Dissertation

Identification of candidate Notch targets in Hydra vulgaris

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Summary

The Notch signalling pathway plays major roles in several developmental processes including embryogenesis and maintenance of adult tissue homeostasis. Conserved core components of the Notch pathway have been identified in the simple cnidarian *Hydra vulgaris*. Previous experiments have shown the successful application of the chemical DAPT in Hydra, which blocks the Notch signaling pathway by preventing the translocation of the intracellular domain of the Notch receptor (NICD) into the nucleus. This inhibition resulted in several severe phenotypical changes in Hydra polyps.

This study focused on the identification of candidate Notch targets in order to unravel the molecular mechanism underlying the observed phenotypes. A comparative transcriptome analysis was performed and the data of the RefSeq experiment of DAPT-treated and control animals were mapped to data from the recent *Hydra* single cell analysis. Differential expression analysis showed that especially cells of the nematoblast lineage were affected by Notch inhibition. About 50% of the identified Notch-responsive genes were found to be expressed in nematoblasts or nematocytes including *HyPOU4*, *NOWA*, *Spinalin*, *CnASH* and several nematoblast-specific genes. The analysis emphasized that for nematoblast development, Notch signaling becomes important when nematoblasts undergo the final mitosis to differentiate into nematocytes. Known genes specific for proliferating nematoblasts, were not affected by Notch inhibition. Since the majority of the Notch-responsive nematoblast genes were down-regulated upon Notch inhibition, it can be concluded that Notch drives nematoblast differentiation.

In addition, cluster analysis showed several small clusters of Notch-responsive genes, that were specifically expressed in distinct cell types, including endodermal or ectodermal epithelial cells or both, tentacle genes, both ectodermal like battery cells and endodermal, head cells and foot cells. Notch-affected head specific genes included Hy*Alx, CnOtx, Cngoosecoid* and Hy*Wnt7*.

Interestingly, Notch-responsive head genes were mainly down-regulated whereas Notch-responsive genes in the foot and basal disc were mainly up-regulated, suggesting that Notch plays opposing roles in these two Hydra extremities.

Zusammenfassung

Der Notch-Signalweg spielt eine wichtige Rolle bei verschiedenen Prozessen der Embryonalentwicklung und für die Erhaltung der zellulären der Homöostase. Konservierte Kernkomponenten des Notch-Signalwegs wurden in dem einfachen Nesseltier *Hydra vulgaris* identifiziert. Frühere Experimente haben die erfolgreiche Anwendung des Presenilininhibtors DAPT in Hydra gezeigt, der den Notch-Signalweg blockiert, indem er die Translokation der intrazellulären Domäne des Notch-Rezeptors in den Zellkern verhindert. Diese Hemmung führte zu einer Reihe von phänotypischen Veränderungen bei den Polypen.

Diese Studie befasst sich mit der Identifizierung von Notch-Zielgenen, um den molekularen Mechanismus, der den beobachteten Phänotypen zugrunde liegt, zu entschlüsseln. Es wurde eine vergleichende Transkriptomanalyse durchgeführt. Die Daten dieses RefSeq-Experiments von DAPT-behandelten und Kontrolltieren wurden den Daten der jüngsten Hydra-Einzelzellanalyse zugeordnet. Die differenzielle Expressionsanalyse zeigte, dass vor allem Zellen der Nematoblasten-Linie von der Notch-Inhibition betroffen waren. Etwa 50 % der gefundenen potenziellen Notch-Zielgene waren in Nematoblasten oder Nematozyten exprimiert, darunter *HyPOU4, NOWA, Spinalin, CnASH* und mehrere nematoblastenspezifische Gene. Diese Analyse unterstreicht, dass der Notch-Signalweg für die Entwicklung der Nematoblasten wichtig wird, wenn die Nematoblasten die letzte Mitose durchlaufen, um zu Nematozyten zu differenzieren. Bekannte Gene, die spezifisch in proliferierenden Nematoblasten exprimiert werden, waren von der Notch-Hemmung nicht betroffen. Da die Mehrzahl der Gene, die auf Notch reagieren, durch die Hemmung von Notch herunterreguliert wurden, kann man daraus schließen, dass Notch die Differenzierung von Nematoblasten steuert.

Darüber hinaus zeigte die durchgeführte Clusteranalyse mehrere kleine Cluster von auf Notch reagierenden Genen, die spezifisch in verschiedenen Zelltypen exprimiert wurden, z.B. in endodermalen und/oder ektodermalen Epithelzellen , sowohl ektodermale als auch endodermale Tentakelzellen, Epithelzellen im Kopf und im Fuß. Zu den von der Notch beeinflussten Kopfgenen gehörten Hy*Alx, CnOtx,CnGsc (Goosecoid)* und Hy*Wnt7*.

Interessanterweise wurden Notch-regulierte Gene im Kopf durch DAPT hauptsächlich herunterreguliert, während Notch-regulierte Gene im Fuß und in der Basalscheibe hauptsächlich hochreguliert wurden, was darauf hindeutet, dass Notch in diesen beiden Extremitäten von Hydra gegensätzliche Rollen spielt.

1. Introduction

1.1 Notch signaling pathway

The Notch signaling pathway plays important roles in apoptosis, cell proliferation, differentiation, embryonic development, homeostasis and cell fate decision (Borggrefe and Oswald., 2009). It is therefore not surprising that alteration of this pathway leads to several diseases including cancer. In fact, Notch was indeed found to be involved in the development of several cancer types including Leukemia, Breast cancer, colon cancer, medulloblastoma, melanoma and pancreatic cancer (Bray, 2006).

1.1.1 Core components

The first Notch studies, which also provided the name of the pathway originate from the year 1914 when a mutant of *Drosophila melanogaster* was discovered that displayed notched wings (Dexter J, 1914). The studies continued in this species to reveal more components of this pathway (Knust and Campos-Ortega, 1989). Meanwhile the core components of the Notch signal transduction cascade have been found to be conserved in many invertebrate and vertebrate species. Astonishing is the simplicity of the core architecture of the pathway in the light of the numerous cell fate decisions in a large array of cell types, for which Notch is responsible.

Although some differences exist in the notch core components between species, the basic model is common for all animals from simple metazoans to humans. The differences include the number of paralogues for receptor and ligands and the number of components that are involved in the transcription machinery. In general, the main Notch core components include a transmembrane Notch receptor and transmembrane ligands of the classes Delta and Serrate/Jagged. Maturation of the Notch receptor includes its cleavage by a Furin-like convertase (S1 cleavage) in the Golgi. Thereafter the receptor is translocated to the cell surface. The extracellular part of the Notch receptors is usually composed of about 30 EGF repeats, several cysteine rich LIN repeats and a linker between the transmembrane region and the intracellular domain. The intracellular region of the receptor includes a RAM domain, several ANK repeats and a PEST domain. Notch ligands are characterized by an extracellular DSL domain at the N-terminus, which is essential for binding to the Notch receptor. This domain is followed by several EGF repeats. Unlike Delta ligand, ligands of the class

Serrate/Jagged contain an additional extracellular cysteine rich (CR) domain (reviewed by (Borggrefe and Oswald., 2009)).

Activation of the notch pathway starts with two enzymatic cleavages of the receptor at two different sites. The first of those cleavages (S2 cleavage) (Figure 1) is promoted by ligand binding and involves members of the ADAM family of metalloproteases (Black and White, 1998; Brou et al., 2000). Ligand binding opens the ADAM cleavage site that is normally hidden in the structure to prevent premature receptor activation. ADAM cleavage then triggers another cleavage (S3 cleavage) (Figure 1), which is mediated by the enzyme complex gamma-secretase, containing presenilin, nicastrin, PEN2 and APH1. The cleavage by ADAM occurs extracellularly at a position just before the transmembrane domain (Black and white, 1998; Brou et al., 2000), whereas the presenilin cleavage is performed inside the transmembrane domain, thereby releasing the Notch intracellular domain (NICD), which is composed of the entire intracellular region of the Notch receptor (Mumm et al., 2000). This enables the NICD to translocate into the nucleus where it regulates gene expression (Figure 1).

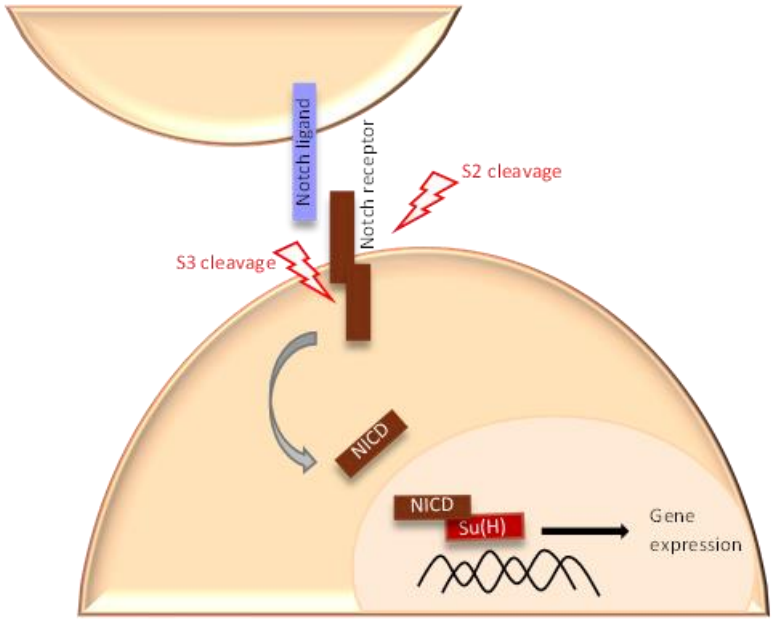

Figure 1: Schematic representation of the Notch core components

1.1.2 NICD as transcriptional activator

In the nucleus, NICD forms a complex with CSL, a DNA-binding protein named CBF-1 in mammals, Su(H) in Drosophila and LAG-1 in C. elegans, and which is highly conserved through all animal species. On its own, NICD is not able to bind DNA. CSL normally acts as a repressor

and occupies the promoter site of Notch targets. Its interaction with NICD attracts co-activators including Mastermind, whereafter the whole complex functions as an activator, promoting Notch-dependent transcription of target genes (Wilson and Kovall, 2006; Petcherski and Kimble, 2000). Repression of Notch targets by CSL is mediated by recruiting factors including SKIP (Ski-interacting protein)(Zhou et al., 2000), Hairless and Groucho in *Drosophila melanogaster* (Nagel et al., 2005) and SMRT, SHARP and CtBP in mammalian species (Oswald et al., 2005).

The switch that is caused by the binding of NICD to CSL is a stepwise process in which molecular changes cause dissociation of repressors and association of activators. One of the first steps upon binding NICD to CSL is a conformational change promoting a higher binding affinity between the Notch ANK (ankyrin) repeats and CSL. This allows Mastermind to bind the NCID/CSL complex whereafter other activators are attracted including chromatin remodeling factors and histone acetyltransferases like p300 (Wallberg et al., 2002). For a long time it was thought that CSL is stably bound to DNA and that it is the binding of NICD which turns the switch from repression to activation. However, this theory has been debated in the last years and a recent model suggests a more dynamic CSL-DNA binding (reviewed by (Falo-Sanjuan and Bray, 2019)). Findings that support this theory include the low DNA binding affinity of CSL and the short residence of CSL at its binding site in absence of NICD. The presence of NICD increases this residence time by 10 fold (Falo-Sanjuan and Bray, 2019).

It is still unknown how NICD initiates transcription, especially with such extremely low nuclear concentrations that are detectable after NICD nuclear translocation. Different hypotheses have been suggested. One of them focuses on the ability of NICD to determine the stability and size of polymerase II clusters. It has been shown that the number of transcribed mRNA of a target gene is correlated with the lifetime of polymerase II clusters and thus with their "stability" (reviewed by (Falo-Sanjuan and Bray, 2019)).

The outcome of Notch activation is highly dependent on the nuclear context at the moment of NICD nuclear entrance. This results in great differences in cellular Notch responses, varying from cell proliferation to apoptosis (Radtke and Raj, 2003). Thus, the Notch signalling outcome depends on the cell-type and developmental stage. A study in *Drosophila* has shown that two different cells types share only very few Notch targets (Krejcí et al., 2009). In case of two possible targets that can be activated, the concentration of NICD seems to be the triggering

factor, indicating a threshold system. This was demonstrated in *Drosophila* where different NICD concentration thresholds activated different enhancers (Falo-Sanjuan et al., 2019). It has also been suggested that the selection of Notch targets depends on the behavior of the Notch signal, which can be pulsed or sustained (Nandagopal et al., 2018).

1.1.3 Notch targets

Despite its broad transcriptional outcome, only a few conserved direct Notch targets have been identified. This is probably due to the fact, that Notch has different targets in different tissues and species and only a few targets are conserved through species and tissues (Bray and Bernard, 2010). One of the first discovered and also the best studied targets of the Notch signaling pathway are the bHLH genes of the HES/HEY family (Fischer and Gessler, 2007). This basic-helix-loop-helix (bHLH) superfamily of proteins comprises transcription factors, which are found not only in metazoans, but also in plants and fungi (Jones, 2004). The highly conserved bHLH region of these transcription factor is composed of a **basic** DNA-binding region, followed by an α-**helix**, a variable **loop** region and another α-**helix** (Jones, 2004). These proteins usually also contain other domains that are necessary for their function. bHLH proteins are mainly involved in metabolic processes in *Saccharomyces cerevisiae*, whereas in metazoans and in plants they play a role in development and cell cycle regulation (Massari and Murre, 2000). In *Drosophila*, the HES/HEY related Hairy and Encancer-of-split regulate segmentation, myogenesis and neurogenesis (Fisher and Caudy, 1998). In mammals several HES/HEY proteins have been identified in various tissues with roles in embryonic and adult development (Fischer and Gessler, 2007).

Other direct Notch targets identified so far include *myc*, which was found to be a Notch target in *Drosophila* and some cancer types and which plays a role in proliferation, and CDK5 and CyclinD, which are also involved in cell proliferation (reviewed by (Bray and Bernard, 2010)). In addition, members of the apoptosis machinery have also been reported as Notch targets, including *p21, reaper and Wrinkeled/hid*. Also several Notch pathway genes were assigned as Notch targets, like DELTEX and NRARP and Notch itself, suggesting autoregulation in this pathway. In addition the MAPK and Ras pathways have been shown to be targeted by Notch (reviewed by (Bray and Bernard, 2010)).

1.2 Notch signaling in *Hydra*

1.2.1 Discovery of Notch in *Hydra*

The Notch pathway was found conserved in the cnidaria *Hydra vulgaris*. Cnidarians form a sister clade to bilaterian animals and arose very early in evolution. Among the cnidarians are the hydrozoans. An intensively and well-studied hydrozoan is the simple freshwater polyp *Hydra vulgaris*. *Hydra* has a single oral-aboral axis with a mouth structure on the oral side and a foot structure at the aboral end (Figure 2). The tip of the oral side is called the hypostome and functions as a mouth by opening for prey ingestion. The head is surrounded by a ring of evenly spaced tentacles, which serve to catch prey. The foot at the aboral side is composed of specialized foot cells that secrete a sticky substance that helps the polyp to attach to substrates. The rest of the animal (i.e. between head and foot) represents a hollow tube that encloses the gastrovascular cavity, where digestion of foot takes place, and is called the body column. The body column is only two cell layers thick, with the ectoderm as the outer layer and the endoderm as the inner one. The two layers are separated by a layer of extracellular matrix (mesoglea). Cells of both layers have stem cell properties as long as they are in the body column. At the extremities, these cells differentiate into specialized head or foot cells. The interstitial space of the body column harbours stem cells of a third lineage, the interstitial stem cells (David and Campbell, 1972; David and Gierer, 1974). These are multipotent stem cells that give rise to neurons, gland cells, germ cells and nematoblasts. The latter are sensory cells that contain the so called cnidocytes, which play a central role in capturing prey. Stem cells of all three cell lineages in the body column are continuously self-renewing, thereby leading to displacement of tissue towards the two ends of the animal, where these cells vanish. This phenomenon results in the maintenance of a certain animal size. Displacement of tissue also occurs towards new buds. *Hydra* have a preference for asexual reproduction by budding. Upon regular feeding, new buds start to grow from the lower part of the body column. Once the appropriate size is reached a foot structure is developed between the bud and the mother whereafter the bud detaches.

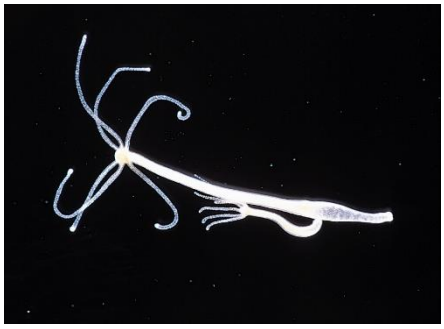

Figure 2: An adult *Hydra vulgaris* with bud

The *Hvnotch*-gene was cloned from *Hydra* cDNA and showed high similarity with the Notch homologue from *C. elegans* (GLP-1). Further analysis of the deduced protein structure revealed the conservation of the characteristic Notch domains including an N-terminal signal peptide, several EGF repeats and LIN repeats at the extracellular region followed by a linker between the transmembrane region and the intracellular region comprising a RAM domain followed by ankyrin repeats and a PEST domain at the C-terminus. Moreover, the proteolytic cleavage sites for members of the ADAM family of metalloproteases and the cleavage site for gamma-secretase are conserved. Although all essential domains are present, Hvnotch is clearly compact in size and contains only six EGF-repeats, which is even less than GLP-1 (Käsbauer et al., 2007).

Transfection of *Hydra* cells with C-terminally GFP-fused *Hvnotch* showed Notch-localization at the cell membrane and to a lesser extent at intracellular membranes, but not in the nucleus. Expressing only the intracellular region (NICD) in *Hydra* cells showed its localization in the nucleus. A Notch mutant that lacked almost the entire extracellular region was also found mainly localized in the nucleus. As mentioned before, the lack of the extracellular region triggers the cleavage by the enzyme complex gamma-secretase. Treatment with the chemical reagent DAPT, which blocks the activity of presenilin prevented translocation of this mutant into the nucleus. It was previously shown that inhibiting the action of gamma-secretase with DAPT in Drosophila and Zebrafish mimicked the phenotypes that were seen in Notch-"loss of function" mutants (van den Brandt et al., 2004; Cheng et al., 2003; Hadland et al., 2001). The block in nuclear translocation in DAPT-treated animals was seen as proof for the conservation of the gamma-secretase enzyme complex in *Hydra*. Moreover, it showed that DAPT could efficiently block Notch-signal transduction in *Hydra* (Käsbauer et al., 2007)

1.2.2 Notch inhibition phenotypes in Hydra

In Hydra, the complete *HyHes* sequence has been found. Phylogenetic analysis has shown that *HyHes* was most related to genes of the *Drosophila* "Enhancer-of-split" subfamily and to the mouse *Hes5* (Münder et al., 2010). Several CSL binding sites were identified in the *HyHes* promoter region and it was also shown than *HyHes* expression was controlled by NICD (Münder et al., 2010).

Notch pathway inhibition with DAPT (Käsbauer et al., 2007) revealed several important roles for Notch signaling in Hydra. Normally, the head structure of the animals is composed of an apical hypostome, formed from hypostomal tissue and underneath the so-called tentacle ring where at regular distances, tentacles are formed. The tentacles are composed of tentacle tissue with battery cells in the ectoderm that are filled with mature nematocytes. Underneath the tentacle ring the body column starts, which has another cell type composition than the hypostomal and tentacle tissues. As constantly dividing cells from the body column are displaced into the tentacles and hypostome, the head pattern of the Hydra is maintained. This indicates that certain patterning signals are continuously present to ensure the correct structure of the *Hydra* head. The head structure is dramatically disturbed upon inhibition of the Notch pathway. An enrichment of tentacle tissue was observed and tentacles were not regularly spaced around the tentacle ring any more. Sometimes a giant tentacle was formed and the old hypostome was pushed aside (Münder et al., 2013).

Tentacles contain nematocytes which are the stinging cells that are characteristic for cnidarians and which help the animal to catch pray. These nematocytes are derivatives of the interstitial stem cell lineage that resides in the interstitial space of the body column. When committed to the nematoblast fate, these interstitial cells undergo synchronous divisions to form nests of 4, 8 and 16 cells before they finally differentiate into nematocytes. Cells after a final mitosis are phenotypically characterized by the appearance of a vacuole. DAPT inhibition experiments have shown a reduction in the number of vacuole-containing nematoblasts and an enrichment of the number of immature nematoblasts without vacuoles. Thus, the final differentiation was blocked in absence of the Notch pathway. This was confirmed by the observation that DAPT-treated animals had shortened tentacles (Käsbauer et al., 2007).

Although tissue of the body column is continuously proliferating, animals do not become larger than a certain size. This is because a large part of the cells is displaced into buds. Budding is the asexual and most preferred way of reproduction in *Hydra,* at least under laboratory conditions. The budding process starts with the evagination of the lower part of the body column and ends with the formation of a foot at the bud side, which forms the eventual boundary between bud and parent. After foot formation the bud detaches from the mother and falls off. DAPT treatment experiments have shown that Notch signaling is indispensible for foot formation in the bud. Notch inhibition at the budding stage, at which a foot-parent boundary is formed prevented foot formation. This effect was irreversible. As a result, the bud did never detach from the parent and Y-shaped animals were formed. It was shown that expression patterns of the FGF receptor at the bud-parent boundary were disturbed upon Notch inhibition in a way that indicated the lack of a boundary between parent and bud which would normally be lined by *kringelchen* expression (Münder et al., 2010).

Hydra can also reproduce sexually and develop oocytes and sperm cells. Induced by unknown signals some animals start developing an oocyte and others start developing testes with sperm cells that are released into the medium. Commitment to the female germ cell lineage starts with the formation of germ cells of the first stage (GCI), which then undergo several differentiation steps to eventually either become an oocyte or one of the nurse cells that provide nutrition to the oocyte. DAPT treatment inhibited the first differentiation of GCI-cells and thus prevented the formation of germ cells of the second stage (GCII) (Käsbauer et al., 2007).

Every animal develops according to a certain pattern that is typical for the organism. This pattern formation or patterning starts in some animals already in the unfertilized egg where RNAs are distributed in a certain manner to get translated after fertilization. In amphibians and echinodermates, the first cleavages of the fertilised egg lead to formation of a morula, which then develops into a blastula. Eventually gastrulation takes place and a dorsal blastopore lip is formed. In 1924, it was found by Mangold and Spemann that this region in the Xenopus embryo has the ability to recruit surrounding tissue to induce patterning of the embryo. Transplantation of this dorsal lip into the ventral side of a host embryo induced the formation of a second axis. Because of its ability to organize the surrounding tissue into a certain pattern, this region was named Spemann's Organizer. However, the ability of a certain

tissue to induce a new axis had actually been described 15 years before by Ethel Brown in *Hydra*. She observed that a small piece of a hydra head is able to recruit surrounding tissue to induce a second axis when transplanted into the body column of a host animal. The organizer function in hydra is set up newly every time when the Hydra head is amputated. This organizer drives the correct patterning of the regenerating head and maintains it in adult animals. Interestingly, many factors that play a crucial role in embryonic patterning also play an essential role in Hydra head regeneration. These factors include members of the Wnt and BMP signalling pathways. It was previously shown that inhibition of the Notch pathway after head amputation impairs head regeneration (Münder et al., 2013). During the course of DAPT treatment, amputated heads are either not regenerated or only unstructured tentacle tissue is formed at the site of the amputated head. It was therefore suggested that Notch signaling is interfering with the organizer function and with head patterning in hydra. (Münder et al., 2013)

1.3. *Hydra* single cell analysis

A powerful tool that has become widely available in the last years and which has been applied for several organisms, including Drosophila, Zebrafish and C. elegans is the single cell analysis (Davie et al., 2018; Farnsworth et al., 2020; Schild et al., 2021). This tool allows to study genomics, transcriptomics and proteomics at the level of single cells. Single cell transcriptome analysis has recently been performed for *Hydra* (Siebert et al., 2019). Transcriptomes of about 25.000 single *Hydra* cells were collected to build stem cell differentiation trajectories. For this purpose, whole polyps were dissociated into single cells, which resulted in a collection of single cell transcriptomes that covered a wide range of differentiation states and their expressed genes (Siebert et al. 2019). This yielded a comprehensive molecular map for each *Hydra* cell lineage, including neurons which shows cell state transitions within each lineage. The outcome of this extensive analysis includes an expression pattern for each cell state and cell lineage. Moreover, it provides information about the extend in which the genes are expressed and it also shows the expressional changes that occur during cell state transitions.

2. Aim of the study

The Notch pathway belongs to the most important developmental pathways and has also been associated with different diseases including many cancer types. Therefore, Notch signaling has become a well-studied pathway. However, the many different and sometimes also contrary outcomes of Notch pathway activation make its investigation a difficult task. Notch has different targets in different cell types and in different nuclear contexts. Each cell type and each tissue seem to have attributed the Notch signaling to a certain purpose that is specific for that cell type and tissue. The power of Notch-signalling is to define cell fates and tissue boundaries very precisely due to the establishment of transcriptional programs in cells that are in physical contact with each other.

In complex animals, it is not possible to examine the Notch pathway in an intact adult animal, because it is difficult to manipulate the pathway in all cells at the same time. Therefore, Notch studies are usually performed on cell cultures of the certain cell type.

The simple body plan and the developmental potential of adult polyps of *Hydra vulgaris* makes it possible to examine Notch targets in an intact, living organism, by applying the Notch inhibitor directly in the *Hydra* medium. Moreover, the contour of the entire animal consists of only two cell-layer and each layer is in direct contact with the aqueous environment. This allows all cells to take up the inhibitor and thus Hydra provides the possibility to manipulate the Notch pathway in whole polyps and to identify Notch targets on the organismic level.

In this work I aimed to identify Notch transcriptional targets in *Hydra*, in the first place to find a molecular mechanism for previous phenotypical observations and in the second place to provide an overview of Notch targets in an intact animal.

3. Results

3.1 Differential gene regulation in DAPT-treated Hydra reveals candidate direct Notch signalling targets

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RESEARCH ARTICLE

Differential gene regulation in DAPT-treated Hydra reveals candidate direct Notch signalling targets

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Abstract

In Hydra, Notch inhibition causes defects in head patterning and prevents differentiation of proliferating nematocyte progenitor cells into mature nematocytes. To understand the molecular mechanisms by which the Notch pathway regulates these processes, we performed RNA-seq and identified genes that are differentially regulated in response to 48 h of treating the animals with the Notch inhibitor DAPT. To identify candidate direct regulators of Notch signalling, we profiled gene expression changes that occur during subsequent restoration of Notch activity and performed promoter analyses to identify RBPJ transcription factor-binding sites in the regulatory regions of Notch-responsive genes. Interrogating the available single-cell sequencing data set revealed the gene expression patterns of Notch-regulated Hydra genes. Through these analyses, a comprehensive picture of the molecular pathways regulated by Notch signalling in head patterning and in interstitial cell differentiation in Hydra emerged. As prime candidates for direct Notch target genes, in addition to Hydra (Hy)Hes, we suggest Sp5 and HyAlx. They rapidly recovered their expression levels after DAPT removal and possess Notch-responsive RBPJ transcription factorbinding sites in their regulatory regions

Contribution to the paper

The qPCR and RefSeq experiment were planned and performed by me. I've also performed all data analyses described in the paper (Figures 1, 2, 3, 4, 5, 6, 7, S1, S2), except for the promoter analysis, which was performed and analyzed by Jack Cazet from Juliano's lab (Figure 8; Table S1, S2). I have independently written the first draft of the paper and also contributed in later editing steps.

3.2 Apoptosis in Hydra: function of HyBcl-2 like 4 and proteins of the transmembrane BAX inhibitor motif (TMBIM) containing family

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Apoptosis in Hydra: function of HyBcl-2 like 4 and proteins of the transmembrane BAX inhibitor motif (TMBIM) containing family

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Abstract

Mechanisms of programmed cell death differ between animals, plants and fungi. In animals, apoptotic cell death depends on caspases and Bcl-2 family proteins. These protein families are only found in multicellular animals, including cnidarians, insects and mammals. In contrast, members of the TMBIM-family of transmembrane proteins are conserved across all eukaryotes. Sequence comparisons of cell death related proteins between phyla indicate strong conservation of the genes involved. However, often it is not known whether this is paralleled by conservation of function. Here we present the first study to support an anti-apoptotic function of Bcl-2 like proteins in the cnidarian Hydra within a physiological context. We used transgenic Hydra expressing GFP-tagged HyBcl-2-like 4 protein in epithelial cells. The protein was localised to mitochondria and able to protect Hydra epithelial cells from apoptosis induced by either the PI(3) kinase inhibitor wortmannin or by starvation. Moreover, we identified members of the TMBIM-family in Hydra including HyBax-Inhibitor-1, HyLifeguard-1a and -1b and HyLifeguard 4. Expressing these TMBIMfamily members in Hydra and human HEK cells, we found HyBax-inhibitor-1 protein localised to ER-membranes and HyLifeguard-family members localised to the plasma membrane and Golgivesicles. Moreover, HyBax-inhibitor-1 protected human cells from camptothecin induced apoptosis. This work illustrates that the investigated Bcl-2- and TMBIM-family members represent evolutionarily conserved mitochondrial, ER, Golgi and plasma membrane proteins with anti-apoptotic functions. The participation of ER and Golgi proteins in the regulation of programmed cell death might be a very ancient feature.

Contribution to the paper

I have analyzed the expression patterns described in the part “Expression patterns of TMBIM genes in comparison with members of Hy-Bcl-2-like familie”. Therefore, I have retrieved expression information from the Hydra single cell analysis and performed cluster analysis (Figure 10). I have also contributed in writing this part of the paper and in later editing of the whole work.

4. Discussion

Developmental pathways, including Notch-, Wnt- and BMP-pathways, play important roles from the very early beginning of an organism's live and throughout adulthood and are highly conserved through animal evolution. An advantage of studying developmental pathways in *Hydra* is the fact that the whole-body structure and tissue homeostasis depend on them throughout adult life.

It remains remarkable how a simple pathway like Notch, with very few core members and with no clear amplification step can cause so many different and sometimes also contrary outcomes.

For this study mRNA of DAPT-treated *Hydra* was isolated at three empirically determined time points and sequenced. The empirical determination of time points had been based on the expression behavior of known Notch targets, *HyHes* and *CnASH* as determined by qPCR. The goal of this experiment was to identify all genes that were affected by a 48 hrs DAPT inhibition, by comparing DAPT-treated animals with control animals. Control animals were treated with the DAPT solvent DMSO in order to rule out any effects caused by the solvent. The two subsequent time points, at which mRNA was isolated were meant to observe the recovering behavior of affected genes in the hours after DAPT removal (see Figure 3 for an overview of the workflow).

For analyzing the data I assembled a *Hydra vulgaris* transcriptome on the basis of all reads that were obtained in this experiments using the de novo Trinity transcriptome assembler. Then I mapped the sequenced reads of each condition and time point to this transcriptome to obtain the level of gene expression for each sample.

Differential gene expression analysis was then applied to identify the expression differences between DAPT-treated and control animals. All differentially expressed genes were annotated and mapped to the single cell data (Siebert et al. 2019) in order to determine expression patterns across all known cell states. The single cell data contains datasets for all *Hydra* cell states and also for the cell states within each cell lineage separately. These include the datasets of only the ectodermal cell lineage, of only the endodermal cell lineage, interstitial cell lineage and also one for only neurons (Siebert et al., 2019). The dataset of all cell states in

Hydra provides a good overview of all cell types, whereas those of individual cell lineages provide a comprehensive map of the transitions between cell states. Mapping of the differential analysis data to the single cell atlas proved a powerful approach to identify the cell states and cell types in which the Notch target genes are expressed.

The analyses performed in both studies constituting my PhD-thesis (Motamedi et al., 2019 and Moneer et al., 2021) are hierarchical cluster analyses, in which genes with similar gene expression patterns were clustered together. Combining this information with the expression pattern of cell states identified the genes that were expressed in each cell state. This made it possible to for example identify which and how many genes were expressed in the different differentiation states of nematoblasts and how they were affected by the DAPT treatment and how they recovered their expression in the hours upon DAPT removal.

This analysis was also applied to investigate the expression patterns of programmed cell death-related genes of the *Hydra* bcl2 and TMBIM-family (Motamedi et al., 2019). Depicting the output of the hierarchical cluster analysis as a heatmap showed the different cell type specificities for each of these genes (Motamedi et al., 2019, Figure 10). This was important information to understand the functional implications of the high diversity of Bcl-2 and Lfg-family members in Hydra, which is similar to human members of these families but surprising given the simple tissue organization of Hydra polyps. One might have expected a gradual increase of protein family members during evolution. However, the number of family members found in *Hydra* showed that the family of programmed cell death-related genes was already extended in pre-bilaterians. This might emphasize not only the importance of programmed cell death but also the need for many different proteins to ensure the fine-tuning and precision of its execution. A clue for that is the fact that each programmed cell death-gene showed a unique expression pattern across the different *Hydra* cell types (Motamedi et al., 2019, Figure 10). Thus, the cell types and the extent in which each gene was expressed was specific for the particular gene.

Only *Hylfg1a, Hybak2 and Hybcl2-7* had similar expression patterns, i.e. they were poorly expressed in *Hydra* and most probably their expression is related to extrinsic triggers. In contrast, *HyBI1* showed the highest overall expression, especially in ectodermal and endodermal stem-, head-, foot- and tentacle cells. Cells at the extremities are continuously

replaced and apparently *HyBI1* is constantly expressed in these sites in order to protect the cells or to slow down the process of programmed cell death.

It is obvious that many of the examined programmed cell death-genes show higher expression at the extremities. Hybak−like1, Hydra vulgaris lfg1b, Hydra vulgaris lfg4 and Hybcl−2−like6 are mainly expressed in head, tentacle and foot cells and poorly in neurons and gland cells. This might be explained by the fact that a high number of apoptotic cells is found at these oral-aboral positions. Only Hybcl−2−like1/3 is mainly expressed in gland cells and neurons and poorly in other cell types. Also obvious is that in contrast to nematoblasts, which are still proliferating and differentiating, post-mitotic nematocytes show higher expression of many different programmed cell death-genes.

Remarkable is the extreme high expression of Hybcl−2−like2 in female nurse cells. Also Hydra vulgaris BI1, Hybcl−2−like4, Hydra vulgaris lfg4 and Hybak−like1 show a high expression in these cells. This is in accordance with previous studies where a arrested apoptosis was observed during oogenesis (Alexandrova et al., 2005, Technau et al., 2003).

Hybcl−2−like2 showed ubiquitarian expression across the different cell types but was totally absent in nematoblasts. *Hybcl−2−like4* on the other hand, also showed ubiquitarian expression, however lacked any expression in zymogen gland cells. It is possible, that *HyBI1, Hybcl−2−like2 and Hybcl−2−like4* are involved in more general cellular functions.

Besides the hierarchical clustering, I also applied NMF (Non-negative matrix factorization) analysis on the differential expression data in order to identify co-expressed genes. This analysis yields modules of putative co-expressed genes, which are called metagenes. In contrast to hierarchical clustering, NMF allows each gene to be in more than one metagene, providing more possibilities to identify gene modules. This may often provide a more realistic picture, since each cell type has its own expressed gene composition (Lee and Seung, 1999).

The first observation that was made in my DAPT-washout study according to hierarchical clustering was that different cell types indeed have different Notch-responsive genes (Moneer et al., (2021) Figure 3, 4A and 6), confirming that each cell type has its own set of Notch targets. The previously observed phenotypes in *Hydra* upon Notch inhibition show that Notch outcomes differ in different cell types (Käsbauer et al., 2007; Münder et al., 2010; Münder et al., 2013). Interestingly, for the majority of *Hydra* cell states we find specific Notch-responsive

genes, meaning that Notch plays a certain role in each of these cell states/cell types (Moneer et al., 2021 Figure 3 and Metagenes).

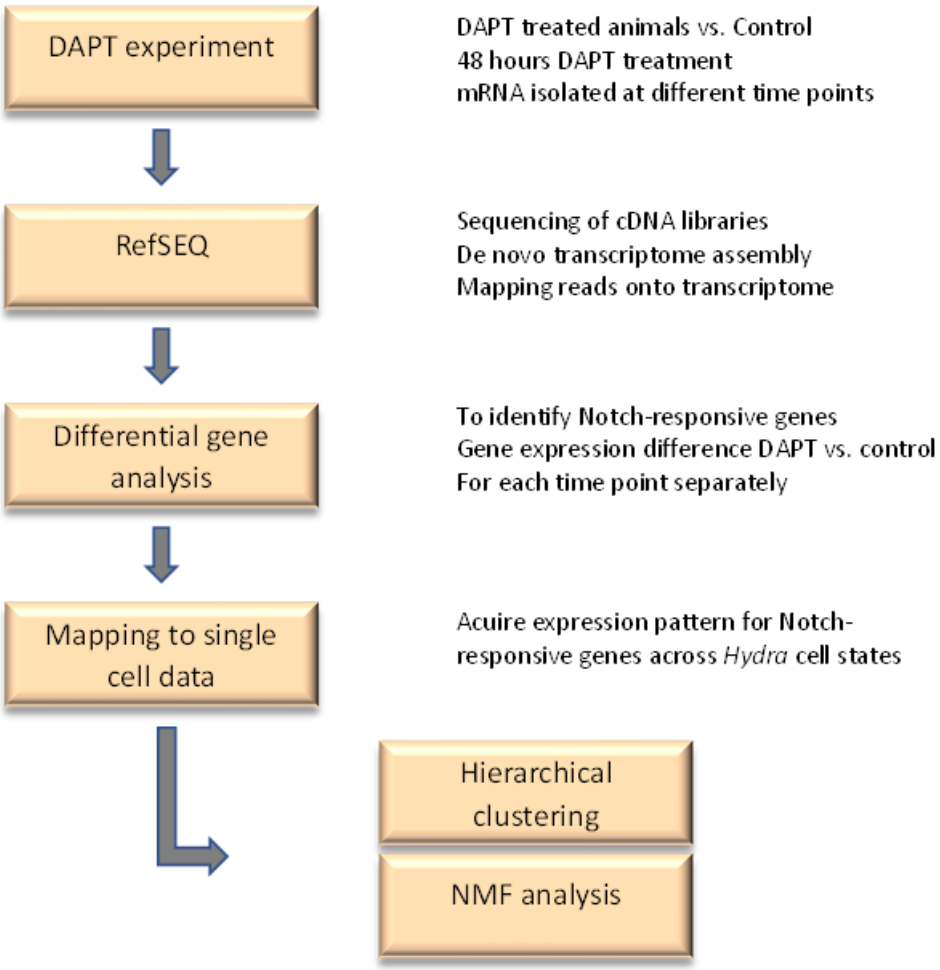

Figure 3: Overview of the applied workflow. The DAPT experiment was followed by the extraction of the total RNA and thereafter of the mRNA of which eventually a cDNA library was created and sequenced

4.1 Notch and Neurons in Hydra

The cell types that were found neither by hierarchical clustering nor by the NMF analysis to be regulated by Notch are neurons. Previous studies could not show any effect of Notch inhibition on neurons. In this analysis, no cluster of Notch-responsive genes specifically expressed in neurons could be found. Studies in *Xenopus*, *Zebrafish*, *Drosophila* and *Chick* have shown the role of Notch signaling in neurogenesis. In *Drosophila* embryogenesis, Notch plays a major role in suppressing the majority of a cluster of equipotent progenitors and allowing only a few cells to become neural precursors. If Notch is inhibited at this point, all cells of the cluster become neural cells. In vertebrates, Notch also plays an important role in embryonic

neurogenesis, but here it controls the timing of neural differentiation rather that the decision to gain a neural fate (Engler et al., 2018). In adult *Hydra*, neurons continuously differentiate from interstitial stem cells and migrate towards the extremities (Hager and David, 1997), where they, like other *Hydra* cells, eventually vanish. In 2007, Käsbauer and colleagues did not observe any change in nerve cell number during DAPT treatment and therefore concluded that Notch inhibition does not affect nerve cell differentiation. However, the question remains unanswered whether replacement of *Hydra* nerve cells is essential for the existing ones to vanish. A lack in supply of new nerve cells might also lead to retention of existing nerve cells and therefore, no difference could be observed during Notch inhibition.

In contrast, Käsbauer and colleagues (Käsbauer et al., 2007) did find a clear difference in another type of neuronal cells, namely nematoblasts, during DAPT treatment. More precisely, the number of nematoblasts with a visible vacuole was drastically reduced while the number of nematoblasts without a visible vacuole was increased. *Hydra* nematoblasts also differentiate from interstitial stem cells and eventually become nematocysts of which the majority is incorporated into tentacle battery cells. Nematocysts play an essential role in prey capturing (David, 2012). Nematoblast differentiation includes several differentiation steps and therefore differentiating nematoblasts have been subtyped into nb1 through nb8 by Siebert and colleagues (Siebert et al., 2019). The cells with a small but visible vacuole represent the cell state immediately after exit from the final mitosis. It was already concluded, that Notch normally controls this differentiation step. In my work (Moneer et al., 2021), we not only confirm this observation but also provide a set of candidate Notch-responsive genes that might be directly involved in driving nematoblast differentiation. This set includes the genes that are expressed in stages nb4 through nb8 and the expression levels of which are recovered within the first 3 hours upon DAPT removal (Moneer et al., 2021, Figure 4B, Table S1), for example two nematoblast-specific genes (see supplementary table 1, Moneer et al., 2021) and Cnido-Jun. The latter has already been assigned a role in driving nematogenesis in *Nematostella vectensis* (Sunagar et al., 2018). Here I show that Cnido-Jun ist most probably directly controlled by the Notch signaling. The analysis has also revealed two transcription factors, *HyPOU* and *CnASH,* of which was already known to be expressed in post-mitotic nematoblast. I now show that these are most probably not driven by Notch signaling directly, since they do not recover their expression quickly after DAPT removal.

4.2 Notch and Oogenesis in Hydra

Oogenesis in *Hydra* starts with the differentiation of germ cells, which proceed through different cell states to eventually give rise to one oocyte (Alexandrova et al., 2005). The first step is the commitment of interstitial stem cell to germ cell lineage and their differentiation into germ cells I (GCI). This process then continuous with the differentiation of GCI into GCII and from GCII into GCIII (Alexandrova et al., 2005). Until the oocyte is formed, the egg patch contains different fractions of GCI, GCII and GCIII and this composition is changed with time, where the number of GCI decreases and that of GCIII increases. It was observed that Notch inhibition blocks the differentiation of GCI into GCII (Käsbauer et al., 2007). GCI then don't stop proliferating and form tumor like structures instead of differentiated egg patches. For the RefSeq study *Hydra vulgaris*, strain Basel were used. In contrast to the AEP strain, these animals rarely produce eggs and mainly reproduce asexually. However, the NMF analysis shows a Metagene composed of Notch-responsive genes, that are expressed in female germ cells (Moneer et al., 2021, Metagenes G). This cluster is not recognized by hierarchical clustering, most probably because these genes show a stronger expression in other cell types/cell states and are therefore attributed to other clusters. However, in this cluster a boule-like gene (XP_012556641.1) appeared to be regulated by Notch-signalling. This encodes an RNA-binding protein important for spermatogenesis and should be investigated further.

Drosophila oogenesis has been used as a model to investigate egg development and studies have shown the involvement of Notch signaling in various steps through oogenesis (Ward et al., 2006). One of the very early steps in *Drosophila* oogenesis, which requires Notch is the formation and maintenance of the germline stem cell niche (Ward et al., 2006). It is possible that even in the asexual *Hydra* strain the very early steps in commitment to the germ cell lineage, which are controlled by Notch do take place. However, this process might be stopped, because signals that are essential for the continuation are lacking. The hypothesis that Notch is involved in the very early steps of germ cell lineage commitment in Hydra was not investigated by Käsbauer and colleagues (Käsbauer et al., 2007) (only animals with clearly visible egg formation were selected) and should be examined in the sexual *Hydra* strain AEP.

4.3 Notch and the Wnt pathway

The RefSeq analysis revealed components of the Wnt- and BMP-pathways as candidate Notch targets (Moneer et al., 2021). Previous Notch studies in *Hydra* have already suggested an interaction between the notch pathway and the Wnt pathway. However, this was only in regenerating heads, which showed no Wnt3 expression after DAPT treatment (Münder et al., 2013). The Differential analysis in intact adult *Hydra* did not show any effect on Wnt3 expression as result of DAPT inhibition, however it did reveal an effect on Wnt components including *Wnt7, TCF* and *Sp5.* This suggests a certain Notch-Wnt crosstalk not only during head regeneration but also in adult polyps. Crosstalk between the Wnt- and Notch- signalling pathways is not new and has previously been suggested as a way to increase outcome diversity with only a few signaling pathways (Muñoz-Descalzo and Martinez Arias, 2012). In human colorectal carcinoma cell line Notch inhibition by DAPT led to the upregulation of the Wnt target *c-myc*, meaning that this Wnt target is normally repressed by Notch in this cell type (Acar et al., 2021). In zebrafish, self-renewal and differentiation of sensory hair cells is controlled by Notch, through inhibition of Wnt-induced proliferation (Romero-Carvajal et al., 2016) and a study by Deregowski and colleagues (2006) suggested Wnt suppression by Notch through Hes1 during osteoblastogenesis.

4.4 Notch and the BMP pathway

A new finding that was revealed by the differential gene expression analysis is the interaction between the Notch- and BMP-signalling pathways in *Hydra* (Moneer et al., 2021, Figure 7B). A member of the BMP signaling pathway, TGF4 was found upregulated in the *Hydra's* foot and recovered its expression level quickly after DAPT removal (i.e. within the first 3 hours). An interaction between the Notch and the BMP pathway was also demonstrated in zebrafish where inhibition of Notch by DAPT led to a significant reduction of *bmp10* expression in regenerating hearts (Wang et al., 2021) and in mesenchymal stem cells, where Notch plays a role in enhancing the BMP9-induced early osteogenic differentiation (Cao et al., 2017). Dahlqvist and colleagues reported that BMP-induced inhibition of muscle stem cell differentiation depends on Notch signaling (Dahlqvist et al., 2003).

5. Outlook

This work (Moneer et al., 2021) provides a tool to study the impact of many different pathways on patterning and cellular homeostasis in Hydra. By combining differential gene expression studies with analysis of data from the single cell analysis it was possible to define the role of Notch-signalling in different cell states and cell types during development, (Siebert et al, 2019). *Hydra* provides a suitable environment for studying developmental pathways because 1) these pathways are conserved in *Hydra*, 2) the body plan of *Hydra* makes it possible to apply drugs to all cells and therefore to study the influence of the drug on an intact animal and 3) developmental pathways are continuously active because of the steady state patterning processes in *Hydra.*

A very important process in organism's life is the onset of the organizer that drives the very early embryogenesis (reviewed by Anderson and Stern, 2016). The *Hydra's* head contains this organizer function and it has already been shown that Notch signaling is indispensable for exerting this function and driving head regeneration (Münder et al., 2010). A follow-up-study to Moneer et al., 2021 should be the elucidation of the Notch targets during *Hydra* head regeneration. This includes narrowing the window during head regeneration where Notch is required and performing a similar RefSeq experiment as shown in the present work (Moneer et al., 2021).

Single cell data provides a reference of the expression pattern of genes across cell states, which subsequently can be compared to the expression upon treatment, injury or during certain developmental stages etc.

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7. Abbreviations

ADAM	A Disintegrin and Metalloproteinase Protein
bHLH	basic-helix-loop-helix
BI	Bax Inhibitor
BLAST	Basic local Alignment and Search Tool
BMP	Bone Morphogenetic Protein
CR	cysteine rich
CSL	CBF1, Suppressor of Hairless, Lag
DAPT	N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester
DMSO	Dimethyl sulfoxide
EGF	epidermal growth factor
HES	Hairy Enhancer of Split
Hv	Hydra vulgaris
nb	nematoblast
NICD	Notch intracellular domain
NMF	Non-negative matrix factorization
PEN2	presenilin enhancer 2
qPCR	quantitive Polymerase Chain Reaction
TCF	T cell factor
TMBIM	The transmembrane Bax inhibitor motif

8. Publications

Moneer J, Siebert S, Krebs S, Cazet J, Prexl A, Pan Q, Juliano C, Böttger A. Differential gene regulation in DAPT-treated Hydra reveals candidate direct notch signalling targets. J Cell Sci. 2021 Jun 14:jcs.258768. doi: 10.1242/jcs.258768. Epub ahead of print. PMID: 34125230.

Motamedi M, Lindenthal L, Wagner A, Kemper M, **Moneer J**, Steichele M, Klimovich A, Wittlieb J, Jenewein M, Böttger A. Apoptosis in Hydra: function of HyBcl-2 like 4 and proteins of the transmembrane BAX inhibitor motif (TMBIM) containing family. Int J Dev Biol. 2019;63(6-7):259-270. doi: 10.1387/ijdb.180199ab. PMID: 31250909.

Bräuer KE, Brockers K, **Moneer J**, Feuchtinger A, Wollscheid-Lengeling E, Lengeling A, Wolf A. Phylogenetic and genomic analyses of the ribosomal oxygenases Riox1 (No66) and Riox2 (Mina53) provide new insights into their evolution. BMC Evol Biol. 2018 Jun 19;18(1):96. doi: 10.1186/s12862-018-1215-0. PMID: 29914368; PMCID: PMC6006756.

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