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Wingless/Notch crosstalk in Drosophila melanogaster
Wingless/Notch crosstalk v Drosophile melanogaster

Bakalářská práce

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Poděkování

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V Praze, 2022 Hana Hajšmanová

Abstrakt

Základním kamenem existence mnohobuněčných organismů je přítomností signalizačních drah, komplexních biochemických kaskád umožňujících vzájemnou komunikaci mezi buňkami. Dráhy Notch a Wnt jsou klíčové pro správný embryonální vývoj, a jejich deregulace vede k vzniku mnoha závažných onemocnění. Mnohaletý výzkum postavený z velké míry na poznatcích z pokusů v modelovém organismu D. melanogaster ukázal, že komponenty těchto drah nepůsobí striktně odděleně, ale naopak vstupují do vzájemné interakce (crosstalk). Tento fenomén je však u savců prozkoumán jen v omezené míře. Tato bakalářská práce má za cíl shrnout dosavadní poznatky o spolupůsobení drah Wnt a Notch u D. melanogaster a nastínit, kde by mohl crosstalk hrát roli u savců.

Klíčová slova

Drosophila melanogaster, Notch, Wnt, Wingless, β-catenin, crosstalk, vývoj, signalizace, signální dráhy, octomilka

Abstract

Fundamental for the existence of multicellular organisms is the presence signalling pathways, complex biochemical cascades, allowing reciprocal communication amongst the cells. Both Notch and Wnt pathways are crucial for embryonic development and their deregulation leads to many severe diseases. Decades of research, largely based on model organism D. melanogaster, showed that components of these pathways do not function strictly separately but on contrary interact with each other (crosstalk). This phenomenon is explored only to a lesser extent in mammals. The aim of this thesis is to recapitulate existing findings of Wnt/ Notch crosstalk in D. melanogaster and outline where crosstalk could play a role in mammals.

Key words

Drosophila melanogaster, Notch, Wnt, Wingless, β-catenin, crosstalk, development, signalling, pathway, fruit fly

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1.Introduction

With the rise of multicellular organisms, signalling emerged as a form of communication between the cells. Received signals can originate from outside the cells, in various physical forms (e.g. light, pressure, temperature, voltage) or can be mediated by chemical agents such as small molecules or peptides. These chemical signalling pathways are series of chemical reactions where molecules work together in order to control cell function (Gilbert, 2010).

Several signalling pathways were identified as crucial for the embryogenesis, amongst them Wnt and Notch, each representing a different form of signalling mechanism. Wnt and Notch pathway, both discovered in Drosophila melanogaster by identification of distinct phenotypes, are core signalling pathways in control of the development and are even functioning in many of the same developmental patterning events (Clevers & Nusse, 2012; Siebel & Lendahl, 2017). Both pathways were studied individually for a long time, but more and more contexts are being discovered, where they intertwine one with another. This mutual influence is called crosstalk and has been reported in many cases between various pathways. Components of one pathway can co-operate in regulations of transcriptional targets; transcriptional target of one pathway can affect expression of other pathway's core components or the crosstalk can occur directly between the signalling proteins - either having positive – activation or negative - inhibitory effect (Collu et al., 2014). All of these crosstalk mechanisms have been observed between Notch and Wnt pathways to such a high extent that common name "Wntch" name has been proposed (Collu et al., 2014a; Hayward et al., 2008). While the role of coaction in Drosophila between both pathways is apparent, up till now the Wntch crosstalk was not systematically tested in mammals. Therefore, we only have a very limited knowledge of its role and the true extent of its importance in our lives.

The purpose of this thesis is to look into existing reports of pathways direct cross-talk, focusing on canonical pathways, summarize findings in the model organism Drosophila melanogaster, less "complex" compared to mammals, and determine functional conservation of mechanisms and contexts, to see where the analogy is extended from Drosophila to mammals.

Gained knowledge might be used to suggest possible mechanisms of augmented Wnt/Notch crosstalk functions in mammals and way of targeting pathway components when treating human diseases and dysfunctions.

2. Drosophila melanogaster

2.1 About D. melanogaster

Drosophila melanogaster, also referred to as a "fruit fly" is a fly species from the Diptera order. It has a short lifespan of approximately 50 days and fast development of about 10 days at room temperature; females can produce up to hundreds of eggs and deliver a large number of offspring, which makes Drosophila a popular model organism (Dietrich et al., 2014).

Its lifecycle consists of two life forms – the larval and adult stages, separated by a period of metamorphosis called pupation. The larval stages are called "instar" and the adult fly preceding pupation is called "imago" as a reference to imaginal discs, which is a "sac-like structure inside the larvae of insect that undergoes metamorphosis" (Aldaz & Escudero, 2010) **(Fig. 1)**.

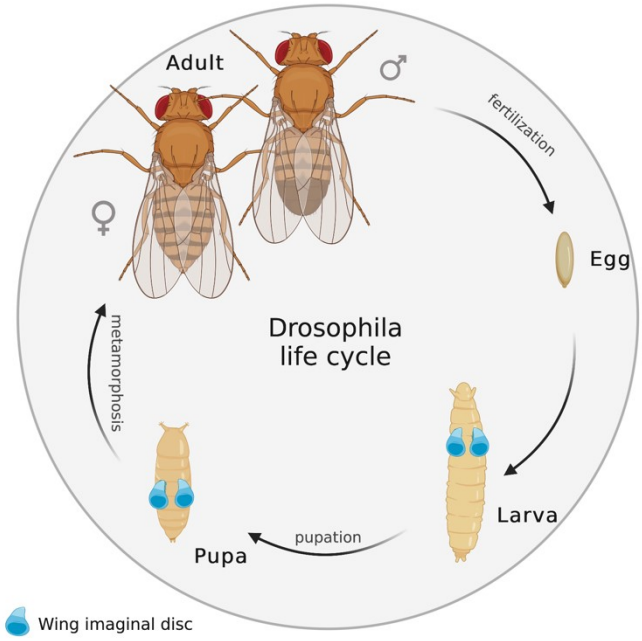


Figure 1. Scheme of Drosophila melanogaster life cycle showing major developmental stages as well as imaginal discs, which later on will give rise to the wings (modified from Barik BK, Mishra M. Nanoparticles as a potential teratogen: a lesson learnt from fruit fly. Nanotoxicology. 2019) Created with BioRender.com

2.2 Use of Drosophila as a model organism

Drosophila melanogaster as a model organism was first used by W. Castle, however, its use is best-know for the work of T. Morgan Hunt (Chromosomal theory of Heredity, 1910), followed by the work of his students C. Bridges (who indicated that genes are located on chromosomes, 1916), A. Sturtevant (creator of the first chromosome map, 1913) and H.J. Muller (who pioneered the induction of mutations by X-ray,1928) in the so-called "Fly room" at Columbian University **(Fig. 2)**. Since then, thanks to the passionate community revolving around this model

organism, Drosophila became extensively studied, and the gathered data and know-how are shared openly at Flybase.org database – serving as a unique resource to thousands of research.

Figure 2. A. Sturtevant working in "Fly room"

2.3 Why Drosophila melanogaster

D. melanogaster has around 14000 genes (Adams et al., 2000), with around 60% of homology to human genome (Mirzoyan et al., 2019). Out of these, approximately 75% correspond to human genes accountable for diseases (Jennings, 2011). For this reason, along with the fact, that fly has a fast generation rate and accessible forward genetic screening, Drosophila is often used as a model for studying human diseases.

Before subdivision of vertebrate lineage, genome of a last common ancestor undergone two rounds of whole genome duplications (WGD) (Holland & Ocampo Daza, 2018), leading to increased complexity in mammals. Drosophila as a model from invertebrate group do not possesses this complexity, for which reason I chose it as an object of this thesis.

Because of the high degree of conservation of both pathways, insights gained through fly analysis can be applied to mammal systems and used for the prediction of component interference in more complex systems.

3. What is signalling and why do cells need it

To create a functional multicellular organism, cells need to be able to control their inner mechanisms, communicate with each other and sense, react, and adapt to their environment. Cells need to interpret a variety of signals, for which they contain different receptors. Usually, each receptor is activated by only one type of signalling molecule that binds to its ligand and causes a particular response e.g. gene expression in the nuclei.

Cells can, and do, utilize multiple pathways at the same time, but the scope of this thesis won't allow mentioning all. I thus aim to summarize what is known about direct crosstalk between Wnt and Notch pathways, interesting for being distinct types of chemical transduction cascades. Wnt/ β-catenin pathway is an example of the paracrine signalling acting in short-range, using a signal molecule to activate the receptor (Gilbert, 2010). Once the receptor become activated, β-catenin become the second messenger, used for amplification of signal and signal conversion into transcriptional activity in the nuclei (Clevers & Nusse, 2012).

Notch, as an example of juxtacrine, or in other words, contact-dependent signalling, acts in the short-range and requires direct contact between ligand and receptor (Gilbert, 2010). Notch is a unique example of signalling receptor that after cleavage, functions also as a signal molecule, followed by translocation to the nuclei, directly driving expression of the Notch target genes. Interestingly, the Notch signalling, unlike the Wnt signalling, does not utilize any amplification step (Kopan & Ilagan, 2009). Hence, Notch signalling is sensitive to perturbations and even its mild deregulation leads to a wide spectra of genetic disorders (Mašek & Andersson, 2017).

3.1. Origin and conservation of the Wnt and Notch pathways

Although orthologs of some Wnt pathway components can be found in unicellular organisms, both pathways arose to its full extent within multicellular organisms (Metazoans), along with the rise of the multicellularity. Origin of β-catenin ortholog is in cell adhesion and this role is preceding its function in Wnt signalling (Dickinson et al., 2011).

Similarly, ancestors of Notch family members are thought to have an origin in adhesion as well, but this is partially maintained up to date (A. Murata & Hayashi, 2016). Components of both pathways are to a large extent conserved from fly to human (Chien et al., 2009; Ntziachristos et al., 2014).

4. Wnt/β-catenin signalling pathway

The term Wnt is a fusion of the names for Drosophila gene *wingless* and its mouse homologue *Integrated* (Int-1) identified as a mouse oncogene (Murine Mammary Tumor Virus (MMTV)) (Nusse & Varmus, 1982). The Wingless pathway is named after a mutant phenotype causing loss of wings and halteres (Sharma, 1973). For purpose of this thesis hereafter referred as Wingless if referring to fly and Wnt if referring to its mammal homologue.

Wnt signalling modes are commonly further distinguished as canonical and non-canonical pathways. Canonical pathway signals through β-catenin and two non-canonical pathways - Planar polarity pathway (PCP) and Wnt/Ca^{2+} pathway - are both β-catenin independent. This thesis focuses on canonical Wnt/β-catenin x Notch crosstalk, but more information about non-canonical Wnts can be found in this article (Corda & Sala, 2017).

4.1 Function

Wnt signalling pathway plays an important role in the development and homeostasis of a vast range of organisms, from D. melanogaster to human which implicates its importance. In Drosophila Wingless pathway plays a role in many imaginal and embryonic developmental processes, including segment polarity (Nüsslein-volhard & Wieschaus, 1980), formation of the wing (Simcox et al., 1989; Williams et al., 1993), head development (Schmidt-Ott & Technau, 1992), patterning of epidermis (Bejsovec & Arias, 1991), leg development (Couso et al., 1993), intestinal homeostasis (Tian et al., 2016), development of sensory organs, including eyes (Cadigan & Nusse, 1996; Treisman & Rubin, 1995), and bristles (Gho & Schweisguth, 1998).

4.2 Key Wnt pathway components

4.2.1 Ligands

There are seven Wnt proteins present in fly (flybase.com) and nineteen in mammals (https://www.web.stanford.edu/group/nusselab/cgi-bin/wnt/). Wnt ligands are secreted glycoproteins, that are cysteine-rich and highly hydrophobic (Mikels & Nusse, 2006), and their expression is tightly spatially and temporally regulated during development. They can act as short-range signalling molecules or long-range morphogens, usually with an inhibitory effect, as seen in wing formation. Wnts also can act as secreted morphogens, often altering the cell fate, depending on their concentration.

4.2.2 Receptors and co-receptors

Receptors of Wnt pathway are called Frizzled. They are seven transmembrane pass receptors, with four *Frizzled* genes in Drosophila, and ten genes in mammals. They are classified as G-protein coupled receptors (Schulte, 2015) and their extracellular tail mediates interaction with Wnts through its cysteine-rich domain (CRD). Frizzled's cytoplasmic tail mediates interaction with Dishevelled (Dsh in fly, Dvl in mammal), through its KTxxxW motif and Dvl's PDZ motif (Umbhauer, 2000), and becomes densely phosphorylated upon this interaction (González-Sancho et al., 2004). Frizzleds are necessary for both Wnt canonical and non-canonical pathways making them a central molecule of the pathway (Ueno et al., 2013).
The co-receptors of the Wnt pathway are also important for signal processing. Arrow in Drosophila and LRP5/6 in mammals are single transmembrane pass proteins from LRP (Low density lipoprotein receptor related protein) family (Wehrli et al., 2000). LRP5/6 phosphorylation is essential for binding Axin (Tamai et al., 2004) and turning pathway to the activated state.

4.2.3 Destruction complex and β-catenin

The so-called Destruction complex is multiprotein complex, playing an important role in Wnt signalling. If Wg/Wnt ligand is not present, this protein assembly phosphorylates Armadillo/β-catenin, targeting it for subsequent degradation in the proteasome, thus limiting Wnt target gene expression.
The Complex consist of Axin, APC (Adenomatous polyposis coli), and two Ser/Thr kinases Shaggy (GSK3β in mammals) and Casein Kinase 1 (CK1α homologue in mammals). Axin acts as a scaffolding protein for assembly of the destruction complex (MacDonald et al., 2009) as well as a binding protein for Dvl, through Dvl's DIX domains (Fiedler et al., 2011; Kishida et al., 1999). Phosphorylation by CK1 serves as a priming phosphorylation (Liu et al., 2002), required for GSK3β dependent degradation of β-catenin (Pai et al., 1997). GSK3β phosphorylates β-catenin at Ser33, Ser37, generating binding sites for subsequent ubiquitination (Liu et al., 2002b).

β-catenin (Armadillo in Drosophila) is key molecule of Wnt/ β-catenin signalling pathway. Activated (hypo-phosphorylated) form of β-catenin accumulates in the cytoplasm and translocates to nuclei, driving transcription of Wnt target genes. Additionally, it does not only

act as a transcriptional coactivator and signal molecule, but also has a structural function in E-cadherin based adherens junctions (reviewed by Valenta, Hausmann, et al., 2012).

4.3 Mechanism of Wnt signal transduction

If present, the extracellular ligand glycoprotein Wingless (wg) binds to its seven transmembrane pass receptor Frizzled, which forms a complex with its co-receptor Arrow (LRP5/6 in mammals) (Wehrli et al., 2000). The complex is formed in Wg dependent manner, and the signal is transduced across plasma membrane to a recruited intracellular protein Dishevelled, which becomes activated (phosphorylated) (González-Sancho et al., 2004) and in that state is bound to Frizzled (Wong et al., 2003).

Dsh interacts with the Destruction complex (Stamos & Weis, 2013) and in active state inhibits kinases from phosphorylating N-terminal S/T residues of Armadillo (Arm), therefore targeting it for subsequent ubiquitination and degradation in the proteasome (Stamos & Weis, 2013).

Inactivation of the Destruction complex allows Arm to accumulate in the cytosol, leading to amplification of the signal, and translocation to the nuclei, where it replaces transcriptional inhibitors Groucho (Daniels & Weis, 2005) and CBP found in complex with Tcf/LEF-1 (T-cell factor) (Cavallo et al., 1998) in Drosophila known as Pangolin (Brunner et al., 1997) and converts it from a repressor to a coactivator, followed by expression of Wg target genes such as *Sp5*, *Axin2*, *Lef1* and others (Kramps et al., 2002) **(Fig. 3).**

Posttranslational modifications (PTMs) are an important part of the Wnt signalling pathway, in some cases essential for activation of the pathway (e.g. phosphorylation of Dsh, Armadillo).

Modification can either activate or inhibit pathway components or both. Protein modification by phosphorylation were reported in case of Armadillo, where N-terminal phosphorylation leads to ubiquitination and degradation, but C-terminal phosphorylation stabilizes it, positively regulating Armadillo‘s accumulation, leading to translocation to the nuclei, interaction with transcriptional factor Pangolin and transcription of target genes (Hino et al., 2005). In mammals, activity of N/C terminus regulates switch between signalling and cell adhesion functions (Bejsovec, 2013; Valenta, Gay, et al., 2012) .This mechanism is not conserved as in Drosophila this switch relies on interaction with Legless (Kramps et al., 2002**)**. Moreover, the pathway can be tuned by ubiquitination (as previously mentioned), glycosylation, palmitoylation, sumoylation and ADP – ribosylation (reviewed by Gao et al., 2014).

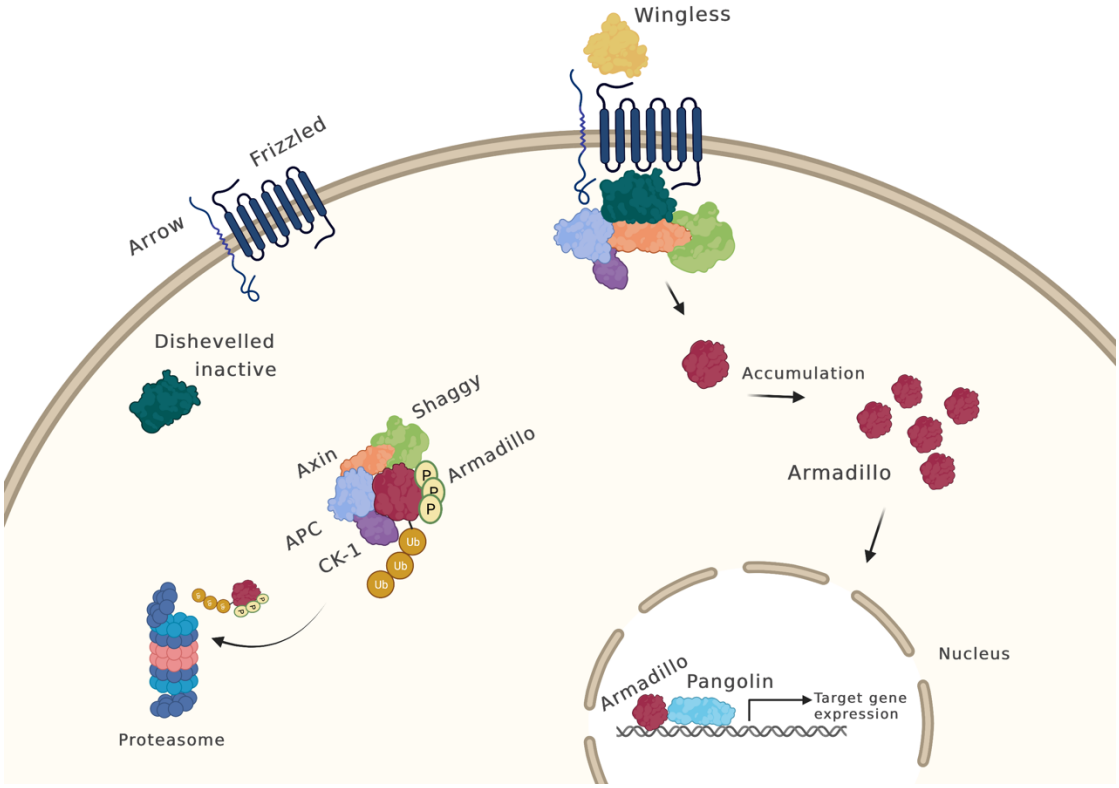


Figure 3. The Wnt/*β*-catenin signalling pathway in Drosophila. If present, Wg ligand binds to receptor-co-receptor complex, anchoring the Destruction complex to the membrane, allowing Armadillo to accumulate in the cytosol and translocate to the nuclei, inducing expression or target genes. When Wg ligand is not present, Dsh is in inactive state and destruction complex targets Armadillo for degradation in the proteasome. Created with BioRender.com

5.Notch signalling pathway

The Notch pathway was named after the mutant phenotype with characteristic nicks “notches” in the D. melanogaster’s wing blade (Dexter, 1914) caused by Notch gene haploinsufficiency (Kidd et al., 1986).

5.1 Function

Notch signalling plays a pivotal role in development, affecting the key cellular processes of cell proliferation (Baonza & Garcia-Bellido, 2000), differentiation (Cheng & Gabrilovich, 2008), apoptosis (Dang, 2012) and adult tissue homeostasis (Fre et al., 2011). It also has a role in

formation of boundaries through distinct cell types of various species (de Celis et al., 1996; del Barco Barrantes et al., 1999; Tossell et al., 2011). In Drosophila, Notch was shown to play an important role in wing development (de Celis & García-Bellido, 1994), development of sensory organs (Hartenstein & Posakony, 1990), fly gut homeostasis (Fre et al., 2011), segmentation of legs (de Celis et al., 1998), muscle development (Rusconi & Corbin, 1998), and oogenesis (Xu & Gridley, 2012).

In mammals, Notch controls liver development by regulating bile ducts development (Zong et al., 2009). It has a role in angiogenesis (Siekmann & Lawson, 2007), inner ear development (J. Murata et al., 2012), limb development (Francis et al., 2005) and epidermis (Panelos & Massi, 2009). Depending on cell type and nature of mutations (Wang et al., 2011; Weng et al., 2004), Notch can either function as an oncogene (Radtke & Raj, 2003) or as a tumour suppressor (Dotto, 2008).

Many Notch associated rare diseases – these are defined as those with prevalence of less than 1:2000 births emphasize the crucial role of Notch signalling during development. For example, loss of function (LoF) of N1 and DLL4 causes Adams-Oliver syndrome, LoF J1 and N2 leads to Alagille syndrome, N2 gain of function results in Hajdu-Cheney syndrome and N3 errors in CADASIL (Reviewed by Mašek & Andersson, 2017).

5.2 Ligands

Ligands are single-pass transmembrane proteins that interact directly with adjacent cell's receptors. In fly were two ligand molecules identified so far - Serrate and Delta - compared to five homologues Jagged1,2 (Jag1, 2), and Delta-like1,3,4 (Dll1,3,4) found in mammals. Interaction of Notch receptors with different ligands can active distinct targets and lead to diverse outputs, sometimes even to opposing effects on tissues (Gama-Norton et al., 2015; Nandagopal et al., 2018) Ligands can have different affinity for receptors, which might results in a different signalling strength (Andrawes et al., 2013). Sometimes role of ligand dwell in cis-inhibition of receptor, as it was reported with DLL3 (Bochter et al., 2022; Ladi et al., 2005) Further activity of ligands can be modified by different PTMs e.g. ubiquitination, glycosylation, endocytosis or proteolysis (D'Souza et al., 2008).

5.3 Receptors

Drosophila Notch protein (Notch1-4 homologue in mammals) is a heterodimeric single-pass transmembrane (TM) receptor, with length over two thousand amino acids, composed of large extracellular (ECD) and intracellular domain (ICD), connected noncovalently by their

transmembrane domains (Blaumueller et al., 1997). The extracellular domain consists of signal peptide (SP), 36-29 EGF-like repeats (Epidermal grow factor) (36 in dNotch and N1), Negative

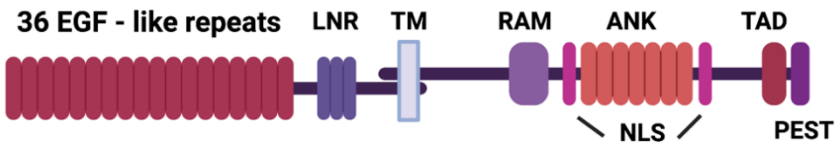


Figure 4. Scheme of Drosophila Notch receptor. Mammal Notch1 and 2 contain the same domains, Notch3 contains 34 EGF and lacks TAD domain, Notch4 contains 29 EGF and lacks TAD as well. EGF, epidermal growth factor; LNR, Lin-Notch repeats; TM, transmembrane domain; RAM, Rbp-associated molecule domain; NLS, nuclear localization signal; TAD, transactivation domain; PEST degradation domain rich in proline (P), glutamic acid (E), serine (S), and threonine (T).

regulatory region (NRR) composed of three LNR motifs (Lin-Notch repeats) and HD (heterodimerization domain), followed by transmembrane domain (TM). Intracellular domain consists of RAM (Rbp-associated molecule) domain, which upon binding of MAML mediates interaction with the CSL (CBF1/RBPJk, Su(H), Lag-1) family of transcriptional factors. It is followed by two NLS (Nuclear localization signal), seven ANK (Ankyrin repeats) which is bind MAML (Mastermind-like) and is generally responsible for protein-protein interactions (Mosavi et al., 2004), TAD (transactivation domain) interacting with general transcriptional factors, and PEST degradation domain responsible for degradation (Gordon et al., 2008) **(Fig.4).**

5.4 Mechanism

There are several proteolytic steps involved in Notch signalling. In mammals, functional Notch receptor is generated by (S1) cleavage from larger precursor protein in Golgi apparatus by Furin-like convertase (Logeat et al., 1998). Unlike in mammals, the majority of Drosophila Notch receptor appears to be “unprocessed”, and the S1 cleavage is not required for function nor the transport of Drosophila Notch to plasma membrane (Kidd & Lieber, 2002). Albeit comparison between Drosophila (dNotch) and mammalian Notch revealed, that lack of S1 cleavage in Drosophila leads to reduction of its transport and signalling strength (Lake et al., 2009).

Second cleavage (S2) is triggered by binding of ligand to the extracellular part of receptor which causes endocytosis of the ligand-generating conformational change and allowing Kuzbanian (Kuz) (ADAM10 in mammals) to access and cleave NECD (Kidd & Lieber,2002).

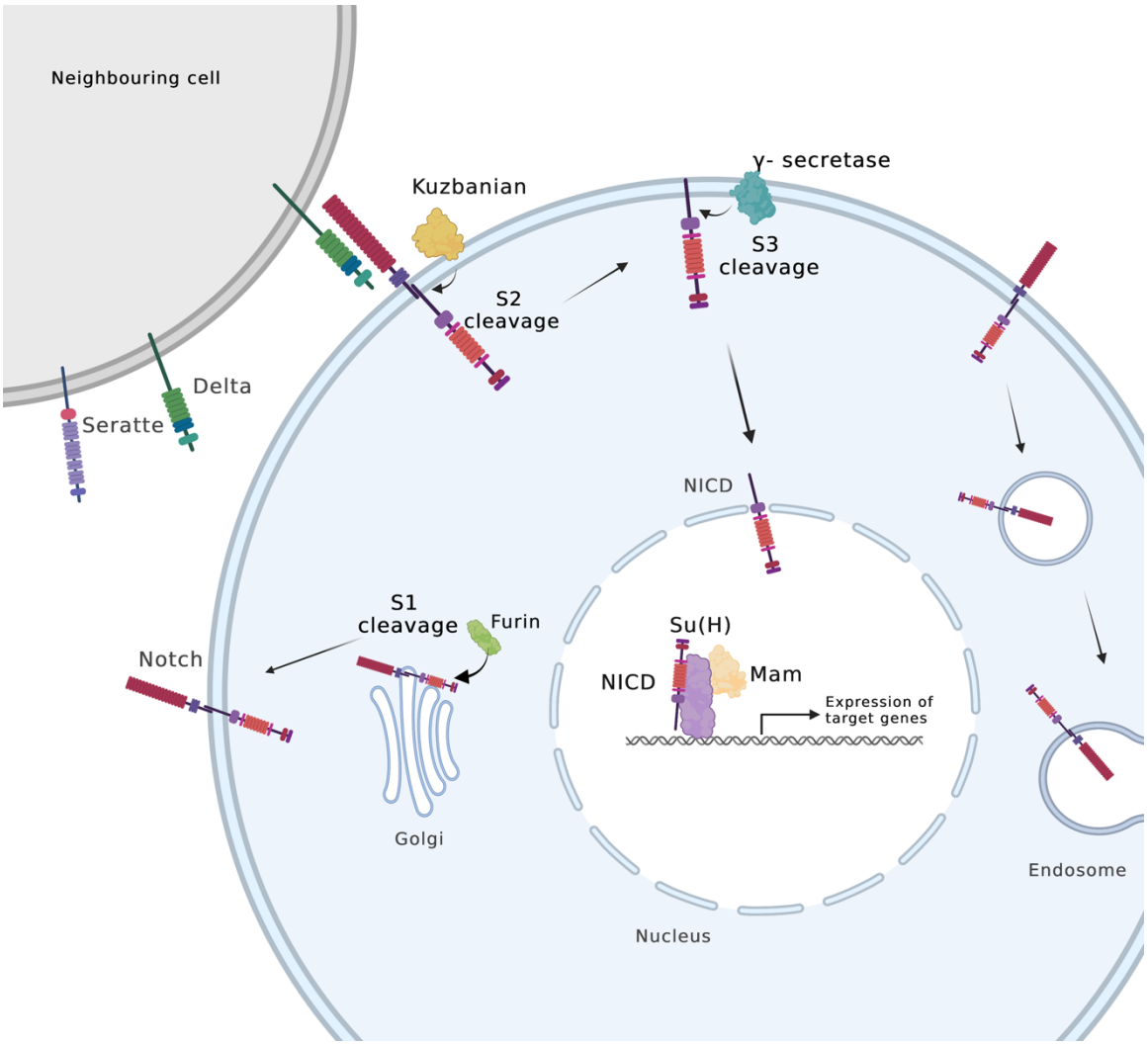


Figure 5. The Notch signalling pathway in Drosophila melanogaster. Upon ligand and receptor binding, Notch receptor undergoes subsequent cleavages (S2, S3) and translocation to the nuclei, leading to expression of target genes. In case of D. melanogaster S1 cleavage generating heterodimer is facultative. (Created with BioRender.com)

Finally, third cleavage (S3) mediated by γ-secretase complex (composed of four polypeptides, Presenilin, Nicastrin, Aph-1 and Pen-2 in Drosophila) (Strooper, 2003) releases NICD (Wolfe, 2006), which traffics to the nuclei where it replaces a transcriptional co-repressor Hairless, and functions as transcriptional trans-activator. In the nuclei, NICD forms a complex with coactivator protein Mam (MAML1-3 in mammals) (Kitagawa et al., 2001) and transcriptional repressor Suppressor Hairless (Su(H); RBPJk in mammals) (Fortini & Artavanis-Tsakonas, 1994). Specifically, Notch binds to CSL (CBF1/RBPJk, Su(H), Lag-1) via RAM and ANK domains and this interaction recruits Mam, which neither of them can bind alone (Nam et al., 2003), initiating transcription of downstream target genes e.g basic helix-loop-helix genes *E(spl)* (*Hes* in mammals) (Akazawa et al., 1995) (**Fig. 5).**

Linear signalling of the Notch pathway might seem simple, but it is quite the contrary.
Alterations can occur by means, such as PTMs, affecting receptors as well as ligands – ubiquitination - priming protein for degradation, phosphorylation - often used as crosstalk mechanism, having variable effects. Glycosylations are essential for Notch signalling as loss of O-fucosyltransferase leads to loss of Notch signalling in fly (Shi & Stanley, 2003) and embryonic lethality in mice (Shi & Stanley, 2003) and act as a substrate for Fringe glycosyltransferases, modulating ligand-receptor interactions (Haines & Irvine, 2003). Modifications can happen via coactivators and inhibitors that function at virtually every step of the pathway (reviewed by Bray, 2016).
Signalling can also be regulated by trafficking of receptors, resulting in receptor endocytosis, recyclation or lysosomal degradation. Indeed, the NICD degradation is key control element of the pathway, actively suppressed by proteosomal degradation, mediated by interaction E3 ligases, Deltex (Matsuno et al., 1995) (DTX in mammals) and Suppressor of Deltex Su(dx) in Drosophila (Cornell et al., 1999), Itch (Chastagner et al., 2008), WWP1 (Flasza et al., 2006), WWP2 (Jung et al., 2014), and adaptor protein Numb (McGill & McGlade, 2003).

6. Wg/Notch crosstalk in Drosophila melanogaster

6.1 What is crosstalk

Crosstalk is a signal-crossing of two or more pathways that are influenced by each other, allowing pathways to regulate components in a different pathway, utilize the same "molecular tools" to achieve different outcomes in different tissues, depending on the context. Metazoan signalling networks are complex and open to modulations, enabling signalling crosstalk to occur. As listed in the introduction components of one pathway can i) co-operate in regulations of transcriptional target genes; ii) employs its transcriptional target to affect signalling components of the other pathway, iii) crosstalk through direct protein-protein interactions, either having positive – activatory, or negative – inhibitory effect (Collu et al., 2014).

Over the time the discovered crosstalk between Notch and Wnt/Wg pathways became so prominent, that a common name "Wntch" has been proposed (Hayward et al., 2008). Together Wntch co-regulate processes in distinct tissues, e.g. wing development, mechanosensory development, gut homeostasis, myogenesis and are often mediating opposite cell fates.

This thesis focus is summarization of current Wntch direct crosstalk findings in Drosophila and mammals, determine their functional conservation and put them into context in relation to each other.

6.2 D. melanogaster developmental processes regulated by Wnt x Notch crosstalk

6.2.1 Wg/Notch in patterning of Drosophila melanogaster wing margin

A combination of direct and transcriptional crosstalk of Wg and Notch drives development and patterning of the wing from wing imaginal discs (Klein & Arias, 1999; Zecca & Struhl, 2007). Process starts by expression of both Notch ligands - Serrate in ventral part of wing imaginal discs (Fleming et al., 1997) and Delta in the dorsal part (Doherty et al., 1996). Ligands induce Notch activation, which in turn activates Wingless expression in boundary cells (Diaz-Benjumea & Cohen, 1995), where wing rim is later formed. Wg signals back, promoting Notch ligands expression along D/V boundary creating a positive feedback loop (de Celis & Bray, 1997).

Direct protein crosstalk mediates this association. Initial Wg expression is broad but as Wg signalling activates Dsh (which was shown to inhibit N) Notch activity is suppressed, refining Wg expression into sharp stripe at the boundary (mechanism of inhibition described below). Wingless ligand is secreted from the D/V boundary and patterns wing margin acting as morphogen, resulting in activation of the target genes in a concentration dependent manner,

with high expression close to the D/V boundary and lower expression further away (Neumann & Cohen, 1997; Zecca et al., 1996). In this case both ligands are necessary, as reduction in one of them results in wing nicks (Guruharsha et al., 2012). This haploinsufficient phenotype which gave name to pathways indicate dosage sensitivity of inductive signalling while forming wing margin (Salazar & Yamamoto, 2018).

6.2.2 Notch/Wg in sensory organ precursors (SOPs)

Notch affects development of peripheral nervous system in Drosophila, specifically mechanosensory bristle development on the notum though mechanism of lateral inhibition and cell fate specification (Simpson, 1990). Bristles are mechanoreceptors that allow fly to sense mechanical forces and reach coordinated movement. Bristle precursors originate from a group of ectodermal cells called proneural cluster, which all have the same potential to become Sensory organ precursor (SOP) and express *Achaete*/*Scute* genes. But only one cell can eventually remain SOP, and other will adopt secondary fate through Notch mediated lateral inhibition (Hartenstein & Posakony, 1990; Heitzler & Simpson, 1991). In this context Notch signalling leads to *E(spl)* expression which functions as a repressor and downregulates *Achaete/Scute* expression. When expressed, *Ac/Sc* promote Delta ligand expression (Kunisch et al., 1994).

Initially Notch maintains cells in a naïve state and *Ac/Sc* genes are not expressed. Wg signalling then overcomes this inhibition promoting a group of cells to "transition state", where cells express lower threshold of *Ac/Sc* (Phillips & Whittle, 1993), triggering on the Delta ligand expression and inducing Notch signalling in surrounding cells. Notch signalling then limits the number of cells maintaining *Ac/Sc* expression and potential to become SOP through this mechanism of Delta-mediated lateral inhibition. Cells that express the highest levels of Delta, signal to the surrounding cells and activation of Notch signalling in those cells prevents them from adopting SOP precursor fate (Kunisch et al., 1994).

Consequently, Notch ligand expression diminishes in surrounding cells which further reduces N signalling in the cell that will adopt SOP fate. Notch induces differentiation of SOP into distinct cell types by repeatedly achieving polarity. Differentiated bristle consists of four distinct cells – socket, shaft, sheath, and neuron. By polarization asymmetric divisions and uneven distribution of cell type-specific genes Notch ensure specification of distinct types. Cell with the highest threshold of Notch activation will become Socket cell, then one with lower threshold will become Shaft, then Sheath and cell with the lowest N threshold will became

neuron. Notch has a similar function on intestinal (van Es et al., 2005; Zecchini et al., 2005) and muscle development (Rushton et al., 1995).

6.3 Underlying molecular mechanisms of the D melanogaster Wnt x Notch crosstalk

6.3.1 Dishevelled as a negative regulator of Notch

Dsh was reported to bind and negatively regulate N through direct interaction by endocytosis and modulation of trafficking in SOP, where Dsh overexpression disrupted Notch mediated lateral inhibition and vein development (Axelrod et al., 1996), ectopic joint regulation (Capilla et al., 2012), and ommatidia photoreceptors R3/4 cell fate determination, where Frizzled is required to recruit Dsh to apical cell cortex, where it downregulates Notch in R3 photoreceptor cells (Strutt et al., 2002).

This interaction is ligand independent (Muñoz-Descalzo et al., 2010) and does not require cleavage of Notch from the membrane (Capilla et al., 2012). The Dsh binding site was mapped to its N terminus, containing the DIX domain (35-84aa) (Axelrod et al., 1996). DIX domain is also responsible for binding Axin (Kishida et al., 1999). Substitution of K46V and Q47A showed reduction of Dsh binding to Notch by 95%, therefore showing specificity for NICD while it is maintaining 65% of interactions with Axin (Capilla et al., 2012).

Dsh interacts with C terminal of the NICD (Axelrod et al., 1996) specifically within its PEST domain to last 114 amino acids (Ramain et al., 2001b). Contradicting report located Dsh interaction to positions 2125-2537 of N, located between ANK repeats and OPA (Glutamine-rich) repeats (Muñoz-Descalzo et al., 2010). Authors claim that possible discrepancy might reflect different 2-yeast hybrid system Y2H systems used (Gal4/LexA) in each experiment although they don't rule out possible existence of secondary interaction.

6.3.2 Nuclear crosstalk - Su(Dx) downregulates Notch upon activation by Dsh

Additionally, in the wing tissue, Dsh was reported to disinhibit Su(Dx) from its autoubiquitination activity leading to ubiquitination of Notch and reduction of nuclear signalling leading to defects in vein specification (Mund et al., 2015). Autoinhibition activity of Su(Dx) can only be disrupted by multiple PY-containing adaptors (Yao et al., 2019) and therefore this "unlocking" activity of Dsh is dependent on its ability to polymerize.

Dsh binds to Su(Dx) WW domains via PPxY motifs, but additional binding sites might be involved, as mutation of these motifs did not abolish Su(Dx) binding. (Mund et al., 2015) Possibly PPLP (suggested by Ramain et. al., 2001).

6.3.3 Multiple roles of Axin

Axin does not only act as a scaffolding protein for assembly of the Wnt Destruction complex but also can act as an anchor for Arm tuning down its activity rather than amount (Tolwinski et al., 2003), in a cooperative fashion with Notch. In 3rd instar imaginal discs, overexpression of Notch partially reduced effects of loss of Axin functions on Armadillo (Hayward et al., 2006). This inhibition is independent of Shaggy (Hayward et al., 2006; Tolwinski et al., 2003). Arrow was shown to recruit Axin to the membrane leading to its degradation so it cannot bind Armadillo (Tolwinski et al., 2003).

Additionally, Axin, together with APC (encoded by Apc1 and 2 in Drosophila), was shown to reduce the amount of Notch present on the cell surface in wing discs. It does so by modulation of ligand independent traffic, targeting Notch to degradation (Muñoz-Descalzo et al., 2011). Neither of these mechanisms of interactions was further investigated in Drosophila nor mammals and potential binding sites remain to be elusive.

6.3.4 Shaggy positively regulates Notch signalling

Although the evidence is limited, Shaggy kinase was reported to act downstream of in transduction of Notch inhibitory signal on *Ac/Sc* genes in determination of SOP fate by lateral inhibition (Ruel et al., 1993). It was shown to be facilitated by interaction with NICD, induced by Delta ligand binding (Simpson et al., 1993). Later studies identified Shaggy as a positive modulator of Notch (Foltz et al., 2002a). Mechanism and potential binding sites were not further investigated and negative effects of Shaggy kinase on dNotch were not reported, but we need to keep in mind that most of fly research were performed in wing discs, so perhaps negative effect mediated by Shaggy might have role elsewhere.

Further, Su(H) was shown to associate with phosphorylated Notch proteins, rather than unphosphorylated (Kidd et al., 1998). Hence, Shaggy kinase might one of the kinases phosphorylate Notch in specific contexts, prompting their association with Su(H). In addition, Notch Deltex dependent pathway appears to be positively regulated by Shaggy (Ramain et al., 2001b).

6.3.5 Notch buffers levels of Armadillo

Notch negatively regulates activity and amount of Arm in Su(H) independent manner by direct interaction (Hayward et al., 2005; Sanders et al., 2009). It does so via promoting endocytic trafficking of an activated (hypo-phosphorylated) form of Arm into endosomal compartments

where it is degraded (Hayward et al., 2005; Sanders et al., 2009), thereby regulating pool of Armadillo available for signalling (Sanders et al., 2009). Overexpressed Notch can suppress levels of Arm, cause by loss of Shaggy in 3rd instar wing discs (Hayward et al., 2005). This interaction is mediated by full-length (preceding S1 cleavage) Notch and has been mapped to its RAM-ANK domains (Hayward et al., 2005). Further research is needed, but the whole interaction might require presence of both Axin (see above) (Hayward et al., 2006) and Deltex (see below) (Acar et al., 2021; Ramain et al., 2001).

7. Wnt/Notch crosstalk in mammals

The *in vivo* evidence of the crosstalk in mammals (vertebrates in general) is very limited and majority of the data, if not stated otherwise, comes from the cell lines. Following chapter builds on the mechanisms discovered in *Drosophila* and aims at identification of the conserved and novel crosstalk mechanisms that emerged in vertebrates.

7.1 WWP2 regulates Notch levels upon Dvl binding

WWP2 (WW domain containing E3 ubiquitin protein ligase 2, homologue of Su(Dx)) was reported to inhibit Notch signalling in humans cell lines as well. WWP2 is adapting the same mechanism of Notch inhibition thus polymerization of Dvl2 is leading to "unlocking" autoinhibition of WWP2 (Mund et al., 2015) targeting its substrate for ubiquitination instead. In the case of NICD leading to mono-ubiquitination and eventual targeting for lysosomal degradation, happening prior to S3 cleavage (Jung et al., 2014). Mono-ubiquitination of the receptor can initiate endocytosis and subsequent localization to the lysosome (reviewed by Hicke, 1999). WWP2 regulates Notch by binding to different proline-rich sequences, mostly PPxY motifs. Notch3 contains PPPY motif in PEST domain (Jung et al., 2014) corresponding to Drosophila's PPxY motif required for direct binding. This negative regulation seemed to be more prominent when N3 was overexpressed in ovarian cancer cells (Jung et al., 2014).

Binding motifs in Notch1 and Notch2 were accessed as well. In Notch2 related LPAY motif was found (Mund et al., 2015), but no so in Notch1. This corresponds with finding that Itch does not directly interact with Notch1 (because N1 lacks Itch binding domain) and negative regulation happens through Numb adaptor protein (McGill et al., 2009).

Dvl mediated WWP2 ubiquitylation of NICDs did not reduce their levels, but their signalling activity. Auto-ubiquitinated WWP2 was reported to be stable (Mund et al., 2015), similar with WWP2 ubiquitinated Dvl2 in (Michael Graeb, PhD thesis, cited in Mund et al.,2015) so

conceivably the same could be happening with Notch. Both Su(Dx) and WWP2 showing the same pattern of behaviour demonstrates, how conserved the activities of mentioned ligases are.

7.2 Effects of GSK3β on Notch receptors

Until this day there have been conflicted reports about the role of GSK3B kinase on Notch receptors.

7.2.1 Effects of GSK3β on Notch1 receptor

In case of Notch1 there are conflicted reports of both positive and negative regulation of N1ICD activity by GSK3β. On one hand, GSK3β was shown to increase stability and transcriptional activity of Notch1 (Foltz et al., 2002a; Guha et al., 2011; Han et al., 2012), causing a positive regulation. On the other hand, it was reported to negatively regulate Notch1 as well (Jin, Kim, Oh, et al., 2009; Zheng & Conner, 2018).

One mechanism of activatory interaction between Notch1 and GSK3β is achieved through kinase binding to NICD S/T-P-S/T phosphorylation domains, leading to its protection from proteasomal degradation and promotion of Notch signalling. In this case GSK3β did not control nuclear localization of Notch and relation was more efficient in lower Notch concentrations (Foltz et al., 2002a). Second mechanism is through increase of NICD levels that can promote CBF1/RBPJk transactivation and enhance expression of downstream Notch target genes in vascular smooth muscle cell (vSMC) not only with Notch1, but also in Notch3 (Guha et al., 2011). GSK3β was shown to upregulate transcriptional activity of N1ICD by phosphorylation of S/T-P-S/T sites and its subsequent nuclear localization in a manner independent of classical NLS localization (Han et al., 2012).

When using mouse *Notch1* cDNA and human HEK293 cell lines, GSK3β bound to N1ICD residues T2122, T2124, S2126 and T2128 albeit it is unlikely, that all these residues would be phosphorylated simultaneously, due to phosphate group repulsions.

In this case nuclear localization and accumulation of N was not sufficient to increase its transcriptional activity and phosphorylation of S/T-P-S/T residues by GSK3β was shown to be required for N1ICD transcriptional activity as well as nuclear translocation (Han et al., 2012). The mechanism requires further investigation, as Foltz et al. (2002b) reported that GSK3β does not control nuclear localization of Notch.

Both receptor and N1ICD levels were reported to be downregulated by GSK3β (Jin, Kim, Oh, et al., 2009). This was proposed to happen via GSK3β-mediated regulation of N1 presence on

the plasma membrane, therefore limiting its availability for signalling by control of receptor transport decisions and inhibition of receptor recycling. (Zheng & Conner, 2018).
Hence, GSK3β can selectively regulate either activated Notch signalling by targeting N1ICD or it can prevent presence of Notch1 available for signalling by targeting full-length receptor, causing either positive or negative effects on Notch1.

7.2.2 GSK3β negatively regulates Notch2

In case of Notch2, GSK3β was reported to bind to a full-length protein as well as N2ICD. Although it was shown to bind more efficiently to full-length receptor, only nuclear forms of N1ICD and N2ICD were shown to be phosphorylated (Inglés-Esteve et al., 2001; Redmond et al., 2000, cited in Espinosa et al., 2003), showing only the processed Notch is a substrate for GSK3β phosphorylation. Thus, ligand binding does not induce only nuclear translocation, but phosphorylation as well.
Activated Notch is mostly nuclear, while GSK3β itself is mainly cytoplasmic, co-localization of the most GSK3β-N2ICD in the nuclei lead to proposal that GSK3β may translocate to the nuclei bound to activated Notch. N2ICD phosphorylation negatively modulates Notch driven transcription (Espinosa et al., 2003). GSK3β inactivates N2 by binding to its C terminal of ANK repeats, within S/T rich domain (STR) of Notch particularly T2068 and/or S2070, T2074, T2093. Importantly, phosphorylation of N2ICD is reversed in presence of Wnt1, simultaneously leading to upregulation of Notch dependent transcriptional promoter of *Hes1* (Espinosa et al., 2003). Role of GSK3β binding to full-length might be in regulation, by competition for binding sites with other coactivators affecting Notch accessibility and transcriptional levels in case of low Wnt levels.
Triggering the Notch signalling was reported to be further increased in a positive feedback-loop with GSK3β activity increase via phosphorylation of its Y216 and S9 to lesser extent (Brack et al., 2008). Intriguingly, dephosphorylation of these sites, is required for phosphorylation of β-catenin (Hagen et al., 2002) and was reported in mouse muscle, where high Notch and low Wnt3A levels switch to high Wnt signalling in muscle stem cell is essential for the muscle regeneration (Brack et al., 2008).

7.3 Notch and β-catenin interactions

7.3.1 Notch negatively regulates β-catenin

There are multiple reports of Notch-mediated inhibition of canonical Wnt signalling through alteration of free β-catenin levels. This could occur via membrane bound Notch(Acar et al., 2021; Hayward et al., 2005; Kwon et al., 2011; Ramain et al., 2001b) or cleaved NICD (Acar et al., 2021; Deregowski et al., 2006; Nicolas et al., 2003).

Membrane bound Notch1 was shown to physically associate with active (unphosphorylated) form of β-catenin and negatively regulate its accumulation, limiting available pool of β-catenin for the Wnt signalling activity in stem and colon cancer cells (Kwon et al., 2011). Interaction does not require cleavage of Notch from the membrane, but does require endocytic adaptor protein Numb (Kwon et al., 2011) (which negatively regulates Notch signalling by trafficking of tethered Notch into the lysosome for degradation), or Deltex (Acar et al., 2021; Ramain et al., 2001b). β-catenin interacts through its Arm domain (Jin, Kim, Ki, et al., 2009) with the RAM domain of N1ICD (not closely specified), but when PEST domain was removed, effects of Notch on β-cat were reduced, showing that PEST degradation domain plays role in this inhibitory mechanism on β-catenin (Kwon et al., 2011). Pursue by Kwon et al., 2011, was so far the strongest attempt to assess crosstalk in vivo in mice. Embryos with *Notch1* deletion showed expansion of cardiac progenitor cells (CPCs).

7.3.2 NICD limits β-catenin transcriptional activity

The NICD can inhibit β-catenin transcriptional activity as well (Acar et al., 2021; Deregowski et al., 2006; Nicolas et al., 2003) possibly to a greater extent than tethered form. It does so by forming complex with β-catenin within the nuclei and limiting β-catenin mediated transcription. Their interaction is direct (presumably using the same binding sites) and is further stabilized by RBPJk (Acar et al., 2021) which is nuclear restricted and cannot form complex with β-catenin itself.

Using these two mechanisms of limiting Wnt signalling can be serve as explanation in SOPs for Wnt inhibition. First round of inhibition occurs through membrane bound Notch and as this inhibition is frail, it can be exceeded by Wnt. Second round occurs through inhibiting β-catenin induced transcription and therefore Wnt is not capable of overcoming this inhibition (Acar et al., 2021). And is released only when levels of NICD decrease as a result of the lateral inhibition.

7.3.3 β-catenin enhances transcriptional activity of Notch1 target genes

Jin and colleagues reported that β-catenin can increase transcriptional activity of Notch on *Hes1* and *CSL* promoter approximately threefold and activate N1 dependent gene expression by direct interaction with NICD (Jin, Kim, Ki, et al., 2009). RBPJk is also present within this complex (Yamamizu et al., 2010), most probably stabilizing this interaction like it was reported to do so in inhibitory crosstalk of nuclear β-catenin/NICD complex (Acar et al., 2021). The NICD/β-catenin/RBPJk complex was shown to co-regulate expression of arterial genes in endothelial cells (ECs) (Yamamizu et al., 2010).

The same work from Jin and colleagues also claimed that LEF-1 can negatively diminish the β-catenin/NICD complex interaction as it competes for the same domain of β-cat as Notch, suggesting β-catenin can act as a switching molecule between the classical TCF/LEF-1 mediated and NICD mediated transcription. This interaction can affect osteogenesis to a different extent, depending on the β-catenin's binding partner (Jin, Kim, Ki, et al., 2009). This is consistent with opposing inhibitory effects of NICD on Wnt/β-cat signalling in cells of osteoblastic lineage (Deregowski et al., 2006).

7.4 N1ICD as a LEF-1 coactivator

N1ICD (and N2ICD to lesser extent) was shown to stimulate LEF-1 activity through direct interaction of Notch C terminal of transactivation domain (TAD) and high-mobility group (HMG) domain of LEF-1. N3 does not have such an effect. The interaction is not mediated by Wnt components and uses different promoters than β-catenin activated LEF-1 and is likely to be seen when levels of NICD are high and functionally surpass CSL levels (Ross et al., 2001).

7.5 Dishevelled as an inhibitor of CSL

Dishevelled was shown to limit Notch signalling by interaction with CSL and reduction of its levels within the active transcription factor pool. This alteration does not require NICD as Dvl binds directly to CSL, and as a result NICD then fails to form complex with CSLs and MAML, so it cannot drive Notch target gene transcription. Dishevelled N-terminal DIX and PDZ motifs are required for this interaction. This was shown both in Drosophila where Dsh inhibited Su(H), but also in *in vivo* assay in Xenopus embryos using rescue experiments with human mRNA, showing Dvl inhibited RBPJk bypassing all 4 Notch receptors (Collu et al., 2012).

8. Concluding discussion

This thesis summarizes and compares findings of direct Notch/Wnt crosstalk in Drosophila melanogaster and mammals to date.

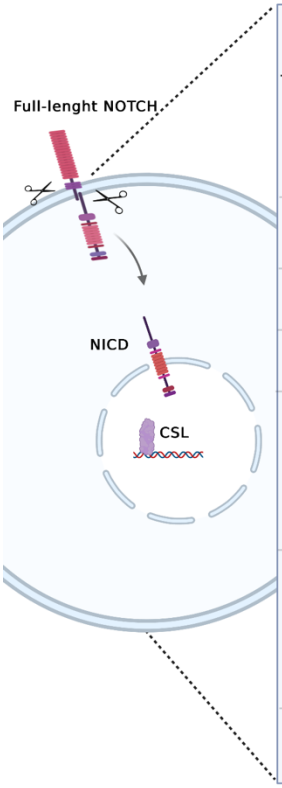
Both pathways were discovered in Drosophila by identification of mutant phenotypes and were extensively studied since then, contributing as a base of our present knowledge about pathways, making Drosophila a great basis for analysis of the two pathway's crosstalk. Acknowledging both invertebrate and vertebrate model organisms, the two pathways - Wnt and Notch, are crucial for embryonic development and homeostasis and play distinct roles in diverse tissues.

Bearing in mind the pre-duplication state of the whole genome, that vertebrate lineage underwent twice (2WGDs), pathways in fly have relatively modest number of components, when only one Notch receptor, and lesser number of Wnt ligand is present i.a., it is natural that fine-tuning of these pathways is occurring, when reaching multiple outputs. It is thus reasonable to use fly as a first step of accessing crosstalk complexity. In mammals the Notch receptors are expressed in different tissues, but can be co-expressed, having a bigger, more dynamic range of interactions with the increased number of Wnt components, leading to more intricate crosstalk that are only poorly understood up to date. There are many reports of Drosophila's Notch and mammalian Notch1,2 and 3 involved in direct crosstalk with Wnt components. The reason for why the involvement of Notch4 in crosstalk has not been studied, is because N4 was reported to lack the signalling capacity and play only inhibitory role in relation to Notch1 (James et al., 2014).

While multiple crosstalk modes were charted, several molecules are likely to play more significant role than others. Wnt's Dishevelled, components of the Destruction complex, and β-catenin interact with full-length, cleaved, and nuclear NICD **(Fig.6).**

The role of Dishevelled in Notch pathway is inhibitory, contrasting with its role in activation of Wnt signalling. In Drosophila, Dishevelled was reported to negatively regulate tethered Notch by endocytosis, interacting with the Notch last 114 amino acids (Ramain et al., 2001b). Further investigation is needed as Muñoz-Descalzo et al. (2010) located this interaction to 2125-2537aa of Notch. Additionaly, Dsh negatively regulates NICD by ubiquitination and lysosomal degradation through E3 ligase Suppressor of Deltex (Mund et al., 2015), using the same mechanism, that was further investigated in human cells where Dvl inhibits Notch through Su(Dx) homologue WWP2. WWP2 binds to Notch3 PPPY motif (Jung et al., 2014) and LPAY in Notch2 (Mund et al., 2015). In Notch1 no motif was found (Jung et al., 2014).

Alternative way of inhibition might be through NICD stabilization by WWP2, as the same is happening for WWP2 itself (Mund et al., 2015) and with less efficiency for Dvl (Michael Graeb, PhD thesis, in Mund et al.,2015), this could explain the reduction of Notch signalling activity. Additionaly, Dvl can limit Notch signalling, by competing for interaction with CSL. This affects Drosophila Notch as well as all mammalian receptors (Collu et al., 2012). If Dishevelled in these acts alone or in synergy with other Wnt components, remains to be solved.



Drosphila's interacting Wnt protein	*BS on Drosophila Notch*	*Mammal's interacting Wnt protein*	*BS on mammal Notch*	*mechanism*	*Drosophila*	*mammals*
Dsh	last 114aa in PEST domain	Dvl	n.t.	negative- increased Notch endocytosis	✓	–
	n.t.		n.t.	negative- competing with Notch for CSL	–	✓
Su(Dx)	PPxY motifs	WWP2	none in N1 LPAY in N2 PPPY in N3	lysosomal degradation of Notch caused by mono-ubiquitination	✓	✓
Axin	n.t.	Axin	n.t.	downregulation of Arm with Notch	✓	–
APC	n.t.	APC	n.t.	degradation of full-lenght Notch with Axin	✓	–
Shaggy	n.t.	GSK3β	T2122, T2124 S2126, T2128 in murine NOTCH1	positive on dN and N1ICD	✓	✓
	n.t.		T2068, S2070, T2074, T2093 in NOTCH2	negative on full-lenght N1,2 and N1,2 ICDs	–	✓
Armadillo	RAM-ANK domains	β-catenin	RAM domain	degradation of β-cat by full-length dNotch and N1	✓	✓
	n.t.		RAM domain	negative- N1ICD limits β-catenin transcriptional activity	–	✓
Pangolin	n.t.	LEF-1	N1 and N2 TAD domains	positive- N1 and 2 ICDs stimulate LEF-1 activity	–	✓

Figure 6. Table comparing functional conversation of crosstalk in Drosophila and mammals. Table listing Drosophila and mammal components showed to interact or be influenced by Notch in the crosstalk. Binding sites are listed, in case if they were identified, *n.t.* stands for *not tested*, when direct interaction or identification of direct binding sites was not performed. Last two columns indicate if mechanism was assessed in organism, if not it is indicated by dash. Created with BioRender.com

Axin, as a scaffold protein for Wnt signalling has multiple roles in crosstalk with Notch. In Drosophila's 3rd instar imaginal wing discs Axin can cooperate with Notch in downregulation of Armadillo independent of Shaggy kinase, acting as an anchor (Hayward et al., 2006). Further Axin and APC in Drosophila reduced number of Notch present on the cell surface of wing discs. It does so by ligand independent trafficking leading to degradation. APC is not enough for this inhibition *per se* and only acts with Axin in this manner (Muñoz-Descalzo et al., 2011). Binding sites of these inhibitions were not investigated, and this issue was not accessed in mammals.

Neither of these mechanisms were investigated further in fly nor mammals and binding sites remain to be elusive.

Role of another Destruction's complex component Shaggy kinase was reported to have a positive influence on Drosophila's NICD (Foltz et al., 2002a). This interaction is specifically induced by Delta ligand binding (Simpson et al., 1993), playing role in transduction of lateral inhibitory signal. Direct binding sites of this mechanism were not investigated.

There are no reports on nuclear crosstalk between Shaggy and NICD, but this thesis pointed at mechanism functioning in mammals, that might also have a role in Drosophila (discussed below). Reports on homologue GSK3β are conflicted, as it has both positive and negative effects on Notch1. In the case of Notch2 there are reports of negative regulations only. This may happen because GSK3β does not have a positive effect on Notch2 specifically, or regulations were not assessed in the physiological context of its function. Phosphorylation by GSK3β can increase the stability of Notch1, protecting it from degradation and promoting its signalling strength in lower Notch concentrations. It does so by direct binding and phosphorylating S/T-P-S/T sites (Foltz et al., 2002a).

The Second mechanism of promotion is achieved by phosphorylation of T2122, T2124, S2126 and T2128 residues, that in mice lead to nuclear localization of Notch1 (independent of the nuclear localization signal), and enhanced expression of downstream Notch target genes (Han et al., 2012). The same was reported in Notch3, although with only moderate effect on transcription (Guha et al., 2011). This mechanism might distinguish between two types of positive regulation, as each of them might have different intensity of promoting Notch induced expression and might be put into effect in different contexts.

On the other hand, GSK3β seems to have negative effects on full-length and cleaved Notch1 as well. It regulates levels of full-length receptor presence on the cell surface, thus limiting its availability for signalling (Jin, Kim, Oh, et al., 2009). GSK3β might negatively regulate full-length Notch2 without phosphorylation by competing for binding sites with Notch coactivators when Wnt levels are low.

When is Notch receptor cleaved from the membrane, GSK3β can phosphorylate T2068, S2070, T2074 and T2093 sites in NICD ANK domain (Espinosa et al., 2003) and lead to inhibition of Notch2 transcriptional activity on target genes. Interestingly, phosphorylation of N2 is reversed in presence of Wnt1 (Espinosa et al., 2003), or Wnt3A (Brack et al., 2008) leading to upregulation of Notch dependent transcriptional promoter of *Hes1* (Espinosa et al., 2003).

Possibly via Wnt induced phosphorylation of GSK3β's S9 and Y216 (Brack et al., 2008). The dephosphorylation of GSK3β is tightly linked to phosphorylation of β-catenin and is a

prerequisite for correct muscle regeneration where the switch from Notch to Wnt signalling is required for the re-activation of the muscle progenitors (Brack et al., 2008), adding to the increased variety of fine-tuning mechanism in more complex organisms.

Positive effects that Shaggy kinase has on Notch receptor are corresponding well to the effects that GSK3β has on mammalian Notch receptors. Negative effects of Shaggy kinase on dNotch were not reported, perhaps they might play role in other tissues than wing discs, where the majority of fly research on Wnt x Notch crosstalk was performed or might be due to a limited function in Drosophila, where the effect of Shaggy kinase on Notch are more constrained. In mammals, GSK3β function can be exceeded beyond its role in Wnt signalling, as similar increased variety was shown with E3 ligases (Revici et al., 2022).

Conflicting reports might depend on numerous proteins, whose involvement may change the nature of GSK3β effects and might lead to suppression of Notch signalling in contexts where it is needed. Or they might reflect each receptor specificity. In this instance, further research on the context where GSK3β acts alone or in synergy with other components is needed. Maybe these outputs can be regulated by interaction with NEDD4 ligases as they were shown to interact with other Thr/Ser kinases (An et al., 2014).

Role of CK1 (CK1α in mammals), as a last component of Destruction complex, was not examined to date.

Crosstalk works in both ways as a central molecule of Wnt signalling, Armadillo in Drosophila and β-catenin in mammals, is affected by crosstalk with Notch as well. Full-length dNotch promotes endocytic trafficking and degradation of activated Armadillo (Hayward et al., 2005; Sanders et al., 2009). Notch binds within its RAM-ANK domains (Hayward et al., 2005), possibly Axin (Hayward et al., 2006; Kim et al., 2013) and Deltex (Ramain et al., 2001a) might play its parts in this process, but it needs to be further tested. It was proposed that dNotch targets the pool of Armadillo in epithelial cells or near adherens junction rather than Shaggy sensitive pool (Sanders et al., 2009). Similar results were reported for N1, activation of which destabilised adherens junctions by inhibition of E-cadherin in NSCLC cells (non-small cell lung cancer) through Snail family of transcriptional repressors. It further inhibited the expression of β-catenin as a way to compensate for increased β-catenin levels caused by E-cadherin decrease (Kim et al., 2013) .Mammalian Notch1 affects β-catenin mediated Wnt signalling similarly way. It can do so via cleaved N1ICD, or membrane-bound Notch. In both cases Notch targets active (hypo-phosphorylated) β-catenin. The β-catenin's accumulation after Wnt accumulation is negatively regulated by simultaneous full-length Notch, possibly via direct interaction of the

two through N1ICD RAM domain (Kwon et al., 2011). Interaction of N1ICD with β-catenin also occurs within the nuclei (Acar et al., 2021; Deregowski et al., 2006; Nicolas et al., 2003) and is more efficient as it limits transcription induced by β-catenin. This interaction is further stabilized by RBPJk (Acar et al., 2021) which is restricted to the nuclei, and most probably, both mechanisms are using the same Notch binding sites. The scheme of combined double inhibition could be used as an explanation for SOP, where first round of inhibition could occur through membrane-bound Notch, and as this inhibition is frail, it can be exceeded by Wnt. The second round occurs through inhibition of Armadillo induced transcription and therefore Wnt is not capable of overcoming this inhibition.

The β-catenin can increase transcriptional activity of N1 on promoter of *Hes1* and *CSL* and activate gene expression by direct interaction with NICD (Jin, Kim, Ki, et al., 2009).

Additionaly, N1 and N2 ICDs were shown to stimulate LEF-1 activity through direct interaction of their TAD domain with LEF's HMG. This uses different promoters than LEF-1 activation by β-catenin and was more likely to be seen in case of high levels of Notch, when levels of NICDs functionally surpass CSL available (Ross et al., 2001).

This thesis pinpointed the conservation of direct crosstalk mechanisms from fly to mammals. The crosstalk components are targeting or targeted by, either activated NICDs or tethered Notch receptors, often with augmented functions and increased variety of fine-tuning, altered by other factors. Seeing the extent of conservation of these mechanisms, it is rather surprising that those two pathways are in most cases studied separately, especially concerning human diseases. In general, Wnt x Notch direct crosstalk implies negative effects on components of the other pathway, driving a rapid switch between them. Needless to say, many components taking part in Wnt signal transduction, were not accounted nor tested as possible candidates for crosstalk with Notch e.g., CK1, Arrow (Lrp5/6), Rnf43 and Znfr3. The last three listed do interact with Dishevelled so they may act in synergy when regulating Notch.

Crosstalk of Notch and Wnt plays a role in many various tissues and regulations are context-dependent, often being influenced by other pathway's components or factors under various circumstances. Further research of situations where Wnt/Notch components act alone or in the synergy with other components is needed, as many more crosstalk between pathways have been reported. For example, crosstalk between both Notch and Wnt with components of Hedgehog pathway, in which there are reports on the presence of more pathways involved like the TGF-β signalling pathway. Therefore, it will be interesting to further access crosstalk mechanism in various pathways and see how they influence each other in development and homeostasis.

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