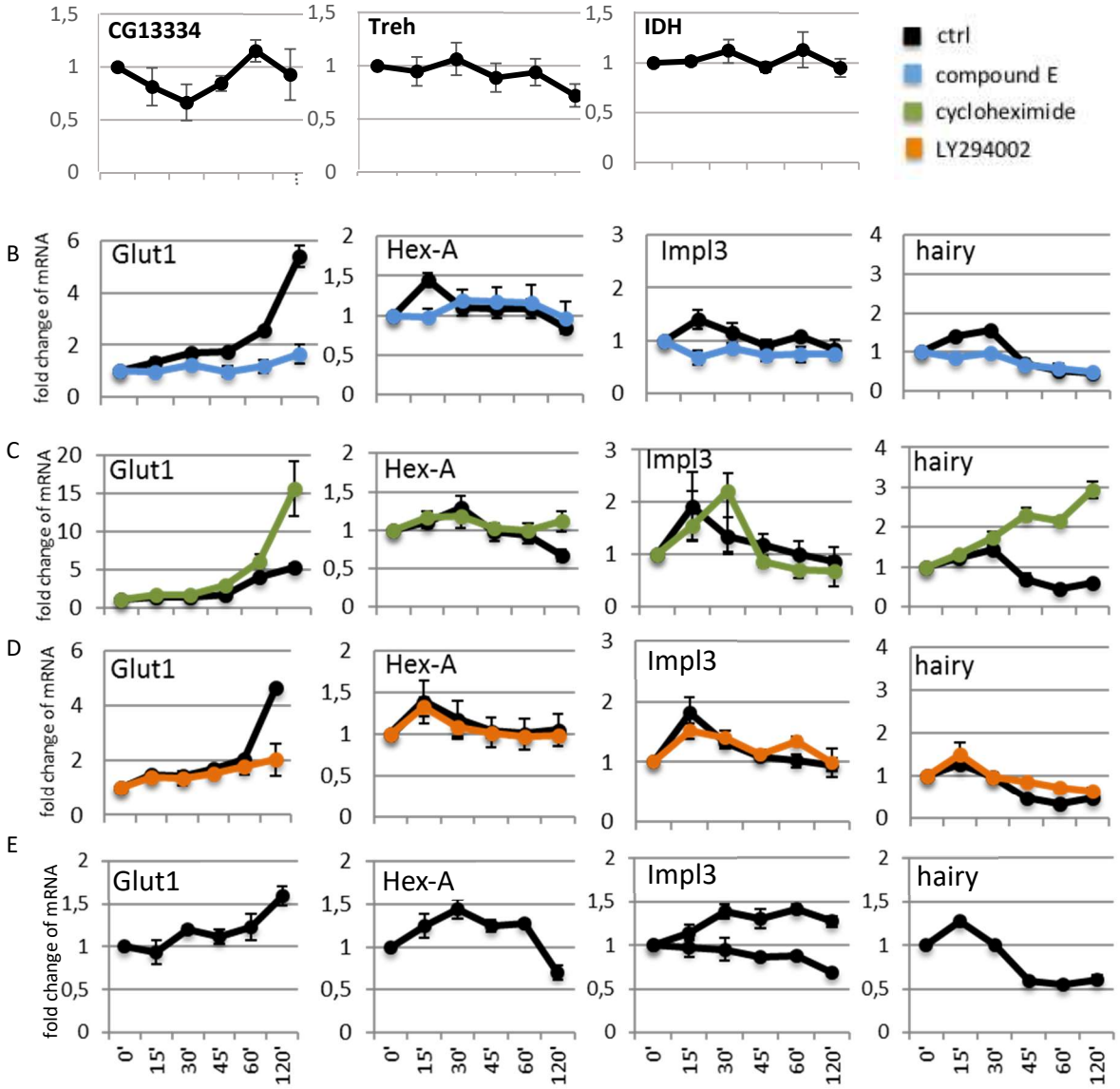


Fig. 14. Short pulse of Notch directly activates *Glut1, Hex-A, Impl3* and *hairy* mRNA expression in *S2N* and *DmD8* cells. A-D. Notch pathway was activated in *S2N* cells by EDTA for 15 minutes and mRNA extracted at indicated time points (minutes, axis labels same for **A-E**). Response of control cells is shown in black. **A** shows expression pattern of non-responsive genes. Compound E was used to block γ-secretase (blue, **B**), cycloheximide blocks protein synthesis (green, **C**) and LY294002 inhibits PI3K/Akt (orange, **D**). **E.** Notch pathway activated in *DmD8* cells by EDTA for 15 minutes and mRNA extracted at indicated time points (minutes). Data in **A-E** are normalized to CG11306 housekeeping gene. Averages of three biological replicate experiments; error bars are standard errors of the mean. Note that each control in drug treated experiments is an independent set of biological triplicates.

4. 1. 4. Notch activation leads to changes in cellular metabolism

Although we demonstrated Notch responsiveness of metabolism related genes on the transcriptional level, the question remained whether this had any impact on cellular metabolism. To address this point, we measured the concentrations of key metabolites of glycolysis and TCA cycle as well the consumption of glucose before and after Notch activation in S2N cells. Using NMR we detected lactate and fumarate in S2N cells at several time points following a short pulse of 15 minutes of Notch activation (Fig. 15 A). We were able to reproducibly detect a gradual increase in lactate production that coincided with a decrease in fumarate levels. Using [2-13C]glucose tracing and detection by NMR at 75 minute time point we observed an increase in extracellular glucose consumption, as well as higher lactate and lower fumarate levels (Fig. 15 B).

To test if Notch can mediate metabolic changes in wing discs we expressed N^{icd} in the flip-out clones that induce hyperplastic growth of the tissue (Djiane et al. 2012) and measured its metabolic parameters on the Seahorse analyzer (Fig. 15 C). We could clearly see higher levels of extracellular acidification rate (ECAR) in the N^{icd} discs indicating larger amounts of lactate secreted into the media. Also the glycolytic reserve, defined as the increase of ECAR after the block of mitochondrial ATPase with oligomycin, was minimal in N^{icd} discs suggesting that these cells already run on their maximal glycolytic capacity. On the other hand, their oxygen consumption rate (OCR), indicating the rate of respiration, was lower than in control discs. They also produced less ATP (defined as the difference between basal state and oligomycin treated conditions) and they had smaller spare respiratory capacity (defined as the increase of OCR after FCCP ionophor). All these metabolic parameters suggest a glycolytic shift in the N^{icd} discs.

Taken together, the metabolic parameters both in cell culture and in wing discs after Notch activation show a metabolic switch towards increased rates of glycolysis and a slower TCA cycle and respiration, thus consistent with the Notch dependent induction of glycolytic shift. In this case the increased activity of the pentose phosphate pathway (PPP) should also be detected. We attempted to measure the fraction of glucose that goes through PPP using [2-13C]glucose (Delgada et al. 2004) but the spectra for PPP derived [3-13C]lactate overlapped with alanine and we were unable acurately determine the PPP flux. Although the low TCA cycle suggests metabolic shift towards he Warburg effect we can not exclude that Notch activation stimulates glycolysis without the increase in PPP.

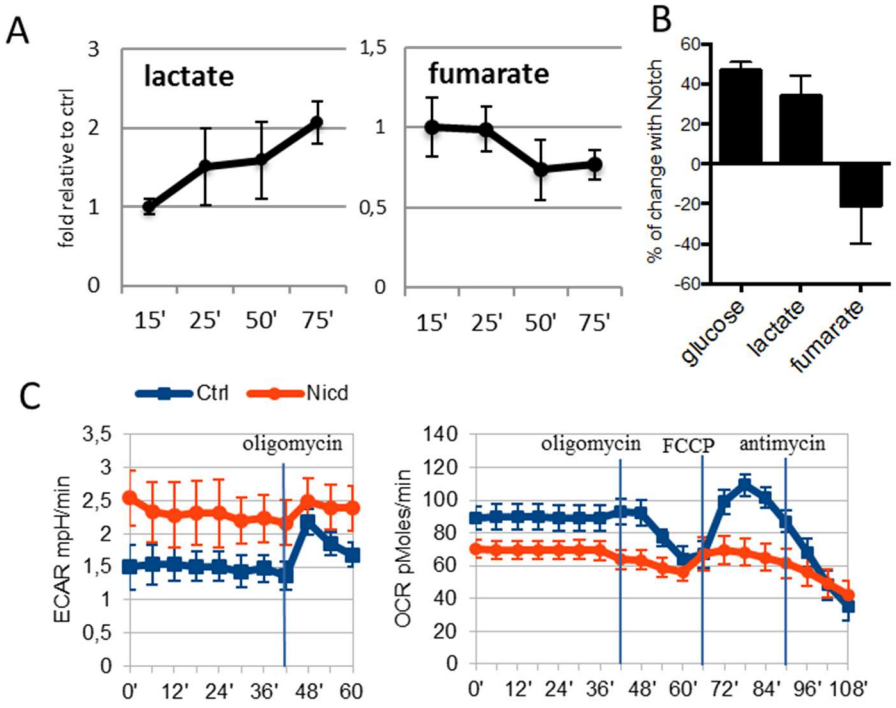


Figure 15: Notch activation mediates changes in cellular metabolism. A. Notch was activated in S2N cells for 15 minutes after which fresh Schneider media was added and lactate and fumarate levels were measured at indicated time points by NMR. Normalized to metabolite levels in S2N cells without Notch expression (without $CuSO_4$ induction) but treated with EDTA to prevent any EDTA non-specific effects. **B.** Notch was activated in S2N cells for 15 minutes after which fresh Schneider media supplemented with [2-13C] glucose was added and glucose, lactate and fumarate levels were measured at 75 min time point by NMR. Change relative to metabolite levels in S2N cells without Notch expression but treated with EDTA was plotted. **C.** Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of wing discs with N^{icd} induced hyperplasia (orange) or in controls (blue). Oligomycin blocks mitochondrial ATP synthase, FCCP is mitochondrial ionophore and antimycin blocks electron transport chain. Hyperplastic discs show higher glycolysis and lower respiration, consistent with the induction of glycolytic shift. Averages of three biological replicate experiments; error bars are standard errors of the mean.

4. 1. 5. Notch downregulates TCA via upregulation of the repressor hairy

The increased glycolysis after Notch stimulation can, at least partially, be explained by the increased expression of *Glut1* and *Hex-A*, the key genes involved in glucose metabolism. How does however Notch stimulation lead to decreased TCA cycle? The increased expression level of the *Impl3* lactate dehydrogenase gene suggest that at least some pyruvate produced by glycolysis is converted to lactate, rather than being transported to mitochondria and consumed by the TCA cycle. On top of that, there may be another way how Notch downregulates the activity of TCA cycle, through hairy.

As we presented above the transcription factor hairy is a direct Notch target in S2N cells. To verify if this regulation happens also *in vivo* we created a hairy-GFP FlyFos construct that recapitulates hairy endogenous pattern (Fig. 16 A, Fig. S4). Its expression in the wing pouch

was decreased after Notch RNAi supporting our previous finding that hairy is a Notch target *in vivo* (Fig. 16 B). On the other hand, its expression in the notum was not affected suggesting its tissues specific regulation by Notch.

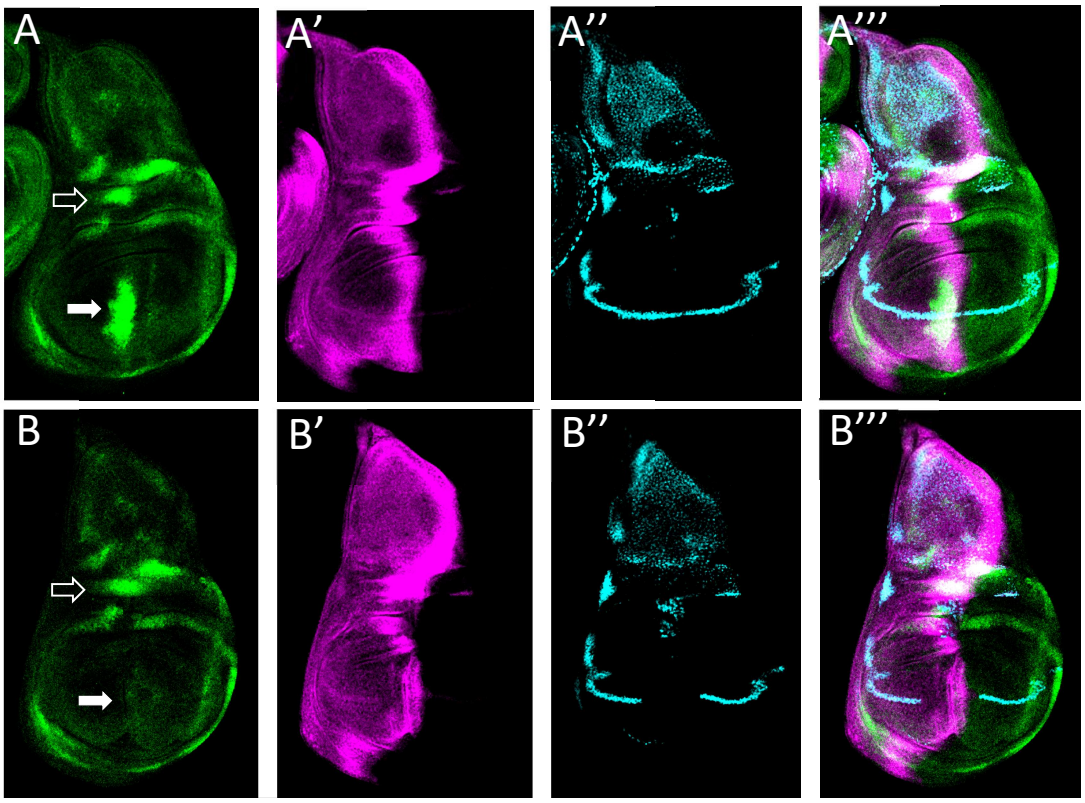


Fig. 16. Hairy is regulated by Notch. A. The expression of Hairy-GFP FlyFos construct (green) in control wing discs. Ci in magenta, cut in blue. **B.** The expression of Hairy-GFP FlyFos construct in

wing discs with Notch RNAi expressed in the patched domain. Ci in magenta, cut in blue. The *Ptc* domain is slightly wider than the intense band in Ci as seen by missing expression of cut gene. White arrow indicates the missing hairy expression in the pouch after Notch RNAi. Immunostainings were performed by R. Perez-Gomez.

Hairy has been shown to be upregulated during hypoxic conditions and act as a metabolic switch by binding to the regulatory regions of several TCA cycle genes and downregulating their expression (Zhou et al. 2008). To test if it is able to act similarly even at normoxic conditions we looked for the expression of TCA cycle genes in hairy mutant wing discs of flies kept at normoxic conditions. We saw upregulation of *Sdhb*, *l(1)G0255* and *Kdn* genes which all have predicted hairy binding motifs close to their promoters according to (Zhou et al. 2008) (Fig. 17 A). We also obtained similar results when we downregulated hairy in S2N cells (Fig. 17 C). Accordingly, the expression of TCA cycle genes is diminished following Notch activation in S2N cells (Fig. 17 E). These results suggest that hairy represses, to some extent, TCA cycle genes under normoxic conditions and that upregulation of *hairy* by Notch supports reduced mitochondrial oxidative metabolism.

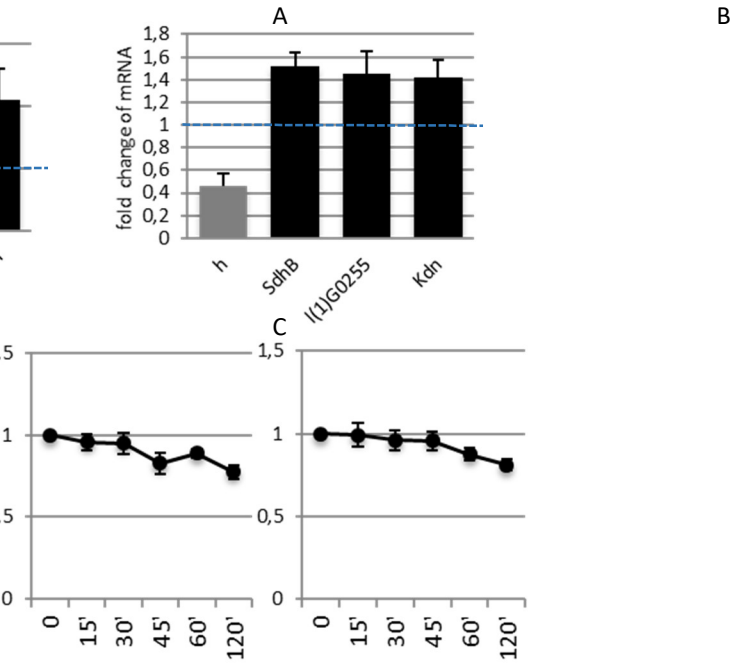
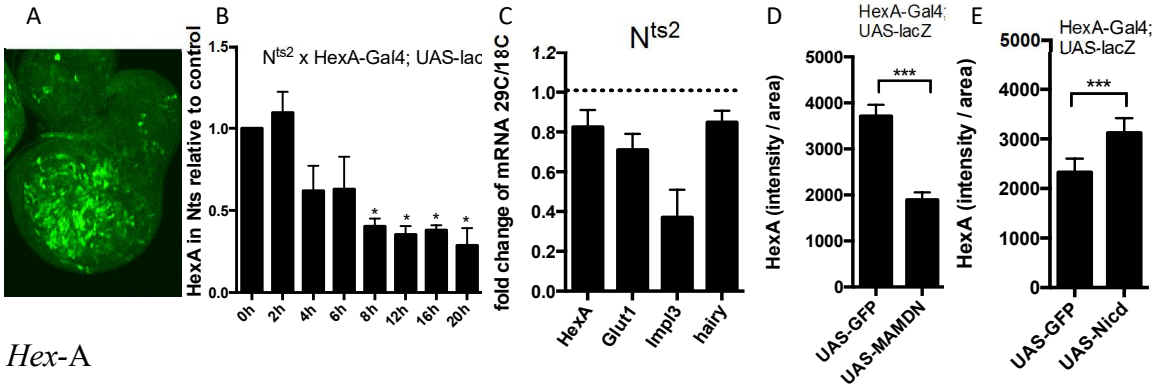


Fig. 17. Hairy represses TCA cycle genes in response to Notch signal. A. The levels of expression of TCA cycle genes with predicted hairy binding sites and amount of hairy mRNA in *hairy* hypomorphic mutant (h1) wing discs in normoxic conditions, relative to the expression in wild type control OregonR wild type flies ('OR'). **B.** The fold of difference in mRNA levels of TCA cycle genes with predicted hairy binding sites and efficiency of hairy RNAi downregultion after RNAi treatment against *hairy* in S2N cells, relative to the expression of the TCA cycle genes in control cells treated with dsRNA against GFP **C.** Transcriptional response of TCA cycle genes after 15 minutes of Notch activation (minutes, as in Fig. 14). **A - C** represent averages of three biological replicates; error bars shows standard error of the mean.

4. 1. 6. Functional significance of Notch dependent metabolic changes

Notch signaling controls cell growth and proliferation in several tissues during normal development including imaginal discs (Estella & Baonza 2015) or T-cells (Joshi et al. 2009) and overactivation of Notch pathway leads to pathophysiological conditions such as wing disc hyperplasia (Djiane et al. 2012) or tumor development (Ling et al. 2010), (Palomero et al. 2006). Does Notch dependent upregulation of metabolic genes help to stimulate tissue growth?

In order to support this hypothesis we first tested its obvious presumption that the metabolic genes identified in this study are regulated by Notch. The lack of suitable reporters for *Impl3* or *Glut1* did not allow us to investigate the Notch dependent regulation of these genes but we could test the regulation of *Hex-A* using a Gal4 enhancer trap line. We used a thermosensitive allele of Notch to reduce Notch signaling and looked for the expression of the



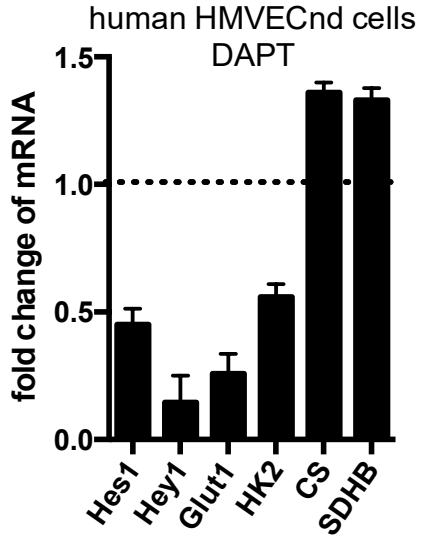
Hex-A reporter in the wing discs after moving the flies from 18°C (with active Notch signaling) to 30°C (with Notch receptor becoming inactive). We saw a gradual decrease of the activity of *Hex-A* reporter in comparison to control supporting the idea that *Hex-A* is regulated by Notch in the wing disc (Fig. 18 B). In agreement with this observation Hex-A expression was downregulated when co-expressed with the dominant negative form of Mastermind (Fig. 18 D) and it was stimulated as a response to N^{icd} activation (Fig. 18 E). As we showed in Fig. 16 *hairy* is also a Notch target in wing disc.

Fig. 18: Hex-A is a target of Notch *in vivo*. A. The expression pattern of HexA–Gal4 reporter in the wing discs (crossed to UAS-lacZ). The strength of the signal from HexA–Gal4 reporter was quantified inIMAGEJby calculating the integrated density of HexA immunostaining signal from the whole disc (sum of pixel values), after background subtraction, using Z-stacks of confocal pictures spanning the whole disc thickness. The integrated density was divided by area of the disc and plotted as 'HexA intensity per area'. **B.** The intensity of HexA–Gal4 reporter in wing discs after deactivation of Notch signaling in flies with thermosensitive allele Nts2, relative to the expression of HexA–Gal4 reporter in wild-type control Oregon R flies. The y-axis represents intensity of HexA reporter per area of the disc, x-axis indicates hours after shifting flies from 18°C to the non-permissive temperature of 30°C. Significance is according to one-tailed Student's t-test, compared with values at time 0. **C.** The change

of mRNA expression of metabolic genes in wing discs with thermosensitive allele Nts2. The ratio of mRNA levels at 30°C against 18°CinNts2 was compared relative to mRNA levels in control wild-type Oregon R flies at the same temperatures. **D.** The intensity of HexA–Gal4 reporter in wing discs after blocking Notch activation via the expression of dominant negative Mastermind. Control flies express UAS-GFP instead of Mastermind. The y-axis represents intensity of HexA reporter per area of the disc. **E.** The intensity of HexA–Gal4 reporter in wing discs after Notch activation via the expression of Notch intracellular domain (N^{icdMH3}). Control flies express UAS-GFP instead of N^{icd}. The y-axis represents intensity of HexA reporter per area of the disc. Significance is according to one-tailed test.

We also extended our investigations to human microvascular endothelial cells (HMVECnd) that are known to have an active Notch pathway and that exhibit a metabolic profile similar to the Wargurg effect (De Bock et al. 2013). In this case, we used the γ-secretase inhibitor DAPT to inhibit Notch (Cheng et al. 2003) and examined the expression of human *hexokinase 2* and *glucose transporter 1*, as well as of two classic Notch-regulated genes, *Hes1* and *Hey1*, that show sequence similarity to hairy. The mRNAs for *Hes1*, *Hey1*, *hexokinse 2* and *glucose transporter 1*, were significantly decreased and mRNA for TCA cycle genes *citrate synthase* (CS, human orthologue of Drosophila Kdn) and *succinate dehydrogenase* (Sdhb) increased under these conditions (figure 19). Such changes in expression could result in lower glycolysis and increased TCA cycle, hence reversal of the Warburg effect phenotype. These observations fit with the hypothesis that Notch is needed for the maintenance of the Warburg effect in these cells, in full agreement with our Drosophila-based model.

Fig. 19: Metabolic Notch targets in primary human cells. The fold changes of mRNA in human microvascular endothelial cells (HMVECnd) after blocking γ-secretase with 10 mM DAPT for 6 h, in comparison with cells treated with DMSO. Eperiment was performed by Alena Krejčí



Next we investigated if the regulation of metabolic genes by Notch plays a role in Notch dependent cell growth and proliferation during normal development of the wing disc. Reduced Notch signaling in the *engrailed* domain of the wing disc causes smaller posterior compartment (Housden et al. 2014). Therefore we asked if downregulation of metabolic genes could be one of the mechanisms contributing to this phenotype. If it is true then downregulation of metabolic genes should also lead to smaller growth of the posterior compartment. When we knocked down single metabolic genes by RNAi in the *en* domain we did not see much of a reduction in the size except a slight reduction with RNAi against *Hex-A*. However, knocking down both *Hex-A* and *Impl3* or *Impl3* and *Glut1* caused significant reduction of the posterior compartment (Fig. 20 B). Similar effect was observed also in adult wings. Here, instead of downregulating metabolic genes by RNAi, we slowed metabolism by keeping flies on low nutrient food. Wings of N^{55e11} heterozygous mutants were smaller in size on both high and low nutrient food. However, *Hairless* mutants kept the size of their wings unchanged on the low nutrient food (Fig. 20 C) and it selectively affected only the wings without having an effect on the growth of the rest of the body (Fig. 21 B). This observation is

consistent with a hypothesis that upregulation of Notch targets in Hairless mutant wing discs includes metabolic genes and this level of upregulation is sufficient to maintain proper disc development under cond[illegible] *Hex-A* reporter was [illegible] and *hairy* were upr[illegible] ctivity is able to buf[illegible]

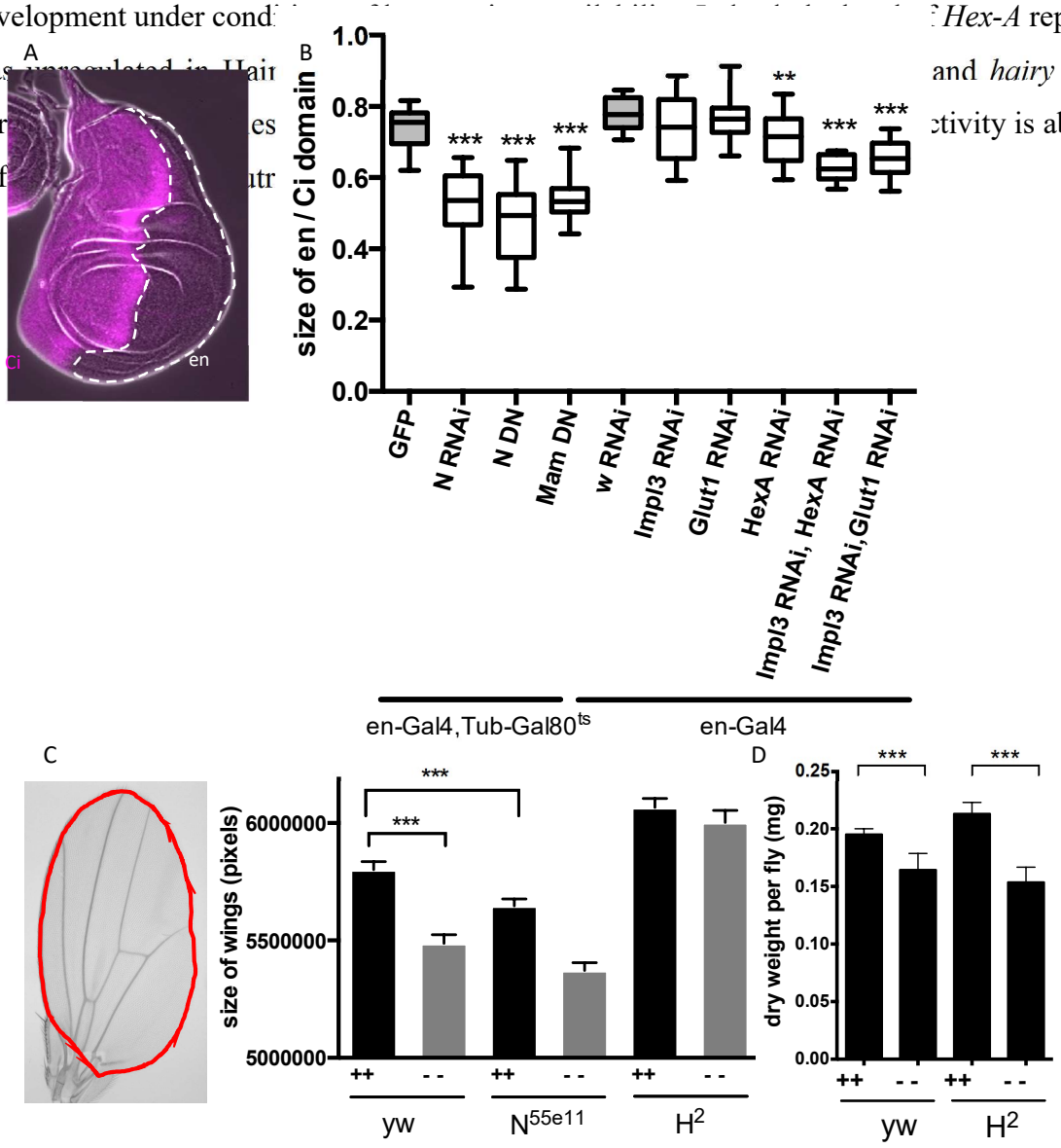


Fig. 20: A. Notch signaling may regulate growth of the wings by regulating expression of metabolic genes. A. Immunostaining of wing disc showing the anterior part (stained with Ci, magenta) and posterior part (Ci negative, engrailed). **B**. The effect of inhibiting Notch pathway or metabolic genes on the size of en domain in wing disc. The thermosensitive form of Gal80 repressor was used with the en-Gal4 to drive expression of N^{RNAi},N^{DN}, Mam^{DN} and GFP constructs at 29°C for 96 h before dissections of L3 larval wing discs. Two copies of en- Gal4 driver were used to drive two copies of UAS-RNAi of metabolic genes or of RNAi against white gene. The ratio between en/Ci domains was plotted. Significance relative to values in control flies (grey). **C.** The size of adult wings when larvae of indicated genotype were raised on nutrient rich (++) or nutrient poor (--) food. The N55e11 and H2 mutants were crossed to yw before scoring the heterozygous progeny. **D.** Dry weight of yw and H2 flies raised on nutrient high (++) and nutrient low (- -) diets. Bodies of 20 males with dissected wings were placed per tube, dried on a lyophilizer, and average weight per fly was calculated from three replicates.Results for Fig. 20C were obteinend by Pavel Steffal and Alena Krejčí. Data from 15 to 30

wing discs or 40 wings; error bars show standard error of the mean or min and max values Significance is according to two-tailed test.

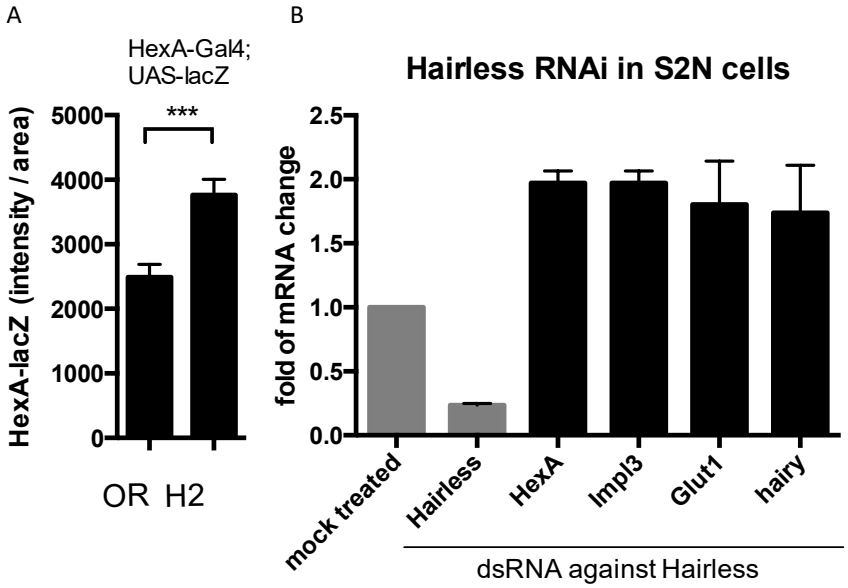


Fig. 21 The Hairless mutants upregulate *Hex-A*. A. The intensity of HexA–Gal4 reporter in wild-type control Oregon R and in Hairless (H2) mutant flies. The y-axis represents relative intensity per area. **B.** S2N cells were treated with dsRNA against Hairless and the expression of mRNA for metabolic genes were quantified relative to their expression in control cells treated with dsRNA against GFP (mock treated, first column). Normalized to the housekeeping gene CG11306. Error bars show standard error of the mean or min and max values. Significance is according to Student's t-test.

Finally, we asked whether we could see upregulation of metabolic genes during Notch-induced hyperplasia and whether we could suppress this phenotype by downregulating metabolism. We used the Su(H)-VP16 construct (thus making Su(H) a constitutive activator) to overactivate the Notch pathway in the patched domain of the wing disc epithelium that is known to induce cell overproliferation, especially in the ventral top region of the disc (Djiane et al. 2012). We analysed the transcriptional response of metabolic genes in this tissue using *in situ* hybridizations. Although this technique lacked the sensitivity to unambiguously describe the endogenous expression patterns of our candidate genes across the wing disc, the upregulation of *Glut1*, *Hex-A*, *Impl3* and *hairy* in the patched domain was clearly evident and especially profound in the most proliferating ventral top region of the disc (Fig. 22 A-D). Importantly, we were able to partially rescue Notch-dependent overproliferation by downregulation of Hex-A or Impl3 expression (Fig. 22 E) or by keeping larvae on low-nutrient food (Fig. 22 F).

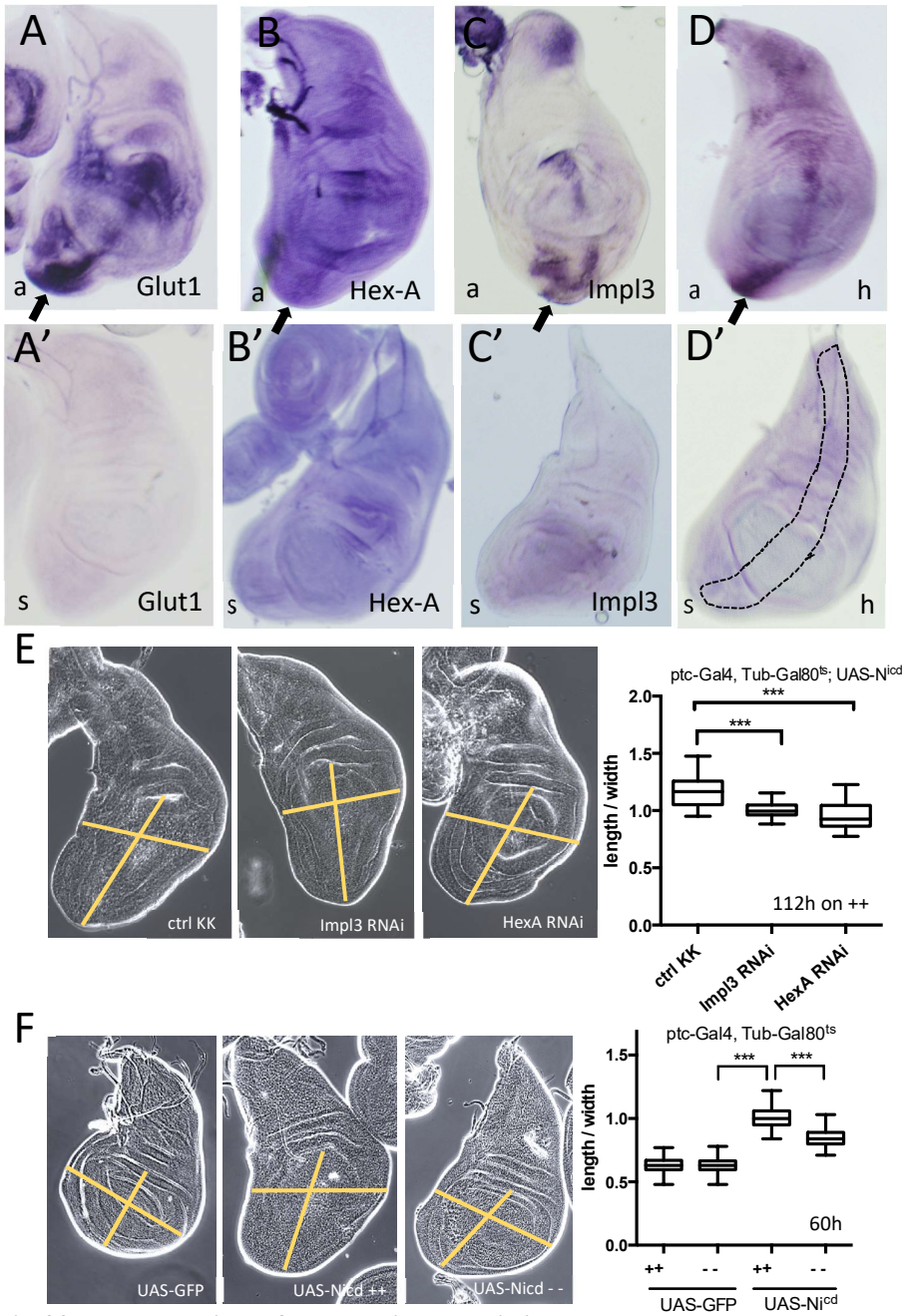


Fig 22. Upregulation of metabolic genes is important during Notch-induced hyperplasia. A-D *In situ* hybridization of Notch-mediated hyperplastic wing disc (using ptc driven Su(H)-VP16) with specific antisense probes ('a', in A-D) or negative control sense probes ('s' in a0 –d0).The ptc domain is schematically indicated in figure d0. **E.** Larvae with N^{icd}-induced hyperplasia in the wing disc (Ptc-Gal4, Tub-Gal80ts; UAS-N^{icdMH3}) crossed to control KK line, Hex-A RNAi or to Impl3 RNAi, kept at 29°C for 112 h before dissection on high nutrition food (++). The size of wing pouch was quantified by measuring the ratio between the length and width of the pouch. **F.** Larvae with N^{icd}-induced hyperplasia in the wing discs (Ptc-Gal4, Tub-Gal80ts crossed to UAS-N^{icdMH3} or control UAS-GFP) were raised on nutrient rich (++) or nutrient poor (- -) food and the size of wing pouch was quantified by measuring the ratio between the length and width of the pouch. (E,F) Data from 23 to 91 wing discs; error bars show min and max values. Significance is according to two-tailed Student's t-test.

Based on our results, we suggest a model where Notch-dependent upregulation of metabolic genes contributes, among other mechanisms, to the stimulation of cell growth in the wing disc during normal development as well as during Notch-induced hyperplasia (Fig 23): Activation of the Notch signaling pathway triggers increased expression of several glycolytic genes such as *Glut1*, *Hex-A*, lactate dehydrogenases *Impl3* or *CG13334* and also transcription factor *hairy*. Overexpression of *Glut1* and *Hex-A* enhances glycolytic rate. Mitochondrial metabolism is then reduced by both increased expression of lactate dehydrogenase which prevents pyruvate from entering mitochondria and hence TCA cycle and overexpression of transcription factor hairy which directly binds to the promotors of several TCA cycle genes and represses their expression.

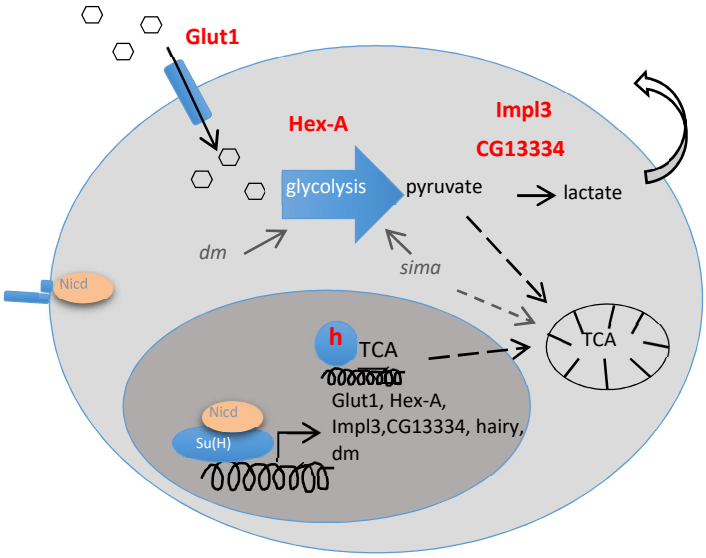


Fig. 23 Model of Notch response leading to changes in cellular metabolic profile. Schematic model of the Notch response leading to increased glycolysis and decreased TCA cycle via the direct transcriptional regulation of metabolic genes. Collaboration with myc (dm), that is also a Notch target, or with Hif-1 (sima) in the case of hypoxic conditions may help the metabolic transition.

4. 2. Results related to Horvath M, Mihajlovic Z, Slaninova V, Perez-Gomez R, Moshkin Y, Krejci A. 2016 The silent information regulator 1 (Sirt1) is a positive regulator of the Notch pathway in *Drosophila*. Biochemical journal, 473, 4129-4143 (2016).

Data are available online in the above mentioned article and journal or in printed version of this thesis

4. 3. Unpublished results

These data are available in printed version of this thesis only.

5. Discussion

During my PhD project I investigated the interplay between Notch signaling and metabolism from two different perspectives. The majority of my effort aimed to reveal the metabolic genes that are direct targets of Notch signaling and that help Notch to mediate metabolic reprogramming in specific tissues. However, I also undertook several experiments to show that Notch signaling output is sensitive to the state of cellular metabolism, particularly the NAD^+: NADH ratio. The *Silent information regulator 1* (Sirt1) seems to be one of the metabolic sensors involved in Notch signaling. Thus there is a reciprocal regulation between the Notch signaling and metabolism, creating a regulatory loop.

5. 1. The model of Notch driven metabolic shift towards the Warburg effect and its general implications

We discovered that Notch activation directly regulates the expression of several metabolic genes, helping to shift the cellular metabolic status towards increased rates of glycolysis and lower TCA cycle, in a manner reminiscent of the Warburg effect. This metabolic transition may be relevant in various context where Notch activation stimulates growth, proliferation or cell survival:

Glycolytic shift was previously observed in MCF7 breast cancer cells with hyperactivaton of Notch signaling where the induction of metabolic genes relies on the PI3K/AKT pathway (Landor et al. 2011). Here the authors showed that Notch signaling drives expression of several metabolic genes, such as *glucose transporter* or *hexokinase* through the activation of PI3K/AKT pathway. Our results point to a more general model where regulation of metabolic genes by Notch is not restricted to cells with overactivated Notch receptor but it happens also in cells with endogenous levels of Notch signaling and it is important outside the cancer model. Therefore, regulation of metabolism by Notch may play an important physiological role in various situations during development or adult homeostasis. Indeed, recent evidence shows that Notch mediated survival of memory CD4+ T cells is dependent on the regulation of glucose uptake through the expression of Glut1 (Maekawa et al. 2015). Glycolytic phenotype is essential for the quiescence and self-renewal of adult stem cells (Takubo et al. 2013) as well as for the rapidly proliferating embryonic stem cells (Zhou et al. 2012; Ochocki & Simon 2013) and Notch has been shown to be critical in both of these processes (Koch et al. 2013) although the direct regulation of metabolism by Notch has not

yet been investigated in this context. Cells during immune response also switch to glycolytic metabolism (Bajgar et al. 2015) and Notch has been shown to govern differentiation of distinct hematopoietic cell types (Radtke et al. 2013) but again, whether Notch can directly promotes metabolic shift in these cells has not been investigated.

5. 1. 1. Responsivness of Su(H) enhancers of metabolic genes in vivo

By „data mining" approach in the previously published ChIP-chip data sets we selected seven metabolism related genes containing Su(H) binding site in their vicinity. Although majority of them responded in a luciferase assay we do not have a bullet-proof evidence that exactly these peaks are responsible for Notch dependent regulation *in vivo*.

Sometimes we could not say for sure which genes these enhancers belong to. For example, the gene *CG13334* and the former gene *CG13335* (*CG42807* and *CG42808* according to the latest release of the *Drosophila* genome) lie in the close proximity to each other with the Su(H) peaks positioned in between of them. Therefore, we cannot say whether they regulate *CG13334* or *CG42807* or *CG42808* gene. Moreover, *CG13335* was identified as a Notch target gene in DmD8 cells (Krejčí et al. 2009) because the mRNA for *CG13335* was upregulated following Notch activation (*CG13334* was not examined on the arrays). However, it is possible that *CG13334* and former *CG13335* genes have a common enhancer. In any case, there is no doubt that the metabolic genes in our study are responsive to Notch signaling, as we showed by several experiments both in cells lines and, importantly, in vivo. However, we were unable to perform experiments that would prove the specific enhancer regions responsibility in vivo. Our plan was to use the FlyFos system (Ejsmont et al. 2009) to create a Hex-A, Glut1 and hairy fly lines where the large genomic fragment would contain a GFP fusion protein of interested, show their responsivness to Notch in vivo and then mutate the specific Su(H) binding sites to lose the Notch dependent regulation. We succeeded in the insertion of GFP into the big bacmid constructs but we failed after several attempts to create the transgenic flies where the construct would be inserted into the genome (both in house and two commercial sources).

Taken together, we showed that several metabolic genes are responsive to Notch pathway *in vivo* (in the wing disc) and the enhancers identified in our study are certainly Notch responsive in a luciferase assay. However, we did not test the function of the specific enhancers responsible for the Notch dependent regulation of the metabolic genes *in vivo*.

5. 1. 2. Notch activation elicits relatively small fold upregulation of metabolic genes

We showed that metabolic genes are responsive to Notch pathway both in Drosophila cell lines and in vivo in the wing discs, as well as in human microvascular endothelial cells (HMVEC). It is remarkable that the relatively low and transient changes in the mRNA expression of the Notch regulated metabolic genes identified in our study are associated with such profound and long lasting changes in the cellular metabolic profile. However, changes of similarly small magnitude have been reported to promote the Warburg effect in cancer cells. For example, in trastuzumab-resistant human breast carcinoma cells, a 2.5-fold induction of Glut1 and 0.5-fold induction of Ldha (lactate dehydrogenase) correlated to increased glucose uptake and increased production of lactate (Lee et al. 2015). Likewise, a 2.5-fold upregulation of Glut1 and 0.5-fold increase in hexokinase 2 caused by downregulation of Hsulf-1 in ovarian cancer cells lead to glycolytic phenotype (Mondal et al. 2015). In human bladder cancer cells, the expression of a long non-coding RNA UCA1 promoted glycolysis via a 0.5-fold increase in hexokinase 2 expression (Li et al. 2014). Our data are therefore fully consistent with these studies in human cells, which lend further support for the functional significance of our results.

Interestingly, after a peak of upregulation, some genes were downregulated following a short pulse (15 minutes) of Notch activation (*hairy* and *Hex-A).* This would suggest that there is a secondary repression mechanism employed, through a protein mediator that is part of the Notch response. Indeed, when we treated S2N cells with protein synthesis inhibitor cycloheximide, not only that this downregulation was abolished but at least in case of hairy the transcription was even more enhanced. It is entirely possible, and our unpublished data points to it, that *E(Spl)* genes are responsible for this later phase of repression. Also late induction of *Glut1* that was upregulated only 45 – 60 minutes after Notch activation was rather unexpected because most of the primary Notch target genes came within 30 minutes. Nevertheless, the treatment of the cells with γ-secretase inhibitor Compound E effectively abrogated the response of *Glut1* to Notch activation and also the treatment with protein synthesis inhibitor cycloheximide lead to even more enhanced induction of *Glut1* transcription. Moreover, there are other examples of Notch primary targets that respond late such as EGFP in DmD8 cells (Krejčí, Bernard, Housden, et al. 2009). Nevertheless, according to our data from experiment with PI3K inhibitor LY294002 it seems that at least in case of *Glut1* a cooperation with AKT/PI3K signaling might be necessary for full transcription induction.

We can speculate that the transcriptional changes after a relatively short pulse of Notch

activation (15 min) in S2N and DmD8 cells lead to increased protein levels that are then stable over a longer time period and thus cause the metabolic changes we observe. How long this metabolic shift lasts and whether sustained or repeated Notch activation would be necessary to maintain it remains an open question. However, our data show that even 75 min after the initial Notch pulse, lactate production is still increasing despite the fact that the mRNA for Hex-A and Impl3 have long returned to their original levels (figure 15 A-B).

5. 1. 3. Notch activation leads to changes in cellular metabolism

Our results showed that Notch pathway regulates expression of several genes involved in metabolism. Nevertheless, the big question remained whether there is a functional connection between upregulation of these metabolic genes and changes in cellular metabolism. Using NMR analysis and also the Seahorse XFe flux analyzer we showed that overexpression of Notch signaling pathway leads to measurable changes in overall cellular metabolism both in cell lines and in imaginal wing discs with the overexpression of Notch signaling pathway. These changes seem to be similar to the Warburg effect where glycolysis is enhanced at the expense of mitochondrial metabolism. Nevertheless, the Warburg effect is usually also associated with increased activity of the pentose phosphate pathway (PPP). We attempted to measure the fraction of glucose that goes through PPP using [2-13C] glucose (Delgada et al. 2004), but the spectra for PPP derived [3-13C] lactate overlapped with alanine and we were unable to accurately determine the PPP flux. Although the low activity of the TCA cycle also suggests a metabolic shift towards the Warburg effect, we cannot exclude that Notch activation stimulates glycolysis without an increase in PPP. At the same time, we can imagine scenarios where in certain contexts Notch can regulate glycolysis and TCA cycle independently of each other.

The increased glycolytic rate we observed after Notch activation both in cells and wing discs can be easily explained by upregulation of several glycolytic genes we identified. To explain downregulation of TCA cycle was more challenging though. The TCA cycle was probably downregulated at least partially by the increased expression of lactate dehydrogenase that converts pyruvate to lactate and therefore restricts the flow of pyruvate into the mitochondria. Nevertheless, we also saw downregulation of several TCA cycle genes upon Notch activation (Figure 17 E). We speculated that hairy might be the candidate transcription factor mediating this repression as it is known to be the main switch between glycolytic and aerobic metabolism during hypoxia (Zhou et al. 2008). Indeed, we showed that the transcription factor hairy is a Noch target *in vivo* and it is capable to repress the expression of

several TCA cycle genes, resulting in the downregulation of the TCA cycle and therefore the whole mitochondrial metabolism in response to Notch activation.

Nevertheless, the hairy FlyFos-GFP reporter we used to determine hairy regulation by Notch in normal wing disc development does not fully reproduce the endogenous hairy expression pattern in the wing pouch using the hairy antibody. Thus, the FlyFos reporter may not truly reflect endogenous regulation of hairy that is known to be unstable on the level of protein as well as mRNA (Hooper et al. 1989). While hairy is our prime candidate to explain how Notch downregulates TCA, there are other possible mechanisms which can be employed. For example, Basak and col. showed that in K562 cells canonical Notch signaling activation leads to decreased expression of *NADH ubiquinone oxidoreductase 75 kDa subunit 1 (NDUFS1*, one of the ETC Complex I subunit) and also *ornithine transferase* (*OAT*) and *glutaminase* (*GLS*) which are involved in glutamine metabolism. While the mechanism underlying Notch dependent downregulation of *NDUFS1* was not uncovered, they proposed that downregulation of *OAT* and *GLS* might be mediated by upregulation of Notch target genes and known repressors *Hes1* and *Hey1*(Basak et al. 2014). Therefore, Notch dependent alteration in oxidative phosphorylation and glutamine metabolism could be mediated by E(spl) genes also in the Drosophila wing disc tissue. Moreover, *c-myc* is another direct target of Notch pathway known to upregulate glutamine metabolism either by directly upregulating transcription of glutamine transporter (Wise et al. 2008) or by post-transcriptional alterations of GLS mediated by c-myc dependent suppression of miR-23 (Gao, Ping 2009).

5. 1. 4. Functional significance of Notch dependent metabolic changes

Our data from cell lines and *in vivo* convincingly supported the idea that activation of Notch signaling pathway regulates the expression of several metabolic genes and subsequently induces changes in overall cellular metabolism. Nevertheless, the significance of these Notch mediated metabolic changes in living organism remained to be investigated. Notch signaling pathway is the main regulator of cell proliferation and growth of the imaginal wing discs in *Drosophila* (Baonza & Garcia-Bellido 2000) and downregulation of Notch signaling in the *engrailed* domain leads to the reduction of size of the posterior part of the wing disc (Housden et al. 2014). In our *in vivo* analysis we observed that knocking down the selected metabolic genes has similar phenotypic consequences on growth as downregulation of Notch signaling. The same effect can be observed in the wings of adult *Drosophila*. Wings of the N^{55e11} flies are smaller compared to the wild type and the same is true for the wings of flies kept on "low" nutrient food and therefore with suppressed cellular metabolism. Moreover, when we induced

Notch dependent overgrowth of the *Drosophila* imaginal wing discs we were able to rescue it by both downregulation of glycolytic genes and dietary restriction which would suggest that a high metabolic rate is vital part of Notch induced hyperplasia. Altogether, these observations are consistent with the model where Notch is able to at least partially stimulate cell growth during *Drosophila* wing disc development by the upregulation of metabolic genes.

Although our *in vivo* data do not unambiguously distinguish between the direct or indirect regulation of metabolic genes by Notch, our cell line based data rather point towards direct effects. Notch has been shown to regulate the growth of wing discs by several other mechanisms, mainly through the regulation of *Wg* signaling, the transcription factors *vg* and *myc* and cell cycle regulators (Djiane et al. 2012; Kim et al. 1996; Wu & Johnston 2010). Although we did not directly measure the metabolic parameters in wild type wing discs, we identified the Notch directed transcriptional regulation of metabolic genes in this tissue that implies the establishing or maintenance of glycolytic shift via Notch signaling. Moreover, it is possible that metabolic regulations are part of the Notch induced proliferation in other contexts.

The regulation of metabolic genes to stimulate tissue growth is certainly not a privilege of Notch signaling pathway. The growth of wing discs is primarily driven by Notch, but in other contexts metabolic genes might be induced by other signaling pathways via different enhancers in their regulatory regions or indirectly through the induction of transcription factors such as myc (De La Cova et al. 2004; Gallant 2013; Wu & Johnston 2010). Also, activation of insulin pathway via insulin-like peptides is one of the main regulators of the cell and organ growth in *Drosophila* (Brogiolo et al. 2001). Moreover, specific chromatin states could determine whether metabolic genes will be responsive to Notch or to a different signal or transcription factor, in a tissue and context specific manner.

The metabolic genes mentioned in our study are not the only ones that Notch may use to mediate changes in cellular metabolism. The transcription factor myc is a direct Notch target but also a master regulator of the cell cycle and growth, stimulating the transcription of ribosomal genes as well as enzymes of glycolysis, glutaminolysis and lipid synthesis (Palomero et al. 2006; Krejčí, Bernard, Housden, et al. 2009). Hif-1 is the key regulator of metabolism under hypoxic conditions, such as in cancer, and its targets also involve genes of glycolysis and glutaminolysis. Mouse NICD1 can physically interact with HIF-1 and potentiate its recruitment to its target promoters (Zheng et al. 2008). Also interaction of Notch signaling and metabolic master regulator, the Insulin pathway, was described. It was shown that Notch target Hes1 directly blocks expression of *PTEN* which leads to activation of PI3K/AKT signaling which is necessary for pre-T cell receptor-induced (Palomero et al. 2007;

Wong et al. 2012). It is therefore possible that the Notch pathway uses several mechanisms to promote glycolytic shift, one of which is the direct transcriptional upregulation of metabolic genes that we describe.

It is apparent form our results that low nutrient availability cannot rescue the mild wing overgrowth of H2 mutants but it is sufficient to at least partially rescue ptc > Nicd-driven wing disc hyperplasia. These results appear contradictory; however, they represent very different conditions. In the former, the reduction in *Hairless* results in very mild increase in Notch activity that is likely to be within a range where homoeostatic buffering can occur. For example, a negative feedback loop limits the extent of Notch pathway activation and restricts expression of metabolic enzymes to a certain threshold, leading to mild disc overgrowth. In the ptc > Nicd discs, there is sustained high level of Notch activity that is no longer subject to the normal feedback regulation (as it is controlled by exogenous Gal4 system). Many genes are upregulated under these conditions (Djiane et al. 2012), which are likely to circumvent the normal feedback regulation allowing maximal metabolic rates and massive over proliferation.

5. 2. Notch signaling pathway is influenced by changes in cellular metabolism and the NAD^+: NADH ratio

These data are available in printed version of this thesis only.

6. Bibliography

Abràmoff, M.D., Magalhães, P.J. & Ram, S.J., 2004. Image processing with imageJ. *Biophotonics International*, 11(7), pp.36–41.

Agani, F. & Jiang, B.-H., 2013. Oxygen-independent regulation of HIF-1: novel involvement of PI3K/AKT/mTOR pathway in cancer. *Current cancer drug targets*, 13(3), pp.245–251.

Ahn, S. & Susztak, K., 2010. Getting a Notch Closer to Understanding Diabetic Kidney Disease. *Diabetes*, 59, pp.1865–1867.

Aihara, H., Perrone, L. & Nibu, Y., Transcriptional Repression by the CtBP Corepressor in Drosophila. *Madame Curie Bioscience Database, Landes Bioscience*, p.Available from: http://www.ncbi.nlm.nih.gov/books/.

Allenspach, E.J. et al., 2002. Notch signaling in cancer. *Cancer biology & therapy*, 1(5), pp.466–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12496471.

Altomare, D. a & Testa, J.R., 2005. Perturbations of the AKT signaling pathway in human cancer. *Oncogene*, 24(50), pp.7455–7464.

Andersen, P. et al., 2012. Non-Canonical Notch Signaling: Emerging Role and Mechanism. *Trends in Cell Biology*, 22(5), pp.257–265.

Armstrong, A.R., Laws, K.M. & Drummond-Barbosa, D., 2014. Adipocyte amino acid sensing controls adult germline stem cell number via the amino acid response pathway and independently of Target of Rapamycin signaling in Drosophila. *Development (Cambridge, England)*, 141, pp.4479–4488. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25359724.

Arnett, K.L. et al., 2010. Structural and Mechanistic Insights into Cooperative Assembly of Dimeric Notch Transcription Complexes. *Nat Struct Mol Biol*, 17(11), pp.1312–1317.

Auer, J.S. et al., 2015. MAPK-dependent phosphorylation modulates the activity of Suppressor of Hairless in Drosophila. *Cellular Signalling*, 27(1), pp.115–124. Available at: http://www.sciencedirect.com/science/article/pii/S089865681400343X.

Azoulay-Zohar, H. et al., 2004. In self-defence: hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death. *The Biochemical journal*, 377(Pt 2), pp.347–55.

Bajgar, A. et al., 2015. Extracellular Adenosine Mediates a Systemic Metabolic Switch during Immune Response. *PLoS Biology*, 13(4), pp.1–23.

Baonza, a & Garcia-Bellido, a, 2000. Notch signaling directly controls cell proliferation in

the Drosophila wing disc. *Proceedings of the National Academy of Sciences of the United States of America*, 97(6), pp.2609–2614.

Barth, J.M.I. & Köhler, K., 2014. How to take autophagy and endocytosis up a notch. *BioMed Research International*, 2014.

Basak, N.P., Roy, A. & Banerjee, S., 2014. Alteration of mitochondrial proteome due to activation of Notch1 signaling pathway. *Journal of Biological Chemistry*, 289(11), pp.7320–7334.

Baysal, B.E., 2003. On the association of succinate dehydrogenase mutations with hereditary paraganglioma. *Trends in Endocrinology and Metabolism*, 14(10), pp.453–459.

Bensaad, K. et al., 2006. TIGAR, a p53-Inducible Regulator of Glycolysis and Apoptosis. *Cell*, 126(1), pp.107–120.

Bergman, B.C. et al., 2009. Myocardial glucose and lactate metabolism during rest and atrial pacing in humans. *The Journal of physiology*, 587(9), pp.2087–99. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2689346&tool=pmcentrez&rendertype=abstract.

Bernard, F. et al., 2010. Specificity of Notch pathway activation: twist controls the transcriptional output in adult muscle progenitors. *Development (Cambridge, England)*, 137(16), pp.2633–42. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2910383&tool=pmcentrez&rendertype=abstract [Accessed January 7, 2014].

Beverly, L.J., Felsher, D.W. & Capobianco, A.J., 2005. Suppression of p53 by Notch in Lymphomagenesis : Implications for Initiation and Regression. *Cancer research*, 65(16), pp.7159–7168.

Bhambhani, C. et al., 2011. The oligomeric state of CtBP determines its role as a transcriptional co-activator and co-repressor of Wingless targets. *The EMBO journal*, 30(10), pp.2031–2043. Available at: http://dx.doi.org/10.1038/emboj.2011.100.

Blair, S.S., 1999. Drosophila Imaginal Disc Development: Patterning the Adult Fly. In V. E. A. Russo et al., eds. *Development: Genetics, Epigenetics and Environmental Regulation*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 347–370. Available at: http://dx.doi.org/10.1007/978-3-642-59828-9_21.

De Bock, K. et al., 2013. Role of PFKFB3-driven glycolysis in vessel sprouting. *Cell*, 154(3), pp.651–663.

Bolós, V., Grego-Bessa, J. & de la Pompa, J.L., 2007. Notch signaling in development and cancer. *Endocrine reviews*, 28(3), pp.339–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17409286 [Accessed December 11, 2013].

Bonnet, M. et al., 2011. Posttranscriptional deregulation of MYC via PTEN constitutes a major alternative pathway of MYC activation in T-cell acute lymphoblastic leukemia. *Blood*, 117(24), pp.6650–6659.

Borggrefe, T. & Oswald, F., 2009. The Notch signaling pathway: transcriptional regulation at Notch target genes. *Cellular and molecular life sciences : CMLS*, 66(10), pp.1631–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19165418 [Accessed December 16, 2013].

Bras, L. & Loyer, N., 2011. The Multiple Facets of Ubiquitination in the Regulation of Notch Signaling Pathway St ephanie. *Traffic*, 12, pp.149–161.

Bray, S.J., 2006. Notch signalling: a simple pathway becomes complex. *Nature reviews. Molecular cell biology*, 7(9), pp.678–89. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16921404 [Accessed December 12, 2013].

Brogiolo, W. et al., 2001. An evolutionarily conserved function of the drosophila insulin receptor and insulin-like peptides in growth control. *Current Biology*, 11(4), pp.213–221.

Brosnan, C.F. & John, G.R., 2009. Revisiting Notch in remyelination of multiple sclerosis lesions. *The journal of clinical investigation*, 119(1), pp.10–13.

Burnstock, G. & Ulrich, H., 2011. Purinergic signaling in embryonic and stem cell development. *Cellular and molecular life sciences : CMLS*, 68(8), pp.1369–94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21222015.

Byun, J.S. & Gardner, K., 2013. C-terminal binding protein: A molecular link between metabolic imbalance and epigenetic regulation in breast cancer. *International Journal of Cell Biology*, 2013.

Carrero, P. et al., 2000. Redox-regulated recruitment of the transcriptional coactivators CREB-binding protein and SRC-1 to hypoxia-inducible factor 1alpha. *Molecular and cellular biology*, 20(1), pp.402–15. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=85095&tool=pmcentrez&rendertype=abstract.

Cave, J.W. et al., 2005. A DNA Transcription Code for Cell-Specific Gene Activation by Notch Signaling. *Current biology*, 15, pp.94–104.

Cohn, M.J. et al., 1995. Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell*, 80(5), pp.739–746.

Colombani, J. et al., 2003. A nutrient sensor mechanism controls Drosophila growth. *Cell*, 114(6), pp.739–749.

Colombani, J., Andersen, D.S. & Léopold, P., 2012. Secreted Peptide Dilp8 Coordinates

Drosophila Tissue Growth with Developmental Timing. *Science*, 336(6081), pp.582–585. Available at: http://science.sciencemag.org/content/336/6081/582.abstract.

Conigrave, A.D., Quinn, S.J. & Brown, E.M., 2000. L-amino acid sensing by the extracellular Ca2+-sensing receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 97(9), pp.4814–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=18315&tool=pmcentrez&rendertype=abstract.

Cornejo, M.G. et al., 2016. Crosstalk between NOTCH and AKT signaling during murine megakaryocyte lineage specification. *Blood*, 118(5), pp.1264–1274.

Crabtree, H.G., 1929. Observations on the Carbohydrate Metabolism of Tumours. *Biochemical journal*, 23, pp.536–545.

Culi, J. & Modolell, J., 1998. Proneural gene self-stimulation in neural precursors: an essential mechanism for sense organ development that is regulated by Notch signaling. *Genes & Development*, 12(13), pp.2036–2047. Available at: http://www.genesdev.org/cgi/doi/10.1101/gad.12.13.2036 [Accessed January 7, 2014].

Dan, H.C. et al., 2014. Akt-dependent activation of mTORC1 complex involves phosphorylation of mTOR (mammalian target of rapamycin) by IkappaB kinase alpha (IKKalpha). *The Journal of biological chemistry*, 289(36), pp.25227–25240.

Dazert, E. & Hall, M.N., 2011. mTOR signaling in disease. *Current Opinion in Cell Biology*, 23(6), pp.744–755.

Dekanty, A. et al., 2005. The insulin-PI3K/TOR pathway induces a HIF-dependent transcriptional response in Drosophila by promoting nuclear localization of HIF-alpha/Sima. *Journal of cell science*, 118(Pt 23), pp.5431–5441.

Delgada, T.C. et al., 2004. Quantitation of erythrocyte pentose pathway flux with [2-13C]glucose and 1H NMR analysis of the lactate methyl signal. *Magnetic Resonance in Medicine*, 51(6), pp.1283–1286.

Djiane, A. et al., 2012. Dissecting the mechanisms of Notch induced hyperplasia. *The EMBO Journal*, 32(1), pp.60–71.

Duvic, B. et al., 2002. Notch Signaling Controls Lineage Specification during Drosophila Larval Hematopoiesis. *Current biology : CB*, 12, pp.1923–1927.

Eddison, M., Roux, I. Le & Lewis, J., 2000. Notch signaling in the development of the inner ear : Lessons from Drosophila. *PNAS*, 97(22), pp.11692–11699.

Edgar, B.A., 2006. How flies get their size: genetics meets physiology. *Nat Rev Genet*, 7(12), pp.907–916. Available at: http://dx.doi.org/10.1038/nrg1989.

Efstratiadis, A. & Klinakis, A., 2007. Notch, Myc and Breast Cancer. *Cell cycle*, 6(4),

pp.418–429.

Eisenberg, M.L. et al., 2005. Cellular Physiology and Biochemistr y Biochemistry Insulin Receptor (IR) and Glucose Transporter 2 (GLUT2) Proteins Form a Complex on the Rat Hepatocyte Membrane. *Cellular Physiology and Biochemistry*, 15, pp.51–58.

Ejsmont, R.K. et al., 2009. A toolkit for high-throughput, cross- species gene engineering in Drosophila. *Nature methods*, 6(6), pp.435–7.

Ejsmont, R.K. et al., 2011. Production of fosmid genomic libraries optimized for liquid culture recombineering and cross-species transgenesis. *Methods in molecular biology (Clifton, N.J.)*, 772, pp.423–443.

Estella, C. & Baonza, A., 2015. Cell proliferation control by Notch signalling during imaginal discs development in Drosophila. *AIMS Genetics*, 2(1), pp.70–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9570768%5Cnhttp://www.aimspress.com/article/10.3934/genet.2015.1.70.

Estívariz, C.F. & Ziegler, T.R., 1997. Nutrition and the insulin-like growth factor system. *Endocrine*, 7(1), pp.65–71. Available at: http://dx.doi.org/10.1007/BF02778066.

Fischer, K. et al., 2007. Inhibitory Effect of Tumor Cell-Derived Lactic Acid on Human T Cells. *Blood*, 109(9), pp.3812–3820.

Freeman, D.J. et al., 2003. PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms. *Cancer Cell*, 3(2), pp.117–130.

Fryer, C.J. et al., 2002. Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. *Genes and decelopment*, 16, pp.1397–1411.

Fryer, C.J. et al., 2004. Mastermind Recruits CycC : CDK8 to Phosphorylate the Notch ICD and Coordinate Activation with Turnover. *Molecular cell*, 16, pp.509–520.

Funasaka, T. et al., 2005. Regulation of phosphoglucose isomerase/autocrine motility factor expression by hypoxia. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 19(11), pp.1422–1430.

Furriols, M. & Bray, S., 2001. A model Notch response element detects Suppressor of Hairless-dependent molecular switch. *Current biology : CB*, 11(1), pp.60–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11166182 [Accessed March 30, 2012].

Gallant, P., 2013. Myc function in drosophila. *Cold Spring Harbor Perspectives in Medicine*, 3(10), pp.1–14.

Gao, Ping, E. Al, 2009. c-Myc suppression of miR-23 enhances mitochondrial glutaminase and glutamine metabolism. *Nature*, 458(7239), pp.762–765.

Garriga-Canut, M. et al., 2006. 2-Deoxy-D-glucose reduces epilepsy progression by NRSF-CtBP-dependent metabolic regulation of chromatin structure. *Nature neuroscience*, 9(11), pp.1382–1387.

Géminard, C. et al., 2006. Control of metabolism and growth through insulin-like peptides in Drosophila. *Diabetes*, 55(SUPPL. 2), pp.5–8.

Géminard, C., Rulifson, E.J. & Léopold, P., 2009. Remote Control of Insulin Secretion by Fat Cells in Drosophila. *Cell Metabolism*, 10(3), pp.199–207. Available at: http://dx.doi.org/10.1016/j.cmet.2009.08.002.

Goetze, K. et al., 2011. Lactate enhances motility of tumor cells and inhibits monocyte migration and cytokine release. *International Journal of Oncology*, 39(2), pp.453–463.

Gottfried, E. et al., 2006. Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. *Blood*, 107(5), pp.2013–2021.

Gross, S. et al., 2010. Cancer-associated metabolite 2- hydroxyglutarate accumulates in acute myelogenous leukemia with isocitrate dehydrogenase 1 and 2 mutations. *Journal of experimental medicine*, 207(2), pp.3–8.

Guarani, V. et al., 2011. Acetylation-dependent regulation of endothelial Notch signalling by the SIRT1 deacetylase ¨. *Nature*, 473(7346), pp.234–8.

Gupta, V. & Bamezai, R.N.K., 2010. Human pyruvate kinase M2: A multifunctional protein. *Protein Science*, 19(11), pp.2031–2044.

Gustafsson, M. V. et al., 2005. Hypoxia requires Notch signaling to maintain the undifferentiated cell state. *Developmental Cell*, 9(5), pp.617–628.

Gwinn, D.M. et al., 2008. AMPK phosophorylation of raptor mediates a metabollic checkpoint. *Molecular Cell*, 30(2), pp.214–226.

Hajduch, E., Litherland, G.J. & Hundal, H.S., 2001. Protein kinase B (PKB/Akt) - a key regulator of glucose transport? *FEBS Lett.*, 492(3), pp.199–203.

van Hall, G. et al., 2009. Blood lactate is an important energy source for the human brain. *Journal of cerebral blood flow and metabolism*, 29(6), pp.1121–1129.

Hao, C. & Haase, V.H., 2010. Sirtuins and their relevance to the kidney. *J Am Soc Nephrol.*, 21(10), pp.1620–1627.

Hartenstein, V. & Posakony, J.W., 1989. Development of adult sensilla on the wing and notum of Drosophila melanogaster. *Development (Cambridge, England)*, 107, pp.389–405.

Hayashi, M. et al., 2004. Induction of glucose transporter 1 expression through hypoxia-inducible factor 1?? under hypoxic conditions in trophoblast-derived cells. *Journal of Endocrinology*, 183(1), pp.145–154.

Hayashi, Y. et al., 2016. A novel non-canonical Notch signaling regulates expression of synaptic vesicle proteins in excitatory neurons. *Scientific Reports*, 6(23969), pp.1–13. Available at: http://dx.doi.org/10.1038/srep23969.

Vander Heiden, M.G. et al., 2010. Evidence for an alternative glycolytic pathway in rapidly proliferating cells. *Science (New York, N.Y.)*, 329(5998), pp.1492–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3030121&tool=pmcentrez&rendertype=abstract [Accessed December 18, 2013].

Vander Heiden, M.G., Cantley, L.C. & Thompson, C.B., 2009. Understanding the warburg effect: The metabolic requirements of cell proliferation. *Science*, 324(5930), pp.1029–1033.

Holness, M.J. & Sugden, M.C., 2003. Regulation of pyruvate dehydrogenase complex activity by reversible phosphorylation. *Biochemical Society transactions*, 31(Pt 6), pp.1143–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14641014.

Hooper, K.L., Parkhurst, S.M. & Ish-Horowicz, D., 1989. Spatial control of hairy protein expression during embryogenesis. *Development*, 107(3), pp.489–504. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2612375.

Hori, K. et al., 2004. Drosophila Deltex mediates Suppressor of Hairless-independent and late-endosomal activation of Notch signaling. *Development*, 131, pp.5527–5537.

Hori, K., Sen, A. & Artavanis-Tsakonas, S., 2013. Notch signaling at a glance. *Journal of cell science*, 126(Pt 10), pp.2135–40. Available at: http://jcs.biologists.org/content/126/10/2135.long.

Horvath, M. et al., 2016. The silent information regulator 1 (Sirt1) is a positive regulator of the Notch pathway in Drosophila. *The Biochemical journal*, 473, pp.4129–4143.

Housden, B.E., Li, J. & Bray, S.J., 2014. Visualizing Notch signaling in vivo in Drosophila tissues. *Methods in molecular biology (Clifton, N.J.)*, 1187, pp.101–113.

Hu, Y.-Y. et al., 2016. Hif-1alpha and Hif-2alpha differentially regulate Notch signaling through competitive interaction with the intracellular domain of Notch receptors in glioma stem cells. *Cancer Letters*, 349(1), pp.67–76. Available at: http://dx.doi.org/10.1016/j.canlet.2014.03.035.

Huang, X., Warren, J.T. & Gilbert, L.I., 2008. New players in the regulation of ecdysone biosynthesis. *Journal of genetics and genomics*, 35(1), pp.1–10.

Chen, Y. et al., 2012. Quantitative acetylome analysis reveals the roles of SIRT1 in regulating diverse substrates and cellular pathways. *Molecular & cellular proteomics : MCP*, 11(10), pp.1048–1062. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3494151&tool=pmcentrez

&rendertype=abstract.

Cheng, H.-T. et al., 2003. Gamma-secretase activity is dispensable for mesenchyme-to-epithelium transition but required for podocyte and proximal tubule formation in developing mouse kidney. *Development (Cambridge, England)*, 130(20), pp.5031–5042.

Cheng, L.Y. et al., 2011. Anaplastic lymphoma kinase spares organ growth during nutrient restriction in drosophila. *Cell*, 146(3), pp.435–447. Available at: http://dx.doi.org/10.1016/j.cell.2011.06.040.

Chopra, I. et al., 2012. Phosphorylation of the insulin receptor by AMP-activated protein kinase (AMPK) promotes ligand-independent activation of the insulin signalling pathway in rodent muscle. *Diabetologia*, 55(3), pp.783–794.

Christofk, H.R., Vander Heiden, M.G., Wu, N., et al., 2008. Pyruvate kinase M2 is a phosphotyrosine-binding protein. *Nature*, 452(7184), pp.181–186. Available at: http://dx.doi.org/10.1038/nature06667.

Christofk, H.R., Vander Heiden, M.G., Harris, M.H., et al., 2008. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature*, 452(7184), pp.230–233. Available at: http://dx.doi.org/10.1038/nature06734.

Ikawa, T. et al., 2006. E proteins and Notch signaling cooperate to promote T cell lineage specification and commitment. *The Journal of experimental medicine*, 203(5), pp.1329–42. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2121213&tool=pmcentrez&rendertype=abstract [Accessed January 7, 2014].

Ishiuchi, T. et al., 2009. Mammalian Fat and Dachsous cadherins regulate apical membrane organization in the embryonic cerebral cortex. *Journal of Cell Biology*, 185(6), pp.959–967.

Itoh, T.Q., Matsumoto, A. & Tanimura, T., 2013. C-Terminal Binding Protein (CtBP) Activates the Expression of E-Box Clock Genes with CLOCK/CYCLE in Drosophila. *PLoS ONE*, 8(4), pp.1–7.

Itoh, Y. et al., 2003. Free fatty acids regulate insulin secretion from pancreatic [beta] cells through GPR40. *Nature*, 422(6928), pp.173–176. Available at: http://dx.doi.org/10.1038/nature01478.

Joshi, I. et al., 2009. Notch signaling mediates G 1 / S cell-cycle progression in T cells via cyclin D3 and its dependent kinases. *Blood*, 113(8), pp.1689–1699.

Kageyama, R., Ohtsuka, T. & Kobayashi, T., 2007. The Hes gene family : repressors and oscillators that orchestrate embryogenesis. *Development*, 134, pp.1243–1251.

Kannan, K. et al., 2001. DNA microarrays identification of primary and secondary target genes regulated by p53. *Oncogene*, 20, pp.2225–2234.

Kawauchi, K. et al., 2008. p53 regulates glucose metabolism through an IKK-NF-[kappa]B pathway and inhibits cell transformation. *Nat Cell Biol*, 10(5), pp.611–618. Available at: http://dx.doi.org/10.1038/ncb1724.

Kelner, M.J. et al., 1995. Heterologous Expression of Selenium-Dependent Glutathione Peroxidase Affords Cellular Resistance to Paraquat 1. *Archives of biochemistry and biophysics*, 323(1), pp.40–46.

Kim, E., 2009. Mechanisms of amino acid sensing in mTOR signaling pathway. *Nutrition research and practice*, 3(1), pp.64–71.

Kim, E. & Guan, K.L., 2009. RAG GTPases in nutrient-mediated TOR signaling pathway. *Cell Cycle*, 8(7), pp.1014–1018.

Kim, J. et al., 1996. Integration of positional signals and regulation of wing formation and identity by Drosophila vestigial gene. *Nature*, 382(6587), pp.133–138. Available at: http://dx.doi.org/10.1038/382133a0.

Kim, J.W. et al., 2006. HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. *Cell Metabolism*, 3(3), pp.177–185.

Kim, M.-Y. et al., 2007. Tip60 histone acetyltransferase acts as a negative regulator of Notch1 signaling by means of acetylation. *Molecular and cellular biology*, 27(18), pp.6506–6519.

Kim, R.-J. et al., 2014. PTEN Loss-Mediated Akt Activation Increases the Properties of Cancer Stem-Like Cell Populations in Prostate Cancer. *Oncology*, 87(5), pp.270–279. Available at: http://www.karger.com/DOI/10.1159/000363186.

Koch, U., Lehal, R. & Radtke, F., 2013. Stem cells living with a Notch. *Development (Cambridge, England)*, 140(4), pp.689–704. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23362343.

Koch, U. & Radtke, F., 2007. Notch and cancer: a double-edged sword. *Cellular and molecular life sciences : CMLS*, 64(21), pp.2746–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17687513 [Accessed January 7, 2014].

Kondoh, H. et al., 2005. Glycolytic Enzymes Can Modulate Cellular Life Span Glycolytic Enzymes Can Modulate Cellular Life Span. *Cancer Res*, 65(1), pp.177–185.

Kondoh, T., Mallick, H.N. & Torii, K., 2009. Activation of the gut-brain axis by dietary glutamate and physiologic significance in energy homeostasis. *American Journal of Clinical Nutrition*, 90(3), pp.832–837.

Kopan, R., Goate, A. & Louis, S., 2002. Aph-2 / Nicastrin : An Essential Component of □ -Secretase and Regulator of Notch Signaling and Presenilin Localization. *Neuron*, 33, pp.321–324.

Kopan, R. & Ilagan, M.X.G., 2009. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell*, 137(2), pp.216–33. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2827930&tool=pmcentrez&rendertype=abstract [Accessed December 24, 2013].

Koressaar, T. & Remm, M., 2007. Enhancements and modifications of primer design program Primer3. *Bioinformatics*, 23(10), pp.1289–1291.

Koyama, T., Mendes, C.C. & Mirth, C.K., 2013. Mechanisms regulating nutrition-dependent developmental plasticity through organ-specific effects in insects. *Frontiers in Physiology*, 4 SEP(September), pp.1–12.

Krebs, H.A., 1967. The redox state of nicotinamide adenine dinucleotide in the cytoplasm and mitochondria of rat liver. *Advances in Enzyme Regulation*, 5, pp.409–434. Available at: http://www.sciencedirect.com/science/article/pii/0065257167900295.

Krejčí, A., Bernard, F., Housden, B.E., et al., 2009. Direct response to Notch activation: Signaling crosstalk and incoherent logic. *Science signaling*, 2(55).

Krejčí, A., Bernard, F., Krejčí, A., et al., 2009. Direct response to Notch activation: signaling crosstalk and incoherent logic. *Science signaling*, 2(55), p.ra1. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19176515 [Accessed December 27, 2013].

Krejčí, A. & Bray, S., 2007. Notch activation stimulates transient and selective binding of Su(H)/CSL to target enhancers. *Genes & development*, 21, pp.1322–1327.

De La Cova, C. et al., 2004. Drosophila myc regulates organ size by inducing cell competition. *Cell*, 117(1), pp.107–116.

Land, S.C. & Tee, A.R., 2007. Hypoxia-inducible factor 1 alpha is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. *Journal of Biological Chemistry*, 282(28), pp.20534–20543.

Landor, S.K.-J. et al., 2011. Hypo- and hyperactivated Notch signaling induce a glycolytic switch through distinct mechanisms. *Proceedings of the National Academy of Sciences of the United States of America*, 108(46), pp.18814–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3219154&tool=pmcentrez&rendertype=abstract [Accessed January 7, 2014].

Lanet, E. & Maurange, C., 2014. Building a brain under nutritional restriction: Insights on sparing and plasticity from Drosophila studies. *Frontiers in Physiology*, 5 MAR(March), pp.1–9.

Lavoie, M.J. & Selkoe, D.J., 2003. The Notch Ligands , Jagged and Delta , Are Sequentially Processed by ⎵ -Secretase and Presenilin / □ -Secretase and Release Signaling Fragments *. *The Journal of biological chemistry*, 278(36), pp.34427–34437.

Lee, K. et al., 2015. ECM1 promotes the Warburg effect through EGF-mediated activation of PKM2. *Cellular Signalling*, 27(2), pp.228–235. Available at: http://www.sciencedirect.com/science/article/pii/S0898656814003507.

Lee, S. et al., 2011. gamma-Secretase-regulated Proteolysis of the Notch Receptor by Mitochondrial Intermediate Peptidase. *The Journal of biological chemistry*, 286(31), pp.27447–27453.

Lefort, K. et al., 2007. Notch1 is a p53 target gene involved in human keratinocyte tumor suppression through negative regulation of ROCK1 / 2 and MRCK ⎵ kinases. *Genes & Development*, 21, pp.562–577.

Levine, A.J. & Puzio-Kuter, A.M., 2010. The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. *Science*, 330(6009), pp.1340–1344.

Lewis, J., 1998. Notch signalling and the control of cell fate choices in vertebrates. *Seminars in cell and developmental biology*, 9, pp.583–589.

Li, Z. et al., 2014. Long non-coding RNA UCA1 promotes glycolysis by upregulating hexokinase 2 through the mTOR-STAT3/microRNA143 pathway. *Cancer Science*, 105(8), pp.951–955.

Lim, J.H. et al., 2004. Ras-dependent induction of HIF-1alpha785 via the Raf/MEK/ERK pathway: a novel mechanism of Ras-mediated tumor promotion. *Oncogene.*, 23(58), pp.9427–9431.

Lin, S. et al., 2010. Lineage-specific effects of Notch/Numb signaling in post-embryonic development of the Drosophila brain. *Development (Cambridge, England)*, 137(1), pp.43–51. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2796933&tool=pmcentrez&rendertype=abstract [Accessed January 7, 2014].

Lindhurst, M. et al., 2011. A Mosaic Acrivating Mutation in AKT1 Associated with the Proteus Syndrome. *N Engl J Med.*, 365(7), pp.611–619.

Ling, H., Sylvestre, J. & Jolicoeur, P., 2010. Notch1-induced mammary tumor development is cyclin D1-dependent and correlates with expansion of pre-malignant multipotent duct-limited progenitors. *Oncogene*, 29(32), pp.4543–4554. Available at: http://dx.doi.org/10.1038/onc.2010.186.

Liu, Y. et al., 2008. A fasting inducible switch modulates gluconeogenesis via activator-

coactivator exchange. *Nature*, 456(7219), pp.269–273.

Lu, L. et al., 2011. Modulations of hMOF autoacetylation by SIRT1 regulate hMOF recruitment and activities on the chromatin. *Cell research*, 21(8), pp.1182–1195. Available at: http://www.nature.com/cr/journal/v21/n8/pdf/cr201171a.pdf.

Lupu, F. et al., 2001. Roles of growth hormone and insulin-like growth factor 1 in mouse postnatal growth. *Developmental biology*, 229(1), pp.141–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11133160.

Ma, C.C. -y. et al., 2014. SIRT1 suppresses self-renewal of adult hippocampal neural stem cells. *Development*, 141(24), pp.4697–4709. Available at: http://dev.biologists.org/content/141/24/4697%5Cnhttp://dev.biologists.org/content/141/24/4697.abstract%5Cnhttp://dev.biologists.org/content/141/24/4697.full.pdf%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/25468938%5Cnhttp://dev.biologists.org/cgi/doi/10.1242/dev.11793.

Maekawa, Y. et al., 2015. Notch controls the survival of memory CD4+ T cells by regulating glucose uptake. *Nat Med*, 21(1), pp.55–61. Available at: http://dx.doi.org/10.1038/nm.3758.

Maniati, E. et al., 2011. Crosstalk between the canonical NF- κ B and Notch signaling pathways inhibits Ppar γ expression and promotes pancreatic cancer progression in mice. *The journal of clinical investigation*, 121(12), pp.4685–99.

Martin, J.F. et al., 2000. Minidiscs Encodes a Putative Amino Acid Transporter Subunit Required Non-Autonomously for Imaginal Cell Proliferation. *Mechanisms of Development*, 92(2), pp.155–167.

Matsuno, H. et al., 2003. Boundary formation by notch signaling in Drosophila multicellular systems: experimental observations and gene network modeling by Genomic Object Net. *Pacific Symposium on Biocomputing*, 8, pp.152–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12603025.

McBride, A. et al., 2009. The Glycogen-Binding Domain on the AMPK β Subunit Allows the Kinase to Act as a Glycogen Sensor. *Cell Metabolism*, 9(1), pp.23–34. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2642990/.

McDaniell, R. et al., 2006. NOTCH2 Mutations Cause Alagille Syndrome , a Heterogeneous Disorder of the Notch Signaling Pathway. *the American joufnal of Human Genetics*, 79, pp.169–173.

Meier-Stiegen, F. et al., 2010. Activated Notch1 target genes during embryonic cell differentiation depend on the cellular context and include lineage determinants and inhibitors. *PloS one*, 5(7), p.e11481. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2900208&tool=pmcentrez&rendertype=abstract [Accessed January 7, 2014].

Mihaylova, M.M. & Shaw, R.J., 2012. The AMP-activated protein kinase (AMPK) signaling pathway coordinates cell growth, autophagy, and metabolism. *Nat Cell Biol*, 13(9), pp.1016–1023.

Miller, A., 2004. Superoxide dismutases : active sites that save , but a protein that kills. *Current Opinion in Chemical Biology*, 8, pp.162–168.

Mirth, C., Truman, J.W. & Riddiford, L.M., 2005. The role of the prothoracic gland in determining critical weight for metamorphosis in Drosophila melanogaster. *Current Biology*, 15(20), pp.1796–1807.

Mishra-Gorur, K. et al., 2002. Down-regulation of Delta by proteolytic processing. *The Journal of cell biology*, 159(2), pp.313–324.

Mondal, S. et al., 2015. HSulf-1 deficiency dictates a metabolic reprograming of glycolysis and TCA cycle in ovarian cancer. *Oncotarget*, 6(32), pp.33705–19. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26378042%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4741796.

Moran, B.M. et al., 2014. Evaluation of the insulin-releasing and glucose-lowering effects of GPR120 activation in pancreatic beta-cells. *Diabetes, obesity & metabolism*, 16(11), pp.1128–1139.

Moran, S.T. et al., 2007. Synergism between NF-KappaB1/p50 and Notch2 during the Development of Marginal Zone B Lymphocytes 1. *the journal of imunology*, 179, pp.195–200.

Morin, A. et al., 2014. Oncometabolites-driven tumorigenesis: From genetics to targeted therapy. *International Journal of Cancer*, 135(10), pp.2237–2248.

Mukherjee, T. et al., 2011. Interaction Between Notch and Hif-alpha in Development and Survival of Drosophila Blood cells. *Science*, 332(6034), pp.1210–1213.

Mulholland, D.J. et al., 2012. Pten loss and RAS/MAPK activation cooperate to promote EMT and metastasis initiated from prostate cancer stem/progenitor cells. *Cancer Res*, 72(7), pp.1878–1889.

Mulligan, P., 2011. A SIRT1-LSD1 Co-repressor Complex Regulates Notch Target Gene Expression and Development. *Mol. cell*, 42(5), pp.689–699.

Murray, R.K. et al., 2012. *Harper´s Illustrated Biochemistry, 29th Edition* 29th ed., MvGraw-Hill Companies, Inc.

Murugan, A.K., Bojdani, E. & Xing, M., 2010. Identification and functional characterization of isocitrate dehydrogenase 1 (IDH1) mutations in thyroid cancer. *Biochemical and*

biophysical research communications, 393(3), pp.555–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2838977&tool=pmcentrez&rendertype=abstract [Accessed January 7, 2014].

Nagao, M., Sugimori, M. & Nakafuku, M., 2007. Cross Talk between Notch and Growth Factor / Cytokine Signaling Pathways in Neural Stem Cells . *Molecular and cellular biology*, 27(11), pp.3982–3994.

Nagel, A.C., Wech, I. & Preiss, A., 2001. scalloped and strawberry notch are target genes of Notch signaling in the context of wing margin formation in Drosophila. *Mechanisms of Development*, 109, pp.241–251.

Nakamura, T., Tsuchiya, K. & Watanabe, M., 2007. Crosstalk between Wnt and Notch signaling in intestinal epithelial cell fate decision. *Journal of Gastroenterology*, 42(9), pp.705–710. Available at: http://dx.doi.org/10.1007/s00535-007-2087-z.

Namihira, M. et al., 2009. Committed Neuronal Precursors Confer Astrocytic Potential on Residual Neural Precursor Cells. *Developmental Cell*, 16(2), pp.245–255. Available at: http://dx.doi.org/10.1016/j.devcel.2008.12.014.

Nellesen, D.T., Lai, E.C. & Posakony, J.W., 1999. Responsiveness of Enhancer of split Complex Genes to Common Transcriptional Activators. *Developmental biology*, 213, pp.33–53.

Nicol, J. et al., 2009. THe integrated genome browser: free software for distribution and exploratoin of genome-scale datasets. *BMC Bioinformatics*, 25(20), pp.2730–2731.

Niemitz, C., Nibbrig, M. & Zacher, V., 2007. Human ears grow throughout the entire lifetime according to complicated and sexually dimorphic patterns--conclusions from a cross-sectional analysis. *Anthropologischer Anzeiger; Bericht uber die biologisch-anthropologische Literatur*, 65(4), pp.391–413.

Nichols, J.T. & Miyamoto, A., 2007. Notch Signaling – Constantly on the Move. *Traffic*, 8, pp.959–969.

Nijhout, H.F., 2003. The control of body size in insects. *Developmental Biology*, 261(1), pp.1–9.

Nordlie, R.C., Foster, J.D. & Lange, A.J., 1999. Regulation of glucose production by the liver. *Annual review of nutrition*, 19, pp.379–406.

Ntziachristos, P. et al., 2014. From Fly Wings to Target Cancer Therapies: A Centennial for Notch Signaling. *Cancer cell.*, 25(3), pp.318–334.

Ochocki, J.D. & Simon, M.C., 2013. Nutrient-sensing pathways and metabolic regulation in stem cells. *Journal of Cell Biology*, 203(1), pp.23–33.

Orgeig, S., Morrison, J.L. & Daniels, C.B., 2011. Prenatal development of the pulmonary

surfactant system and the influence of hypoxia. *Respiratory Physiology & Neurobiology*, 178(1), pp.129–145. Available at: http://dx.doi.org/10.1016/j.resp.2011.05.015%5Cnpapers3://publication/doi/10.1016/j.resp.2011.05.015.

Osawa, H. et al., 1996. Analysis of the signaling pathway involved in the regulation of hexokinase II gene transcription by insulin. *J.Biol.Chem.*, 271(28), pp.16690–16694.

Osawa, H. et al., 1995. Regulation of Hexokinase II Gene Transcription and Glucose Phosphorylation by Catecholamines, Cyclic AMP, and Insulin. *Diabetes*, 44(12), pp.1426–1432. Available at: http://diabetes.diabetesjournals.org/content/44/12/1426.abstract.

Oswald, F. et al., 2005. RBP-J κ /SHARP Recruits CtIP/CtBP Corepressors To Silence Notch Target Genes. *Molecular and cellular biology*, 25(23), pp.10379–90.

Palmer, W.H. & Deng, W.M., 2015. Ligand-Independent Mechanisms of Notch Activity. *Trends in Cell Biology*, 25(11), pp.697–707. Available at: http://dx.doi.org/10.1016/j.tcb.2015.07.010.

Palomero, T. et al., 2007. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. *Nat Med*, 13(10), pp.1203–1210. Available at: http://dx.doi.org/10.1038/nm1636.

Palomero, T. et al., 2006. NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *PNAS*, 103(48), pp.18261–18266.

Parks, A.L. et al., 2000. Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. *Development*, 127, pp.1373–1385.

Peng, X. ding et al., 2003. Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. *Genes and Development*, 17(11), pp.1352–1365.

Pillai, V.B., Sundaresan, N.R. & Gupta, M.P., 2014. Regulation of Akt signaling by Sirtuins: Its implication in cardiac hypertrophy and aging. *Circ Res.*, 114(2), pp.368–378.

Popko-Scibor, A.E. et al., 2011. Ubiquitination of Notch1 is regulated by MAML1-mediated p300 acetylation of Notch1. *Biochemical and Biophysical Research Communications*, 416(3–4), pp.300–306. Available at: http://dx.doi.org/10.1016/j.bbrc.2011.11.030.

Porporato, P.E. et al., 2012. Lactate stimulates angiogenesis and accelerates the healing of superficial and ischemic wounds in mice. *Angiogenesis*, 15(4), pp.581–592.

Qiang, L. et al., 2011. HIF-1α is critical for hypoxia-mediated maintenance of glioblastoma stem cells by activating Notch signaling pathway. *Cell Death and Differentiation*,

19(2), pp.284–294. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3263503&tool=pmcentrez&rendertype=abstract.

Radtke, F., MacDonald, H.R. & Tacchini-Cottier, F., 2013. Regulation of innate and adaptive immunity by Notch. *Nature reviews. Immunology*, 13(6), pp.427–437.

Rangarajan, A. et al., 2001. Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. *The EMBO Journal*, 20(13), pp.3427–3436.

Reitman, Z.J. & Yan, H., 2010. Isocitrate dehydrogenase 1 and 2 mutations in cancer: alterations at a crossroads of cellular metabolism. *Journal of the National Cancer Institute*, 102(13), pp.932–41. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2897878&tool=pmcentrez&rendertype=abstract [Accessed January 2, 2014].

Riddle, S.R. et al., 2000. Hypoxia induces hexokinase II gene expression in human lung cell line A549. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 278(2), pp.L407–L416.

Rizzo, P. et al., 2008. Cross-talk between Notch and the Estrogen Receptor in Breast Cancer Suggests Novel Therapeutic Approaches. *Cancer research*, 68(13), pp.5226–5235.

Rosen, E.D. & Spiegelman, B.M., 2014. What we talk about when we talk about fat. *Cell*, 156, pp.20–44.

Rusten, T.E. et al., 2004. Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K Pathway. *Developmental Cell*, 7(2), pp.179–192.

Saj, A. et al., 2010. A combined ex vivo and in vivo RNAi screen for notch regulators in Drosophila reveals an extensive notch interaction network. *Developmental cell*, 18(5), pp.862–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20493818 [Accessed December 28, 2013].

Sam, A.H. et al., 2012. The role of the gut/brain axis in modulating food intake. *Neuropharmacology*, 63(1), pp.46–56. Available at: http://www.sciencedirect.com/science/article/pii/S0028390811004631.

Sancak, Y. et al., 2010. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell*, 141(2), pp.290–303.

Saravanamuthu, S.S., Gao, Chun, Y. & Zelenka, Peggy, S., 2009. Notch signaling is required for lateral induction of Jagged1 during FGF-induced lens fiber defferentiation. *Developmental biology*, 332(1), pp.166–176.

Satoh, A., Stein, L. & Imai, S., 2011. The role of mammalian sirtuins in the regulation of

metabolism, aging, and longevity. *Handb Exp Pharmacol*, 206, pp.125–162. Available at: http://www.springerlink.com/index/10.1007/978-3-642-21631-2.

Segal, B.H. et al., 2012. Regulation of innate immunity by NADPH oxidase. *Free Radic Biol Med.*, 53(1), pp.72–80.

Sellick, C.A. et al., 2009. Effective quenching processes for physiologically valid metabolite profiling of suspension cultured Mammalian cells. *Analytical chemistry*, 81(1), pp.174–183.

Semenza, G. et al., 1996. Hypoxia Response Elements in the Aldolase A, Enolase 1,and Lactate Dehydrogenase A Gene Promoters Contain Essential Binding Sites for Hypoxia-inducible Factor 1. *Journal of Biological Chemistry*, 271(51), pp.32529–32537.

Shestov, A.A. et al., 2014. Quantitative determinants of aerobic glycolysis identify flux through the enzyme GAPDH as a limiting step. *eLife*, 3, pp.1–18.

Shi, S. & Stanley, P., 2003. Protein O-fucosyltransferase 1 is an essential component of Notch signaling pathways. *PNAS*, 100(9), pp.5234–5239.

Shim, H. et al., 1997. c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. *Proceedings of the National Academy of Sciences of the United States of America*, 94(13), pp.6658–63. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=21214&tool=pmcentrez&rendertype=abstract.

Shimada, K. et al., 2013. TORC2 Signaling Pathway Guarantees Genome Stability in the Face of DNA Strand Breaks. *Molecular Cell*, 51(6), pp.829–839. Available at: http://dx.doi.org/10.1016/j.molcel.2013.08.019.

Shimizu, H. et al., 2014. Compensatory flux changes within an endocytic trafficking network maintain thermal robustness of notch signaling. *Cell*, 157(5), pp.1160–1174.

Shingleton, A.W. et al., 2009. Many ways to be small: different environmental regulators of size generate distinct scaling relationships in Drosophila melanogaster. *Proceedings. Biological sciences / The Royal Society*, 276(1667), pp.2625–2633.

Shirazi-Beechey, S.P. et al., 2014. Role of nutrient-sensing taste 1 receptor (T1R) family members in gastrointestinal chemosensing. *The British journal of nutrition*, 111, pp.8–15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24382171.

Schell, J.C. et al., 2014. A role for the mitochondrial pyruvat carrier as a repressor of the Warburg Effect and colon cancer cell growth. *Mol. cell*, 56(3), pp.400–413.

Schonbrun, M. et al., 2009. TOR complex 2 controls gene silencing, telomere length maintenance, and survival under DNA-damaging conditions. *Molecular and cellular*

biology, 29(16), pp.4584–94. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2725747&tool=pmcentrez&rendertype=abstract.

Schwanbeck, R. et al., 2011. The Notch signaling pathway : Molecular basis of cell context dependency. *European Journal of Cell Biology*, 90, pp.572–581. Available at: http://dx.doi.org/10.1016/j.ejcb.2010.10.004.

Schwartzenberg-bar-yoseph, F., Armoni, M. & Karnieli, E., 2004. The Tumor Suppressor p53 Down-Regulates Glucose Transporters GLUT1 and GLUT4 Gene Expression The Tumor Suppressor p53 Down-Regulates Glucose Transporters GLUT1 and GLUT4 Gene Expression. *Cancer Res*, 2(1), pp.2627–2633.

Slaninova, V. et al., 2016. Notch stimulates growth by direct regulation of genes involved in the control of glycolysis and the tricarboxylic acid cycle. *Open biology*, 6.

Sonveaux, P. et al., 2008. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. *Journal of Clinical Investigation*, 118(12), pp.3930–3942.

Spinner, N.B., 2000. CADASIL : Notch signaling defect or protein accumulation problem ? *The journal of clinical investigation*, 105(5), pp.561–562.

Stambolic, V. et al., 2001. Regulation of PTEN transcription by p53. *Molecular Cell*, 8(2), pp.317–325.

Stieper, B.C. et al., 2008. Imaginal discs regulate developmental timing in Drosophila melanogaster. *Developmental Biology*, 321(1), pp.18–26.

Tabata, T. & Takei, Y., 2004. Morphogens, their identification and regulation. *Development (Cambridge, England)*, 131(4), pp.703–712.

Takahashi, H. et al., 2006. Nucleocytosolic Acetyl-Coenzyme A Synthetase Is Required for Histone Acetylation and Global Transcription. *Molecular Cell*, 23(2), pp.207–217.

Takubo, K. et al., 2013. Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. *Cell Stem Cell*, 12(1), pp.49–61. Available at: http://dx.doi.org/10.1016/j.stem.2012.10.011.

Tang, H.Y. et al., 2011. FOXO regulates organ-specific phenotypic plasticity in Drosophila. *PLoS Genetics*, 7(11).

Tapon, N. & Harvey, K.F., 2012. The Hippo pathway-from top to bottom and everything in between. *Seminars in cell & developmental biology*, 23(7), pp.768–769.

Tato, I. et al., 2011. Amino acids activate mammalian target of rapamycin complex 2 (mTORC2) via PI3K/Akt signaling. *Journal of Biological Chemistry*, 286(8), pp.6128–6142.

Thorens, B., 2014. GLUT2, glucose sensing and glucose homeostasis. *Diabetologia*, 58(2),

pp.221–232.

Tiberi, L. et al., 2012. BCL6 controls neurogenesis through Sirt1-dependent epigenetic repression of selective Notch targets. *Nat Neurosci*, 15(12), pp.1627–1635. Available at: http://dx.doi.org/10.1038/nn.3264.

Tiebe, M. et al., 2015. REPTOR and REPTOR-BP regulate organismal metabolism and transcription downstream of TORC1. *Dev Cell.*, 33(3), pp.272–284.

Toro, J.R. et al., 2003. Mutations in the fumarate hydratase gene cause hereditary leiomyomatosis and renal cell cancer in families in North America. *American journal of human genetics*, 73(1), pp.95–106.

Tsurugizawa, T., Uneyama, H. & Torii, K., 2014. Brain amino acid sensing. *Diabetes, Obesity and Metabolism*, 16, pp.41–48.

Tsutsumi, S. et al., 2003. Regulation of cell proliferation by autocrine motility factor/phosphoglucose isomerase signaling. *Journal of Biological Chemistry*, 278(34), pp.32165–32172.

Ulrich, E.L. et al., 2008. BioMagResBank. *Nucleic Acids Research*, 36(SUPPL. 1), pp.402–408.

Vaccari, T. et al., 2008. Endosomal entry regulates Notch receptor activation in Drosophila melanogaster. *Journal of Cell Biology*, 180(4), pp.755–762.

Vasquez-Del Carpio, R. et al., 2011. Assembly of a Notch transcriptional activation complex requires multimerization. *Molecular and cellular biology*, 31(7), pp.1396–408. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3135300&tool=pmcentrez&rendertype=abstract [Accessed January 7, 2014].

Vilimas, T. et al., 2007. Targeting the NF-[kappa]B signaling pathway in Notch1-induced T-cell leukemia. *Nat Med*, 13(1), pp.70–77. Available at: http://dx.doi.org/10.1038/nm1524.

Vucenik, I. & Stains, J.P., 2012. Obesity and cancer risk: Evidence, mechanisms, and recommendations. *Annals of the New York Academy of Sciences*, 1271(1), pp.37–43.

Wang, J.-C., 2005. Finding primary targets of transcriptional regulators. *Cell cycle (Georgetown, Tex.)*, 4(3), pp.356–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15711128.

Wang, R.N. et al., 2014. Bone Morphogenetic Protein (BMP) signaling in development and human diseases. *Genes & Diseases*, 1(1), pp.87–105. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4232216&tool=pmcentrez&rendertype=abstract.

Wang, X. et al., 2007. Pyruvate Protets Mitochondria From Oxidative Stress in Human Neuroblastoma SK-N-SH Cells. *Brains res.*, 1132(1), pp.1–9.

Warburg, O., 1956. On the Origin of Cancer Cells. *Science*, 123(3191), pp.309–14.

Watanabe, H. et al., 1991. Purification of human tumor cell autocrine motility factor and molecular cloning of its receptor. *Journal of Biological Chemistry*, 266(20), pp.13442–13448.

Watanabe, I. & Okada, S., 1967. Effects of temperature on growth rate of cultured mammalian cells (L5178Y). *The Journal of cell biology*, 32, pp.309–323.

Webster, G.A. & Perkins, N.D., 1999. Transcriptional Cross Talk between NF-κB and p53. *Molecular and cellular biology*, 19(5), pp.3485–3495.

Webster, N.J.G. et al., 1996. Repression of the Insulin Receptor Promoter by the Tumor Suppressor Gene Product p53 : A Possible Mechanism for Receptor Overexpression in Breast Cancer Repression of the Insulin Receptor Promoter by the Tumor Suppressor Gene in Breast Cancer1. *Cancer research*, 56, pp.2781–2788.

Weinhouse, S., Millington, R.H. & Wennerf, C.E., 1951. Metabolism of Neoplastic Tissue I . The Oxidation of Carbohydrate and Fatty Acids in Transplanted Tumors. *Cancer research*, 11, pp.845–850.

Wellendorph, P. et al., 2005. Deorphanization of GPRC6A : A Promiscuous L - Amino Acid Receptor with Preference for Basic Amino Acids. *Mol. Pharmacol.*, 67, pp.589–597.

Weydert, C.J. & Cullen, J.J., 2010. Measurements of Superoxide Dismutase, Catalase, And Glutathione Peroxidase in Cultured Cells And Tissue. *Nat protoc.*, 5(1), pp.51–66.

Whelan, J.T. et al., 2009. Notch-1 Signaling Is Lost in Prostate Adenocarcinoma and Promotes PTEN Gene Expression. *Journal of cellular biochemistry*, 107(5), pp.992–1001.

Wise, D.R. et al., 2008. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proceedings of the National Academy of Sciences of the United States of America*, 105, pp.18782–18787.

Wise, D.R. & Thompson, C.B., 2010. Glutamine Addiction: A New Therapeutic Target in Cancer. *Trends Biochem Sci*, 35(8), pp.427–433.

Wong, G.W. et al., 2012. HES1 opposes a PTEN-dependent check on survival, differentiation, and proliferation of TCR??-selected mouse thymocytes. *Blood*, 120(7), pp.1439–1448.

Woo, H. et al., 2009. Alzheimer ' s disease and Notch signaling. *Biochemical and Biophysical Research Communications*, 390(4), pp.1093–1097. Available at: http://dx.doi.org/10.1016/j.bbrc.2009.10.093.

Wu, D.C. & Johnston, L.A., 2010. Control of wing size and proportions by Drosophila Myc. *Genetics*, 184(1), pp.199–211.

Xie, G. et al., 2013. Cross-talk between Notch and Hedgehog Regulates Hepatic Stellate Cell Fate. *Hepatology*, 58(5), pp.1801–1813.

Xie, M., Liu, M. & He, C.S., 2012. SIRT1 Regulates Endothelial Notch Signaling in Lung Cancer. *PLoS ONE*, 7(9).

Yamada, K. et al., 2011. Roles of Drosophila Deltex in Notch receptor endocytic trafficking and activation. *Genes to Cells*, 16, pp.261–272.

Yamagata, K. & Kitabayashi, I., 2009. Sirt1 physically interacts with Tip60 and negatively regulates Tip60-mediated acetylation of H2AX. *Biochemical and Biophysical Research Communications*, 390(4), pp.1355–1360. Available at: http://dx.doi.org/10.1016/j.bbrc.2009.10.156.

Yang, L. et al., 2014. The mTORC1 effectors S6K1 and 4E-BP play different roles in CNS axon regeneration. *Nature communications*, 5, p.5416. Available at: http://www.nature.com/ncomms/2014/141110/ncomms6416/abs/ncomms6416.html.

Yeung, S.J., Pan, J. & Lee, M.H., 2008. Roles of p53, MYC and HIF-1 in regulating glycolysis - The seventh hallmark of cancer. *Cellular and Molecular Life Sciences*, 65(24), pp.3981–3999.

Young, S.H. et al., 2010. Amino acid sensing by enteroendocrine STC-1 cells: role of the Na(+)-coupled neutral amino acid transporter 2. *American Journal of Physiology - Cell Physiology*, 298(6), pp.C1401–C1413. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2889636/.

Yu, F. et al., 2012. Regulation Of The Hippo-YAP Pathway By G-Protein- Coupled Receptor Signaling. *Cell*, 150(4), pp.1–11.

Zecca, M. & Struhl, G., 2010. A feed-forward circuit linking wingless, Fat-Dachsous signaling, and the warts-hippo pathway to drosophila wing growth. *PLoS Biology*, 8(6).

Zeng, Q. & Hong, W., 2008. The Emerging Role of the Hippo Pathway in Cell Contact Inhibition, Organ Size Control, and Cancer Development in Mammals. *Cancer Cell*, 13(3), pp.188–192.

Zhao, Y. et al., 2015. SoNar, a Highly Responsive NAD+/NADH Sensor, Allows High-Throughput Metabolic Screening of Anti-tumor Agents. *Cell Metabolism*, 21(5), pp.777–789.

Zheng, X. et al., 2008. Interaction with factor inhibiting HIF-1 defines an additional mode of cross-coupling between the Notch and hypoxia signaling pathways. *Proceedings of the National Academy of Sciences of the United States of America*, 105(9), pp.3368–73.

Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2265116&tool=pmcentrez&rendertype=abstract.

Zhou, D. et al., 2008. Mechanisms underlying hypoxia tolerance in Drosophila melanogaster: hairy as a metabolic switch. *PLoS genetics*, 4(10), p.e1000221. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2556400&tool=pmcentrez&rendertype=abstract [Accessed January 7, 2014].

Zhou, W. et al., 2012. HIF1α induced switch from bivalent to exclusively glycolytic metabolism during ESC-to-EpiSC/hESC transition. *The EMBO Journal*, 31(9), pp.2103–2116. Available at: http://emboj.embopress.org/cgi/doi/10.1038/emboj.2012.71.

Zhu, C.T. & Rand, D.M., 2012. A Hydrazine Coupled Cycling Assay Validates the Decrease in Redox Ratio under Starvation in Drosophila. *PLoS ONE*, 7(10).

Zou, Y. et al., 2010. IDH1 and IDH2 mutations are frequent in Chinese patients with acute myeloid leukemia but rare in other types of hematological disorders. *Biochemical and biophysical research communications*, 402(2), pp.378–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20946881 [Accessed January 7, 2014].

7. Supplement

Supplement 1: Sequences of cloned enhancer with marked mutated Su(H) binding sites. Mutated Su(H) binding sites are marked in red (or blue in case of one of the Hex-A (1) construct). Red (blue respectively) brackets bound mutagenesis primer sequences. Mutations in Su(H) bindig sites are showed underneath of each of the mutated sequence.

Glut1

GAAGACGACGACATGACTGCCTTTTATTGCTCCTACGCCTCTGC{CTAGCCCCTCCGTTCCC
ATTTTCCCACGCCCCTCCCTTTTGTTCAGC}TGTCGTGGCCGTTGAACAAATGAACAACATA
AATAATTGCAGAGGCACACACACTGACACACACACACACACGCGTTCTGAGGAAACTCGCAC
GCACACTGACAAAGAGTGGGAAAATCGCAGGACTCCCAAGGAACGTTTGCAGCCTTCGAATT
GAGATTAATGAGCAGCAAAAGTGATTC AAAGTCAGCATCCTCG

TTCCCAC → TAACAAC

Hex-A (1)

TGTGCTACAAGCGAAAGCAGACATCGCAAGTATAACAGTTGAAGGTGACACTGGAGACCCTG
GCGATAGGACACGTATAACAGTTCTGTAATCC{CAACATTGGCTGACGAAATCATGGGAATG
CAAGGCGT{GGTTGGGCGG}GTTAAGCCACTTCTCCCAAAAAGTTATAAAAACCCATC}TGG
AACCAGTTGATTTTTTGTATTTATCTGGTTCTGTTGACATGGTGATTTTACTTCAAAGAACC
CGTTTATGAAATTCGAAACCTACAATGTAAATGGAAATTCCCTGAGTCAATTTTTCCAATAT
GCAACTCCTTTGGA

ATGGGAA →ATTGTTA
TTCTCCC →TAATACC

Hex-A (2)

CAGCACCGAATGGAAATTGCACATGAAATCTAGCTGCAAAAATATGAAACAACAAAATTCCA
TTGAATGGAGAGCCAGAAAGAGCGAAGTAGAGGGGAAGAGAGTGAGAGAAGGAGGGAGCGGA
ACTTCAAACTGGGCGGCACGTACACACACAAAAGAGGGCAGGGCGGGGAGACGAGCCCCACG
GACACTGAACAAAAATACATACAAATGCAAAGATATATGTATTTGAGGATGTTGACGCGTCG
CTAGAACTTGAGAATTCCAAGAAGCGGTAAAAACGGGTGAGACGATGATGGTAGGGGAGTTG
GGGAGGGGAAGGTAGCTGTCAACAGCTACGCAAACTTGCTCTCCCATTTTTCTGGGTAAACC
TTGCTCTTTCTCATCTAAAACGGCATTGTTATACTAACACCGATGCCGAG{GGAGAGCGAGA
TCGCTGAGAGTGGGAAAGCGGGTGGTGGGGCCCGGC}GCCGAACAGGGGGTTCAA
AGAACGAAGCCAA

GTGGGAA →GTTGTTA

Hex-A (3)

GCGACGCATAAGGGTTTCCGCATAAACACGCGTTTGACTCATTCTCATTCATAGCCACTCTC
TCGCCCCCTCTCTCACGCGCACCCTATCAATATGCCATTTGTGGACCCCTCAGCGTCGCACA
TATACACGCCATATCTCCAACCATGCCG

Treh

CGTAAACGAAAGGAAAAGTGCATAAAAGCGAGCAGAGATAGCGTCAGAATGACTTATCGCG{
TATACGCCCCGTTGAGCCACGTGAAAAATGTTTAGTTCTTATGAAAT}CGTTCAAAAGGCCC
AAAGCAAATCAGCTGAAAGCATAAAAATAAACCAATATAAACATACCTATGTGTGTGTGCCT
GACGAGCCCCCATACTTATGTACTTCGCTCACGCGCTAGTGTGCGTGTTAAACTTTTGTATG
TCTGCGAAATGTTATAAACTGGTTTTCATGTTACTCTCTCGATCTAAAAACAGGCAAAGGGA
TAAGATACAAACGTGATTGAGTGTTTCTTCCGATTGATTCGACTTCGGAACCCTTTGATATT
TAAATATAACTTTTGAATAATGCAACTGCCGCTCGATCGACAATGGAGTTGTTCTTCTTTCG
ATAAGTAATGCTTATCTTTTGTCTGGCTCTTGCTCTTTATATGTGTGTGTTCACGAGTGGTT
GCCCAAAGGTCACCGACGAAGGTCGTTGGTATTTCGAGATGAAAACGTGCTGTTGTTTGGGA
GAAACTTGGCAGTCTCTGTGGATAGAGTCTGTAGACTGGCATAATTTTCGGAACC
GGTTTGTCTGGCAGAAAAAGGAGGCAGAAA

GTGAAAA → GTTATTA

ImpL3

TCAGTTTCGTTTGGGGAGAGCTAAACTCAAGACCAACTGAAGTTCACTTCAGTTTTCAGCTC
CCGAGCAGCAGCAGCAGCAGCAACTTTCGAGATATACGAGCATTTGTCACATAAACTGCAAC
GCCTTTCCCATTGTCAGCTCGCCACGAAGACTTGGTCGCTGTTTGCTTTTGCTTTTGGCCAT
AAAATATATGGCCCCGTATCGCAAAAAGCTGCGAGTGTTGAAATTTAAGTTTTAATTTAAGC
GATATGAAAAAAAAAGGGCAAGACGGCAAACGGCGGTCTGCAGTCGTCGAATCGTGCACGAA
ATGAAGCCATTTTCATATTTATGAATTTTGCCCCTTATCGAAAAACGCTTCAATTGGCCATC
GACTTGGGGCCGGCAAATTAAATATGGGCAGTCATAAACGATA
{CTGACAGGTCGGTGGGATCTGTGGGAAAAGGGGCAAGATTCCGGGGA}ACTTGAGATCCAG
AATCCAGAGCGTAACATCCTCGCCGACTTAGCTGAGTTATTAAGGCATCTTTATAGCCAATC
TCAATGAGGTGTGAAGTGCAATCGTTGTTGGCCTTTTTATTAATACGCGAAAATATATTTTA
TTTGTTGTTCCGTCTGCGATCGACTGCGATATTAAG C

GTGGGAA → GTAGAAA

CG13334 (1)

CTGCTCCATTTGCTGTTGACCTATTGAAATGCGACAGTTTCACATGTGTTTTCGGGATTGCG
TGGGATGATCGTGGAGCACGTGTGTGTTGACATACTGACCGCAGAGAGTGTACTTGAGCAAG
GGACTTCCCCGTGAATCTGCCCGCCTGCTCAAGTGCTACGTAATTTGCATGGTCTGGATTTT
TTCAAATTACATGACATTTTCCCACACTGATTAGCCCGCACATGCCATTCTCTATTTTCGAT
GTGCATCCGAGTGCGACCTTCGAACAGGCAAACAGCCAGTGCAGCCTGCATCCGATGCACAT
CCATTGCATCCAGTGCAACGAAGTGCAACGCCATCATTTTTTCCCACAGCCGAGGGCTCTGG
GTTATATTTAACAAATGCATGAGAGATTTGGGTGCGCTACGTTTTCGTTTTCGTTTCGGA

CG13334 (2)

CCCTGGATACAGACGATTGCGCTCCACTGATTTTTCCCATCATCGAGCGATGCTCCCTGCCT
TTGGGCACTTTCTTCATCTCCTGGCTGGCCTCCTGCAATTTGCTGTGAACCTGTTCGAAAAT
TATGCATCGATGCGCATCAGTGGCAATCATGGATGGCAACATAATAATGGGATGCTGCTGAT
GAGTTTGCTCATAAACTGATGAGGGTACCTGGCTGTCTACCTAGCTACCTGTACCTGTGATT
CATTTCGATTGCCTAGCTGCTGATTCTGTTCACCTGTTCGTTTGCTACACTTTATGCATGCT
TCGGCAGTTTTCTAATTATGACTTATGATCTCTGGGATTAGACAAACAATGGATCGTGGCAC
GTGGTTCCCAAGATGCTTTCGCTTGTGCCTGACCTTTTCCCACGATCTGTTTGCGGTTGAGC
GCTCGAGGCCCATGATCACCGCAAATCCCCTGCAGACAAAACAGCCAAAGG

CG13334 (3)

TGGACGCAACCATCATATTCTTCTGCGCTCCTGATCCTTTTTCCAATACCTCGATTCGGAGT
TCTCACGCGAGCACATGCCAAAGCGAAAAAAAGGGACAGGGAAACAATTAACAACTAATTTA
GGCCTCTGCATATAATTTTATGGAGAGTGGTGCGGAGAGTCGCAGTCCCACAGGGAGATGCC
TATCTGCAGGAGCTGTGGGAAAAAATACGTTACATTCCTTTCCTTTGCTTTCCTTCTCTCTC
TGCCTGTCGTTGCGGCATTTATTCCCACGCGATCCTCGACTCTCGTTTTTGTTGGTTTTCGG
GGTTTCGCTTTCGTGGAGTA

IDH

GTAAATAGCTGGGCGGAATGCGATCGGAAGTGTGGGTGTTTGGCGTGGAGTGTGAACCGGTT
TATATAAGGCTAAACAAATGCTCCACTTACCTTTTAGAATATTTAAAATGCCGAATTTAGAA
TGTTAGAGCACAGACTAATTAAATTGCAGACCGAAAGATCTCGAACCACTCCCAATCAGACG
CAGCGAATAACTGAAACTGATTTGGCGACGACAACAATAAAGAGCAAGAGGTGGCGCGTGTG
GAAGATAATTCCGTAGATAAAAACATGTTATTCAA{TCAGCTGCTTGCGAAGAGCTTTCTCA
CGAACTTTAAGCTTTACTCTC}GAGTTCTGCAGCACTCAGTATTAGTTCGTCTTGCTGAACC
GCCAAACATAAAATTTGAATTCTCAATTACCGCAACTTGTGAGAGCAGCGTGCGTTCGTTTG
CTTTAATGACGCTAAAAGAAAATCCATAAAATGTGCTCCAAAAGTGAAATGTTAGCCGG

TTCTCAC → TAATAAC

hairy

AGCAACAACACCAACACCACCGCGACCATCACCAACAGCACAGCCAGAAACACAGCCTCTTG
TGAATCCCTCAGTTAGCAGAGCCCAGCAGAGTCAAGCCAAACCGATCGCTGATCGACCGACC
GACCGACGATCACCAATGGGGGTTTCGCAGTGTGATTTC{CAAAAAGGAAGAAATGCCCATT
CCCGCGAGCCACGGGGGCGTATGAG}TAAC GCGGTG

TTCCCGC → TAACAGC

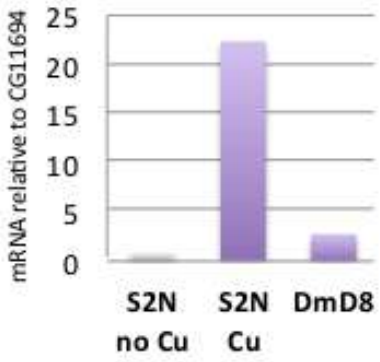
Supplement 2: List of all used oligonucleotides

Oligonucleotides used for cloning of enhancers		
Gene	**Primer name**	**Sequence**
Glut1	Kpn1 Glut1_fw	GATCA GGTACC GAAGACGACGACATGACTGC
	Bgl2 Glut1_rev	GACTC AGATCT CGAGGATGCTGACTTTGAATC
Hex-A	Kpn1 Hex-A_1fw	GATCA GGTACC TGTGCTACAAGCGAAAGCAG
	Bgl2 Hex-A_1rev	GACTC AGATCT TCCAAAGGAGTTGCATATTGG
	Kpn1 Hex-A_2fw	GATCA GGTACC CAGCACCGAATGGAAATTG
	Bgl2 Hex-A_2rev	GACTC AGATCT TTGGCTTCGTTCTTTGAACC
	Kpn1 Hex-A_3fw	GATCA GGTACC GCGACGCATAAGGGTTTCC
	Bgl1 Hex-A_3rev	GACTC AGATCT CGGCATGGTTGGAGATATG
Treh	Kpn1 Treh_fw	GATCA GGTACC CGTAAACGAAAGGAAAAGTGC
	Bgl2 Treh_rev	GACTC AGATCT TTTCTGCCTCCTTTTTCTGC
CG13334	Kpn1 CG13334_1fw	GATCA GGTACC CTGCTCCATTTGCTGTTGAC
	Bgl2 CG13334_1rev	GACTC AGATCT TCCGAAACGAAAACGAAAAC
	Mlu1 CG13334_2fw	GACTC ACGCGT CCCTGGATACAGACGATTGC
	Bgl2 CG13334_2rev	GACTC AGATCT CCTTTGGCTGTTTTGTCTGC
	Mlu1 CG13334_3fw	GACTC ACGCGT TGGACGCAACCATCATATTC
	Bgl2 CG13334_3rev	GACTC AGATCT TACTCCACGAAAGCGAAACC
Impl3	Bgl2 ImpL3_fw	GACTC AGATCT TCAGTTTCGTTTGGGGAGAG
	Kpn1 ImpL3_rev	GATCA GGTACC GCTTAATATCGCAGTCGATCG
IDH	Mlu1 Idh_fw	GACTC ACGCGT GTAAATAGCTGGGCGGAATG
	Kpn1 Idh_rev	GATCA GGTACC CCGGCTAACATTTCACTTTTG
Hairy	MluI hairy_fw	GATCA GGTACC AGCAACAACACCAACACCAC
	Bgl2 hairy_rev	GACTC AGATCT CACCGCGTTACTCATACGC
-	RV3	CTAGCAAAATAGGCTGTCCC
Oligonucleotides used for mutation of Luciferase vectors		
Gene	**Primer name**	**Sequence (mutated nucleotides in red)**
Glut 1	Glut mut fw	TAGCCCCTCCGTTCCCATTTAACAACGCCCCTCCCTTTTGTTCA
	Glut mut rev	TGAACAAAAGGGAGGGGCGTTGTTAAATGGGAACGGAGGGGCTA
Hex- A	Hex mut patser fw	GTTGGGCGGGTTAAGCCACTAATACCAAAAAGTTATAAAAACCC
	Hex mut patser rev	GGGTTTTTATAACTTTTTGGTATTAGTGGCTTAACCCGCCCAAC
	Hex mut both fw	ACATTGGCTGACGAAATCATTGTTATGCAAGGCGTGGTTGGGCG
	Hex mut both rev	CGCCCAACCACGCCTTGCATAACAATGATTTCGTCAGCCAATGT
	HexA2 mut s	GGAGAGCGAGATCGCTGAGAGTTGTTAAGCGGGTGGTGGGGCCCGGC
	HexA2 mut a	GCCGGGCCCCACCACCCGCTTAACAACTCTCAGCGATCTCGCTCTCC
Treh	Treh mut s	TATACGCCCCGTTGAGCCACGTTATTAATGTTTAGTTCTTATGAAAT
	Treh mut a	ATTTCATAAGAACTAAACATTAATAACGTGGCTCAACGGGGCGTATA

CG13334	CG13334_2mut s	TTTCGCTTGTGCCTGACCTTTAACAACGATCTGTTTGCGGTTGAGCG
	CG13334_2mut a	CGCTCAACCGCAAACAGATCGTTGTTAAAGGTCAGGCACAAGCGAAA
ImpL3	ImpL3 mut s	CTGACAGGTCGGTGGGATCTGTTGTTAAAGGGGCAAGATTCCGGGGA
	ImpL3 mut a	TCCCCGGAATCTTGCCCCTTTAACAACAGATCCCACCGACCTGTCAG
IDH	IDH mut s	GAGAGTAAAGCTTAAAGTTGTTATTAAGTCTTCCGCAAGCAGCTGA
	IDH mut a	TCAGCTGCTTGCGAAGAGCTTAATAACGAACTTTAAGCTTTACTCTC
hairy	Hairy mut fw	AAAAAGGAAGAAATGCCCATAACAGCGAGCCACGGGGGCGTATG
	Hairy mut rev	CATACGCCCCCGTGGCTCGCTGTTATGGGCATTTCTTCCTTTTT
Oligonucleotides used for Time course Q-RT-PCR analysis		
Gene	**Primer name**	**Sequence**
Glut1	Glut1 fw	TCGACCATGGAGCTGATATG
	Glut1 rev	TGAACAGCGACGTGGAATAG
Hex-A	Hex-A fw	GATGTGCACAGCATCAATCC
	Hex-A rev	CTTCGGAATCCTGTCCATTG
Treh	Treh fw	CACCAACGATGACAAGTTCG
	Treh rev	GAATCGCGGTACACACACAG
CG13334	CG13334 fw	ACTCCAAGGATTCCGATGTG
	CG13334 rev	ACTGAGCTCCACCAGTTTGG
ImpL3	ImpL3 fw	TGGTCTGGAGTGAACATTGC
	ImpL3 rev	AGCTTGATCACCTCGTAGGC
IDH	IDH fw	TTGCTTATGCCATGAAGTCG
	IDH rev	ATAGCAGCACGGAGGTCATC
hairy	h fw	ACAAATTCAAGGCCGGATTC
	h rev	TCTTAACGCCATTGATGCAG
Kdn	RT Kdn fw	AAATTCGCCAAGGCATACTC
	RT Kdn rev	CGCGATAGGTGTTGCAGTAG
SdhB	RT SdhB fw	GCGTCTGAACAAGTTGAAGG
	RT SdhbB rev	AGAGAGCTTCTTGATTG
lethal	RT leth fw	GAGCTCCAACGATACCTTCC
	RT leth rev	CTTGAACTCCTCCGACTTG
Thor	RT Thor fw	AGTTGCTTGCTGAGGGGAAC
	RT Thor rev	CACACCCCCTACAGATCCAG
Oligonucleotides used for *hairy* dsRNA production and subsequent Q-RT-PCR analysis		
Gene	**Primer name**	**Sequence**
hairy	h RNAi fw	T7 + TGCTACGCACCTGAGCAAC
	h RNAi rev	T7 + ATGTGTGCGAGTTGGATGAG
IDH	IDH fw	TTGCTTATGCCATGAAGTCG
	IDH rev	ATAGCAGCACGGAGGTCATC
Kdn	RT Kdn fw	AAATTCGCCAAGGCATACTC
	RT Kdn rev	CGCGATAGGTGTTGCAGTAG

SdhB	RT SdhB fw	GCGTCTGAACAAGTTGAAGG
	RT SdhbB rev	AGAGAGCTTCTTGATTG
lethal	RT leth fw	GAGCTCCAACGATACCTTCC
	RT leth rev	CTTGAACTCCTCCGACTTG
Oligonucleotides used for *in situ* hybridizations		
Gene	**Primer name**	**Sequence of primer**
Glut1	Glut1 probe s	GGAGATAGCGCCACTGAA
	T7 Glut1 a	T7 + ATTAGCGGAATGGACACGAG
	T7 Glut1 s	T7 + GGAGATAGCGCCACTGAA
	Glut1 probe a	ATTAGCGGAATGGACACGAG
Hex-A	Hex-A probe s	TGTGTACAAGGAGCGTTTGC
	T7 Hex-A a	T7 + GGCTCGTCAGCTTCAATT
	T7 Hex-A s	T7 + TGTGTACAAGGAGCGTTTGC
	Hex-A probe a	GGCTCGTCAGCTTCAATT
Treh	Treh probe s	AACGGCGGTCGAGTCTACTA
	T7 Treh a	T7 + CCTTCACCCACAGTGGAGAT
	T7 Treh s	T7 + AACGGCGGTCGAGTCTACTA
	Treh probe a	CCTTCACCCACAGTGGAGAT
ImpL3	ImpL3 probe s	GTGTGCCTCATCGATGTCTG
	T7 ImpL3 a	T7 + CCCAGGAGGTGTATCCCTTT
	T7 ImpL3 s	T7 + GTGTGCCTCATCGATGTCTG
	ImpL3 probe a	CCCAGGAGGTGTATCCCTTT
CG13334	CG13334 probe s	TCCGGAATCTCACCAAGAAC
	T7 CG13334 a	T7 + TGTCCTGGATTCCGTTTAGG
	T7 CG13334 s	T7 + TCCGGAATCTCACCAAGAAC
	CG13334 probe a	TGTCCTGGATTCCGTTTAGG
IDH	IDH probe s	CCAGGTCACCATTGACTGTG
	T7 IDH a	T7 + TGCACATCACCGTCGTAGTT
	T7 IDH s	T7 + CCAGGTCACCATTGACTGTG
	IDH probe a	TGCACATCACCGTCGTAGTT
hairy	hairy probe s	TGCTACAGCACCTGAGCAAC
	T7 hairy a	T7 + ATGTGTGCGAGTTGGATGAG
	T7 hairy s	T7 + TGCTACAGCACCTGAGCAAC
	hairy probe a	ATGTGTGCGAGTTGGATGAG
Oligonucleotides used for hairy FlyFos preparations		
Gene	**Primer name**	**Sequence of primer**
hairy	hairy FF GFP fw	TGGTGATCAAGAAGCAGATCAAGGAGGAGGAGCAGCCCT GGCGGCCCTGGGAAGTGCATACCAATCAGGACCCGC
	hairy FF GFP rev	TTCGATAGGGGTGGCTATGCTATATGATATGCATATGCA GACACCCTCTACTTGTCGTCGTCATCCTTGTAGTCA
	hairy fw internal	AACAGCAACCCATGTTGGTC
	GFP rev inteernal	GCCGGGTCTGATTGGTATGCACTTC
Oligonucleotides used for bacmid preparations		
Gene	**Primer name**	**Sequence of primer**
-	pUC/M13 rev	AGCGGATAACAATTTCACACAGGA
-	SV40 rev	GTGGTATGGCTGATTATGATC

Supplement 3: Comparison of Notch mRNA levels in uninduced S2N cells (no Cu), induced S2N cells (Cu) and DmD8 cells



Supplement 4: The expression pattern of hairy A. The expression pattern in wild type control Oregon R wing discs using anti hairy antibody (Abcam 20165) **B.** The expression pattern of hairy-FlyFos construct tagged with GFP stained with GFP antibody. **C.** The expression pattern of Hairy-Gal4 (BL30876 y1 w* ; PBac{hEGFP.S}VK00037 x UAS-GFP) using GFP antibody. Hairy is expressed in the pouch as well as in the notum of the wing disc. While the relative intensities of the two bands in the pouch (arrows) differ depending on the reporter used (possibly due to a different timing and perdurance of the constructs) the overall expression pattern is preserved, although not fully recapitulated.