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UNIVERSITÀ DI SIENA 1240

Department of Medical Biotechnologies
Doctorate in Genetics, Oncology and Clinical Medicine (GenOMeC)

Cycle: XXXV

Coordinator: Prof./ssa Alessandra Renieri

Characterization of a Pygo2 splicing variant in zebrafish and potential Pygo2 roles in mediating melanoma in the zebrafish animal model

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ABSTRACT

Pygopus is an essential transcription co-activator with the β-Catenin (β-Cat) complex for the canonical-Wnt (c-Wnt) signaling pathway in Drosophila, while in mammalian cells it could work either within or independently of the c-Wnt pathway. With a highly conserved domain (PHD) in its C-terminus, Pygo2 binds to BCL-9, which in turn binds β-Cat and activates c-Wnt–dependent transcriptional regulation. Emerging data indicate its role in multiple cancers, in which it acts through the c-Wnt-dependent signaling pathway. The role of c-Wnt has been more ambiguous in the case of melanoma, and the role of Pygo2 in this cancer type has not been tested yet. Melanoma is a serious type of skin cancer that develops in the melanocytes and dedifferentiates these cells into neural crest-like cells. c-Wnt signaling is determinant for neural crest cells proper behaviors and differentiation into melanocyte, and up or down regulation of c-Wnt signaling, and/or low/high levels of nuclear β-Cat have been shown in some melanoma studies. Previously in our laboratory we had identified in the zebrafish a new splicing variant of *pygo2*, which lacks the Plant-Homeo-Domain (PHD), and my goal was to finish the characterization of this splicing variant in the context of c-Wnt signaling and set up, with molecular genetic approaches, the fishlines that can be used to analyze the role of Pygo2 in the context of a melanoma model in the zebrafish. Using the TOP-FLASH assay, I showed that Pygo2ΔPHD has a dual role in cooperating with β-Cat in activating the reporter gene, and this depended on the time of cell transfection. Similarly, I discovered that Pygo2ΔPHD has the opposite effect when working with Pygo1 or Pygo2 full-length on the same process; again, this was dependent on the length of time the cells were transfected with the plasmids. This transcriptional behavior correlates with the cellular localization of the transfected protein, in fact I showed Pygo2ΔPHD entered in the nucleus only at 3 days from transfection. I have also created a mutant of the *pygo2 full-length* gene variant in the zebrafish by means of CRISPR/Cas9-based technology, which can still generate Pygo2ΔPHD but not Pygo2 full-length. These fish can survive through development, reach adulthood, and are fertile. Furthermore, in a p53-/- genetic background, I established a zebrafish transgenic line that expresses the mutated form of BRAF in the melanocytes. The resulting fishline has a high chance of developing nevi and melanomas.

CHAPTER 1

INTRODUCTION

Homeostasis in adult tissues and proper embryonic development both depend on the skillful control of signaling molecules and cell-autonomous factors (Langdon and Mullins, 2011) (Katoh and Katoh, 2007). During embryonic development, when a gene's expression is decreased, increased, or a protein's activity is broadly up- or down-regulated, away of its physiological states, it may cause birth defects or cause development to fail entirely. Adult tissues and organs that undergo the same modification frequently develop cancer. The Wnt pathway is arguably one of the most relevant pathways responsible for these effects.

1.1 **Wnt-pathway**

One of the prominent signaling mechanisms controlling both embryonic development and adult tissue homeostasis is canonical-Wnt (c-Wnt) signaling. While there is clear evidence for the c-Wnt pathway's role in regulating embryonic development and tissue homeostasis, we still have much to understand about how this pathway mechanistically regulates gene expression. A better understanding of this signal is very important in light of the fact that changes in c-Wnt signaling are linked to birth defects and cancer (Anastas and Moon, 2012; Morin *et al*., 1997; Liu *et al.,* 2000; Zardawi *et al*., 2009; Takebe *et al*., 2011).

Under normal physiological conditions, in the absence of c-Wnt signaling, the β-Catenin (β-Cat) (Fig.1) protein is found in the cytoplasm and is labeled for degradation by the activity of a multi-protein complex that includes glycogen synthase kinase 3 (GSK3), casein kinase1(Ck1), the tumor suppressor *adenomatous polyposis coli* gene product (APC), and Axins proteins, among others. This complex is disassembled when c-Wnt ligands bind the receptor at the membrane of the cell, causing the disassembly of the described multi-protein complex. This causes an increase in the cytoplasmic concentration of β-Cat and subsequent translocations into the nucleus, where it binds to Lef/Tcf family DNA-binding factors and regulates transcription of Wnt target genes (Cadigan and Waterman, 2012). Pygopus (Pygo) was recently discovered to be a nuclear cofactor of the Lef/Tcf-β-Cat complex.

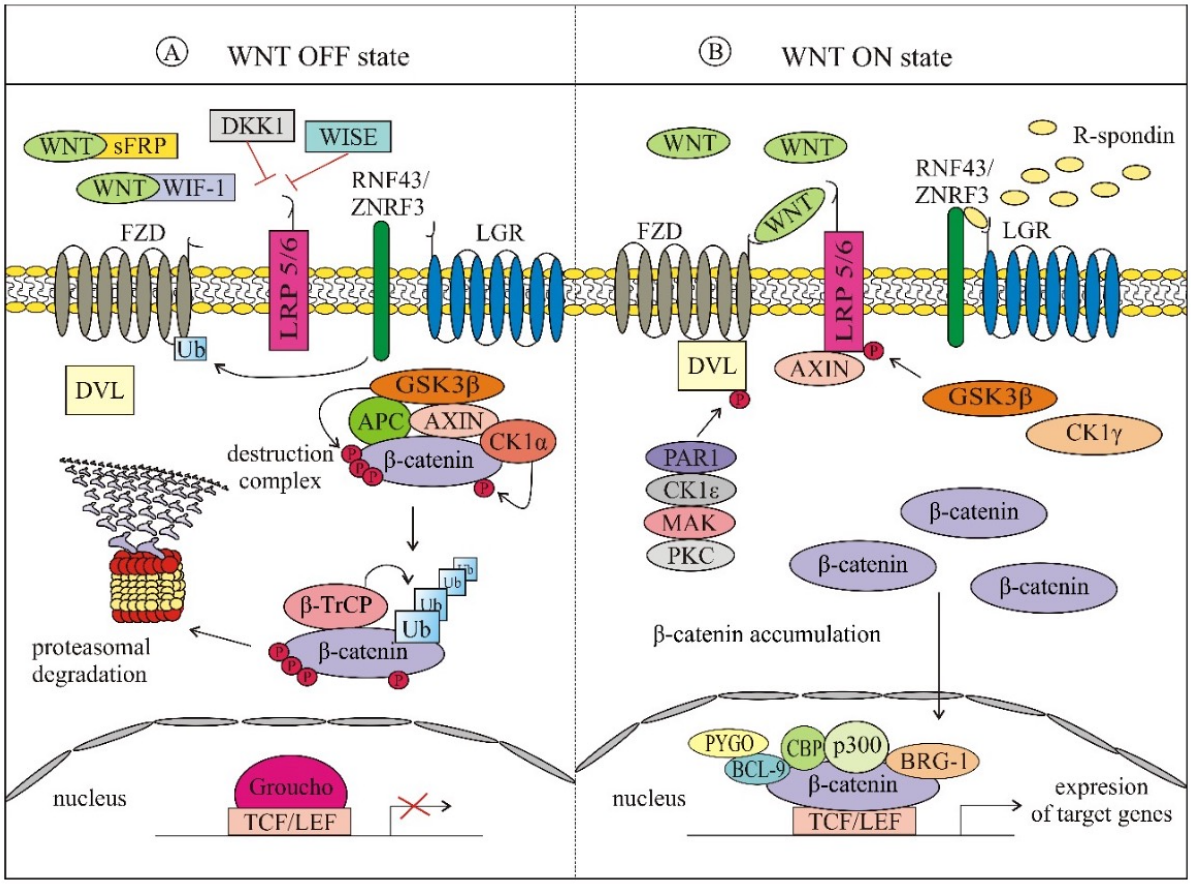


Fig.1: Canonical (β-catenin dependent) Wnt signaling pathway: This figure shows the canonical-Wnt signaling pathway either in absence (left) or presence (right) of Wnt signaling molecule. Adapted from Gajos-Michniewicz A et al., 2020.

1.2 **Pygopous family of nuclear co-factors**

Preliminary studies in the *Drosophila* model system revealed Pygopus' involvement in the anchoring of TCF to Armadillo (*Drosophila* β-Catenin) with the aid of legless (lgl is *Drosophila* BCL9) (Kramps *et al*., 2002). Later, it was demonstrated that BCL9 and BCL9-2, the two mammalian orthologues of Lgl, form a complex with β-Cat that, in response to Wnt molecules binding their cell receptors, translocate into the nucleus, promoting the transcription of c-Wnt-target genes.

Early studies identified a crucial distinction between the function of pygopus in vertebrates and *Drosophila*, namely that Pygo1 and Pygo2 in the latter may also play c-Wnt-independent roles. However, more recent research reported that Pygo acts in a way during heart development in Drosophila that is independent of β-Cat (Tang *et al*. 2013, 2014). In the testis (Nair *et al*., 2008; Cantù *et al*., 2013) and lens (Song *et al*., 2007) of vertebrates, Pygo2 performs tissue-specific, c-Wnt-independent functions.

Looking at the protein structure Pygopus proteins share a N-terminal Homology Domain (NHD) that contains a nuclear localization signal (NLS) as well as a NPF (asparagine-proline-phenylalanine) domain. At the C-term region is present a Plant Homology Domain (PHD), which presents 60 amino acids arranged in zinc-finger motifs defined by seven cysteine and a histidine, and with two Zn ions coordinated with them (Nakamura *et al*., 2007).
Previously in the laboratory we had identified in the zebrafish a new splicing variant of *pygo2*, which lacks the Plant-Homeo-Domain (PHD) (Fig.2)

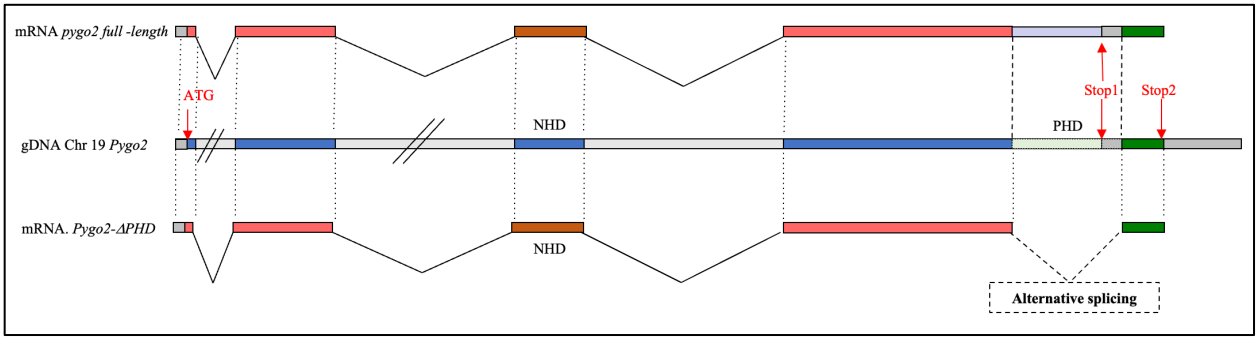


Fig.2: Schematic representation of the gDNA locus containing the *pygo2* (in the center), and the mRNAs for *pygo2 full-length* (above) and *pygo2 ΔPHD* (below).

It has been shown that the NPF sequence binds directly TCF-3 (Fig.3.). The NHD shows also binding with the Mediator complex, MLL2 histone Methyltranferase and GCN5 Histone Acetyltransferase complex (Chen *et al*., 2010). The PHD was shown it can also bind the mediator complex and di- and trimethylated lysine 4 on histone H3 (H3K4me2/3) as well as the proteins BCL9 and BCL9/2 (Gu *et al.,* 2009; Cantù *et al*., 2013) (Fig.3).

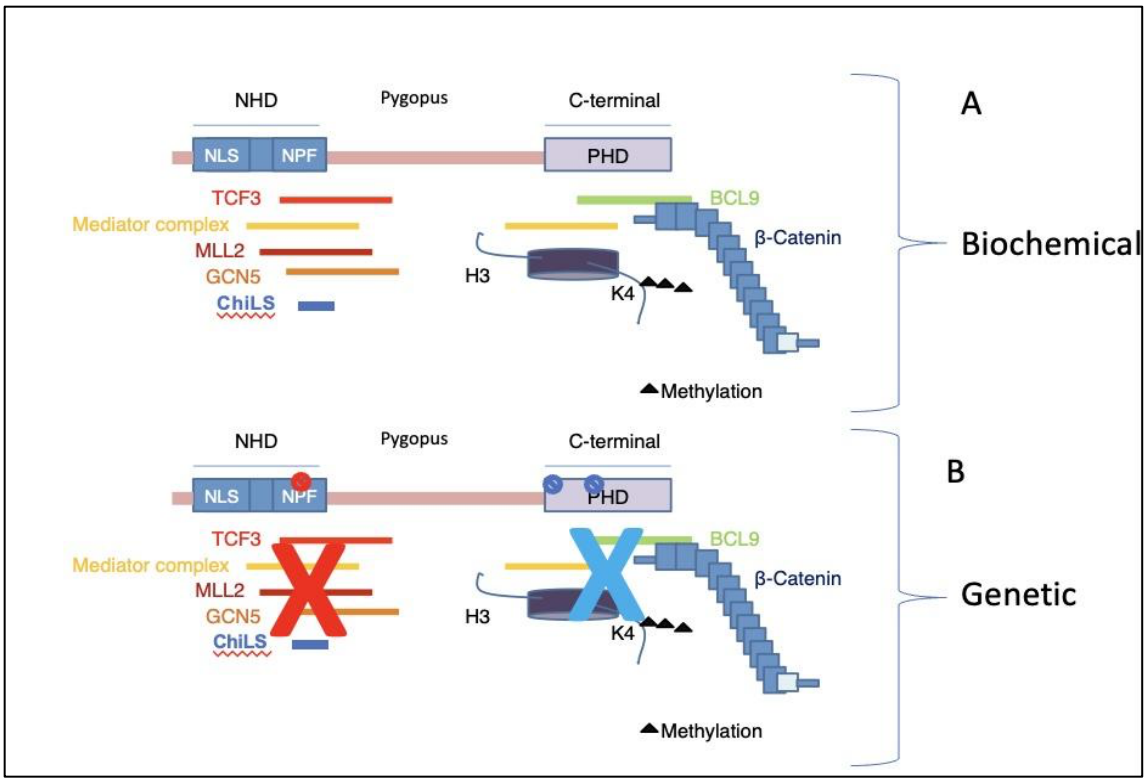


Fig.3: This figure, modified from the thesis of J. Ibetti, shows a full length Pygo2 with all its domains. In A. are shown the regions of interaction with other proteins identified by biochemical studies. In B. are shown the results of genetics studies, as blue dots the point mutations that are changing amino acids that are involved in interactions that are not dramatically affecting the phenotype, blue X. The red doth indicates the interactions that are necessary, identified by genetic evidence obtained by different knock-out/ knock-in or mutations in *pygo2*. the red sign in here indicates regions of pygo2 and relative interacting proteins that are necessary for embryo development and in blue those that can be compatible with development.

In mouse it has being identified an alanine within the PHD (A342) that is required for interactions with H3K4me2/3 (Cantù *et al*., 2013 and 2017), and a leucine (L368) necessary for the BCL9/BCL9-2 binding (Cantù *et al*., 2016). Equivalent amino acids are present in *Drosophila* Pygopous. Only more recent studies have clarified the importance of Pygo and BCL9 during Wnt/β-Catenin-mediated responses in vertebrates. The existence of a complex, multi-protein Wnt enhanceosome that can activate the expression of target genes in response to c-Wnt signaling and in coordination with TCF/LEF factors was first identified by Fiedler and colleagues (Fiedler *et al.,* 2015). For the first time, the ligand of Pygo's N-terminal asparagine, proline, and phenylalanine (NPF) motif was discovered by these authors to be a complex made up of the proteins Chip/LDB ((Lin-11 Isl-1 Mec-3-)/LIM-domain-binding protein) and SSDP

(single-stranded DNA-binding protein), which they dubbed ChiLS. It is interesting to note that ChiLS can bind Groucho and other NPF motifs found in RUNX proteins, defining the main structural elements of the Wnt enhanceosome, which can integrate various signals from factors that are lineage specific. Groucho protein binds to TCF and inhibits the expression of genes activated by c-Wnt signaling, as was already mentioned above. This interaction can keep β-Cat target genes silenced in the absence of c-Wnt ligands. Genetic studies have shown that knock-out mice for Pygo1 (*Pygo1*$^{-/-}$) are viable and fertile, while knock-out mice for Pygo2 (*Pygo2*$^{-/-}$) and Pygo1/2 double knockout mice die during embryonic development between 13.5 dpc and birth due to a series of developmental defects (Li *et al*., 2007; Schwab *et al*., 2007; Song *et al*., 2007; Cantù *et al*., 2014), including heart defects (Cantù *et al*., 2018). This is consistent with the fact that Pygo2 is expressed more often and in more places than Pygo1 during mouse development. Similar heart defects were reproduced in the zebrafish double CRISPR-induced mutants for *pygo1* with an indel at the beginning of the NHD region and *pygo2* with an indel at the end of its NHD sequence (Cantù *et al*., 2018). However, in this animal system, the single homozygous mutant had no evident developmental phenotype. All together, these results indicate an important role for Pygo2 during vertebrate development and possibly a co-requirement for Pygo1.

Elegant genetic approaches, on the other hand, have identified the role of specific interactions of Pygo2 with H3K4me2/3 and BCL9/BCL9l during mice development (Cantù *et al*., 2013; 2016 and 2017). While the biochemical analysis offered a clear scenario of interactions between Pygo2, BCL9, β-Cat, and other factors on the promoter of c-Wnt-responsive genes, these genetic knockout approaches targeting the Pygo2 PHD domain produced surprising results. Mice homozygous for a *pygo2* point mutation (Pygo2-L368A) that transforms L368A, which drastically reduces Pygo2 binding to BCL9/BCL9l, produce embryos that are apparently indistinguishable from those of wild-type littermates. A double mutant, *Pygo1*$^{-/-}$; *Pygo2*$^{L368A/L368A}$, is also making apparently normal animals able to reproduce (Cantù *et al*., 2013; 2017). Further protein interactions studies clarified this contradiction; these works focused on the role of the BCL9 factor within the c-Wnt enhanceosome. BCL9 was shown to have a significant role in the enhanceosome, also prior to c-Wnt signaling and independently of β-catenin (van Tienen *et al*., 2017). In line with this refined model, BCL9 represents a core component of the c-Wnt enhanceosome, undergoing three constitutive interactions with distinct

components of the complex, Pygo, ChiLS, and Groucho, and having an important scaffolding function (Fig. 4) (van Tienen *et al*., 2017).

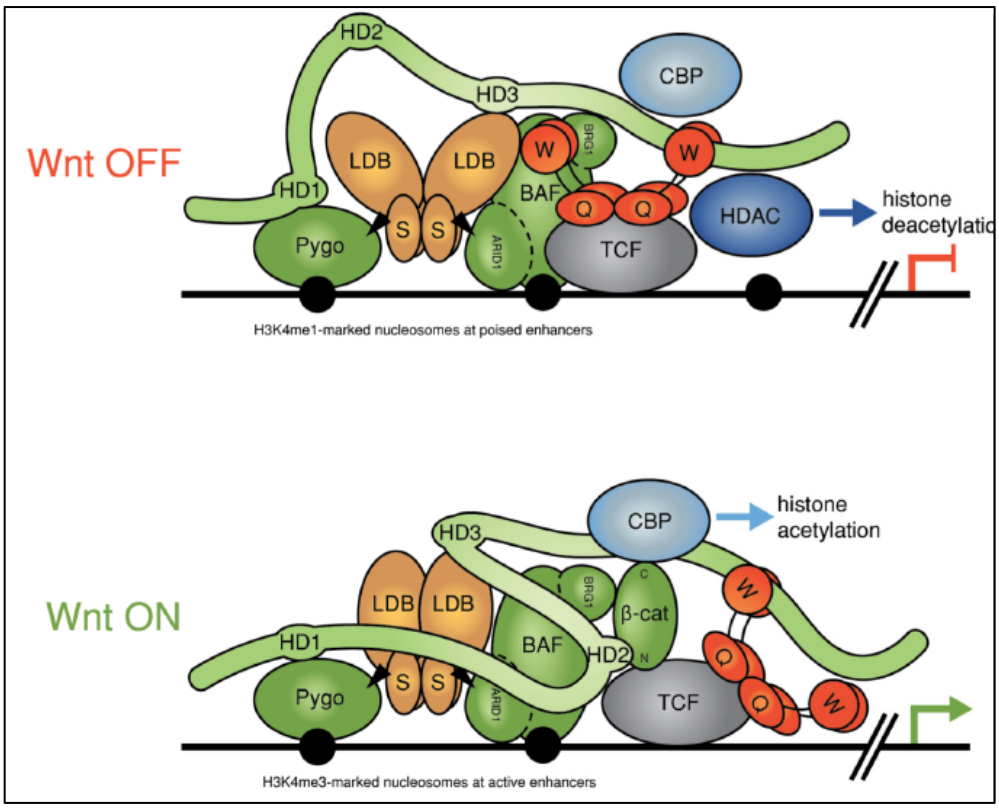


Fig.4. This figure is derived from Van Tienen et al. (2017), showing the enhanceosome either in absence or in presence of c-Wnt sygnaling. LDB and S dimers all together form ChiLS. Chils binds HD3 from BCL9 and the NPF domain (not shown) in Pygo.

Hence, these results describing a scenario in which BCL9 appears to be a core component of the Wnt enhanceosome, able to confer Wnt responsiveness to it by stabilizing the binding with β-Cat and thus facilitating its access to TCF factors and activating its transcriptional complex, showing a scenario more consistent with the genetic data. Thus, even when the association with Pygo is abolished, like in the mouse mutant line for Pygo PHD domain, it is possible that the BCL9 factor might retain both its scaffolding function and, at the same time, its capacity of priming the enhanceosome complex for c-Wnt responses: BCL9 capacity for constitutively binding the other core components of the complex (Chils and Groucho) via a different site might compensate for the lack of interaction with Pygo2.

1.3 Role of Pygo2 in cancer

Since it was discovered that Pygo2 was involved in the regulation of the c-Wnt pathway and the binding of proteins required for the transcription of β-Cat-dependent genes, considerable effort has gone into determining PYGO2's potential roles in tumorigenic processes. Indeed, the c-Wnt cascade and β-Cat nuclear accumulation have previously been linked to the development of various types of cancer, most notably colorectal carcinoma, breast cancer, and glioma (Prasad et all., 2008) (Zardawi *et al*., 2009). Different studies present in the literature have highlighted the particular relevance of Pygopus proteins for human health studies. Even though the role of Pygo in c-Wnt signaling appears to be secondary to that of BCL9, significant research has revealed that Pygo2 appears to be over-expressed in various types of cancer, with this being directly associated with a poor prognosis. Thompson et al. (2002) made the first attempts to link PYGO2 with cancer development using the colorectal cancer cell lines SW480 and HCT116, which have a mutated APC and an oncogenic β-Catenin mutation, respectively. In these cell lines, knocking down *PYGO1* and *PYGO2* transcripts, alone or together, resulted in a significant decrease in transcription rate mediated by the β-Catenin / TCF complex (Thompson *et al*., 2002).

Interestingly, in later studies, *pygo2* was found to be up regulated in adenomas and in almost all murine and human colon tumor cells analyzed, even if in colon cancer cells it did not turn out to be a c-Wnt target (Brembeck *et al*., 2011). Another study by Nguyen *et al*., (2010) revealed that samples from patients' villous colonic adenomas (that present a higher malignant potential) were characterized by an increase in expression of different c-Wnt pathway-related genes, among which *PYGO2*. This suggests that c-Wnt signaling regulation might change during the progression from normal mucosa to tubular adenomas and then to villous adenomas (Nguyen *et al*., 2010). Similar results have been observed in malignant breast tumor samples; *PYGO2* was overexpressed in a high proportion of breast malignant tumors, and it was required for the growth of several cell lines derived from these carcinomas; its activation was associated with a more malignant phenotype (Katoh and Katoh, 2006), (Andrews *et al*., 2008), and PYGO2 was frequently found to be essential for the expression of the c-Wnt target gene cyclin D1, which is known to be involved in tumor growth in the context of this type of tumor (Andrews *et al*., 2007), (Orsetti *et al*., 2006). In addition, PYGO2 was also shown to be required for an epigenetic histone modification program required for c-Wnt-dependent transcriptional activation and the consequent

expansion of breast cancer stem-like cells in culture (Chen *et al*., 2010). Moreover, experiments with (MMTV)-Wnt1 transgenic mice (Watanabe *et al*., 2014) suggest that Pygo2 is involved in the start of cancer and the choice of tumor subtype. Zhang *et al*., (2016) found that *PYGO2* is one of the genes responsible for the development of chemoresistance in breast cancer cells. Glioma is another known c-Wnt/β-Cat-dependent tumor that has been studied for Pygo involvement in tumorigenesis: *PYGO2* has been shown to be significantly up-regulated in the majority of human brain glioma tissues and cell lines (Wang *et al*., 2010), (Zhou *et al*., 2016). Moreover, these studies confirmed that its overexpression was correlated with the patient's age, the WHO tumor classification, and the patient's prognosis (Zhou *et al*., 2016). In addition to this, Chen *et al*., (2011) also discovered that *PYGO2* and cyclin D1 were highly expressed in 67 rat glioma tissue samples. Focusing on hepatic carcinoma, different researchers performed a gene expression profiling analysis on hepatocellular carcinomas and adenomas that revealed the up regulation of some genes, among which *PYGO2*; this situation correlated with poor outcomes in patients (Skawran *et al*.,2008) (Zhang *et al*., 2015). Interestingly, in these specific malignant cellular types, PYGO2 dependent acquisition of a more aggressive behavior is dependent on the inhibition of *E-CADHERIN* expression (Zhang *et al*., 2015), and evidence of a correlation between *E-CADHERIN* downregulation, increased c-Wnt signaling, and carcinoma progression have been reported in different studies and models (Yook *et al*., 2005) (Heuberger and Birchmeier, 2010). Moreover, *PYGO2* was also found to be significantly overexpressed in lung cancer, both in human lung cancer tissue samples and in cell lines (Liu *et al*., 2013), (Zhou *et al*., 2014). In this case as well, an increase in *PYGO2* expression was correlated with a more malignant cancer phenotype and a poor patient prognosis (Liu et all., 2013). Finally, *PYGO2* has been found to be up regulated in a variety of cancers, including epithelial ovarian malignant tumor, cervical cancer, and esophageal cancer (Andrews *et al*., 2008) (Popadiuk *et al*., 2006) (Moghbeli *et al*., 2016). Even more relevant to note, in each type of cancer in which *PYGO2* transcription was activated, its specific knockdown resulted in a decrease in proliferation rates, tumorigenicity, and invasiveness of the carcinoma cell lines considered (Wang *et al*, 2010) (Chen *et al*., 2011) (Andrews *et al*., 2013) (Jiang *et al*., 2015). This nuclear factor was also suggested as a possible target for the local design of anti-cancer drugs (Zhou *et al*., 2014) (Ali *et al*., 2016).

While the data from development suggested that Pygo2 may play a secondary role to BCL9/BCL9l in regulating the expression of c-Wnt dependent genes, the data from cancer suggest that PYGO2 may be more important in those processes. Quite interestingly, it has been shown that PYGO2 plays an important role in breast cancer because of its interaction with H3K4me2/3 (Saxena *et al*., 2020). Moreover, PYGO2 needs to interact with BCL9-β-Catenin to promote breast cancer (Vafaizadeh *et al*., 2021). The discrepancy between the structural studies presented previously and the functional roles of PYGO2 discovered in several cancer lines suggests that in some processes the entire set of PYGO2 functions may be required, and that PYGO2 may be the mediator of the fine regulation of c-Wnt dependent gene expression in cancer induction and progression.

To date, most studies concerning *PYGO2*'s role in cancer development have been conducted in human or murine cell lines. However, it is nowadays known that *in vivo* animal models of tumor development are one of the most efficient approaches: directly exploiting the animal model allows the researcher to better investigate the onset of cancer as well as its evolution over time (Gallo and Bellipanni, 2017). Moreover, we know that for a lot of different animal models, for example the zebrafish, developing tumors show a very significant histological similarity compared to their human counterparts (Liu and Leach, 2011) (Amatruda *et al*., 2002).

1.4 **Zebrafish and Melanoma**

1.4.1 ***The zebrafish***

Animal models play a crucial role in the research aiming to identify the mechanisms of tumor onset and spread as well as in discovering new therapeutic approaches. After four decades of research by Dr. George Streisinger at the University of Oregon (Streisinger *et al*., 1981), the teleost (bony) fish zebrafish (*Danio rerio*) has become one of the most powerful model organisms. The use of this vertebrate system has many advantages: it has a short generation time, high fecundity (up to 200 eggs per mating), external fertilization, the transparency of its small embryos developing outside the mother's body and the possibility of manipulating and growing them very easily under standard conditions, and the high similarity in organ and tissue organization with humans (Streisinger *et al*., 1981) (Kimmel *et al*., 1995) (Detrich *et al*., 1999). All these features make the zebrafish a very practical and inexpensive model.

The sequencing of the zebrafish reference genome has been completed, and more importantly, approximately 70% of the human genes have at least one zebrafish orthologue (Howe *et al*., 2013). The use of the zebrafish as an animal model has been fostered by the easy-to-use molecular-genetic approaches to this animal species. Initially, it was the use of a forward genetic approach via treatment with chemicals, irradiation, or insertional mutagenesis that was classically used for large-scale mutagenesis screens, with the aim to identify vertebrate genes responsible for the development of the embryo (Mullins *et al*., 1994) (Balciuniene *et al*., 2013). Interestingly, many of the pathways involved in embryonic development are directly involved in cancer induction and development when dysregulated in the adult animal. As a result, Zebrafish have emerged as one of the best vertebrate models for modulating the expression of cancer-specific genes and assessing their impact, for example, during the early stages of embryonic development (Liu and Leach, 2011). Then, scientists developed the transposon-mediated approach for the establishment of consistent transgenic zebrafish models, and the expression of specific genes selectively in the tissues of interest or to express different fluorescent proteins in specific cell types became an easy task (Santoriello and Zon, 2012). Finally, the genome editing technique based on the CRISPR-Cas9 system has made reverse genetics approaches in zebrafish more efficient and affordable, with the possibility of obtaining a large number of mutants with reduced efforts in terms of time and laboratory resources, thus permitting the evaluation of the effects of specific gene mutations on cancer development (Blackburn *et al*., 2013; Hwang, *et al.,* 2013; Liu, *et al.,* 2017; Burg *et al*., 2016). In addition to these molecular-genetic approaches, zebrafish are very amenable to more classical experimental studies, like the powerful possibility of performing specific tumor cell transplantation (White et al., 2013). This technology, called xenograft, allows us to study human cancer cell behavior *in vivo*, determining the ability of these cells to grow and metastasize and inducing angiogenesis (Vitale *et al*., 2014; Chen *et al*., 2017; Nicoli and Presta, 2007). All these features establish the zebrafish as a unique *in vivo* system for modeling human cancers. Initially, it was used to study non-solid tumors (Le *et al*., 2007; Smith *et al*., 2010). Later, with the establishment of the homozygous mutant fish for the onco-suppressor factor p53 (Berghmans *et al*., 2005), it also became extremely suitable for the analysis of solid tumor onset and spreading, due to the higher frequency of tumor occurrence in this line; among these types of tumors, one of the most studied and best characterized is certainly melanoma.

1.4.2 ***Zebrafish skin and melanocytes***

Zebrafish skin apparently looks different than human skin; however, it retains many similarities with the human skin organization. It contains the three layers of epidermis, dermis, and hypodermis. Zebrafish and human skin epidermis contain melanocytes, express similar genes, and retain similarities in skin inflammation components and processes, as reviewed by Russo I. *et al*., (2022) (Gallo and Bellipanni 2017).

Recent studies in melanocyte development and malignancies have revealed the zebrafish to be an effective model for studying melanoma biology. Zebrafish melanomas are strikingly similar to their human counterparts, both in terms of genetic features and phenotypic characteristics. Since its early specification, knowledge of the melanocyte lineage has provided essential insights about genetic pathways that might be altered during nevi formation and melanoma occurrence. In brief, the melanocytes that become first nevi and then melanomas are reprogramming themselves towards a Neural Crest (NC)-like type of cell (Ceol *et al*., 2008; Gallo and Bellipanni 2017). Furthermore, melanocytes are externally visible, and single cells can be easily visualized in a living zebrafish, which also lacks consistent adipose tissues that could mask the tumor mass, as seen in rodent models, making melanomas in the zebrafish very detectable (Ceol *et al*., 2008). The melanocytes of vertebrates, including human and zebrafish, derive from the highly motile population of the NC cells (Rawls *et al.,* 2001), a temporary embryonic structure induced during gastrulation by the signaling of BMP-and Notch-pathways, originating from the ectoderm cell layer (Kanzler *et al*., 2000; Simões-Costa *et al*., 2015).

Most NC cell fates are already specified very early in development, prior to the start of migration (Raible and Eisen, 1994). NC cells undergo a so called Epithelial-Mesenchymal Transition (EMT) process, during which they lose adhesion to the neighboring cells and become motile (Ceol *et al*., 2008), (Giarnieri *et al*., 2015). After this event, they begin to migrate to reach their final fate. In zebrafish, melanocyte precursors start to express melanin around 24hpf (hours post-fertilization), and the typical embryonic pattern of pigmentation is mostly completed by 48hpf (Rawls *et al*., 2001). It was shown that c-Wnt signals are necessary and sufficient to direct the specification of the neural crest to a pigment cell fate; this occurs before the migration process begins (Dorsky *et al*., 1998).

During zebrafish development, the *mitfa/nacre* gene, a transcription factor homologous to MITF, is expressed in differentiating neural crest-derived melanophores as well as in the retinal

pigmented epithelium (Lister *et al*., 1999; Wojciechowska *et al*., 2016). Functional inactivation of *mitfa/nacre* causes neural-crest-derived melanocytes to fail to differentiate (Lister *et al*., 1999; Dorsky *et al*., 1998). The discovery of three Tcf/Lef (family of high-mobility-group transcription factors co-activated by Wnt-dependent β-catenin accumulation in the nucleus) binding sites in the promoter region of the zebrafish *mitfa/nacre* gene required for interaction with zebrafish Lef1 in vitro as well as proper expression in zebrafish embryos identified *mitfa/nacre* as a direct target of Wnt signaling (Dorsky *et al*., 1998; Gajos-Michniewicz A, Czyz M. 2020).

1.4.3 ***Nevi and melanomas***

Among the factors that have been shown to be directly linked to the formation of nevi (groups of melanocytes clustered together) and their malignant transformation to melanoma, we have BRAF and RAS mutations, which are a feature of approximately 80% of the cases (Tuveson *et al*., 2003) (Chin, 2003). Patton *et al*. were able to establish a zebrafish model for melanoma development by analyzing BRAF function and *in vivo* monitoring nevi formation (Patton *et al*., 2005). The authors used a stable transgenic approach to drive the expression of the human *BRAF* gene carrying the mutation V600E most found in human nevi and melanoma specifically in the epidermis of the fish, placing it under the control of the melanocyte-specific promoter *mitfa/nacre* (Dorsky *et al*., 2000). Using this approach, scientists were able to demonstrate that the expression of this mutant form of BRAF led to the occurrence of ectopic melanocytes and nevi in the fish, starting at 8 weeks post fertilization (Patton *et al*., 2005). Moreover, the same transgenic approach performed using a p53-deficient zebrafish background resulted in a significant increase of melanocyte lesions that were highly prone to degenerate into invasive melanomas, thus demonstrating that the BRAF factor, in a p53-depleted environment, is sufficient for nevi formation, and it represents one of the primary actors in play during melanoma occurrence, interacting with the p53 pathway (Patton *et al*., 2005).

Interestingly, Kauffman *et al*. (2016) showed that the expression of neural crest progenitor (NCP) genes in neural crest cells and in both zebrafish and human melanomas was correlated to the activation of specific super-enhancers in the context of a complex epigenetic control, leading to the reemergence of an NCP identity in these particular cellular populations. Altogether, the results presented in that report and reviewed in Gallo and Bellipanni (2017) demonstrate that melanoma precursor cells restart an embryonic neural crest developmental program, and that this

reemergence of an NCP-specific feature represents a key step occurring during cancer initiation. Thus, these data show how only sporadic cells within a larger nevus have a gene expression profile that allows the transition to the malignant state. The authors called this a "cancerized field." According to the authors, the acquisition of a tumor fate is very likely due to a combination of gene dysfunction occurring within the single cells together with aberrant signals originating from the niche environment (Kauffman *et al*., 2016).

1.5 **Thesis aims**

It is plausible that the tumorigenic and migratory potential of melanoma cells depends on the reversion of the melanocyte profile into the NC profile. Therefore, it remains to be investigated how far back in the NC developmental program we can find the original step leading to melanoma induction. For instance, could Wnt, BMP, or Notch signaling, or a combination of those early signaling molecules, affect the "cancerized field" such that it induces the transition to a malignant state? Following this same reasoning, Varum *et al*. (2019) have discovered a transcription factor, Yin Yang1 (YY1), required for regulation of the NC transcriptional program and for melanoma initiation in a mouse model. Intrigued by this, I decided to make it the main aim of my thesis. I liked to focus on the nuclear regulation of c-WNT-dependent gene expression by means of the interaction of β-catenin with the Pygo2 nuclear factor. To accomplish my goal of shedding light on the role of c-Wnt signaling to induce the transition of nevi into a malignant state, I divided my effort into two parts. In one hand, I looked at the specific role of Pygo2 in regulating c-Wnt signaling. I first investigated the role of a splicing variant of Pygo2 lacking the PHD domain, *pygo2ΔPHD*, that we had identified in the laboratory. To complete this task, I tested the role of this variant *in vitro* by assaying its ability to regulate β-catenin activity as a transcription factor in a TOP-FLASH assay as well as determining its expression pattern during embryogenesis. On the other hand, I created molecular genetic tools in zebrafish to study the role of the Pygo2ΔPHD variant in the formation of nevi and melanomas, as well as transgenic lines that produce many nevi and melanomas in the fish.

CHAPTER 2

MATERIAL AND METHODS

2.1 Database Resources

The National Center for Biotechnology Information's (NCBI) Internet databases were regularly mined to find DNA/RNA/Protein accession numbers and their associated sequences that specifically matched the entered gene of interest. NCBI databases were used to retrieve both metadata (such as gene annotations, chromosome information, gene IDs, and descriptions) and sequence data (raw sequence, regio/feature labels).

2-2 Zebrafish strains

Tübingen Long Fin (*TLF*), *Brass* (*Brs),* and p53 homozygous mutants (*tp53*^{zdf1}) fish strains were used during this work. They were maintained in a closed system at 28.5 °C, following standard husbandry procedures (*Westerfield M., 2000*). *TLF and Brs* were selected as *wild type*. *tp53*^{zdf1} contains a single T-to-A point mutation that changes Met to Lys at residue 214 of the Tp53 protein (Berghmans *et al*., 2005). All experimental procedures were approved by IACUC (Institutional Animal Care and Use Committee).

2.3 Plasmid preparation

Bacterial transformations with plasmid DNA were completed as per general protocol (Sambrook and Russel, 2006). Several colonies from a LB plate containing the desired transformed bacteria were inoculated in 5 ml or 20 ml of LB plus the appropriate antibiotic and allowed to grow at 37 °C with shaking at around 200 rpm. The day after, the plasmids were recovered using the Roche High Pure Plasmid Isolation Kit. The concentration was measured with the NanoDrop ND-1000.

2.4 **Synthetic mRNA in vitro transcription**

mRNA to be used for microinjection on zebrafish embryos was produced using linearized template plasmid DNA (Table.1). The digested DNA was purified via phenol/chloroform and precipitated with 0.5 volumes of ammonium acetate plus 2 volumes of 100% ethanol. Resuspended linearized DNA was used to prepare the synthetic mRNA with the mMESSAGE mMACHINE kit (Ambion) in accordance with the kit protocol. The synthetic mRNA obtained was quantified at the spectrophotometer.

Table 1: Constructs used for mRNA synthesis with digestion enzymes for linearization of the plasmid, polymerase used and reference

Plasmid	**Restricted Enzyme**	**Polymerase**	**Reference**
pT3TS-nCas9n	*XbaI*	T3	Jao *et al.*, 2013
pCMV-Tol2	*NotI*	SP6	Balciunas et al., 2006

2.5 **Microinjections on zebrafish embryos**

The microinjections were performed in all blastomeres of zebrafish embryos at the 1-2 cell stage. All mRNAs were injected at 100 ng/µl and DNA were injected at 50 ng/µl. The injection solution was made by mixing the double-concentrated mRNA with an equal volume of Dulbecco's modified phosphate-buffered saline (PBS) containing 0.5% phenol red (Sigma). Between 1 and 3pL of solution were injected per embryo.

2.6 **Probe preparation reaction**

For probe synthesis, we linearized the plasmids with restriction enzymes and used the appropriate RNA polymerase; *chordin* was in pCS2+ (Miller-Bertoglio *et al*., 1997) and *goosecoid* was in pBS-SK (Stachel *et al*., 1993); (Table.2). Thus, we set up the mRNA Digoxygenin-labeled probe reaction for each gene using the appropriate RNA polymerase with 5X buffer, 0.1M DTT, 10X NTP DIG mix, and 1ml of RNasin. This mixture was incubated at 37°C for 2-3 hours.

Then, the probe mixture was mixed with a 20mM Tris/HCl pH 7.5, 20mM EDTA, 100mM NaCl TEN buffer and purified through centrifugation for 3' at 3000 rpm in a probe QuantG50 Micro column (Biolab), previously balanced with 0.3M NaOAc (pH 5.2) and 0.1% SDS equilibration buffer. After precipitation with 3M sodium acetate, the dry probe was dissolved in 30 ml of DEPC.

Table 2: constructs for antisense RNA probe synthesis

Plasmid	Restricted Enzyme	Polymerase	Reference
pBS-chd	*SpeI*	T7	Miller-Bertoglio et al., 1997
pBS(SK)-gsc	*HindIII*	T7	Stachel et al., 1993

2.7 **LNA Probe**

Locked nucleic acids (LNAs) contain a methylene bridge between the 2' O and the 4' C on the ribose ring that "locks" the structure into a high binding affinity. These nucleotides have an RNA-mimicking conformation (Koshkin *et al*. 1998; Wengel *et al*. 2003). It has been shown (McTigue et al. 2004) that DNA oligonucleotide probes containing such modified nucleotides at every third position (LNA probes) present a dramatically enhanced hybridization specificity. We designed an LNA probe as described in Kloosterman *et al*. (2006) and ordered the Dig-labeled D20-mer modified nucleotide from EXIQON. We also purchased a similar probe for the miRNA

that would work as a positive control for the WISH (whole mount in situ hybridization); see Table.3 for sequences. The *pygo2ΔPHD* target sequence for the LNA was a region overlapping the putative splice site of *pygo2ΔPHD* mRNA after the region of the PHD had been removed. The sequence was: 5'-aACAACAGCAGTGTCCTGAa -3'. The underlined regions are part of a sequence that is common to both ends of the full-length mRNA of *pygo2*. Thus, our LNA has 13bp of annealing at 5' and 13 at 3' with *pygo2 full-length*. These overlaps unlikely would produce even a week staining. According to Plasterk' laboratory protocol, a 14-bp LNA produces weak staining, however, this is a 20bp LNA with only 13bp sequence overlapping either side of the full-length mRNA. Furthermore, there is no staining (Kloosterman *et al*., 2006) when 22 pb LNA contains only 2-3 bp of MisMatch.

Table 3: names, sequences and melting temperatures of LNA probes used

LNA Name	Sequence	Size	Tm°C
z-pygopus2 ΔPHD	5'-TTCAGGAACACTGCTGTTGTT-3'	20bp	83°C
mdo-miR-206	5'-TGGGGATATAAAGAAGCATGT -3'	21bp	73°C

2.8 In Situ Hybridization

For regular WISH, samples stored in 100% methanol, were rehydrated by rinsing in an increasing dilution of methanol/PBS and then washed with PBT (PBS+Tween-20). The embryos were pre-hybridized for 2 hours with a hybridization solution containing 60% formamide (Roche). The digoxigenin-labeled probes were applied to the respective samples, and hybridization was carried out overnight at 70°C. Embryos were washed in decreasing formamide and citrate buffer (SSC) solutions and then in PBT to remove unbound probe. The solution was replaced with an antibody against DIG, diluted 1:200. This antibody is conjugated to alkaline phosphatase (AP). The 2-hour incubation was followed by 8 PBT washes of 15 minutes each, followed by 3 washes of 5 minutes each with AP buffer (100 mM TrisHCl pH 9.5, 50 nM MgCl2, 100 mM NaCl, 0.2%

Tween 20 and 0.2% Triton-X100). The DIG-AP antibody was detected with BM purple substrate (Roche) kept in the dark at RT with little agitation for coloration. The reaction was stopped (generally after 1–2 hours) at the same time for experimental and control samples with PBS buffer washes and then fixed with 4% PFA. The images were captured with the stereomicroscope SMZ800 (Nikon) and analyzed with the software NIS-Elements BR3.0 (Nikon). For LNA-WISH hybridization, I used the same protocol as above (65% formamide in the hybridization buffer), but with a 52ºC hybridization temperature.

2.9 **Transfections and Luciferase Assays**

~2.0 x 10^6 HEK293T cells were plated in 12-well plates 24 hours prior to transfection. Cells were transfected using a calcium phosphate kit (Clontech) according to the manufacturer's instructions. Cells were transfected with two reporters: 40ng pRL-SV40 and 250ng TopFlash, along with other plasmids. One or more of the following expression constructs was used: pCS2$^+$*Flag-zPygopus-1,* pCS2$^+$*Flag-zPygopus-2,* and pCS2$^+$*Flag-zPygopus-2ΔPHD* expression vectors (200 ng - 400 ng) and myc-*β-catenin* expression plasmids (300 ng) (gift from Dr. Habas laboratory). The total amount of plasmid DNA transfected was made equivalent with the pCS2$^+$-GFP empty vector. Luciferase activities were determined either at 24 or 72 hours after transfection (hat) using the Promega DUAL Luciferase reporter assay system according to the manufacturer's protocol (Promega). Firefly luciferase luminescence was normalized to *Renilla* luciferase luminescence. For this experiment, two rounds of experiments were done, and each sample was only performed once in each round.

2.10 **Protein extraction**

The HEK293 cell line was used to make total protein preparations. ~10^7 cells were resuspended in PBS. Then, proteins were extracted with RIPA buffer plus protease inhibitors (Sigma). Protein concentration was measured using the Bradford protein assay with a biophotometer (Eppendorf), then the protein samples were aliquoted and stored at -80°C.

2.11 **SDS-Page and western blotting**

All western blot experiments were essentially carried out as previously described (Bellipanni *et al.,* 2000). Protein samples were melted on ice, mixed with 2x Laemmli loading buffer, boiled for 10 minutes, and then loaded on polyacrylamide gel, using a Pageruler-prestained ladder (Fermentas) as a marker. The run was at 100V constant for an hour and a half, then the gel was blotted with the Trans-Blot Semi-Dry System (Bio-Rad), using the NuPage Transfer Buffer (Invitrogen) for 25–40 min at 20V constant.

The membrane was then rinsed in blocking buffer (3% milk in PBS-Tween 0,1%) for an hour at RT with shacking. After an hour, it was incubated with the primary antibody, mouse anti-Flag (Rockland) (1/500 dilution), dissolved in 3% milk on PBS-Tween 0,1% overnight (ON) at 4°C with shacking. The following day, after a few washes in PBS, anti-mouse and anti-rabbit peroxidase-conjugated (Amersham) as secondary antibodies were added at 1/10000 dilution for 1 hour. Blot was detected by using Pierce ECL Western Blotting Substrate according to the manufacture protocol description (Pierce) and exposing films for different time lengths.

2.12 **CRISPER-Cas9 Mutagenesis**

2.12.1 ***Target selection***

As described by (Hwang *et al*., 2013) and as already verified by Dr. Gianfranco Bellipanni and Dr. Darius Balciunas (Temple University, Philadelphia, USA; personal communication), the only requirement to be strictly followed for CRISPR target selection is the presence of a 5'-NGG-3' PAM site. I identified a region with these characteristics within the NHD and one within the PHD of the Pygo2 genomic sequence, that is just downstream of the putative splicing site that forms the Pygo2DPHD splicing variant, which would eliminate both the BCL9/9l binding site and the ability to recognize H3K4me2/3. Indels in such region would make the Pygo2 full-length not complete due to a frameshift in the ORF but would not affect the Pygo2ΔPHD variant.

2.12.2 ***sgRNAs synthesis***

The sgRNAs were synthesized after a two-step PCR using the DR274 guide RNA expression vector as a template (Addgene plasmid # 42250). DR274 was a gift from Keith Joung (Hwang *et al*., 2013). To synthesize our sgRNAs, we designed specific short guide primers (see Table 4) containing, in addition to the sequence complementary to the target, a T7 promoter for in vitro transcription, and a homology sequence to the sgRNA component of the DR274 vector. sgRNAs were in vitro transcribed using the "MegaShortscript T7" kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

Table 4: Primers used for CRISPR synthesis against *pygopus2 NHD* and *pygopus2* PHD. The sequences are all 5'to 3' direction, the lowercase in green is the sequence that matches the genome, the G in red was add for improving T7 promoter activity. The sequences in upper case are matching the plasmid DR274 guide RNA expression vector.

Primers name	**Primers sequence 5'to 3'**
GG-CRISPRpygo2-PHD	CGCTAGCTAATACGACTCACTATAGgagcccagagaaaaagaagGTTTTAGAGCTAGAAATAG
GG-CRISPRpygo2-NHD	CGCTAGCTAATACGACTCACTATAggctctggccccccaaagctGTTTTAGAGCTAGAAATAG

2.12.3 ***cas9 mRNA synthesis***

I used the pT3TS-nCas9n vector as a template to in vitro transcribe zebrafish-optimized nCas9n mRNA (Addgene plasmid # 46757). pT3TS-nCas9n was a gift from Wenbiao Chen (Jao et al., 2013). The mRNA was in vitro transcribed using the "mMessage mMachine kit" (Ambion) in accordance with the manufacturer's instructions after the vector was linearized using the XbaI enzyme (New England Biolabs Inc.) (Table 1).

The mRNA was purified by phenol:chloroform extraction (Sigma-Aldrich), precipitated and resuspended in sterile MilliQ water, then quantified and stored at -80°C.

2.12.4 ***CRISPRs microinjection***

The solution to inject was prepared by mixing sgRNAs together with cas9 *mRNA* and rhodamine dextran (Molecular Probes, Life Technology) as a tracer, diluted in RNase-free water.

This injection mix was pressure-injected into 1 to 2-cell-stage embryos using a Narishige micromanipulator. The optimal dose for each CRISPR was selected based on the evaluation of the toxicity and the phenotypic alterations resulting in the embryos, as well as the mortality rate.

2.12.5 ***Mutations detection and screening***

Genomic DNA (gDNA) was extracted from pools of embryos or adult fin clips using DNA extraction buffer, which was prepared according to instructions provided by the ZFIN community (Bradford et al., 2022). Proteinase K (Sigma-Aldrich) was then used to digest the samples at 65°C. Potassium acetate (8 M) was then used to remove proteins from DNA; after transfer of the supernatant phase to new clean tubes, DNA was precipitated using isopropanol and washed with 70% cold ethanol. gDNA was resuspended in nuclease free water. PCR was then performed on gDNA using specific primers amplifying the region we targeted with our CRISPRs (see primers list below) and Taq DNA Polymerase (Thermo Fisher Scientific). To screen for mutations, PCR products were first processed using the "Surveyor Mutation Detection Kit" (IDT Technologies) according to the manufacturer's instructions.

After digestion with Surveyor nuclease, samples were analyzed by 2% agarose-gel electrophoresis to check if multiple bands derived from mismatch-directed cleavage were present. Surveyor-positive PCR samples from F1 pools of embryos needed to be confirmed by Sanger sequencing. They were purified using the "DNA Clean-Up & Concentration Kit" (Zymo Research) according to the manufacturer's instructions and sent out for sequencing (Genewiz-DNA sequencing service).

When screening for heterozygous F1 adult fish, surveyor-positive PCR amplicons derived from fin clips were first purified using a "DNA Clean-Up & Concentration Kit" (Zymo Research) and then sent for sequencing (Genewiz-DNA sequencing service). All electropherograms were analyzed as mentioned above. The presence of indels was tested with two sets of primers, one for the NHD and one for the PHD regions (Table.5). The solution was prepared with Taq DNA polymerase recombinant (Thermo Fisher Scientific). and the protocol was set with these conditions: pre-denaturation 94 °C 4 min; amplification 94°C 30 sec, 55°C 20 sec, 72°C 75 sec, repeated 15 cycles, then amplification 94°C 30 sec, 55°C + 0.5°C each cycle for 20 sec, 72°C 75 sec, repeated 15 cycles; final extension 72°C 10 min. The PCR products were loaded into a 2% agarose gel.

Table 5: Primers used for screening CRISPR-induced indels in the *NHD* and PHD regions of *pygopus2*, and predicted product size

Primers	Sequence	Product Size
5'z-pygopus2 NHD Forward 3' z-pygopus2 NHD Reverse	5'-AACGAGGCAAAGGT-3 5'-GGACCTGGAAGTGGACAG-3	308bp
5'z-pygopus2 diff. Forward 3' z-pygopus2 diff. Reverse	5'-ACCCAACTCCAACCAGAACA-3' 5'-TTCTGCGATTCATTGTCAGC-3'	1137bp

2.13 **Construct preparation**

2.13.1 ***Mitfa promoter isolation***

The pGB-*mitfa:Axin2DN* mini-Tol2 construct was created by amplifying the mitfa sequence from the zebrafish genome and cloning it into the pCS2+ *Flag-Axin2-DN* construct, after which the entire insert was cut with XmaI -HpaI, purified, and inserted into pDB773 cut also with XmaI -HpaI (Fig.5).

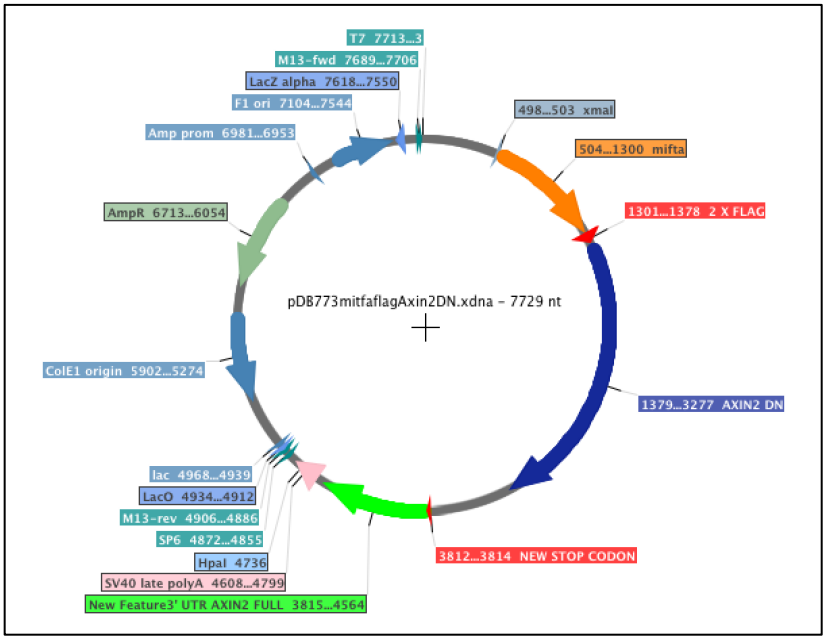


Figure 5: *pDB773 miftaflagAxin2DN*. Mifta enhancer in orange, Axin2DN coding in blue, SV40 Late polyA in pink.

2.14 **Creation of transgenic fish**

The plasmid was co-injected with 100ng/µl of a mRNA coding for the Tol2 transposase into 1 or 2 cell stage embryos T*p53* $^{zdf1/zdf1}$. At 2.5 months of age, F0 fish were visually screened for the presence of nevi or melanomas. MiniCoopR *mitfa:BRAF* was a gift from Leonard Zon (Addgene plasmid # 118846; http://n2t.net/addgene:118846; RRID:Addgene_118846). This plasmid contains the cDNA for the human gene BRAF with the mutation V600E under the control of the epidermis-specific *mitfa* promoter. These sequences are flanked by *tol2* transposon sequences that are necessary for the transposition. For the injections, a solution containing either 50ng/µl of MiniCoopR *mitfa:BRAF* plasmid or 50ng/µl of pDB773-*mitfa:Flag-Axin2DN* was co-injected with 100ng/µl of a mRNA coding for the transposase into 1-2 cell stage embryos T*p53* $^{zdf1/zdf1}$. This line of fish has nevi and melanomas due to BRAFV600E expression in the melanocytes (Ablain *et al*. 2018). At 2.5 months of age, F0 fish were visually screened for the presence of nevi or melanomas, and the fish that tested positive were isolated and later inbred to establish a stable line.

CHAPTER 3

RESULTS

3.1 **Background**

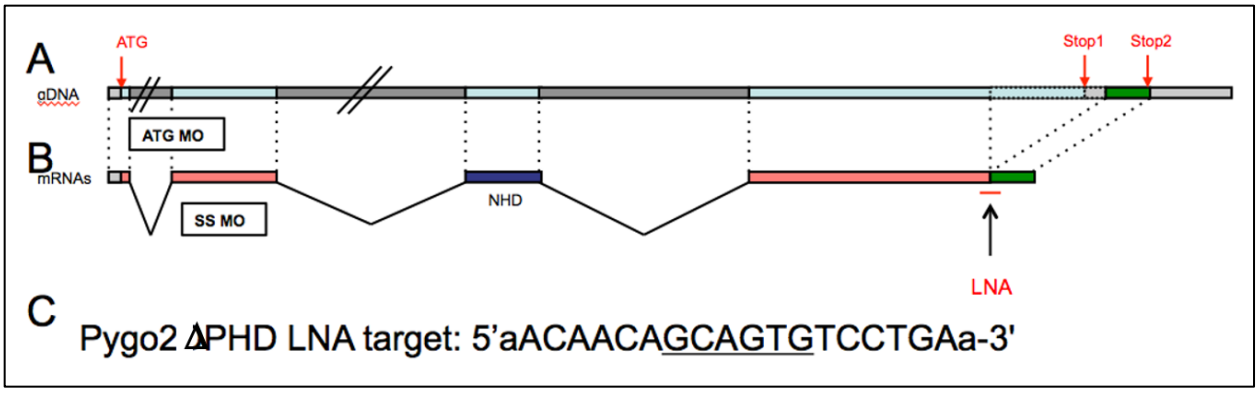
Previous work in our laboratory (Valenti *doctoral* thesis, 2011) have shown by means of the RT-PCR method that both the *pygo2 full-length* and the *pygo2ΔPHD* forms are expressed during early embryogenesis. Over-expression of *pygo1* or *pygo2ΔPHD* mRNAs was described in the doctoral thesis of J. Ibetti completed also in our laboratory, which showed that both factors, when overexpressed, were able to dorsalize a zebrafish embryo. In Dr. Ibetti thesis was also described both *pygo1* and *pygo2* knock-down, that consistently with the over-expression results, had a ventralization effect on the expression pattern of genes expressed at 30% epiboly in the zebrafish embryo. Thus, hat work suggested that Pygo2ΔPHD, despite the lack of the PHD domain, that is the known interface involved in the binding in promoter regions of WNT-dependent genes and the BCL9/β-Catenin, is involved in the c-Wnt-mediated induction of Vox and Vent in the medio-ventral margins of the zebrafish embryo at 30% epiboly.

While my doctoral thesis aimed to investigate the role of Pygo2 in melanoma, I had to complete a series of experiments that would confirm the role of Pygo2ΔPHD in the context of the c-Wnt pathway as well as develop new tools for this research, namely the creation of *pygo2* mutants and the establishment of transgenic lines that consistently developed melanomas. First, I needed to determine the expression pattern of the *pygo2ΔPHD* mRNA, to see if there was a specific region of the early embryo that expressed it. Then, using the Top-FLASH assay, I investigated the role of Pygo2ΔPHD in activating c-Wnt-dependent genes in vitro. The results of this experiment led me to investigate the cellular localization of the zebrafish Pygo2ΔPHD transcript in human cell lines (HEK-293), with the intriguing finding that Pygo2ΔPHD is not only present in the nucleus but also in the cytosol and that it is starting to shift from the cytosol into the nucleus only at three days after the transfection. In another set of experiments, I tested the role of Pygo2ΔPHD by inducing a target mutation, by CRISPR/Cas9 technology, in the PHD domain of *pygo2* in the zebrafish. The hypothesis behind this experiment was to determine if the Pygo2 protein carrying the PHD domain was dispensable for zebrafish development. Finally, I created a transgenic line of zebrafish that rapidly produces nevi and then melanomas.

All together, these experiments helped us not only get a better picture of the role of Pygo2ΔPHD in the zebrafish but also develop new molecular-genetic tools to test the role of this factor and c-Wnt signaling in melanoma development.

3.2 ***pygo2ΔPHD*** **expression during early embryogenesis and at 24hpf in the zebrafish embryo.**

While it was very simple to show the expression of *pygo2ΔPHD* mRNA by using RT-PCRs and sequencing of the fragments (Valenti F. doctoral thesis, 2011), the identification of the expression pattern of *pygo2ΔPHD* during the early development of the zebrafish embryo is not an easy task. In fact, *pygo2-full length* and the *pygo2ΔPHD* forms share 100% of the sequence except for the deletion in the PHD domain. As a result, any standard 400- to 600-bp-long *pygo2ΔPHD* probe would not be specific for the splicing form and would recognize the full-length *pygo2* mRNA as well. However, Plasterk's laboratory (Kloosterman *et al*., 2006) had developed a protocol using LNA oligos to identify the expression pattern of miRNAs of about 20-bp in animal embryos, including the zebrafish. I took advantage of this protocol and designed a 20-bp probe specific for *pygo2ΔPHD* mRNA. This probe matches 10-bp at either side of the putative splicing junction in the *pygo2ΔPHD* mRNA, with an overall overlap with the *pygo2 full* length of 13-bp for each side (Fig.: 6A-C). According to Plasterk's laboratory protocol (Kloosterman *et al*., 2006), this 13-bp overlap with the *pygo2 full-length* is not sufficient to produce any staining. To further confirm that the protocol I was using was correct, I also used a LNA probe for *miRNA 206* from zebrafish as a positive control probe in my WISH analysis (Fig.: 6D).



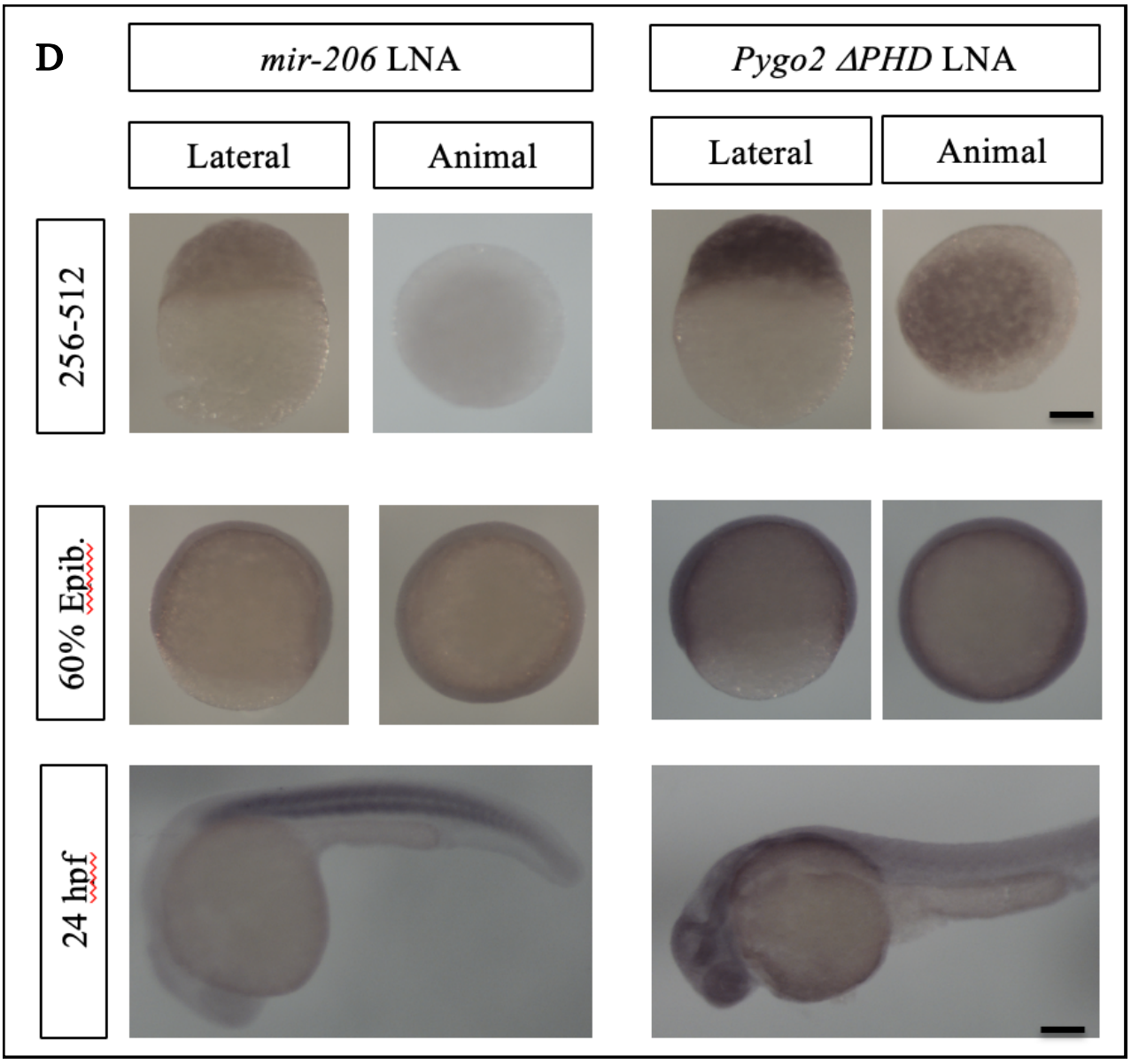


Figure 6: In A is the schematic representation of the pygo2 gene, below is the *pygo2ΔPHD* mRNA with the position of the LNA for the in-situ hybridization (B). In C is the sequence of the LNA. In D embryos at 256-512 cell-stage, 60%epiboly and 24hpf hybridized with LNA probe against *mir-206* (left) and *pygo2ΔPHD* (right).

For the WISH analysis, I used embryos at 256–512 cell stages, 60% epiboly, and 24hpf.

Figure 6D shows that the expression of *pygo2ΔPHD* mRNA is rather intense at 256-512 cell-stages and 60% epiboly stages, compared to the expression of the positive control probe, which is lighter but also present at those stages (Liu *et al*. 2012). At 24hpf, the positive control probe is more visible in the somite, whereas our *pygo2ΔPHD* mRNA is visible in the brain and trunk and in a superficial and widespread light staining.

Overall, this experiment confirmed the expression of *pygo2ΔPHD* mRNA at the analyzed developmental stages; however, it showed that *pygo2ΔPHD* mRNA is expressed ubiquitously at all stages analyzed. Therefore, this result does not offer a clear explanation of the possible roles of Pygo2ΔPHD in the dorsoventral patterning seen with the overexpression experiments.

3.3 ***In-vitro*** **analysis of Pygo2ΔPHD role the Canonical-Wnt pathway**

3.3.1 **Pygo2ΔPHD can mediate c-Wnt signaling**

To gain more insight on how Pygo2ΔPHD may mediate c-Wnt signaling, I moved towards some in vitro assays to test if Pygo2ΔPHD can cooperate with the c-Wnt pathway. For this purpose, I used the TOP-FLASH assay in human cell lines; in this assay, HEK293T cell lines are transfected with two reporter plasmids: pRL, which expresses renilla luciferase cDNA under the control of a basal promoter and is used for normalization, and pTOP-FLASH, which expresses Luciferase under the control of transcriptional activation of the c-Wnt Response Element (WRE) containing TCF/LEF binding sites.

The experiment included co-transfections with other plasmids; one containing the *β-catenin* cDNA is always present, while pCS2^{+}*Flag-zPygopus1,* pCS2^{+}*Flag-zPygopus2 full-length,* and pCS2^{+}*Flag-zPygopus2ΔPHD* were transfected alone or in combination, and analysis was conducted after 24 or 72 hours after transfection (hat) in triplicate (Fig. 7A and B).

When transfected alone, the results will indicate whether the Pygopus variant is interacting with β-Cat for the induction of WRE, while in combination, the results will shed light on possible interferences or interactions towards WRE between Pygo1 and Pygo2ΔPHD or Pygo2 full-length and Pygo2ΔPHD.

The results of this experiment are time dependent; 24 hours after transfection (hat) analysis shows that Pygo2 did not induce the luciferase reporter signal when transfected into the cells alone or in combination with 0.3 µg *β-catenin*. While Pygo1 increased reporter gene activity in the presence of 0.3 µg *β-catenin*, Pygo2ΔPHD appears to suppress luciferase reporter induction when

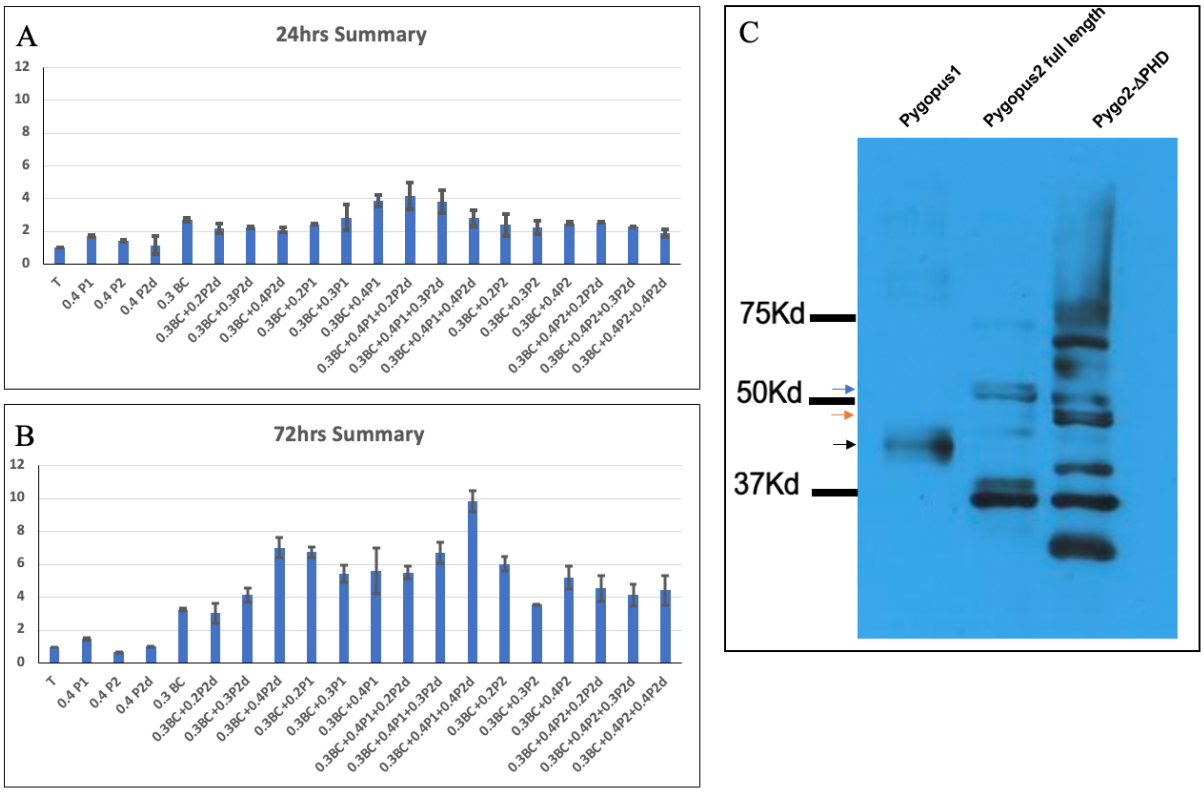


Fig 7: In A is the chart reviewing the TOP-FLASH experiment at 24 hours after transfection (hat). In B is the chart reviewing the TOP-FLASH experiment at 72h hat. P1 is Pygopous 1, P2 is Pygopous 2 full-length; P2D is Pygopus2ΔPHD; BC is β-Cat. In C the results of western blot analysis of the three pygopous used for cell transfection and TOP-Flash analysis; black arrow indicates Pygo1, red arrow indicates Pygo2ΔPHD, blue arrow indicates Pygo2 full-length. Numbers indicate µg used for transfection.

co-transfected with 0.3 µg *β-catenin* and 0.4 µg *pygo1*, and to a lesser extent when co-transfected with 0.4 µg *pygo2 full-length* in a dose-dependent manner. Furthermore, when transfected alone, Pygo2ΔPHD does not trigger reporter signal induction, nor does it affect reporter signal induction when co-transfected with 0.3µg *β-catenin.*

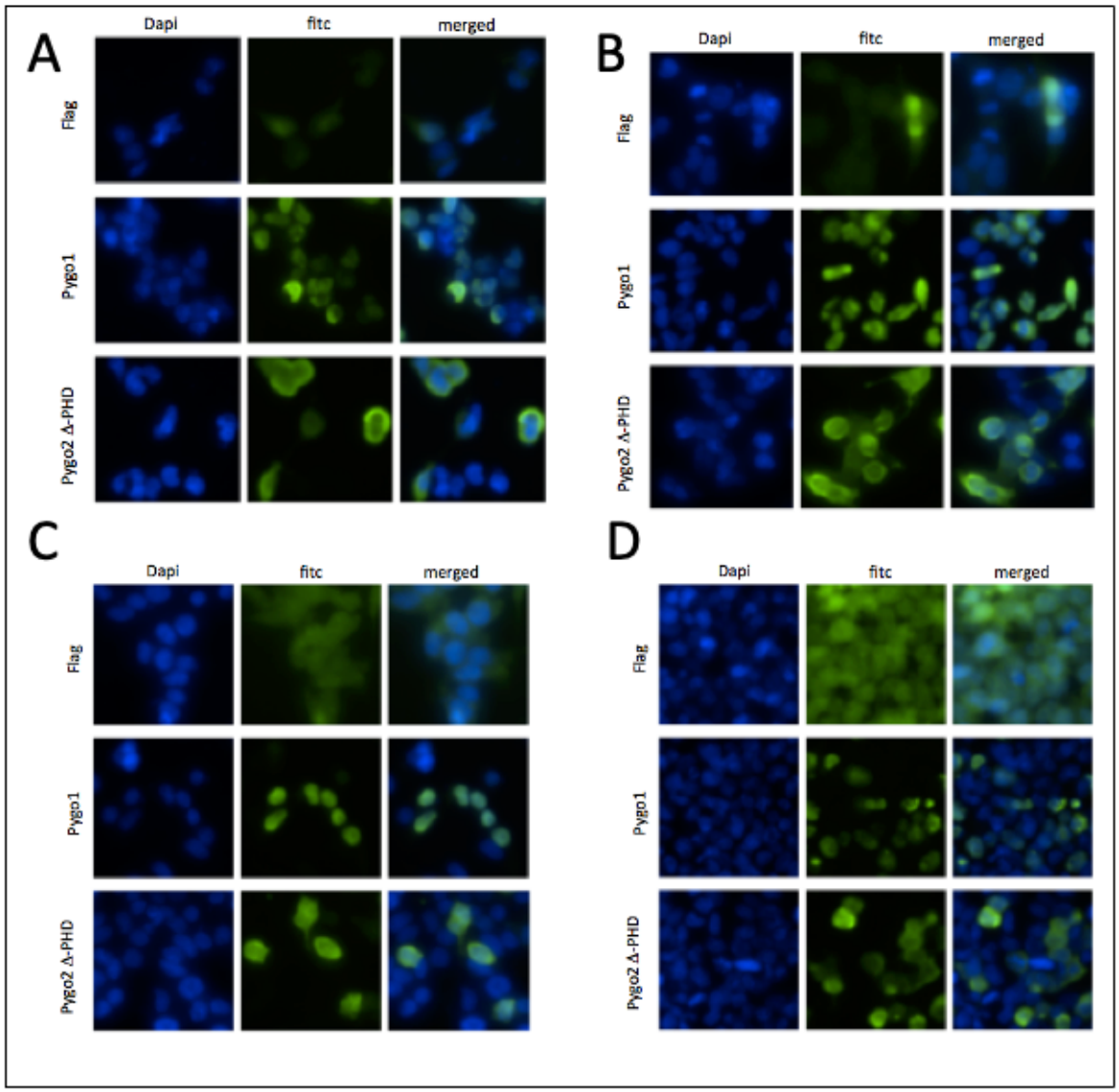
To test if it is necessary to wait a longer time after transfection to be able to see the effects of our Pygopous constructs on the activation of the reporter gene for c-Wnt signaling I repeated the experiment and tested samples at 72hat (Fig.7B). When transfected with 0.3µg *β-catenin*, all Pygo1, Pygo2 full-length, and Pygo2ΔPHD mildly induced the induction of the luciferase reporter, but neither Pygo1 nor Pygo2 full-length showed a dose-dependent effect, whereas Pygo2ΔPHD clearly activates the expression of the reporter gene in a dose-dependent manner, indicating that

this protein somehow works differently than Pygo1 and Pygo2 full-length. Moreover, Pygo2ΔPHD can increase the expression of the reporter gene in a dose-dependent manner when it is co-expressed with Pygo1. While Pygo2ΔPHD may slightly reduce the induction of luciferase reporter by Pygo2 full-length and β-Cat. Given the results of the assay at 24 hours, these findings were both surprising and unexpected. To check that all three zebrafish variants of Pygopus were correctly expressed in the HEK293K cell line, I tested their expression levels in a western blot (Fig.7C). In figure 7C there is the western blot analysis showing the correct expression of the three forms of Pygopus from zebrafish in the HEK293K cell line. To this extent, I took advantage of the fact that all three constructs used were tagged with a Flag-Tag in their N-Term region. The figure 7C shows that all three constructs were expressed at comparable concentrations; the Pygo1 protein had one major band at ~43Kd (black arrow), consistent with the molecular weight and pattern of other western blots of commercially available anti-hPygo1 antibodies. The line containing the extract of the HEK293K expressing the Pygo2 full-length isoform has five visible bands consistent with the commercially available anti-hPygo2 antibodies. The correct size of zebrafish Pygo2 full-length should be around 50Kd and in the form of a double band (see blue arrow). The line containing the extract of the HEK293K expressing the zebrafish Pygo2ΔPHD isoform has a similar pattern to that seen for Pygo2 full-length, but the bands are slightly smaller in size (red arrow), consistent with the absence of the PHD domain. In conclusion, the three constructs appear to be expressed at similar concentrations; at this moment I did not investigate further the nature of the other bands.

3.4 **Zebrafish Pygpous proteins localization *in vitro* in human cell lines**

The TOP-FLASH assay results were intriguing: Pygo2ΔPHD was able to reduce the β-Cat + Pygo1 activation of the reporter gene in a dose-dependent manner at 24h, but reverted this behavior at 72h, when it increased the activation of the reporter gene by β-Cat + Pygo1. In the attempt to clarify what could happen between 24hat and 72hat that triggered these changes, one possible hypothesis was a different localization within the cell of either Pygo1 or Pygo2ΔPHD at those different time points. To test this hypothesis, I used a 2x Flag-tag at the 5' of pygo1 and pygo2 constructs and transfected the HEK293T human cell line with these plasmids. After transfections, I assayed at different time points the localization in the cell of the translated proteins by means of the anti-Flag antibody (Fig. 8A-D).

Figure 8A-D depicts the time course of immunohistochemistry to examine the localization of the Pygopus variants. While Pygo1 begins to be clearly nuclear localized as early as 24hat by overlapping with DAPI nuclear staining, Pygo2ΔPHD is more peri-nuclear and cytosolic at 24 hat and 48 hat (Fig.:8 A and B) and does not become more nuclear localized until 72 and 96 hat (Fig.: 8 C and D).



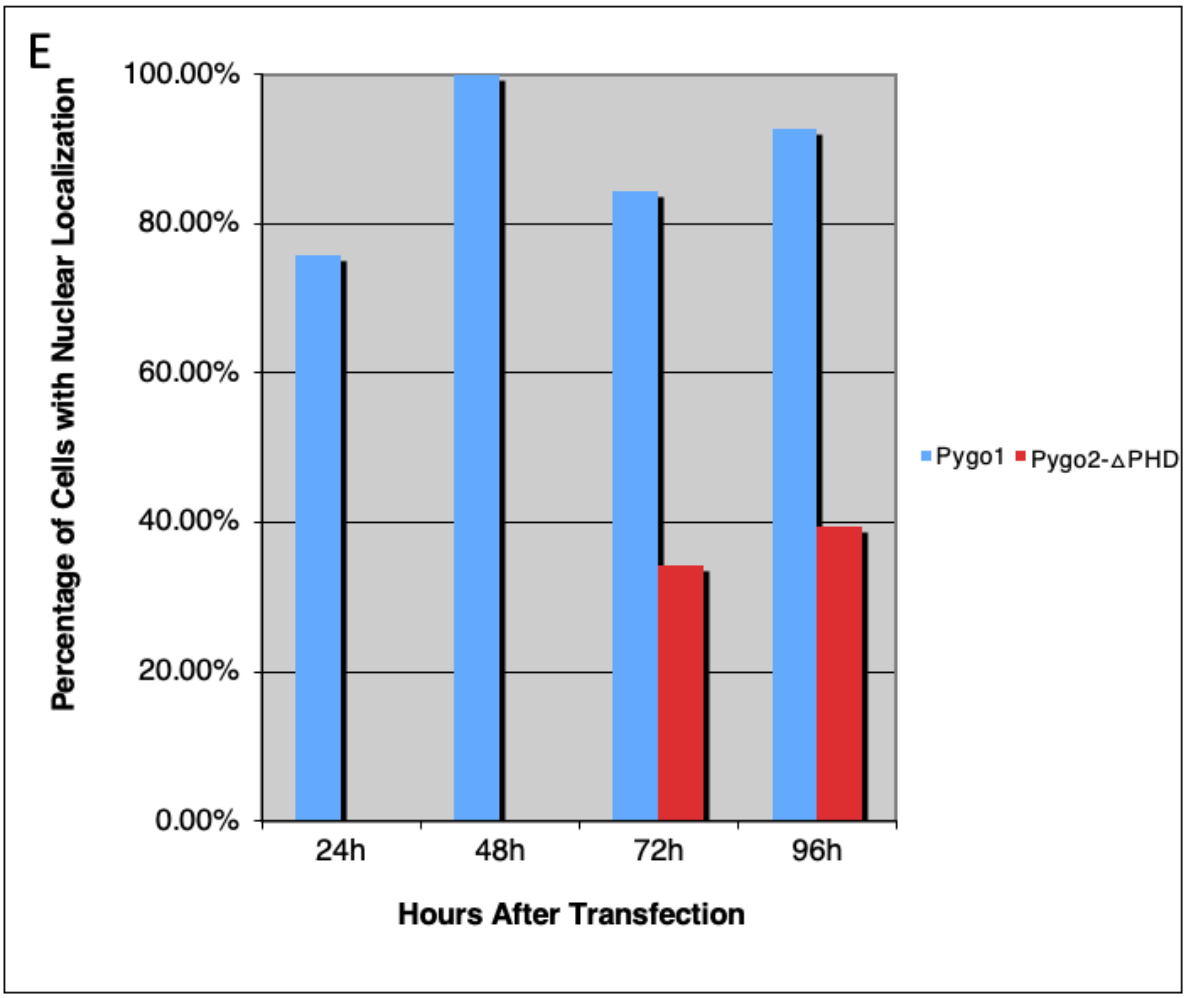


Fig. 8: A-D fluorescent microscope pictures of cells expressing with the three Pygopus of the zebrafish animal model: Pygo1, Pygo2 full length, Pygo2ΔPHD, after 24hat A, 48hat B, 72hat C, and 96 hat D. Nuclei labeled in blue by DAPI, proteins are always labeled in green. In E, chart with the percentage of cell with nuclear localization.

This is reviewed in the chart (Fig.: 8E) where cells from 2 or 3 areas of the well were analyzed for the nucleus and/or cytoplasm localization of the Pygopus isoforms. A total of ~40 cells in each well, at each time point, were analyzed for the nucleus versus cytoplasm localization of each construct. While Pygo 1 was always found in the nucleus, Pygo2ΔPHD was always found in the cytosol from 24 to 48hat and was both cytoplasmic and nuclear at 72 and 96hat.

These results may help to explain the results of the TOP-FLASH assay, where the Pygo2ΔPHD was able to induce the expression of the WRE only at 72hpf and worked in synergy with Pygo1 at that time point, but against Pygo1 at 24hat.

This cytosolic localization data set is supported by results in mice (Cantù *et al*. 2017); in that work, scientists performed cytoplasmic versus nuclear protein fractionation in ameloblasts of mice and found that Bcl9 is cytoplasmic and Pygo2 is both nuclear and cytoplasmic. Also, Bcl9 and Pygo2 appeared to co-localize only in the proximity of the most apical surface of ameloblasts.

The authors of that work suggested a potential role for the Bcl9-Pygo2 complex in the secretion of the enamel components independent of the c-Wnt pathway. Our data instead appear to suggest that cytosolic Pygo2ΔPHD is involved in negatively regulating Pygo1' c-Wnt-dependent activity. To finally clarify this point, I will need to test if the cytosolic localization of Pygo2ΔPHD determines the same localization for BCL9/β-Catenin in the cytosol.

3.5 **CRISPRs induced mutation of *pygopous2 full-length* in the zebrafish.**

Morpholinos have been very useful for loss of function studies over the last two decades, but they do have some drawbacks (Kok FO *et al*. 2015). In previous studies (Valenti doctoral thesis 2011, Ibetti doctoral thesis 2013), I was able to obtain strong evidence that the results obtained with the morpholinos against Pygo1 and Pygo2 were specific. However, morpholinos are injected at the 1cell stage, and their effect either hinders the embryo's development in such a way that it never reaches adulthood or causes it to become faint by the third day post-fertilization and injection. Both effects did not allow us to study pygopus in later embryological stages or adulthood. Therefore, I decided to knock out *pygo2 full-length* by means of CRISPR/Cas9 mediated mutagenesis. I designed two targets within the coding region of the *pygo2* gene. One CRISPR is targeting the NHD domain (CRISPR-NHD) which should produce a null mutation, meaning no *pygo2 full-length* and *pygo2*Δ*PHD* will be correctly translated into a protein. The second CRISPR targets the PHD domain (CRISPR-PHD) and is expected to produce a normal *pygo2*Δ*PHD* splicing variant but a non-functional *pygo2* full-length variant (Fig. 9A-C). For the screening of animals carrying indels, I followed the procedures reviewed in Lawson and Wolfe (Lawson and Wolfe, 2011) (Fig.10).

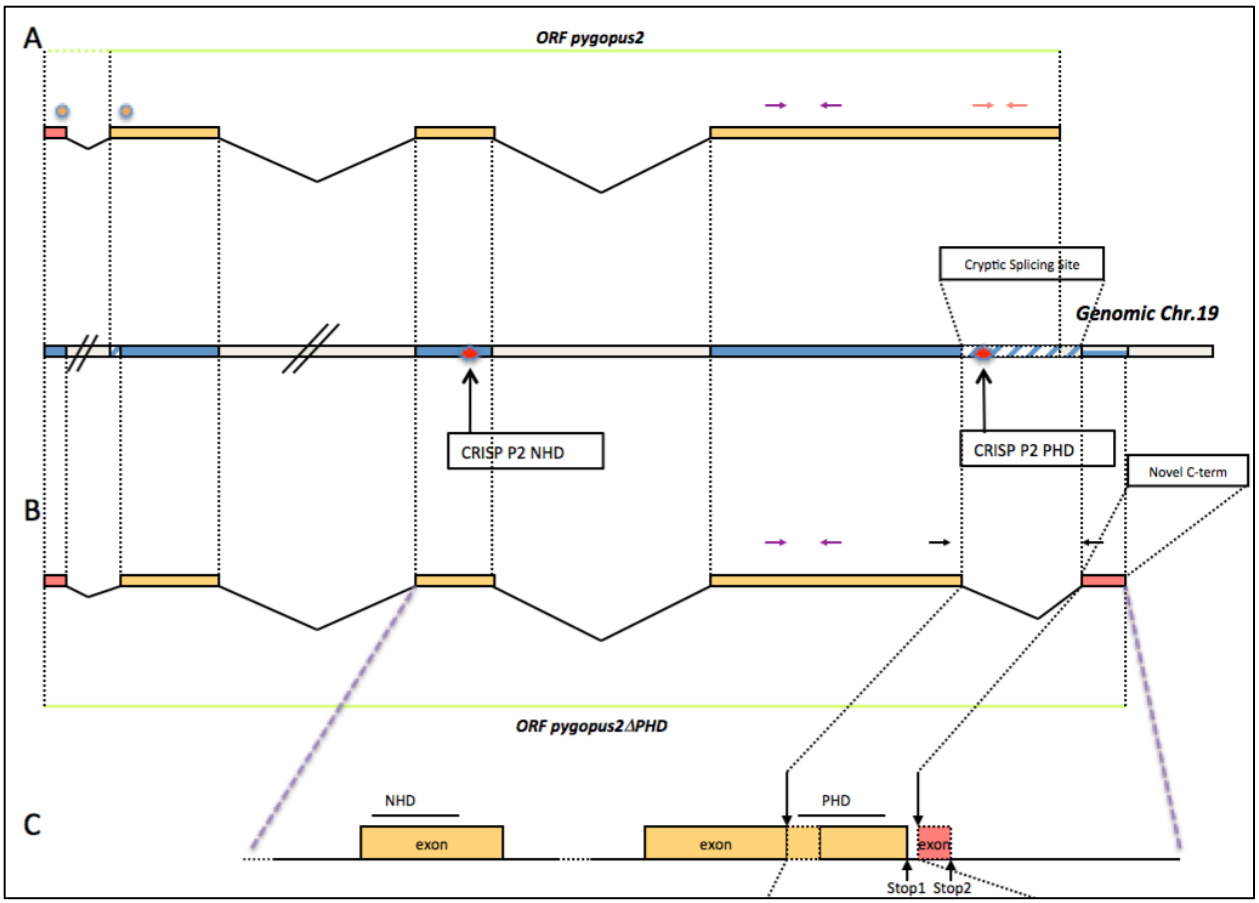


Fig. 9: Schematic representation of the genomic organization of *pygo2* in the center with the two target sites for the CRISPR in red, above, and below are the two mRNAs for the full length (A) and for the ΔPHD (B). Little arrow indicates the primers used for screening for the ΔPHD (black), the full length (orange) and for both variants (red). In C is a subset of the translated protein with the two possible variants

After injection, I tested the activity of the CRISPR on a subset of embryos using the surveyor assay, which detects indels. I obtained positive results only for the CRISPR-PHD; while CRISPR-NHD never gave positive results with the surveyor assay, despite many injections. Thus, CRISPR-PHD was the only mutant line I further pursued.

I raised the F0 embryos injected with CRISPR-PHD up to adulthood. Then I crossed those F0 fish together to find F0 incrosses that also produced a large number of deformed embryos. I used the surveyor assay to test single F1 embryos that had a deformed phenotype, and then I raised the rest

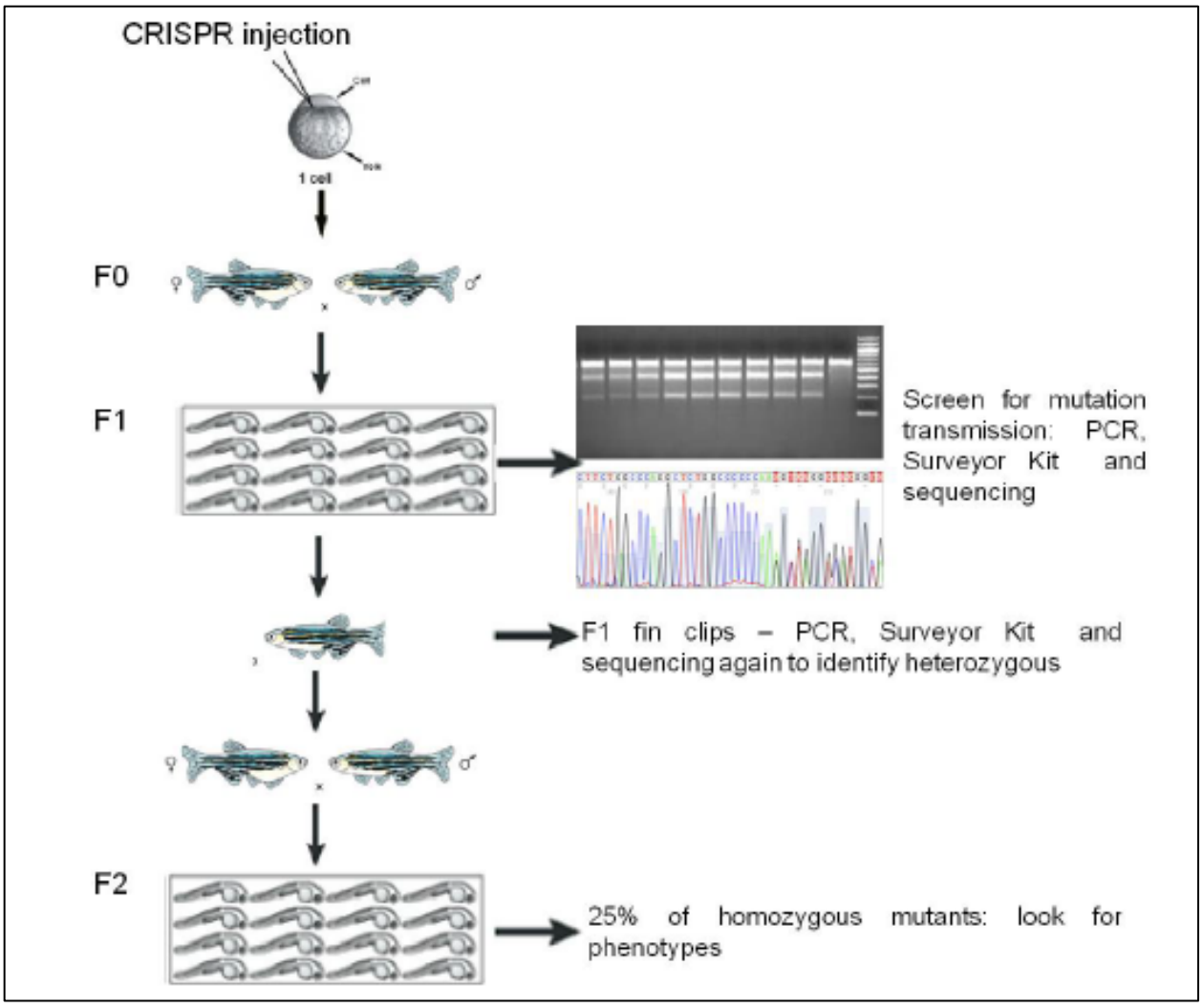


Fig.:10. Figure adapted from Lawson and Wolfe, 2011. F0 is the fish generation that was injected at 1 cell stage with the CRISPR mix, F0 fish were incrossed and their embryos analyzed by surveyor and following sequence of positive clones, F1 sibling were growing till adult. PCR followed by sequence of the fragment was done from PCRs that used DNA derived from single fish fin-clip. Heterozygous F1 fish were crossed to produce an F2 that would contain 25% of homozygous mutant embryos.

of the F0 cross embryos that were siblings of those that had been tested and tested positive for the indel using the surveyor assay. Finally, I wait for the F1 embryos to become adults. When the F1s reach adulthood, I begin screening single fish by analyzing fin-clip DNA with the surveyor assay. In the case of CRISPR-PHD, I initially genotyped 3 fish (2 female and one male) with different types of deletions or missense mutations in the region targeted by the CRISPR. Figure 11 shows the sequence of two of the F1 adult fish analyzed for the CRISPR-PHD mutation compared to a

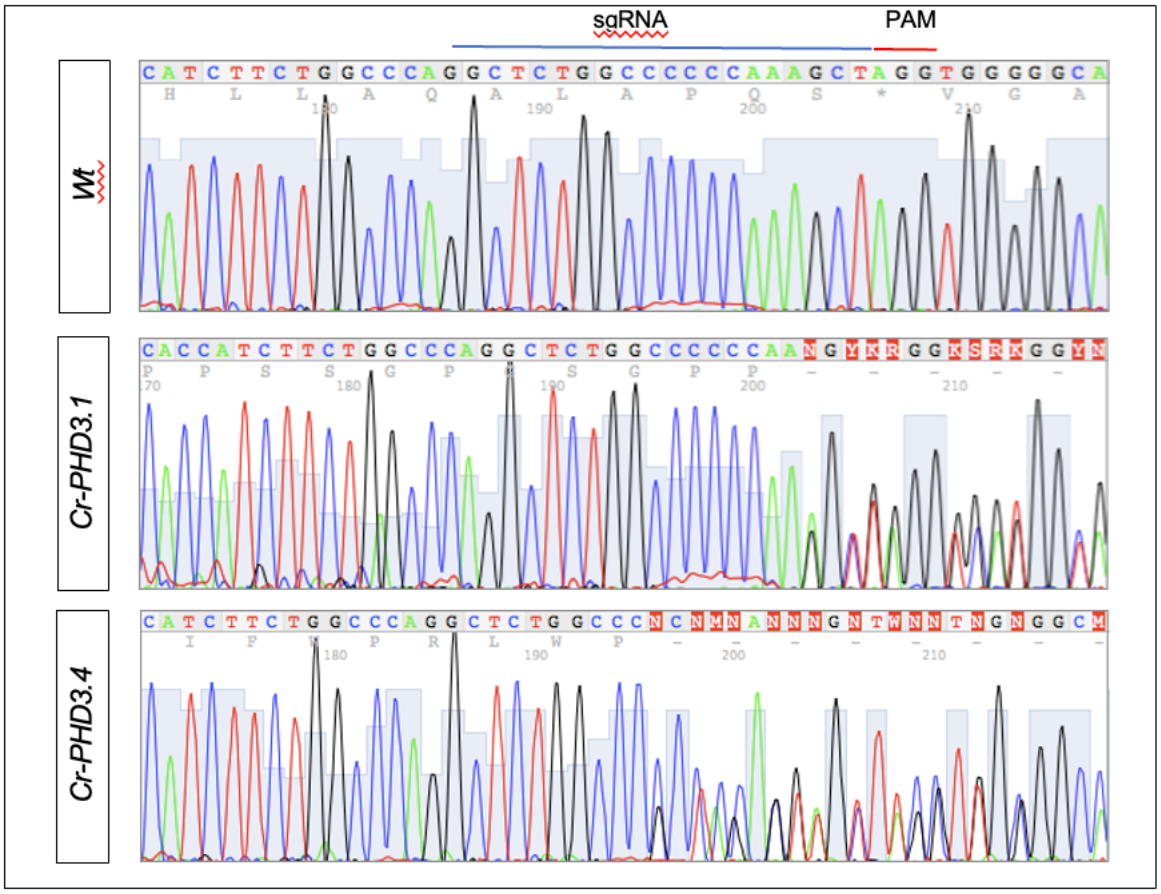


Fig.11: *Wild type* and two heterozygous indels in the PHD region targeted by our CRISPR, the fact that the indel is in one of the two sister chromosomes is determining the presence of double picks.

wild-type (*Wt*) sequence. One carried a 5bp deletion and another a 29bp deletion; a third mutation with a 3bp deletion was not further pursued because the mutation was in frame and determined the loss of only one amino acid. I called the other two mutations *pygo2*-Δ*5* and *pygo2*-Δ*29*.

When I did the first crosses of heterozygous fish for those mutations, I could not identify clear early embryonic phenotypes; however, genotyping of those fish never produced homozygous mutants, suggesting that those homozygous mutant fish were dying at some point during development or rearing. I determine the effects of these individual mutations using molecular marker analysis via *in-situ* hybridization (Fig. 12). I tested the expression of the dorsal specific markers, *chordin* and *goosecoid,* as shown in figure 12 all the embryos obtained from a cross of two *pygo2*-Δ*29 +/-* fish showed a normal expression pattern at 60% epiboly.

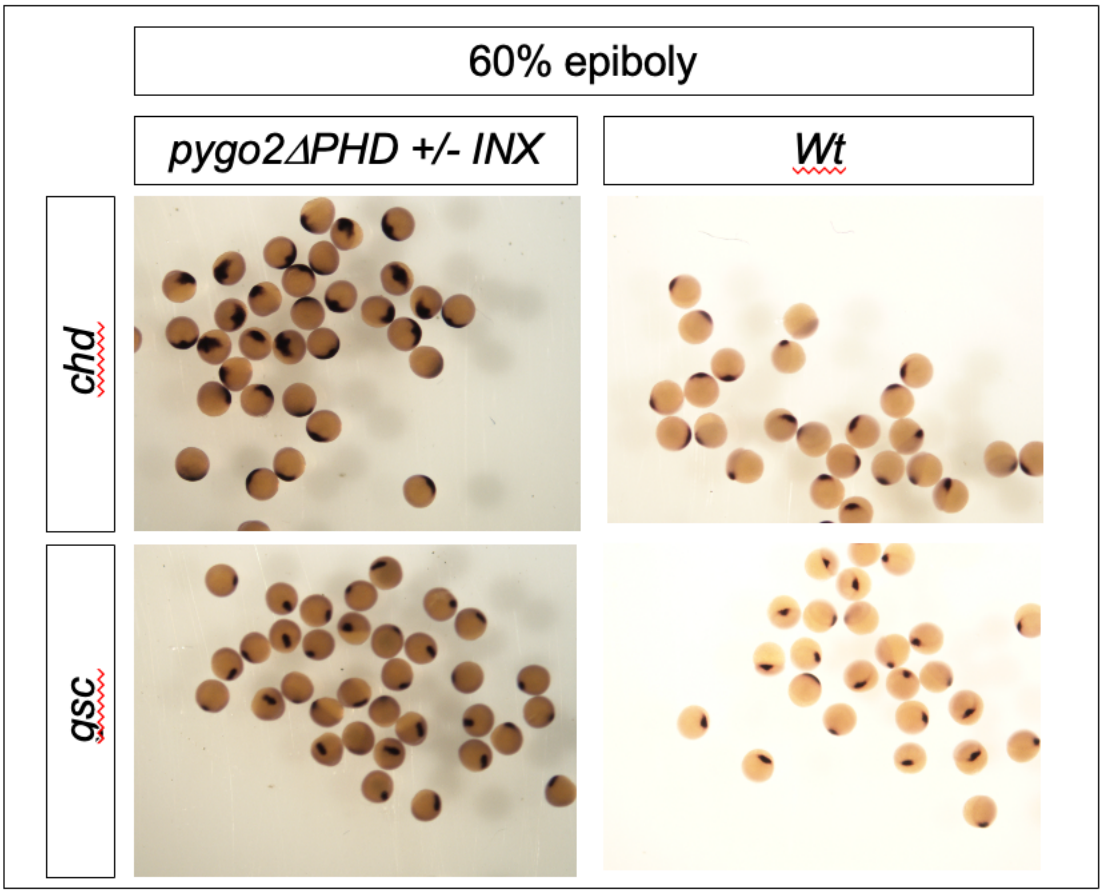


Fig.12: WISH analysis of wild-type and pygo2ΔPHD +/- incrosses. Embryos are at 60% epiboly and are hybridized either with *chordin* (*chd*) or *goosecoid* (*gsc*) probes. The pictures represent batches of 20+ embryos.

This was consistent with the absence of clear early developmental phenotypes. When I reduced the number of larvae in a single tank and kept checking them daily, I was able to raise some homozygous fish for both the *pygo2*-Δ5 and *pygo2*-Δ29 mutants' lines (Fig.13). It may be possible that those mutations make the larvae weaker and more likely to die or be killed in a crowd tank. Most of the homozygous fish were fertile.

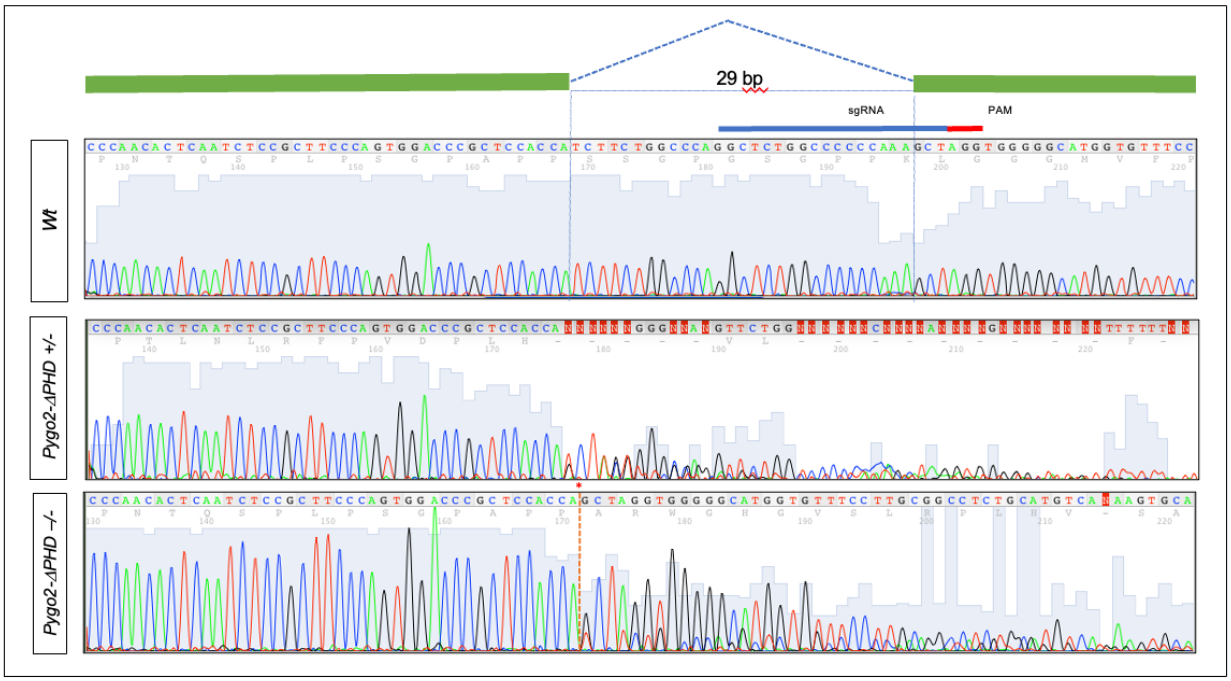


Fig.13: on top is shown the 29bp deletion and the position of the sgRNA and PAM sequences, the top sequence is from wild-type fish, the middle is from a heterozygous fish and the bottom is from one homozygous mutant for the 29bp deletion. The red dotted line identifies the point of fusion between the sequences of *pygo2* 5' and 3' the indel.

I identified a very mild and not fully penetrant phenotype in the ventral fin of 24hpf homo and heterozygous embryos that strongly resembled a mild phenotype obtained by morpholino injections (Fig. 14) however being present also in some heterozygous mutants is not completely convincing, so I did not used this for screening for *pygo2*Δ*PHD* homozygous fish.

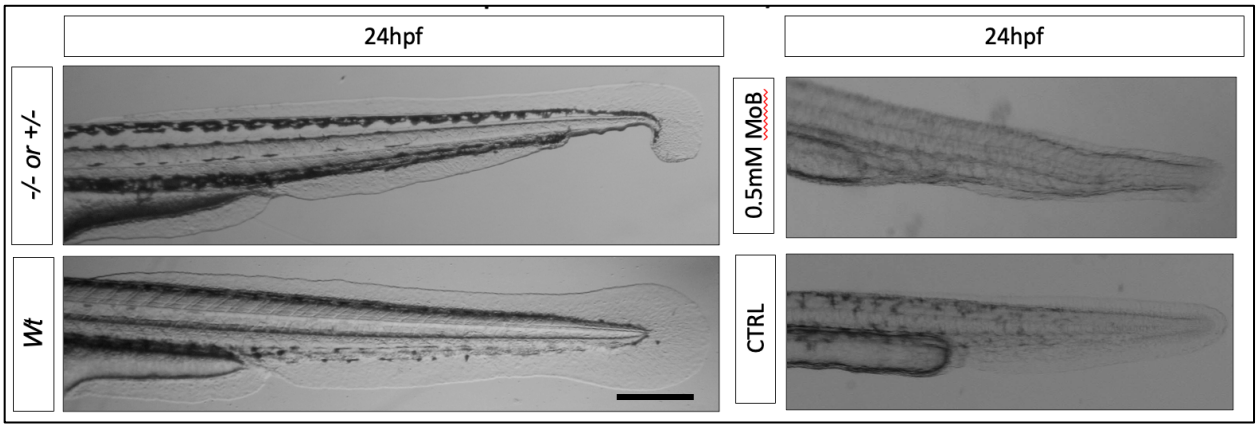


Fig.14: 24hpf embryos posterior tail region showing a loss of the ventral tailfin for embryos that later were genotyped as homo or heterozygous for *pygo2*Δ*PHD* mutation. Top right fin is from an embryo injected with low concentration of ATG-Morpholino against *pygo2*.

These results give strong genetic information on the role of the Pygo2ΔPHD variant. Homozygous fish do not show a strong phenotype, and this indicates that Pygo2ΔPHD could be a splicing form of Pygo2 regularly expressed in the zebrafish. This could explain why our pygo2 morphants, which likely lacked both full-length and ΔPHD isoforms, had a much worse phenotype than the *pygo2-Δ5* and second *pygo2-Δ29* mutants.

3.6 **Transient and established lines for Transgenic fish developing nevi and melanoma**

Melanoma cells dedifferentiate from melanocytes to a neural crest-like cell that starts to migrate (metastasis). We know that c-Wnt signaling is involved in the neural crest's induction and migration during development. Despite this information, the role of c-Wnt on melanoma is not yet completely understood. My hypothesis is that c-Wnt signaling from the epidermis surrounding the melanocytes could also mediate the dedifferentiation steps of the melanocytes through the process to become a melanoma and their migration to metastasize.

To increase the set of tools that could later be used to study the role of Pygopus and c-Wnt signaling in melanoma, I decided to create fish transgenic lines that would consistently produce fish with this cancer type. I focused on two genes, one aiming to directly test the role of dysregulated (constitutively activated) c-Wnt-signaling in the melanocytes. The other has been already shown to cause nevi melanomas in zebrafish with a defective p53 gene. In the first case, I created, pDB773-*mitfa:Axin2DN*, it is a plasmid containing mini-Tol2 sequences (kindly provided by Dr. Balciunas). This construct included a *mitfa* promoter sequence and a mutated form of *axin2*, which had a mutation upstream of the protein's dimerization domain, the DIX domain (created in collaboration with Dr. Daniele Castiglia IDI, Rome). Axin2 cannot form homo- or heterodimers in the absence of this domain, and its overexpression disrupts endogenous Axins ability to dimerize, this results into the stabilization of β-Cat, which then moves into the nucleus and acts as a constitutive c-Wnt pathway activation. I injected several times with this construct and the mRNA for the relative transposase a fish $p53^{zdf1/zdf1}$ mutant line, which has a single T-to-A point mutation that changes Met to Lys at residue 214 of the Tp53 protein ($Tp53^{zdf1/zdf1}$) (Berghmans et al., 2005).

To make the second transgenic line, I obtained the construct MiniCoopR *mitfa:BRAF*, as a gift from Leonard Zon. This construct is a pBS-SK plasmid containing a mutated *BRAF* cDNA (BRAFV600E) (Patton *et al*. 2008), controlled by the epidermis-specific *mitfa* promoter (Dorsky

et al. 2000), flanked by Tol2 sequences (Kawakami K. 2007). Of the lines that are transgenic for this gene or that transiently express this construct, nevi and melanomas are present (Ablain *et al*. 2018). I injected fish homozygous for the *zdf1* allele of p53, with either 50 ng/µl of MiniCoopR mitfa:BRAF plasmid or 50ng/µl of pDB773-*mitfa:Axin2DN* and 100ng/µl of mRNA coding for the transposase. Injected embryos and their siblings were allowed to grow for 2.5 months and then analyzed for the presence of nevi or melanomas. For MiniCoopR *mitfa:BRAF*, I totally injected 72 embryos in three different injections that reached 2.5 months of age. At 2.5 months of age 49 out of 72 presented nevi or melanomas (68%). The results of each injection and the total are reviewed in Table.6.

Injection	**Number of injected embryos**	**Number of control embryos**	**Number of injected F0 with nevi or melanomas at 2.5 months post injection (%)**	**Number of controls F0 with nevi or melanomas at 2.5 months post injection (%)**
1	33	50	25 (76%)	0 (0%)
2	24	20	16 (67%)	0 (0%)
3	16	30	8 (50%)	0 (0%)
total	72	100	49 (68%)	0 (0%)

Table 6: results of three experiments of injections of MiniCoopR *mitfa:BRAF* in $p53^{zdf1/zdf1}$ fish line

Despite several injections, I was never able to identify fish injected with pDB773-*mitfa:flag-Axin2DN* with nevi and melanomas, while the injections of MiniCoopR *mitfa:BRAF* were very successful. It is possible that the pDB773-*mitfa:flag-Axin2DN* had some sequence issues that I did not detect, but the genotyping of random samples of F0 fish or pools of F1 embryos coming from F0 incrosses always failed to show the presence of the transgene (data not shown). This makes it likely that the problem was with the transposase mRNA or the *tol2* sequences on the plasmid.

I crossed 5 pairs of F0 fish carrying large nevi and identified several fish presenting large nevi at 4-5 weeks to isolate a stable line of fish *p53*$^{zdf1/zdf1}$ and transgenic for *mitfa:BRAFV600E* (Fig.15).

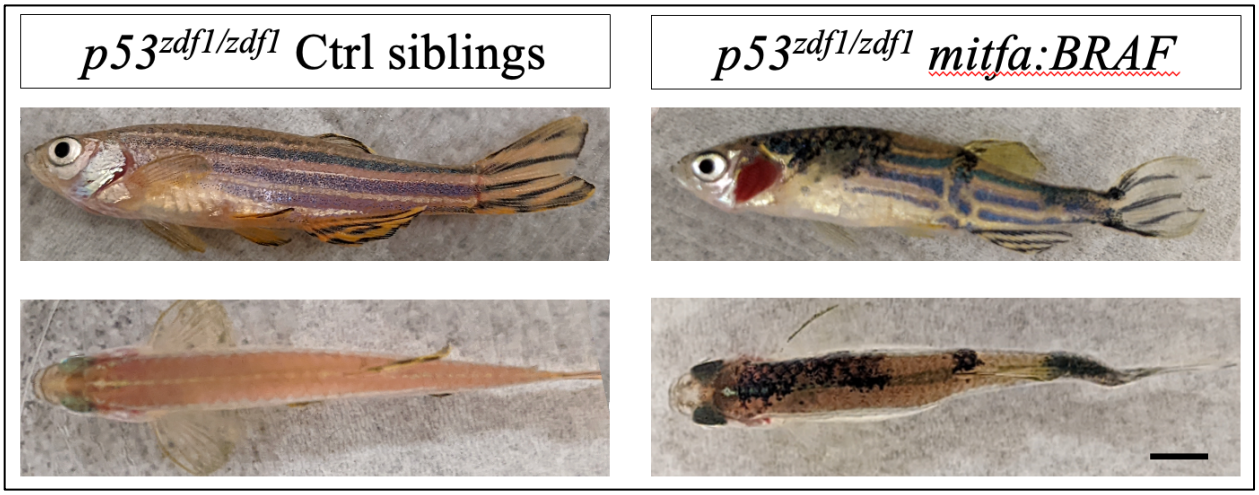


Fig.15: lateral (top) and dorsal (bottom) views of *p53*$^{zdf1/zdf1}$ fish transgenic for *mitfa:BRAFV600E* (right) that present extensive nevi, and control siblings that are not transgenic for that gene.

I am still investigating the reasons why that could happen, and for now we cannot draw any conclusions about the role of increased c-Wnt in melanocytes and melanoma development.

Discussion and Conclusion

Cell physiology, embryonic development, and tissue homeostasis are processes that require a balanced activity of the c-Wnt/β-Cat pathway, while many cancers and birth defects can be caused by an excess or insufficient activity of this pathway (Clevers and Nusse, 2012; Nusse and, Clevers, 2017). Pygopus gene family codes for nuclear proteins that are components of the c-Wnt pathway but also may have other c-Wnt pathway-independent functions in vertebrates. One of the most common ways to regulate the activity of a pathway at the nuclear level is to regulate the level of transcription and the locations of transcription factors, co-factors, and signaling molecules that activate or repress transcription of those genes. For example, chordin and other genes that specify the dorsal side of the zebrafish embryo are expressed in the dorsal side during the Shield-Organizer formation (Miller-Bertoglio et al 1997).

As a result, the first goal of my thesis work was to learn more about the expression pattern of *Pygo2ΔPHD*. We had previously tested *pygo1* and a generic probe for *pygo2*, able to detect both *pygo2* splicing variants, which had a very diffuse expression pattern in all the embryo.
Even though the generic probe for pygo2 isoforms did not show enrichment in any specific region of the embryo, I performed the final and conclusive in situ hybridization assay in zebrafish embryos to examine the expression pattern specific for *Pygo2ΔPHD*. Using a special type of short probe, LNA, I was able to demonstrate that *pygo2ΔPHD* transcripts are also ubiquitously expressed in the early stages of embryogenesis, and that at 24hpf, this was not the specific and localized expression that I expected to see, at least for this variant of Pygo2. However, these findings suggest that Pygo2 activity may be regulated at other levels and not by regulating where *pygo2* gene is expressed, for example c-Wnt during early embryogenesis is first inducing nuclear localization of β-Cat in what will be the dorsal side of the embryo, and later is inducing nuclear localization of β-Cat in the medial side of the embryo, inducing ventral fates (Bellipanni et al., 2006), but β-Cat is always present in all the cells and when in the cytosol may have other functions.

Prompted by the results of the in-situ hybridization with an LNA probe, the second set of the experiments were designed to verify whether the splicing variant Pygo2ΔPHD could regulate *in-vitro* the c-Wnt pathway or not. I conducted two experiments to shed light on this question. One was by using a TOP-Flash test in human cell lines, I determined whether Pygo2ΔPHD is specifically capable of regulating the c-Wnt/β-Cat pathway. The other was a time course analysis

of Pygopous isoforms cellular localization that was performed using fluorescent immunohistochemistry to determine where the Pygopous isoforms were localized following transfection in cell lines. This experiment allowed me to show that zebrafish Pygo2ΔPHD expressed in human cell lines is mostly cytoplasmic on days 1 and 2 after transfection and starts to become nuclear only on day 3 after transfection, whereas Pygo1 is always nuclear. TOP-Flash experiments revealed that Pygo2ΔPHD regulated the c-Wnt/β-Cat pathway in a manner that was dependent on the time after transfection when the analysis was completed. When Pygo2ΔPHD was cytoplasmic, it was unable to activate the c-Wnt/β-Cat reporter gene expression, but it did so after 72hat. Strickling TOP-Flash results of Pygo1 were also consistent with Pygo2ΔPHD localization. Pygo2ΔPHD reduced, β-Cat or β-Cat+Pygo1 co-transfection activation of the reporter constructs at 24hat and did it in a concentration dependent fashion. When Pygo2ΔPHD was nuclear at 72hat, it worked in tandem with Pygo1 to increase expression of the c-Wnt-dependent reporter gene, whereas *pygo2 full-length* transfections at 24hat or 72hat did not result in significant activation of the reporter gene, and neither did co-transection with Pygo2ΔPHD have a significant effect.
These findings suggest that when Pygo2ΔPHD is nuclear, it can activate the c-Wnt/β-Cat pathway alone or in collaboration with Pygo1, but not with Pygo2 full length. When Pygo2ΔPHD is present in the cytosol, it inhibits Pygo1 activation of the c-Wnt/β-Cat pathway in a concentration-dependent manner.

Mice embryos with both pygo1 and pygo2 knockouts have dramatic phenotypes and do not survive, (C. P. Prasad, et al., 2008; C. S. Chim, et al., 2011; N. Takebe, et al., 2011; S. J. Zardawi, et al., 2009). While total *pygo1* and *pygo2* depletion has these strong effects, double mutants *Pygo1* $^{-/-}$*; Pygo2* $^{L368A/L368A}$ with *Pygo2* $^{L368A/L368A}$, which can form a complete protein but cannot bind BCL9, show a normal phenotype, as if the interaction with BCL9 is not required. Furthermore, the role of Pygopous isoforms in zebrafish, as outlined by the mutant so far, is very similar to that seen in mice. Cantù and collaborators (2016; 2018) showed that in a double *pygo1* and *pygo2* mutant with Pygo1 not forming at all and Pygo2 being cut off after the NHD domain, gastrulation can be finished and have only cardiac defects. In our laboratory, it was found that the morpholino *knock-down* of *pygo1*and *pygo2ΔPHD* produced ventralized embryos. Consistently with this, the induction of *mkp3* mRNA by *β-catenin* mRNA injection in *ichabod* (*ich*) mutant embryos was reversed if both Pygo1 and Pygo2 translations were blocked with morpholinos oligonucleotide

injections, indicating that at least for this event, both Pygo1 and Pygo2 work in synergy (Doctoral thesis, Ibetti, 2013). These data from literature could be explained either with a sort of compensantig effect of Pygo1 when P2 is not expressed or by the assumption that these two pygopous may work in tandem on the same promoter.

During my thesis work, I created mutant fish lines using CRISPR/Cas9 methodologies that have deletions within the PHD domain and thus cannot express Pygo2 full-length but can express Pygo2ΔPHD. The embryos with this homozygous mutation could still grow and once adult reproduce, they had a very mild phenotype that was not fully penetrant in the ventral tailfin. Those fish had no obvious heart phenotype, but they were more likely to die during the first three weeks of life in a crowed tank. These findings suggest that Pygo2ΔPHD is an expressed isoform of Pygo2 in the zebrafish and, despite lacking the PHD finger, can still function in the c-Wnt/β-Cat pathway. My research results, along with those of other laboratories, point to a model in which the main form of pygo2 does not require the PHD domain, but Pygo2ΔPHD can still interact with the ChiLS complex, a key cofactor of the enhanceosome involved in the proper activation of c-Wnt downstream genes. ChiLS regulates enhancers in Drosophila that respond to both c-Wnt and Notch signaling (Bronstein and Segal, 2011). ChiLS also regulates distal enhancers of globin and other erythroid genes in mammals (Love et al., 2014). Fiedler et al. (2015) demonstrated that ChiLS directly binds the Pygopus NPF sequence in a 25 amino acid region containing it. As I showed in the introduction, the NPF sequence is adjacent to the Nuclear Localization Signal, and both are found in Pygo2's NHD domain. Consistent with the findings, NHD is also present in the pygo2 mutation that causes heart phenotypes in mice and zebrafish (Cantù et al., 2018) but not the most severe phenotypes seen when pygo2 was completely unactive. According to figure 3 (derived from van Tienen et al., 2017), BCL9 has three HD domains that aid in enhanceosome binding: HD1 binds pygo2, HD2 binds β-Catenin, and HD3 binds ChiLS (van Tienen et al., 2017).

My results suggests that the interaction between the NPF region of Pygo2ΔPHD and ChiLS would be stronger than the interaction between ChiLS and the HD3 domain of BCL9/BCL9l, or between ChiLS and Pygo1. As a result, I could describe a working model in which ChiLS interaction with Pygo2ΔPHD is required for ChiLS positioning in the enhanceosome, ChiLS can move in and out of the nucleus following Pygo2ΔPHD, or ChiLS can simply be disassembled and removed from the enhanceosome. This in turn would lead to the removal of BCL9/BCL9l from

the enhanceosome, resulting in a less-than-ideal binding of β-Catenin to the enhanceosome (Fig.16).

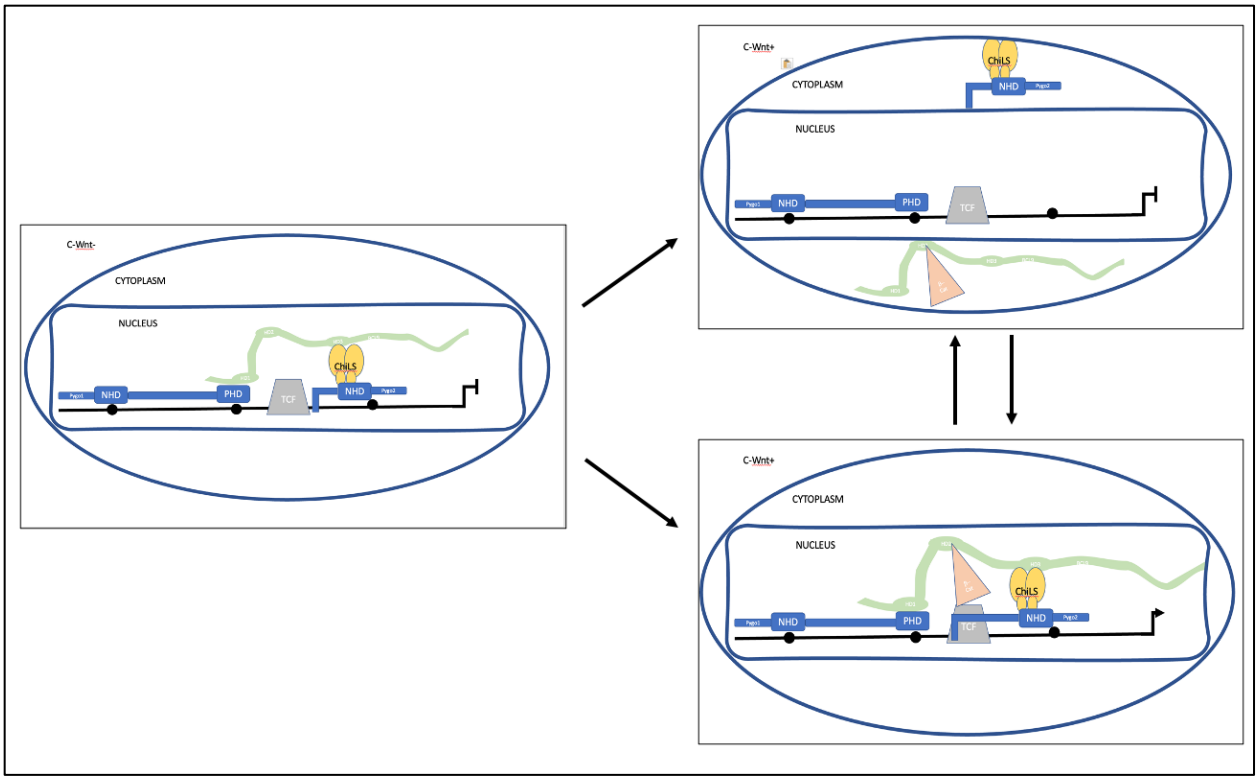


Fig.16: is showing on the left a schematic cartoon of the enhanceosome in absence of c-Wnt signaling and therefore without nuclear β-Cat, as result there is not gene transcription. In the right c-Wnt is present and therefore β-Cat could move into the nucleus, however the top box shows when Pygo2ΔPHD is not nuclear localized, thus the enhanceosome should be unassembled and transcription not active while at the bottom

This model could explain the TOP-Flash results I obtained in my experiments, which show synergistic and concentration-dependent activation of the reporter gene by co-transfection with Pygo1 and Pygo2ΔPHD at 72hat because Pygo2ΔPHD is in the nucleus, but also the opposite effect of Pygo2ΔPHD at 24hat because it will be in the cytoplasm. While Pygo2ΔPHD may interact with Pygo1 in the enhanceosome (Fig.16), it is unlikely that it does so with a direct bunding as Chen et al. (2010) demonstrated that Pygopus does not form dimers. But it could offer its PHD domain to further stabilize BCL9 in the enhanceosome

Finally, by inducing the expression of a mutated form of BRAF, I was able to create a transgenic line that produces zebrafish with nevi and melanomas (BRAFV600E). This line will be

placed in the pygo2ΔPHD mutant background in the future to see if the full length Pygo2 is required to support the formation of nevi and melanomas.

This research help to shed light on the two Pygo2 splicing variants in the zebrafish. It provides genetic evidence that Pygo2ΔPHD is an expressed variant in zebrafish, and that while its expression levels and localization are constant and ubiquitous, it may be involved in the regulation of the c-Wnt pathway via nucleus/cytoplasm shifting. As a direct consequence, it can control the assembly of the enhanceosome's ChiLS cofactors and thus the binding of BCL9/β-Cat to the enhanceosome. It also provided molecular-genetic tools such as the *pygo2ΔPHD* mutants and the *mitfa:BRAFV600E* transgenic line, which could help shed light on the role of c-Wnt signaling in the induction and progression of nevi and melanoma.

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