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Wnt/β-Catenin dependent roles of the Pygopus family of nuclear factors during Zebrafish early dorso-ventral specification

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Candidato

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1.Introduction

The roles played by β-Catenin (β-Cat) in cellular physiology and differentiation has been widely described in literature, going from embryonic development and stem cells renewal to differentiation and cell homeostasis. This protein is a crucial component of Wnt pathway and mediates, in a complex with α-Catenin-typeI Cadherins, cell-cell adherence junctions adhesion (Angst et al., 2001; Li et al., 2011). The stability of β-Cat in the cytoplasm is regulated by several proteins that create a multiproteic complex called <signalosome>. When Wnt proteins bind their cognate receptors the signaling cascade is activated. This cascade leads to an increase of β-Cat in the cytoplasm by inhibition of the signalosome activity. Increased concentration of β-Cat in the cytoplasm causes its translocation in the nucleus. In the nucleus β-Cat activates gene transcription via interaction with DNA binding factors of the lef/tcf family. Deregulation of the c-Wnt/β-Catenin pathway has been linked to embryo malformations as well as several kinds of cancer (Morin et al., 1997; Liu et al., 2000; Zardawi et al., 2009; Takebe et al., 2011), and the study of the mechanisms regulating β-Cat stability and nucleus-cytoplasm trafficking are relevant for understanding the role of this pathway in many tumor types (Clevers H, Nusse R., 2012)

2. Wnt pathway

The signaling cascade is activated when the Wnt ligands, of which have been known at least 16 members in vertebrates, interact with Frizzled (FZD/LRP) receptors and low density lipoprotein receptor-related protein 5 or 6 (LRP5/6) (Riggleman et al., 1990; Peifer et al., 1994; Larabell et al., 1997). These receptors belong to a class of seven-pass transmembrane receptors that, when activated, associate with the cytoplasmic Disheveled protein (Bhanot et al., 1996; Yang-Snyder et al. 1996; He et al. 1997). Such event triggers the phosphorylation of the disheveled, with consequent sequestration of protein Axin through their DIX domains (Mao et al., 2001; Bilic et al., 2007). Axin sequestration has been suggested to be one the causes of β-Cat stabilization and escape from degradation (Mao et al., 2001). This is a consequence of its role in the signalosome complex. Axin (Axin1 and Axin2/Axil/Conductin) together with adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3β and α), casein kinase 1 (CK1) and protein phosphatase 2A (PP2A) composes the signalosome complex that has the specific rule of leading β-Cat to degradation (Ikeda et al., 1998; Su et al., 2008).

The Axin-APC association promotes the β-Cat phosphorylation by Glycogen synthase kinase 3 (GSK3β) and CK1 on key amino-terminal Ser and Thr residues (Ser33, Ser37, Thr41 and Ser45). Hyperphosphorylated β-Catenin is targeted for ubiquitylation by

TRCP, a component of an E3 ubiquitin ligase, and sent to degradation in the proteasome (Ikeda et al., 1998; Liu et al., 2002; Su et al., 2008).
Once the cytoplasmic pool of β-Cat is stabilized it can translocate into the nucleus and, binding to DNA-binding factors of the Lef/Tcf family, regulates transcription of Wnt target genes, of which the most well-known are *c-myc*, *D1 cyclin* and *mkp3* (He et al., 1998; Shtutman et al.,1999; Tetsu et al., 1999; Tsang et al. 2004).

2.1 β-Catenin a crucial conserved protein

β-Cat structure analysis revealed 12 imperfect ARM repeats (R1-12) for with the fly β-Cat was named Armadillo, the fly β-Cat. The protein structure is completed with a N-terminal domain and an helix between the last R12 and the carboxy-terminal domain (NTD and CTD) (Orsulic and Peifer, 1996; Valenta et al., 2012). Every region of this molecule is critical for interactions with different proteins involved, in various grades, in gene transcription activation. The central region, specifically R3-R10, links the transcription factor TCF. In absence of Wnt, TCF, helped by the well-known chromatin repressor Groucho (orthologous TLE1 in mammals) (Cavallo et al., 1998; Roose et al, 1998; Brantjes et al., 2001), binds the Wnt response elements (WREs) and repress any Wnt-independent expression (Brannon et al., 1997). The remaining R11-12 and C-terminal can interact with several kinds of proteins involved in chromatin-modification such as the histone acetyltransferase enzymes CBT and p300 (Hecht at al., 2000; Takemaru and Moon, 2000) activating the expression of specific genes. More, studies suggest an interaction between the mixed lineage leukemia complex (MLL complex) and β-Cat (Sierra et al., 2006). Such complex recruits COMPASS (complex proteins associate with Set1) to mono, di or trimethylate histone H3 tails at Lys4. Acetylations and methylations of Lys residues in the histone N- termini (i.e. H3K3me3) have been established as markers of active transcription (Shilatifard, 2008).
Another protein has been found to play a crucial role in Wnt signaling at nuclear level, Drosophila Hyrax (HYX) and his human orthologue Parafinbromin. A transcriptional platform function has been hypothesized for this component because of its ability to bind, with its N-terminus, β-Cat at R12-C and the polymerase-associated factor 1 complex (PAF1 complex) by its C-terminus (Mosimann et al., 2006).
In the cytoplasm β-Cat R1 repeat interacts with the homology domain 2 (HD2) of Legless/BCL-9, this heterodimer can freely move in and out of the nucleus. In the nucleus this heterodimer binds via its HD1 to the PHD finger of Pygopus (Pygo). It has been proposed that interaction of Pygo with Lgs/BCL9 in a promoter region of the DNA would help anchor β-Cat in the nucleus. However, this is not the only role that BCL9/Pygopus play in combination with β-Cat.

2.2 Pygopus a β-Catenin adaptor

Pygo is a nuclear localized protein that shows a PHD domain in its C-terminus and a N-terminal homology domain (NHD). Its nuclear localization is coded by a nuclear localization signal (NLS) present in the N-terminal region. The N-terminus also shows affinity for the Mediator complex, MLL2 histone Methyltranferase and GCN5 Histone Acetyltransferase complex (Chen et al., 2010). Moreover, Pygo is able to directly bind TCF-3 via a NPF (asparagine-proline-phenylalanine) sequence and interacts by PHD domain with the Mediator complex and with all three methylation states of H3K4 (Gu et al., 2009; Cantù et al., 2013). All these evidences support a proposed function as an anchor for β-Cat in the nucleus and c-Wnt transcription enhancer (Townsley et al. 2004; Hoffmans et al. 2005) (Figure 1).

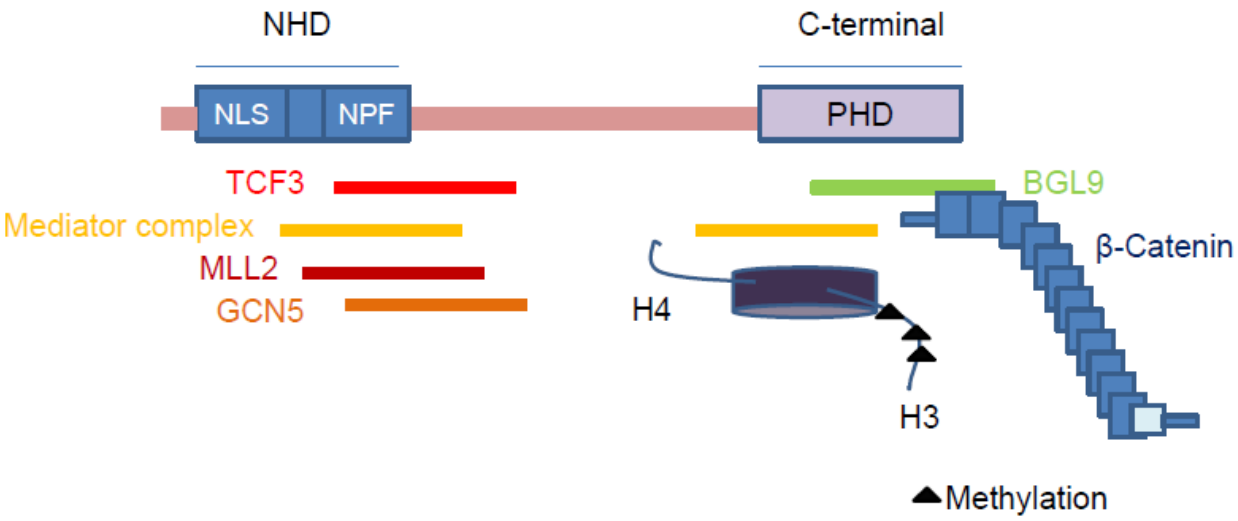

Figure1. **Schematic representation of Pygopus structure**. Pygopus is a philogenetically conserved protein. It presents at the N-terminus a nuclear localization sequence that allows his nuclear localization and a NPF sequence important for interaction with several proteins involved in chromatin remodeling and transcription regulation. The PHD finger interacts via Bgl9 with β-Catenin and recognizes the different state of methylation of lysine K4 of Hystone 3.

The PHD finger of Drosophila Pygo presents 60 aminoacids arranged in C4HC3 motives defined by seven cysteine and a histidine, two Zn ions coordinated in a cross-brace scheme stabilize the entire structure.

Other proteins involved in chromatin remodeling present similar but distinct zinc-binding motifs, i. e. RING finger (Cys3-His-Cys4) and LIM domain (Cys2-His-Cys5) (Pascual et al., 2000; Capili et al., 2001; Belenkaya et al., 2002), and they have also been implicated in human diseases (Matthwen et al., 2007; Baker et al., 2008; Chen et al., 2010). For these reasons, Pygo proteins have been studied with particular interest.

Pygo protein has been shown to play essential roles in the Wg pathway in *Drosophila* and enhancing roles in the c-Wnt pathway in mammals (Brody et al., 2002). In *Xenopus*, none *pygo1* transcript has been yet identified, while there are two *pygo2* mRNAs, *Xpygo-2α* and *Xpygo-2β*, which differ for a 5' coding region. It is not clear if these transcripts result from the differential transcription of a single gene or from the transcriptions of two genes. However, they have been found to have specific temporal patterns. *Xpygo-2α* message is detected in the anterior area, specifically in the neural plate, after gastrulation, whereas *Xpygo-2β* presents an earlier expression during oogenesis and early stages decreasing before and through gastrulation (Lake et al., 2003). While *Drosophila*, Pygo appears to be always linked to c-Wnt signaling, in chordates Pygo proteins seem to play also c-Wnt independent roles (Song et al., 2007). Mammals have two *pygopus* homologs, *Pygo1* and *Pygo*2 (Li et al., 2004; Song et al., 2007; Li et al., 2007). *Pygo1* transcripts are localized only in the heart (Lin et al., 2004), whereas *Pygo2* transcript has been detected in almost all mouse adult tissues analyzed and in embryological stages as early as ES 7 (Lin et al., 2004). Mouse ES 7 stage is equivalent to zebrafish shield stage, thus later to the stages we will analyze in our work. Is it not clear if Pygopus transcripts are present before stage ES 7. Knock-out studies have shown that if the mice lacking of *Pygo2* exhibit defects in ectoderm and endoderm derived tissues morphogenesis, including brain, eyes, hair follicles, and lung. However, the penetrance of such defects is mild compared to the ones observed in mice lacking of *β-catenin* alleles (Schwab et al., 2007) and have reduced the interest to study this gene family. More recently research in Konrad Basler laboratory was able to discriminate better between cell-adhesion and gene activation (c-Wnt dependent) roles of β-Cat during mouse development, showing that a milder phenotype for *β-catenin* (transcription (-); cell adhesion (+)) KO mutants (Valenta et al., 2012).

Further studies in mammals have revealed non-Wnt related functions of Pygo, like for eye development in mice, where it has been shown that Pygo2 affects lens formation independently from its role in Wnt/β-Cat signaling (Song et al., 2007). Nevertheless, there are mammalian Wnt-driven processes that have been shown to require functional *Pygo2*, including lung and mammary gland morphogenesis, as well as kidney and pancreas development (Belenkaya et al., 2002; Schwab et al, 2007, Li et al., 2007; Jonckheere et al., 2008). Taken together, these studies show that Pygo2 plays c-Wnt dependent and independent roles during embryonic development (Li et al., 2007; Valenta et al., 2012). It is not clear if Pygopus Family of nuclear factors play any role during dorsoventral patterning and specification in *Xenopus* and mice. Studies in both animal models failed to address this important question because it is technically difficult and complicated by Pygo maternal expression (Parker at al., 2002; Thompson at al., 2002; Lake at al., 2003).

The ability of Pygo in binding different proteins involved in transcription of mitogen genes and indirectly β-Catenin has suggested its possible role in tumorigenic processes (Thompson et al., 2002; Orsetti et al., 2006; Popadiuk et al., 2006; Andrews et al., 2007; Chen et al., 2010; Chen et al.,2010; Liu et al, 2013).
In fact, c-Wnt cascade activation and β-Cat nuclear accumulation have been shown to be critical factors in several kinds of cancers, such as colorectal carcinoma, breast cancer and glioma (Korinek et al., 1997; Liu et al., 2000; Morin et al., 1997; Prasad et al., 2008; Chim et al., 2008; Takebe et al., 2011; Zardawi et al., 2009).
Studies have been carried out on cells lines and murine models in order to detect a possible role of Pygo in different kinds of tumor at different stages (Calaf et al., 2005; Ozaki et al., 2005; Wang et al., 2010; Chen et al.,2010; Liu et al, 2013; Watanabe et al., 2013). The first experiments found in literature that attempt to correlate *PYGO2* with cancer have been performed on colorectal cancer cells lines SW480 and HCT116, which present respectively a mutated APC and an oncogenic mutation of β-Catenin. In such cells lines, the knock down of *PYGO1* and *PYGO2* transcripts singularly or together by 21 mer RNA oligonucleotides leads to a significant decrease of transcription mediated by β-Catenin-TCF complex (Thompson et al., 2002).
In later studies, *PYGO2* has been found to be over-expressed in malignant ovarian cancer lineages compared to non-malignant cells. Moreover, its co-localization with β-Cat in the nucleus has been shown by immunohistochemistry in human biopsies of different stage of ovarian cancer but not in benign tumor or non-malignant cells (Popadiuk et al., 2006). Similar results have been observed in malignant breast tumors. The over-expression of such nuclear factor has been shown in five breast cancer cell lines (Orsetti et al., 2006; Andrews et al., 2007). In this forms of cancer, not only was *PYGO2* over-expressed in the cells, it was also shown to be required for transcription of *cyclin D1*, gene notoriously involved in tumor growth and directly induced by cWnt/ β-Catenin signaling (He et al., 1998; Shtutman et al.,1999; Tetsu et al., 1999; Orsetti et al., 2006; Andrews et al., 2007). Tissue samples at different cancer stages have been analyzed to confirm *Pygopus* transcription increase and PYGO subcellular colocalization with β-Cat. Another well-know Wnt/β-Cat dependent tumor as gliome has been took in consideration to study Pygo roles in tumorigenesis. Immunohystochemistry analysis has been performed on rat glioblastoma C6 cells and human tissues samples. Moreover, flow-cytometry effectuated on C6 cells transfected with pygopus2 shRNA shows a decrease in proliferation. Furthermore, *PYGO2* involvement in cancer initiation and tumor subtype specification has been suggested by *in vivo* experiments on (MMTV)-Wnt1 transgenic mice (Watanabe et al., 2013).

Thus, took together, these data strongly suggest that Pygo proteins have a particular relevance in human health studies.

3. Role of c-Wnt/ β-Cat in zebrafish embryonic development.

Embryonic development is a combination of several cellular and signaling interactions. In the non-mammalian vertebrate, zebrafish the c-Wnt/β-Cat pathway has been shown to be one of crucial pathways regulating the development of the embryo through two sequential phases, the first, just before midblastula transition (MBT), determines the prospective dorsal side of the embryo. The second acts at early gastrula stage in the ventro-lateral margin of the embryo specifying meso-endoderm territories (Bellipanni et al., 2006; Langdon and Mullins, 2011) (Figure 2A,B).

Several studies support the critical role played by c-Wnt/β-Cat during zebrafish development. In fact, the maternal depletion of *β-cat2* transcript in zebrafish causes a ventralized phenotype known as *ichabod* (Kelly et al., 2000; Bellipanni et al., 2006) (Figure 3A). The inhibition of transcription of both *β-cat1* and *β-cat2* also determines a new phenotype denominated "*ciuffo*", results from the abolition of the events carried out by these proteins during the two development stages (Bellipanni et al., 2006) (Figure 3A,B). Even though β-Cat is widely expressed in the blastoderm at 128-256 cells stage it can be detected in the nucleus of only dorsal marginal cells. This nuclear translocation of β-Cat following c-Wnt pathway activation is important to determine the dorsal cell fates and dorsal organizer formation. The maternal factor Kaiso zinc finger-containing protein (Kzp) activates the transcription of *wnt8* that, in turn, triggers the Wnt/ β-Cat cascade (Yao et al., 2010) (Figure 2A,B). Once in the nucleus, β-Cat directly activates the expression of genes such as *bozozok, squint* and *mkp3* (Melby et al., 2000; Tsang et al., 2004). While *chordin*, and *goosecoid* expression (Melby et al., 2000) is activated indirectly. In particular *bozozok* starts to be expressed just before midblastula transition, MBT, by direct link of β-Cat and its DNA-binding cofactor Tcf/Lef1 to a region within the gene promoter (Ryu et al., 2001; Leung et al., 2003) (Figure 2 A).

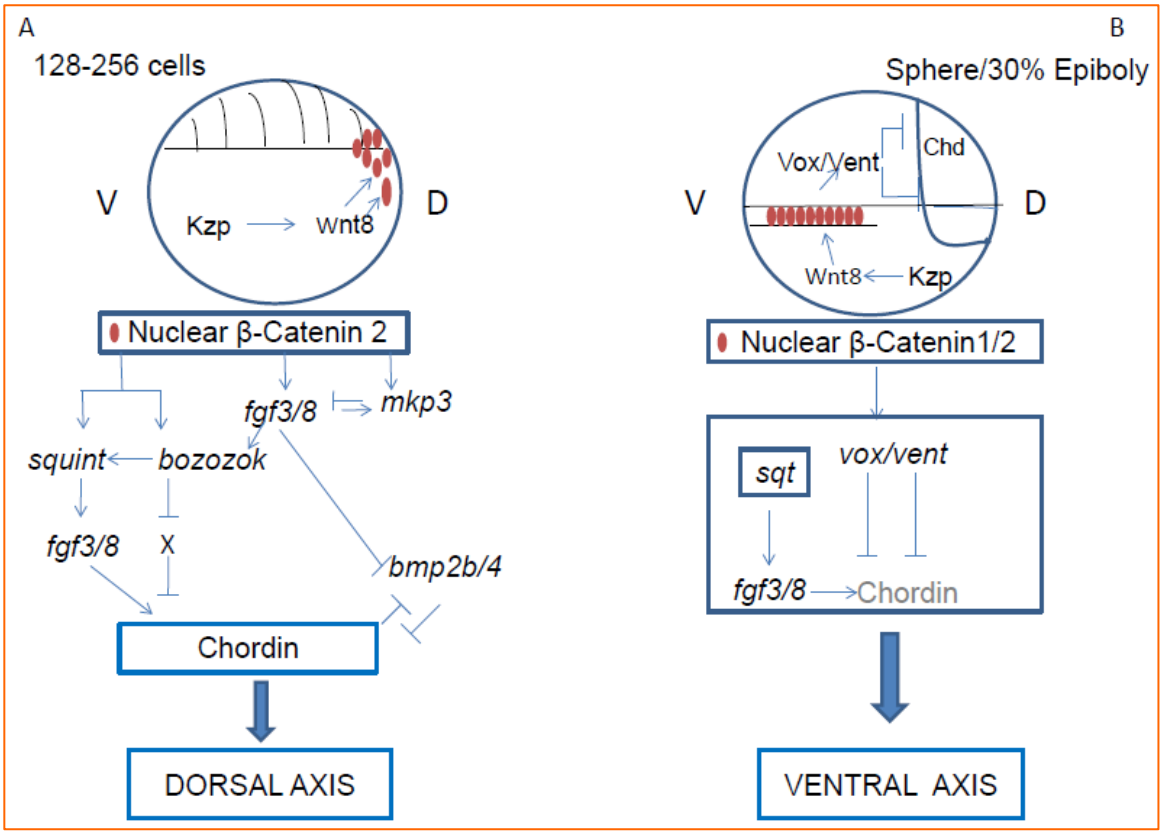

Figure2. **C-Wnt/β-Cat roles in zebrafish embryos early dorsovental induction.** Schematic of events mediated by wnt/β-Cat pathway during 128-256 cells stage (blastula) and sphere (early gastrulation) in zebrafish. A. At blastula stage β-Cat2 translocate in the nucleus of dorsal blastomers activating the transcription of several genes, i.e. *squint*, *bozozok*, *fgf,* and *mkp3*. This first wave of transcription regulates indirectly the expression of dorsal inductor *chordin* with induces in turn the dorsal axis. B. At 30% di Epiboly, instead, β-Cat plays an opposite role establishing the ventral axis. At this stage it is nuclear localized in the medio-ventral area, β-Cat leads to the activation of ventralizing genes *vox* and *vent* that inhibit *chordin* expression restricting its patterning to the dorsal zone.

Bozozok is a transcriptional repressor that acts directly binding the *bmp2b* promoter and tightly confines the expression of the *bmp2b* in the ventral side of the embryo. Its proper turnover is regulated through ubiquitination by the maternal protein *lnx2b* and subsequent degradation via proteasome (Ro and Dawid, 2009; Ro and Dawid, 2010).
β-Cat-dorsal fate establishment does not acts only promoting *bozozok* expression but through several other pathways. A critical pathway involved in dorsal axis formation and tightly regulated by β-Cat is the fibroblast growth factor, FGF, signaling which has been proved to be downstream *squint* activation (Figure 2A).

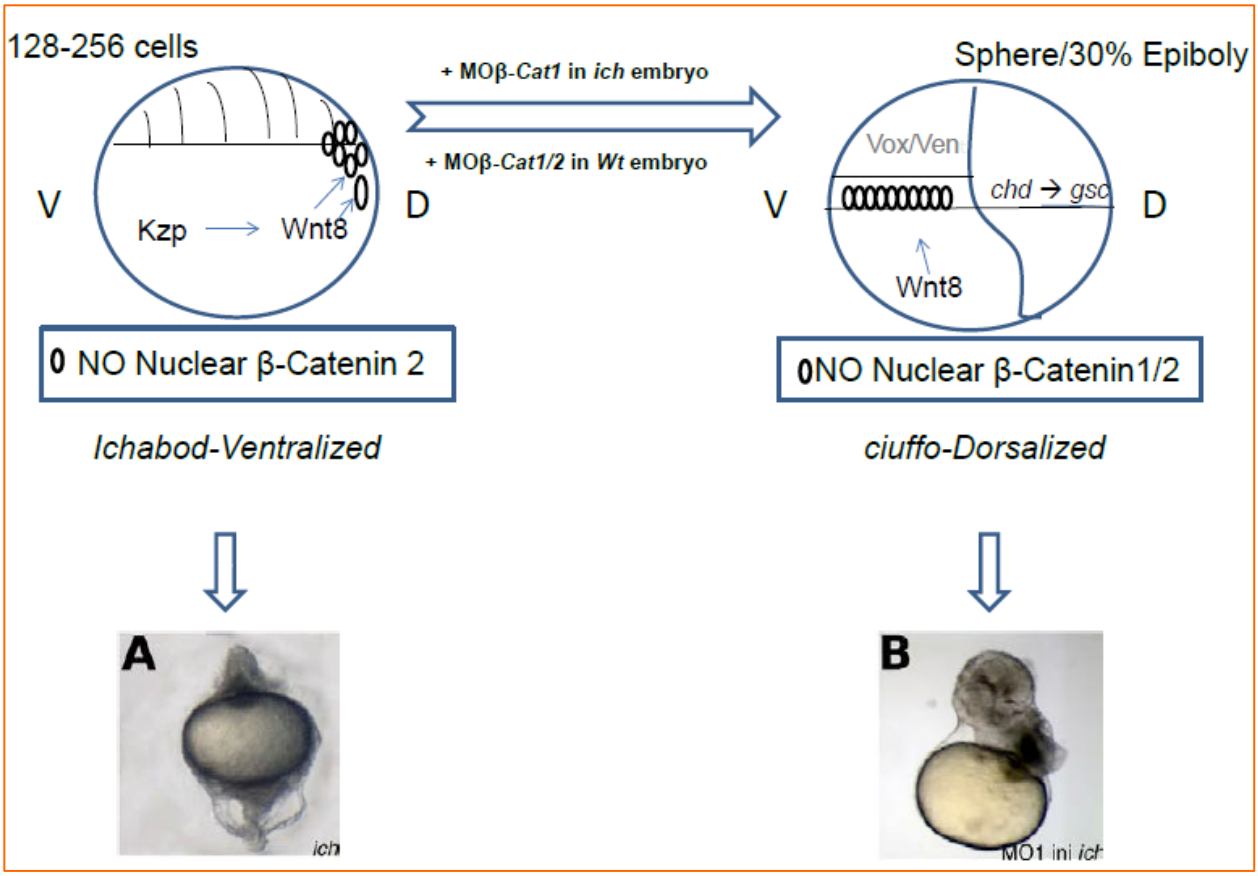

Figure3. **Embryological defects of zebrafish embryos determined by β-Catenin depletion.** (A) The crucial role carried out by wnt/β-Catenin has been widely studied. The maternal mutation *Ichabod* causes a decreased amount of *β-catenin* transcript and results in a ventralized phenotype. (B) At sphere stage the double knock down of *β-catenin 1* and *2* induces a peculiar phenotype called "*ciuffo*". Such phenotype is the result of the combinate abolition of the opposite roles of organizer inductor and ventral axis-specifying factor played by Wnt/β-Catenin signaling during early development stages.

In zebrafish development, the interaction of extracellular FGFs secreted proteins with one of four tyrosine kinase FGF receptors (FGFRs) triggers the receptor dimerization and activation of FGF pathway branches, i. e. RAS/MAPK, phospholipase C gamma (PLCY), and phosphatidylinositol 3-kinase (PI3K) pathways (Thisse and Thisse, 2005; Bottcher and Niehrs, 2005; Beenken and Mohammadi, 2009; Knights and Cook, 2010). Such event ultimate brings to the transcription of dorsal inductor genes such *chordin* and *bozozok* during organizer establishment, as well *otx2* and *hoxb1b* (Furthauer et al., 1997; Tsang et al, 2004; Maegawa et al., 2006). Thus, both c-Wnt/β-Cat and FGF signaling contribute to dorsal specification, with FGFs subordinate to c-Wnt/β-Cat. In addition, several studies report *mkp3* gene expression involvement in the development

of mouse, chick, *drosophila* and zebrafish embryo (Klock and Herrmann, 2002; Dickinson et al., 2002; Eblaghie et al., 2003; Kawakami et al., 2003; Rintelen et al., 2003; Tsang et al., 2004; Maegawa et al., 2006). In zebrafish embryo *mkp3* expression was detected immediately after MBT, at the high stage (3.3hpf) and colocalize in the dorsal region with β-Cat. No maternal *mkp3* RNA was found and *fgfs* transcripts appear only later at 3.7 hpf. Furthermore, β-Cat stabilization via LiCl treatment determines a significant increase of *mkp3* expression. Together these evidences suggest that the first *mkp3* transcription is mediated by the maternal β-Cat signaling (Tsang et al., 2004). MKP3 acts at RAS/MAPK level, it has been shown to dephosphorylate active MAPK1 and MAPK2 (p42/p440) proteins by binding them throw its MAPK-binding domain and subsequent conformational activation of the C-terminal phosphatase domain, thus resulting in *fgf* down-regulation (Zhao and Zhang, 2001) (Figure 2A).

Experiments carried out on *ichabod* embryos have highlighted a linear pathway in with FGFs fulfills an important role during the 128-256 cells stage. Squint, a Nodal protein notoriously under control of β-Cat in the dorsal side (Kelly et al., 2000; Shimizu et al., 2000), acts downstream β-Cat inducing the expression of *fgf3/8* genes. The transcription of these genes leads to a transcriptional activation of *chordin,* a well-known dorsal axis inductor and bmp2b/4 inhibitor (Sasai et al., 1994, 1995; Piccolo et al., 1996). Such studies also show how *fgfs* expression is necessary to maintain a Bozozok feedback by β-Cat in the organizer area after its first transcription period at 3.5/4hpf sphere stage (Maegawa et al., 2006) (Figure 2A).

The later role of c-Wnt/ β-Cat signaling has been shown to specify embryo ventral fates (Ramel et al., 2004, Varga et al., 2007). The *vox/vent/ved* genes expression in ventrolateral gastrula regions at 30% epiboly is supported by Wnt8 signaling and BMPs gradient (Ramel et al., 2005; Varga et al., 2007; Flores et al., 2008; Baker at al., 2010) (Figure 2B). In particular, Wnt8 has been detected in the ventrolateral region in zebrafish (Kelly et al., 1995) and is necessary for several structures, such as ventrolateral and posterior mesoderm, spinal cord and posterior brain (Lekven et al., 2001; Erter et al., 2001; Momoi et al., 2003; Ramel and Lekven, 2004).

In contrast with what happen in the early stage and dorsal patterning the only depletion of β-Cat2 is not sufficient to stop the ventral axis development. Both β-Cat1 and β-Cat2 work redundantly for the expression of ventralizing gene *vox/vent.* In turn Vox and Vent act repressing *chd* (Figure 2B). Experiments of *β-cat1* and *β-cat2* knock-down carried out in *Wt* and *ich* embryos support such mechanism. The characteristic "*ciuffo*" phenotype, defined by a dorsalized protrusion and neurectodermal-markers expression, is obtained after co-injection of morpholino against *β-cat1* and *β-cat2* in *Wt* embryos and morpholino against *β-cat1* in *ich* embryos (Figure 3B). In these morphants *chd*

expression is increased and visible around all the germ-ring (Bellipanni et al 2006; Varga et al., 2007) (Figure 3B).

4. Aims of the Thesis

In my Doctorate Thesis work I aimed to identify c-Wnt/β-Catenin-dependent roles of the Pygopus family of nuclear factors during early zebrafish development. The use of the zebrafish as animal model is particularly advantageous for studies, like this, focusing to dorso-ventral axis specification during early and late blastula stages. These studies are very difficult to carry on in mice, therefore the analysis of the knock-out of these genes in not able to address early Pygo functions in mammals (Cantu' et al., 2013). Similarly, studies in frog lacked to address and identify early c-Wnt/ β-Cat-dependent roles of the Pygopus family of nuclear factors (Lake et al., 2003).

In particular, during my thesis work, I have provided a series of experimental evidences that strongly suggest that Pygo1 and/or Pygo2 play key c-Wnt/ β-Catenin-dependent roles during the two earliest stages in which c-Wnt/β-Catenin signaling is acting in order to establish the dorso-ventral axis of the zebrafish embryo. This is, to our knowledge the first analysis of c-Wnt/ β-Catenin-dependent roles of Pygo in vertebrates.

5.Materials and methods

5.1 Zebrafish strains

Tübingen (*Tü*)*, Brass* (*Brs*) and *Ichabod* (*ich*) 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*). *Tü and Brs* were selected as "*wild type*". *Ich* embryos were obtained by breeding homozygous *ich* females with heterozygous or *Wt* males. For this study was used only *ichabod* embryos obtained from homozygous females that reproducibly gave severely ventralized embryos.

5.2 Cloning

The pCS2+*2xFlagPygopus2* was obtained by two-step PCR approach. The sequence at 5' end of *pygopus2* was recovered using the plasmid pCS2+2xFlag*Pygopus2ΔPHD* as template. Instead, genomic DNA extracted with the phenol/chloroform method from fin-clip (Brass strain) was used to amplify the sequence at 3' end of gene *Pygopus2*. Appropriate primers carrying EcoRI at 5' and XhoI at 3' restriction enzyme sites were used for the PCR (Table1). The solution for each PCR was made with a mix 0,125:1 of cloned PFU DNA Polymerase (Stratagene) and Taq DNA polymerase recombinant (Fermentas) respectively. PCR conditions: pre-denaturation 94°C 5 min; amplification 94°C 30 sec, 53°C 20 sec, 72°C 1:30 min. for the 3' and 2:0 min. for the 5' end, repeated 30 cycles; final extension 72°C 10 min.

The PCR products were loaded in a 1% agarose gel, the band was cut and the DNA recovered with Zymoclean Gel DNA Recovery Kit. A final PCR was made in order to obtain the 5'-EcoRI-pygopus2full-Xho-3' fragment that has to be cloned in the plasmid pCS2+2XFlag. This PCR was performed with the Thermo Scientific Phusion Hot Start II High-Fidelity DNA Polymerase. The two fragments 5' EcoRI and 3' XhoI were mixed with a 2:1 ratio and the primers, table 1, were added after 10 cycles to the master mix made accordingly to the manufacturing instruction. The condition adopted were: 98°C 30 sec, 98°C 8 sec, 58°C 20 sec, 72°C 1 min repeated for 10 cycles, than 98°C 30 sec, 58°C with a gradient of + 0.8 each cycle up to 67°C 8 sec, 72°C 1 min repeated for 15 cycles, final extension 72°C 10 min. The fragment obtained was loaded in a 1% agarose gel, the band was cut and the DNA recovered with Zymoclean Gel DNA Recovery Kit. Than it was double cut with EcoRI and XhoI (Roche). The same double digestion and purification was done for the plasmid pCS2+2xFlagPygopus2*ΔPHD* that was also dephosphorylated with the rAPid alkaline phosphatase kit (Roche). The dephosphorylated pCS2+2xFlag was ligated with the fragment z*-pygopus2* with T4 DNA Ligase kit (Roche).

PCR product	Primers name and Sequence	Size
5' z-pygopus2	-5' zpygopus2 2077 EcoRI : 5'-GGAATTCCTTGGCCGACGAG-3' -3' pygopus2 1530 : 5'-TGGGGGATGAATTAGGATTAGGG-3'	1276bp
3' z-pygopus2	-5'zpygopus2 1530 Forward: 5'-TCCTAATTCATCCCCCACAG-3' -3'zpygopus2XhoI: 5'-ATGCCTCGAGCTTCCTCCCTGTCTCAGCC-3'	633bp
5' fragment **+** **3' fragment**	-5' zpygopus2 2077 EcoRI : 5'-GGAATTCCTTGGCCGACGAG-3' -3'zpygopus2 XhoI: 5'-ATGCCTCGAGCTTCCTCCCTGTCTCAGCC-3'	1879bp

Table1.Primers used for cloning z*pygopus2*.

5.3 Plasmid preparation

Several colonies from an LB plate in which was plated the desired transformed bacteria were inoculated in 5 ml of LB plus the correct antibiotic, and let grow ON at 37°C shacking. The day after, the plasmids were recovered using the High Pure Plasmid Isolation Kit (Roche).

The concentration was measured with NanoDrop ND-1000. The insertion of fragment was tested cutting the isolated plasmids with EcoRI and XhoI (Roche) and running them on a 1% gel. The clones that shown the right size- insert were sequenced.

5.4 Synthetic mRNA in vitro transcription

mRNA to be used for microinjection on zebrafish embryos was produced using as linearized template plasmid DNA (Table2).

The digested DNA was purified via phenol/chloroform and precipitated with 0.5 volume of ammonium acetate plus 2 volume of 100% ethanol. Resuspended linearized DNA was used to prepare the synthetic mRNA with the mMESSAGE mMACHINE kit (Ambion) in accord with the kit protocol. The synthetic mRNA obtained was quantified at the spectrophotometer and then stored in aliquots at -80°C.

Plasmid name	**Restricted Enzyme**	**Polymerase**	**Reference**
pCS2+2xFlag zpygopus1	NotI	SP6	Bellipanni lab unpublished.
pCS2+2xFlag zpygopusΔPHD	NotI	SP6	Bellipanni lab unpublished.
pCS2+2xFlag zpygopus 2	NotI	SP6	Bellipanni lab unpublished.
pZL1 β-catenin1	Eco RI	SP6	Kelly et al 1995/ Moon lab
pBS(SK)β-catenin 2	XhoI	T3	Bellipanni et al 2006
pCS2Myc-β-catenin1	Xba I	SP6	Bellipanni lab unpublished.
pCS2Myc-β-catenin2	XhoI	SP6	Bellipanni lab unpublished.

Table2. Constructs used for mRNA synthesis.

5.5 Microinjections on zebrafish embryos

The Microinjections were performed in all blastomes of zebrafish embryos at 1-2 cell stage or in a single blastome at 8-16 cell stage. All mRNAs were injected at 200 ng/µl. The injection solution was made mixing the double concentrated mRNA and an equal volume of Dulbecco's modified phosphate-buffered saline (PBS) containing 0.5% Phenol Red (Sigma). 1pl to 3pL of solution was injected per embryo.

5.6 Total RNA isolation for Semi-Quantitative PCR

RNA was isolated from between 10 and 20 embryos for each group were moved to a 1,5ml tube and washed two times with cold PBS. Then 1 ml of cold TRIzol (Invitrogen) was added and the embryos were smashed with a pastel. The samples were vortexed for 1 min and centrifuged at 4°C with a refrigerated table centrifuge (Eppendorf 5415 R) at max speed (13200 RPM) for 15 min. The aqueous phase was recovered for each sample and 200 µl of chloroform:isoamyl alcohol 24:1 (Acros) were added then vortexed 1 min, and centrifuged at 4°C with at max speed (13200 RPM) for 10 min. The recovered aqueous phase was precipitated with 1 µl Glycogen (Glycoblue, Ambion) and 500 µl of isopropanol, 10 min at Room Temperature (RT). After this, the sample was centrifuged at 4°C for 15 min at max speed, the liquid phase removed and the obtained pellet was washed with cold 70% ethanol and air dried. The pellet was then dissolved in autoclaved Milli-Q sterile water and treated with DNase (Promega) for one hour. Followed a classic phenol basic/chloroform extraction. The total RNA was tested by PCR for DNA contamination, and if pure, aliquots were stored at -80°C.

5.7 cDNA synthesis

cDNA from 1 µg of total RNA extracts was obtained with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen), using Random hexamers. the cDNA was diluted to a final concentration of 100 ng/µl, and then used for the subsequent experiments. At the same time, using the same reaction mixture and the same conditions, were prepared samples with the same total RNA, without adding the Superscript RT enzyme. These samples were called RT- and were used as negative controls.

5.8 Semi-quantitative PCR

The cDNA obtained from the six groups was analyzed by semi-quantitative PCR. The expression of two *pygo2* variants and *pygo*1 were tested with three set of primers (table3). The solution were prepared with Taq DNA polymerase recombinant (Fermentas) and the protocol was set with this conditions: pre-denaturation 94°C 4 min; amplification 94°C 30 sec, 55°C 20 sec, 72°C 45 sec, repeated 15 cycles, than amplification 94°C 30 sec, 55°C + 0.5°C each cycle for 20 sec, 72°C 45 sec, repeated 15 cycles; final extension 72°C 10 min.

Primers name	Primers sequence	Product Size
5' z-pygopus1 Forward	5'-AAAACGCGACTCAGGACAAC-3'	112bp
3' z-pygopus1 Reverse	5'-GAAAACAGGCTCGGATGAAG-3'	
5'z-pygopus2 NHD Forward	5'-AACGAGGCAAAGGT-3	308bp
3' z-pygopus2 NHD Reverse	5'-GGACCTGGAAGTGGACAG-3	
5'z-pygopus2 diff. Forward	5'-ACCCAACTCCAACCAGAACA-3'	Pygo ΔPHD: 207bp
3' z-pygopus2 diff. Reverse	5'-TTCTGCGATTCATTGTCAGC-3'	Pygo2: 1137bp

Table3. Primers used for testing transcripts *pygopus1* and variants *pygopus2* with semi-quantitative PCR.

5.9 Probe preparation reaction

For probes synthesis, we linearized the plasmids with restriction enzymes (Table4) and used the appropriate RNA polymerase (Table4). Thus, we set up the mRNA Digoxigenin-labeled probe reaction for each gene using the appropriate RNA polymerase with 5X buffer, 0.1M DTT, 10X NTP DIG mix and 1ul of RNase. This mix was incubated for 2-3h at 37^0C. Then the probe mixture was then mixed with a 20mM Tris/HCl pH 7.5, 20mM EDTA, 100mM NaCl TEN buffer and purified through centrifugations for 3' at 3000 rpm in a probe QuantG50 Micro column (Biolab), previously balanced with 0.3M NaOAc (pH5.2), 0.1% SDS equilibration buffer. After precipitation with 3M sodium acetate the dry probe was dissolved in 30μl of DEPC.

Plasmid	Enzyme	Polymerase	Reference
pBS (SK) *goosecoid*	*BamHI*	T7	Stachel et al., 1993
pCS2 *chordin*	*HindIII*	T7	Miller-Bertoglio et al., 1997
pBS (SK) *squint*	*EcoRI*	T7	Erter et al., 1998
pGEM *bozozok*	*XbaI*	T7	Koos and Ho 1998
pBS *vox (vega1)*	*BamHI*	T3	Kawahara et al., 2000a
pBS *vent (vega2)*	*EcoRI*	T3	Kawahara et al., 2000b
pCS2 *fgf3*	*Sal I*	T7	Kudoh et al., 2001
pCS2 *fgf8*	*Sal I*	T7	Kudoh et al., 2001
pCS2 *mkp3*	*Sal I*	T7	Tsang et al., 2004

Table4. Constructs for antisense RNA probe synthesis

5.10 In Situ Hybridization

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 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 at decreasing formamide and citrate buffer (SSC) solutions and then 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 hours incubation was followed by 8 PBT washes, of 15 minutes each, and then AP buffer (100mM Tris-HCl pH9.5, 50nM

MgCl2, 100mM NaCl, 0.2% Tween 20 and 0.2% Triton-X100) was applied in 3 washes, 5 minutes each. 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 stereo-microscope SMZ800 (Nikon), and analyzed with the software NIS-Elements BR3.0 (Nikon).

5.11 Phenotype analysis

For the analysis of the phenotypes at 24hpf we used the classification adopted in Kelly et al., 2000 and Bellipanni et al., 2006, with V4A as most ventralized phenotype. For the analysis of the dorsalized phenotype we adopted the classification used by Kishimoto et al. (Kishimoto et al., 1997)

6.Results

In order to carry on knocking-down experiments we designed antisense oligonucleotides against *pygo1* and *pygo2* mRNA, that are schematically represented in the figure 4. We called MOA*pygo2* and MOB*pygo2* the antisense morpholinos targeting the regions at the beginning of *pygo2*. We used two different morpholinos targeting two different potential ATGs of *pygo2*. MOA*pygo2* recognizes the ATG region of the predicted full-length transcript of *pygo2*, as results from Zv9 genomic sequences, whereas MOB*pygo2* is direct against a novel ATG present in two different cDNAs for *pygo2* that we found in the databases. The genomic sequence containing the exon coding for the novel ATG is, indeed not annotated in the same chromosome of *pygo2*. Thus, to determine if this novel ATG is really belonging to *pygo2* and therefore the genomic sequence has been wrongly assembled, we decided to use a morpholino designed against this novel ATG and compare the resulting morphants with that one obtained by MOA*pygo2*. This novel ATG region belongs to a cDNA that also contains new and uncharacterized splicing variants of *pygo2*, which we called *pygopus2ΔPHD* and has a new exon for the ATG and a cryptic splicing site that eliminates the PHD domain and generates a novel C-terminal region of the protein. The Doctorate work of Dr. Fabio Valenti in our laboratory has shown that, indeed, this splicing variant is expressed during zebrafish development. This was done by RT-PCR and the position of primers used to distinguish between full-length *pygo2* and *pygo2ΔPHD* are indicated in the figure 4 with different colored arrows. The black arrows represent the primers NHD that recognize both the variants because this region is conserved. The red arrows indicate the primers that are designed on the ends of splicing site at the PHD domain, thus can differentiate between the two splicing variants (Table3, Figure 4).

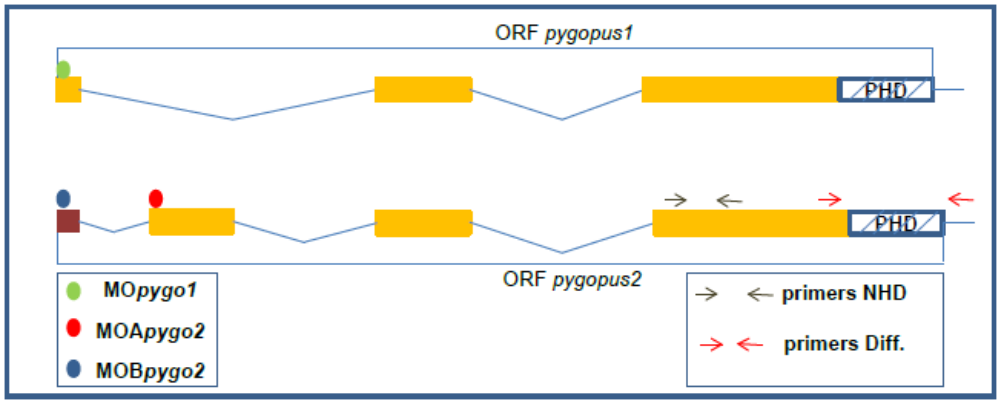

Figure 4. Schematic rapresentation of *pygo1* and *pygo2* RNA. The different colored bullets indicate the position on the mRNA sequences of three different morpholinos denominated MOA *pygo2*, MOB*pygo2*, and MO*pygo1*. The couples of arrows rapresent the primers used for RT-PCR.

It is not yet clear what is the role of *pygo2ΔPHD* during early zebrafish development. Interestingly it has been shown in mice that Pygo2 carrying a point mutation in the PHD domain such that the histone acetyltransferase activity of this domain is destroyed, were perfectly viable, but showed some spermatogenesis defects (Cantu' et al., 2013).

6.1 *pygo1* and *pygo2* expression pattern during early zebrafish development stages.

c-Wnt/β-Cat signaling has been shown to be involved in the early zebrafish development (Kelly et al., 2000; Bellipanni et al., 2006; Yao et al., 2010) as well as in other animal models (Kelly et al 1995) . Previous studies in *Drosophila*, *Xenopus*, and mouse reported that the Pygopus family components are expressed through different embryonic stages (Parker et al. 2002; Thompson et al. 2002; Lake et al., 2003, Li et al., 2004). In *Xenopus*, homologous *xpygo-2α* and *xpygo-2β* transcripts have been found to have specific temporal patterns. *xpygo-2α* message is detected in the anterior area, specifically in the neural plate, after gastrulation, whereas *xpygo-2β* presents an earlier expression during oogenesis and early stages decreasing before and through gastrulation (Lake et al., 2003). In order to identify the patter expression of *pygo1* and *pygo2* in zebrafish we performed whole mount in situ hybridizations on embryos at different stages. Both transcripts of *pygo1* and *pygo2* accumulate starting from the 4-8 cells stage up to the blastula stage (30% epiboly) (Figure 5A-F). However, *pygo2* shows a visible higher expression level than *pygo1* (Figure 5D-F). Their expression at 4-8 cells stage supports a maternal origin and suggests a role in events that act upstream the zygotic gene expression at MBT (Figure 5A,D).

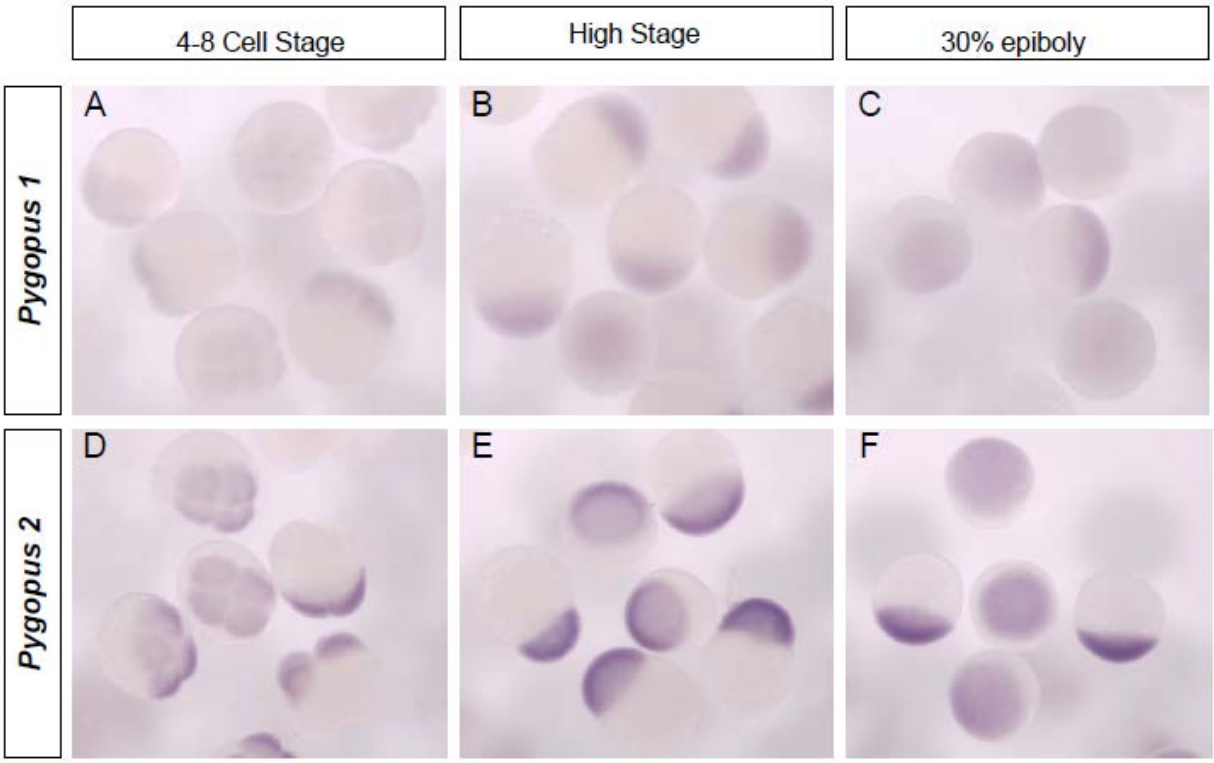

Figure5. A-C,D-F Both *pygo1* and *pygo2* are ubiquitous from 2-4cell stage (maternal contribution) through 30% epiboly stage. D-F However, *pygo1* shows a visible lower expression level than *pygo2*.

6.2 Pygopus family of nuclear factors is mediating c-Wnt/β-Cat induction of the D/V axis during zebrafish blastula stage

6.2.1 Gain-of-function assay for *pygo1* and *pygo2ΔPHD*

We began to test the role of Pygopus in D/V induction by gain-of-function experiments in *Wt* zebrafish embryos or in the maternal recessive mutation *ichabod* (*ich*), in which a second *β-catenin* (*β-cat2*) gene is maternally depleted. We have injected in 2-8 cell stage zebrafish embryos; *pygo1* mRNA or *pygo2ΔPHD* mRNA. *pygo1* overexpression produced ~80% of the embryos with a dorsalized phenotype while the remaining 20% were wild type. This was unexpected, as prior studies over-expressed x*pygo-2β* in *Xenopus* had effect on D/V induction only if co-injected with BCL9 (Kennedy et al., 2010). Injection of *pygo2ΔPHD* mRNA dorsalized ~25% of the embryos, however the remaining embryos had a *Wt* phenotype (Figure 6A). Neither expression of *pygo1* or *pygo2ΔPHD* was able to rescue *ichabod* embryos (data not shown).

We next investigate the effects of the overexpression of *pygo1* and *pygo2ΔPHD* on the expression of genes downstream the c-WNT/β-Cat pathway during dorso-ventral specification in early development of the zebrafish embryo. As it has been shown that Chordin and Goosecoid are crucial in dorsal patterning and organizer establishment (Melby et all., 200; Sasai et al., 1994, 1995; Piccolo et al., 1996), whereas Vox and Vent are ventral inducers (Ramel and Lekven, 2004; Varga et al., 2007) by inhibiting *chd* transcription, we tested whether Pygo1 or Pygo2 are able to regulate the expression of these factors.

The mRNAs coding for *pygo1* and *pygo2ΔPHD* were injected into 2-8 cell-stage embryos and derived embryos were followed until 30% epiboly. The resulting phenotypes were analyzed by in situ hybridization. The injection of *pygopus1* partially reduced the expression of *vox* and *vent* in 30% epiboly embryos in respect to the un-injected *Wt* control embryos, (Figure 6B',C',F',G'). The *chd* expression is also partially expanded in the lateral sides, while the overall intensity was reduced (Figure 6 A',E'). Injection of mRNA for *pygo2ΔPHD* determined a reduction of intensity of *chordin* expression but an expansion of expression in the medio-lateral region of the embryo (Figure 6I'). While, *vox* expression in the margins is reduced (the dark staining in Figure 6K' is beckground due to longer staining time) (Figure 6K'). However, the expression of *goosecoid* is not affected by the overexpression of neither of two mRNAs (Figure 6H',L'). These results confirm the analysis of the over-expression of *pygo1* and *pygo2ΔPHD* mRNAs at 24hpf (Figure 6A), gain-of- function experiments using either *pygo1* and *pygo2ΔPHD* result in dorsalized embryos (Figure 6A).

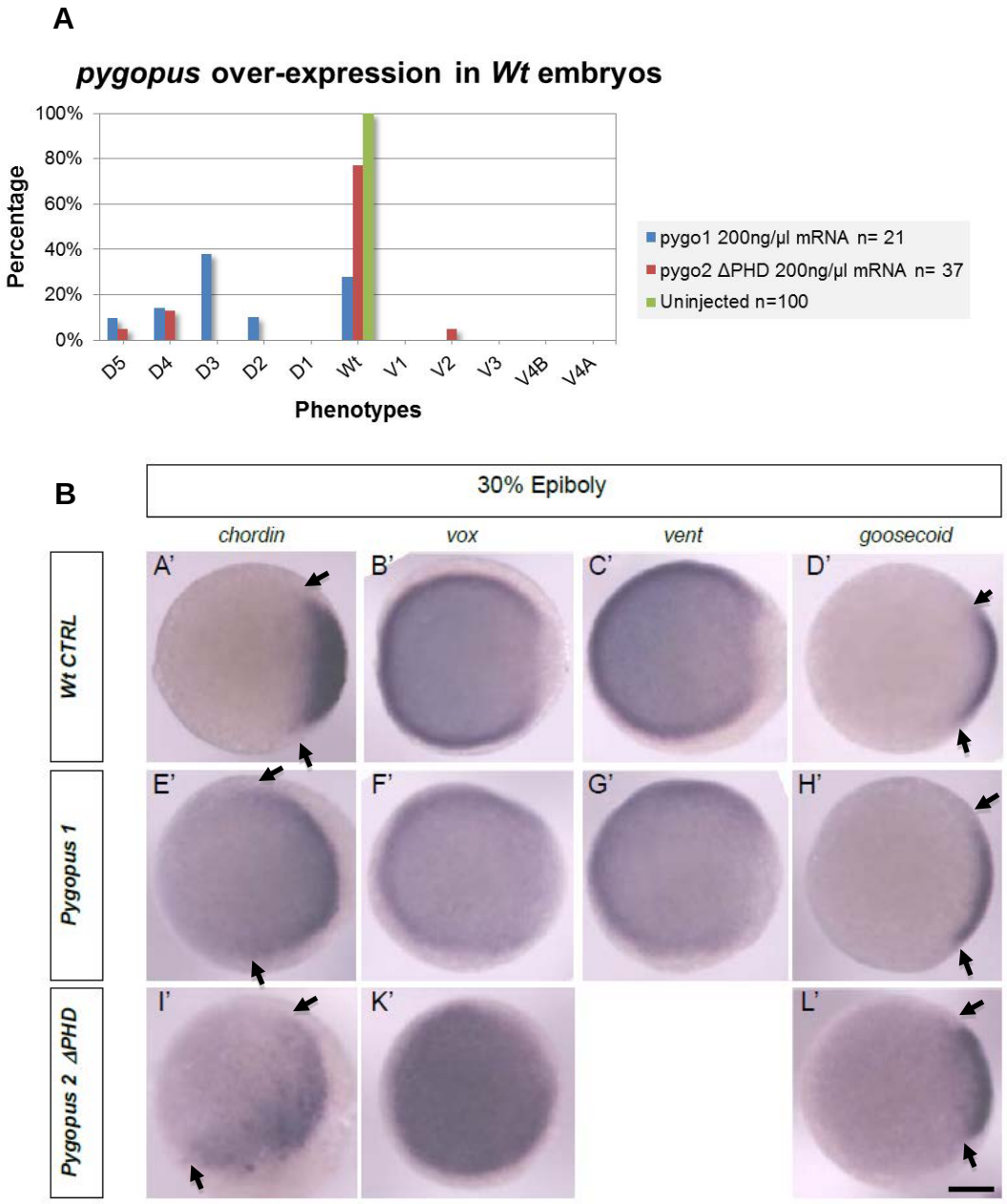

Figure 6.A. Over-expression of MO*pygo1* (blue) or MO*pygo2* in *Wt* embryos results in dorsalived phenotypes compared to uninjected control. A',D'. In *Wt* embryos, at 30% epiboly, the expression of *chordin* is limited to the dorsal side and co-localize with *goosecoid* transcript. B',C'. *vox* and *vent* instead are visible at the dorsal area. E',H'. Injection of mRNA for *pygo1* determined a slight radialization (expression along the margins of the embryo) of *chordin* expression in the ventro-lateral region of 30% epiboly embryos but not affect *goosecoid* expression. F',G'. *vox* and *vent* expression is not altered by *pygo1* overexpression. I',K'. Injection of mRNA for *pygo2ΔPHD* determined reduction of *chordin* and *vox* expression in the margins. L'. *goosecoid* expression is unaffected. Arrows indicate the extent of expression. Staining time was the same for control and injected embryos. Embryos are in their animal view and dorsal is to the right. The size bar is 10µm.

6.1.2 Loss-of-function assay for *pygo1* and *pygo2*

Since β-Catenins has been proved to be crucial for dorso-ventral establishment of vertebrate embryos and Pygopus family members have been shown as components of cWnt/β-Catenin pathway we examined whether Pygo1 and Pygo2 are involved in processes linked with early embryonic development in the zebrafish. To test this hypothesis we examined the effect of *pygo1* and *pygo2* single or double knock down on *Wt* and *ich* embryos (Figure 7A',B'). As previously reported *ich* is a mutant lacking of sufficient maternal *β-cat2* (Bellipanni et al. 2006). Injection of MOA*pygo2* or MOB*pygo2* (Figure 4) in *wild-type* embryos determines various degree of dorsalized phenotypes with MOB*pygo2* always inducing the most severe phenotypes (Figure 7 A, B, C), while in *ich* it results in a full reversion of the ventralized phenotype into a *ciuffo* phenotype (69% of the embryos, n=13) (Figure 7B',D',F',G') peculiar of the double knock-down of both *β-catenins* (Bellipanni et al., 2006) (Figure 3B and Figure 7F',G'). We obtained dorsalized embryos either injecting MOA*pygo2,* that localize in the ATG region predict for *pygo2* from genomic sequencing, and MOB*pygo2* that is complementary to a novel ATG discovered in different cDNAs coding for *pygo2.* Indeed, the phenotypes obtained with MOB*pygo2* were stronger, suggesting that the novel ATG region is real and normally present in *pygo2* mRNA. Since we cannot exclude the existence of two different splicing variants for the ATG region (like in the case of *X. leavis*), the milder phenotypes obtained with MOA*pygo2* may be due either to different functions played by the two splicing variants or, if there is only one variant (that with the new ATG), the MOA*pygo2* will work as a splicing morpholino and show only zygotic, therefore milder, phenotypes.

To confirm the role of the Pygopus family of nuclear factors during dorso-ventral specification of zebrafish blastula we injected both MO*pygo1* and MOB*pygo2* in *wild-type* embryos, this also resulted in the *ciuffo* phenotype (Figure 7E', Figure 7D). In the *ciuffo* phenotype induced by MOB*pygo2* in *ich* embryos and MO*pygo1*+MOB*pygo2* in *Wt* embryos the somite structures appear lost (Figure 7D',E'). Instead the double knock-down of both *β-catenins* produces a *ciuffo* phenotype with clear somite structures (Figure 3B). This is suggesting that Pygopus family of nuclear factors may play c-Wnt independent roles in somite specification. In addition, a large number of injected embryos died during gastrulation before to reach the stage of analysis, either for high toxicity of the morpholinos or for the difficulty to inject the right morpholino amount and/or localize it in the proper region.

These two experiments taken together strongly suggest that the Pygo2 plays c-Wnt/β-Cat dependent role during the second wave of c-Wnt/β-Cat activity at sphere-30% epiboly stages. Apparently, *pygo1* is dispensable for the induction of the medio-lateral fates at this stage. The resulting *ciuffo* phenotype obtained with double injection of

MO*pygo1*+MO*pygo2* in *Wt* embryos but not with the knocking-down of single Pygo transcript also delineates a possible involvement for both Pygo1 and Pygo2 in dorsal induction during the stage of early blastula.

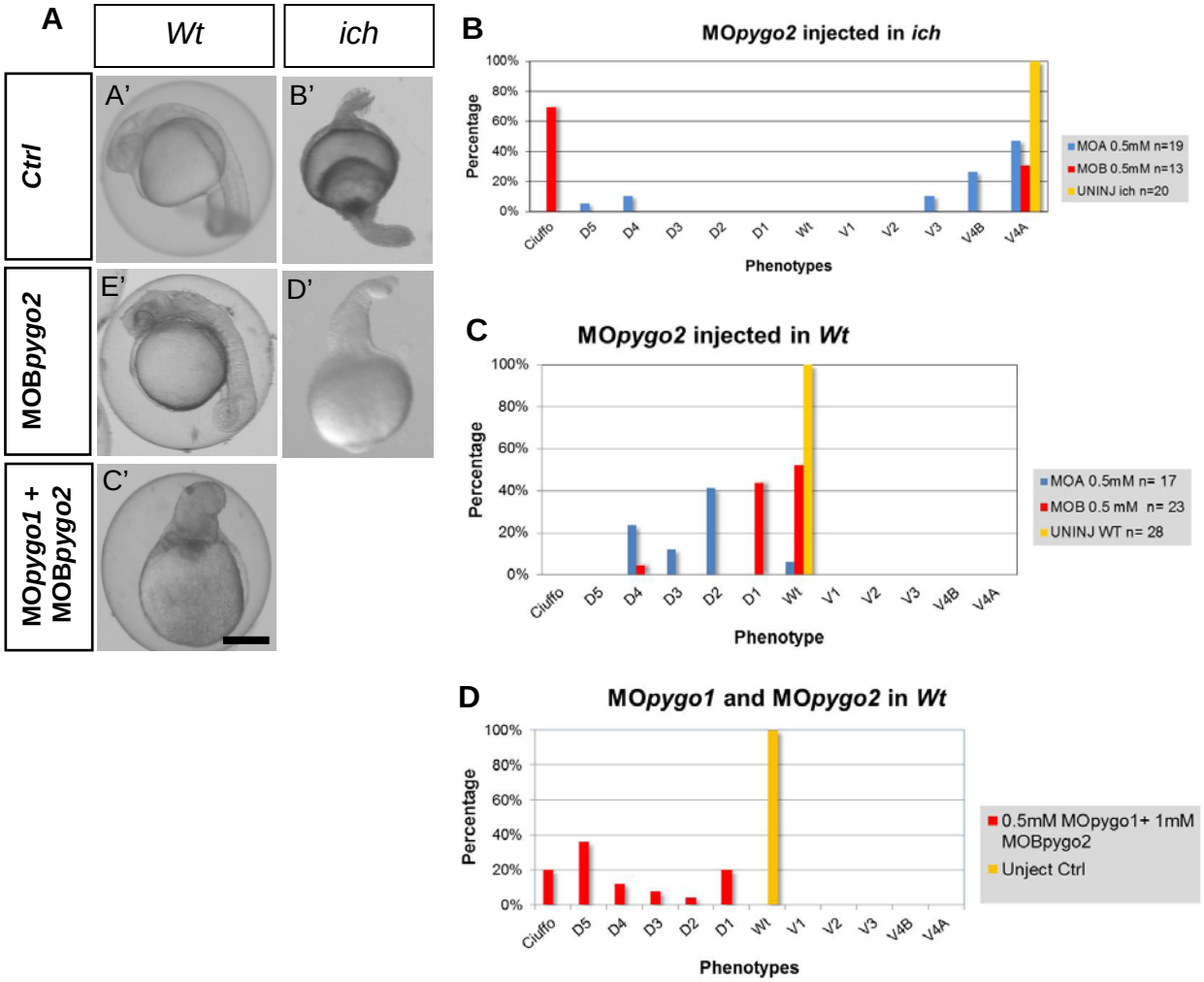

Figure 7A. A',B'. Un-injected embryos *Wt* and i*ch*. C'. Injection MOB*pygo2* in *wild-type* embryos determines a dorsalized phenotype, D'. Know down of *pygopus2* in *ich* results in a full reversion of the ventralized phenotype into a *ciuffo* phenotype. E'. Both MO*pygo1* and MO*pygo2* when injected in *wild-type* embryos resulted in the *ciuffo* phenotype. B. Injection of MOB*pygo2* (blue) in *wild-type* embryos induces a dorsalized phenotype. C. In *ich* it results in a full reversion of the ventralized phenotype into a *ciuffo* phenotype. D. injection of both MO*pygo1* and MOB*pygo2* in *wild-type* embryos resulted in the *ciuffo* phenotype. Scale bar is 10 μM

To confirm the specificity and functionality of our morpholinos we tested if the morpholino phenotype could be rescued by co-expression of the relative mRNA. Injections of mRNA for *pygo1* or *pygo2DPHD* (mRNA coding for a form of Pygo2 lacking the PHD domain) are not able to rescue the *ciuffo* phenotype determined by MOB*pygo2*

(Figure 8A). We just cloned a full-length version of *pygo2* that will be used to complete this rescue panel.

Similarly, injection of *pygo1* mRNA cannot rescue the phenotype determined by the knocking down of *pygo1* (Figure 8B). A possible explanation for these results is that *pygopus* works in tightly controlled stoichiometrical complexes and both increase or decrease of this molecule negatively affects the proteins complex and therefore the pathways they controls. This is not surprising, in *drosophila* gain and loss of function of *pygopus* also results in similar phenotypes (Parker at al., 2002, Thompson et al, 2002). Consistently with this hypothesis, when we over-expressed *pygo1* mRNA in *Wt* embryos and analyzed its effect on 24hpf embryos (Figure 7B) and in patterning on mid-blastula embryos (Figure 6E-H) we obtained also a dorsalized phenotype.

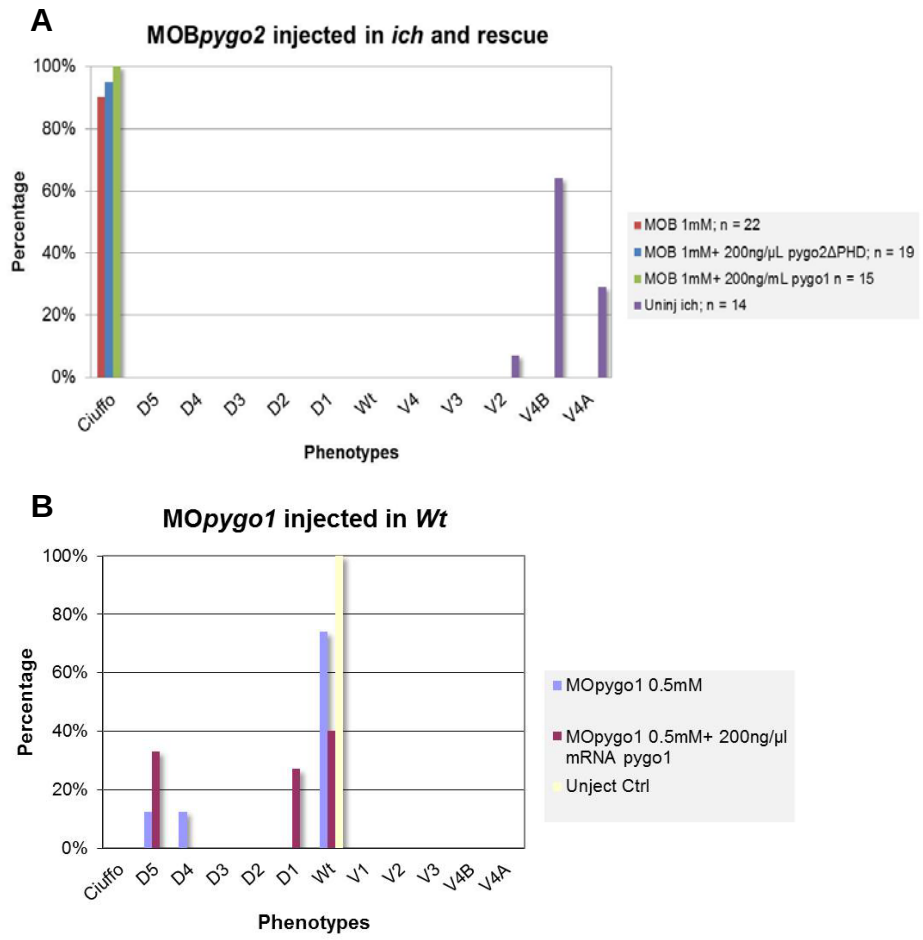

Figure 8. A.B. Diagrams show the percentages of embryos for each phenotype on y axis and the range of phenotypes from *ciuffo* to grade 4 ventralized on x axis. A. Injections of mRNA for *pygo1* (red) or *pygo2ΔPHD* (blue) are not able to rescue the *ciuffo* phenotype determined by MOB*pygo2.* B. Injection of MO*pygo1* (blue) induces dorsalized and Wt phenotypes and the severe types of dorsalized embryos are not rescued by co-expression of *pygo1* mRNA (violet).

6.3 Both Pygopus are required for the c-Wnt/β-Catenin dependent induction of the dorsal side of the early blastula embryo

To confirm if both Pygopus are involved in the dorsal specification at blastula stage we took advantage of the fact that β-Cat2 directly activates the transcription of several genes during early dorso-ventral patterning. Among these genes there is *mkp3* (Tsang et al., 2004). This is supported from the fact that the injection of *β-cat2* mRNA in *ich* embryos, rescues the expression of *mkp3* (Tsang et al., 2004; Maegawa et al., 2006) (Figure 9A,B). Since our previous experiments suggest a c-Wnt/β-Cat-dependent role for Pygopus family components, we checked if they can affect *mkp3* transcription. We injected 50ng/μl of *β-cat2* mRNA in *ich* and completely rescued the expression of *mkp3* at high stage (100%, n=13). Co-injections of 50ng/μl of *β-cat2* RNA and 0.5mM MO*pygo1* reduced the level of mpk3 expression but kept at 100% the percentage of embryos expressing this marker (n=12). While, Co-injection of 50ng/μl of *β-cat2* mRNA and 0.5 mM MOB*pygo2* into *ich* embryos partially blocked the induction of *mpk3* by injection of *β-cat2* RNA such that ~30% of the embryos expressed very low levels of *mpk3* (Figure 9C,D) (n=13). When both the antisense oligonucleotides MO*pygo1*+ MO*pygo2* were injected along with *β-cat2* mRNA the β-Cat2-dependent *mkp3* transcription activation was fully inhibited (n= 7) (Figure 9E). These results strongly indicate that Pygo proteins are required for c-Wnt/β-Cat2 signaling during zebrafish early blastula stage.

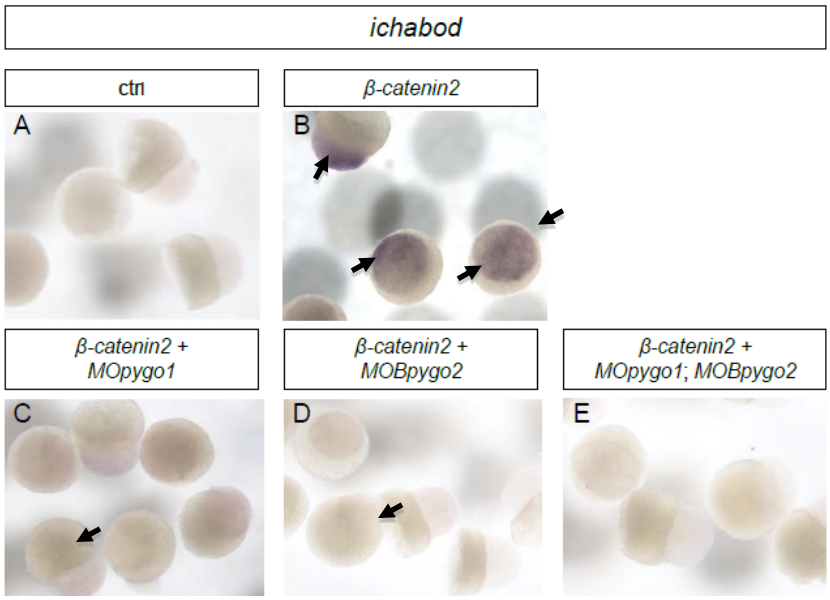

Figure 9.A. *Ichabod* embryos lack of *mkp3* RNA. B. The injection of *β-catenin* RNA rescues the *mkp3* expression. C,D. Injections of MO*pygo1* or MOB*pygo2* alone have a partial repressing effect on mkp3 expression. E. Co-injection of MO*pygo1* and MOB*pygo2* completely repress β-Cat-induced expression of *mkp3* in the prospective dorsal side of high stage embryos.

6.4 Effects of loss of function of the Pygopus family of nuclear factors at late blastula stage

Injections of morpholinos against *pygo2* produced a large number of dead embryos by the end of gastrulation. This early embryo mortality could be either due to the toxicity of the morpholinos or the strong changes they induced upon injection. Therefore in order to unambiguously determine the role of this factor during zebrafish blastula stage we next examined the expression patterns in *pygo2* morphants of key factors of dorso/ventral patterning, which are acting during early gastrulation.

The morpholinos were injected into 2-8 cell-stage *Wt* embryos and the resulting phenotypes were analyzed by in situ hybridization at 30% epiboly. Derived embryos were grouped into classes, depending on the extension of generated variations in the expression and localization of the markers considered (Table5). Since embryos with radialized expression of *chd* or *gsc* could also present down-regulated of these markers, the total percentage of embryos with radicalized or down-regulated expression may result more than 100%. This result is not surprising since we have shown that Pygo2 is involved in the initial establishing of the dorsal side of the embryo at early blastula stage. The dorsal side of the embryo later will express *chd* and then *gsc*. Thus, embryos depleted of Pygo2 may present a reduced *chd* expression, but the later inhibition of *vox* and *vent* in *pygo2* morphants determine also the radialization of *chd* expression fostered by FGF signaling (Figure 2 and 11).

The injection of MO*pygo1* causes only an increase of *chordin* (compare Figure 10A and 10E) with no significant alterations for *goosecoind* and slight reduction of *vox/vent* gene expression (Figure 10B-D,F-H) (Table5). In contrast, *pygo2* knock-down leads to reduced expression of *vox* and *vent* (Figure 10 L,N*),* 80% of injected embryos show a reduced *vox* expression (Table5). Moreover, both *chordin* and *goosecoid* transcripts result to be radialized along the equatorial margins of the embryo, respectively in 31.2% and 42.9% of injected embryos (Table5) (Figure 10 I,M). As expected the double injection of both morpholinos triggers the radialization of the dorsal factors *chordin,* 66.7%, *and goosecoid* with reduction of ventral inductors *vox/vent* expression, in 80% and 100% of embryos analyzed (Figure 10 O-R) (Table5).

These numbers are consistent with the percentage of *ciuffo* phenotype seen in our phenotype analysis of 24hpf embryos (Fig 7AD', AE' and C).

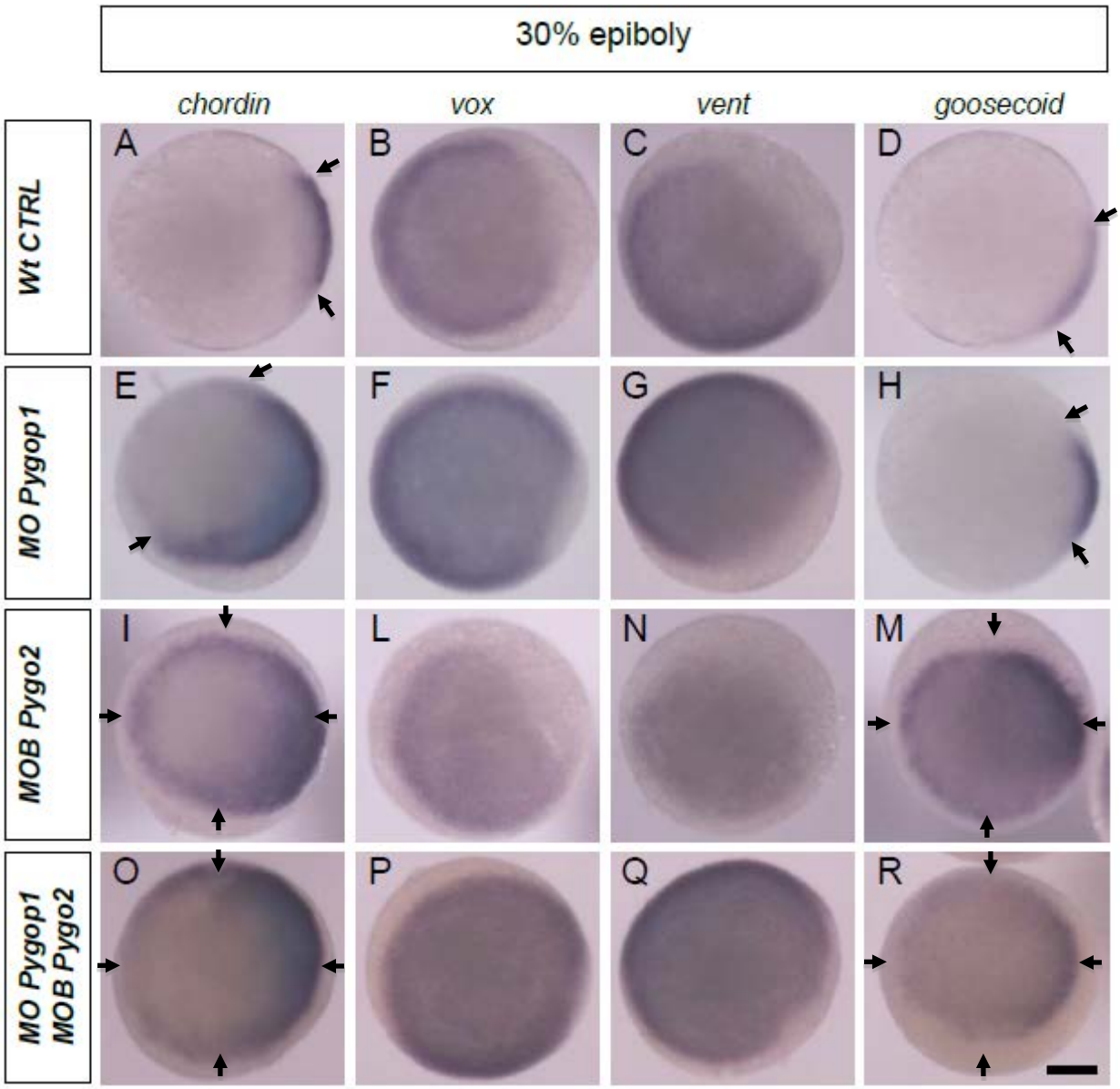

Figure10. A-D Localization of *chordin*, *vox*, *vent*, and *goosecoid* in Wt embryos at blastula stage. E-H Injection of MO*pygo1* determined an expansion of *chordin* expression in the ventro-lateral region of 30% epiboly embryos but not relevant change in other genes expression. I-M Injection of MOB*pygo2* determined radialization of *chordin* expression, while *vox* and *vent* expression in the margins are reduced. Similar results are obtained by the double injection of MO*pygo1* and MOB*pygo2*.The size bar is 10µm.

Table5. Expression of dorso-ventral markers and their localization are affected in embryos injected with MO*pygo1*, MOB*pygo2*, MO*pygo1*+MOB*pygo2*.

Injection MO*pygo1*

	Injected				CTRL
	Wt	**UP-REGULATED**	**DOWN-REGULATED**	**Radialized**	***Wt***
chd	42.8% (n=14)	0%	7.2%	50.0%	100% (n=18)
vox	57.1% (n=14)	0%	42.9%	0%	100% (n=18)
vent	0% (n=9)	0%	100.0%	0%	100% (n=11)
gsc	50.0% (n=14)	0%	50.0%	0%	100% (n=19)

Injection MOBpygo2

	injected				CTRL
	Wt	**UP-REGULATED**	**DOWN-REGULATED**	**Radialized**	***Wt***
chd	12.5% (n=16)	56.3%	0%	31.2%	100% (n=20)
vox	0% (n= 14)	0%	100%	0%	100% (n=21)
vent	0%(n= 15)	0%	0%	0%	100% (n= 21)
gsc	21.4% (n=14)	0	50%	42.9%	100% (n=18)

Injection MOpygo1+MOBpygo2

	Injected				CTRL
	Wt	**UP-REGULATED**	**DOWN-REGULATED**	**Radialized**	***Wt***
chd	0%(n=12)	0%	33.5%	66.7%	100% (n=18)
vox	20.0% (n=15)	0%	80.0%	0%	100% (n=24)
vent	0%(n=12)	0%	100.0%	0%	100% (n=23)
gsc	33.3% (n=15)	26.7%	40.0%	0%	100% (n=18)

7.Discussion

c-Wnt/βCat is a relevant pathway in cell physiology, embryo development and tissues homeostasis (Clevers and Nusse 2013). Pygo is one of the components of this pathway that has been shown to be crucial in Drosophila. *Xenopus* and mouse late embryonic development also require in some but not all cases Pygo downstream c-Wnt/ βCat signaling (Parker et al, 2002; Thompson et al., 2002; Lake at al., 2003). In this study we were interested in analyzing in zebrafish both expression patter and early embryology roles of homologous transcriptional factors Pygo1, Pygo2. Pygo in Drosophila and orthologous *xpygo*-2α and *xpygo-2β* in *Xenopus* are expressed during pre-blastula and post MBT stage supporting a maternal origin. Is not clear if *Pygo* is expressed maternally in mouse. Our in situ hybridization shown an ubiquitous expression patter for *pygo1* and *pygo2* from 4-8 cells stage to gastrula stage in zebrafish indicating that both *pygopus* are maternally accumulated in the zebrafish embryo and with *pygo2* more abundant then *pygo1* (Figure 5).

Previous studies carried out on *Xenopus* found that two putative splicing variants of Pygopus2 are crucial for brain and eyes specification in a β-Cat-depending manner (Lake at al., 2003). Consistently with these results we have shown that overexpression of *pygo1* or *pygo2ΔPHD* results dorsalized phenotype embryos. Moreover, in zebrafish we were able to confirm the phenotypes obtained at 24hpf by analyzing, in morphants at early stages, the expression of genes downstream the c-WNT/β-Cat pathway that regulate the dorsal axis formation (*chordin; gsc*) and ventral specification (*vox/vent*) in late blastula stage. The zebrafish embryo showed respectively expanded *chordin* expression in the margins whereas expressions *vox* and *vent* were reduced (Figure 6).

We have identified a novel ATG present in two different cDNAs for *pygo2* that we found in the databases. The genomic sequence containing the exon coding for the novel ATG is, indeed, not annotated in the same chromosome of *pygo2*. Thus, to determine if this novel ATG is really belonging to *pygo2* and therefore the genomic sequence has been wrongly assembled, we decided to use a morpholino designed against this novel ATG (MOB*pygo2*) and compare the resulting morphants phenotypes with that one obtained by a morpholino specific for the previously known ATG (MOA*pygo2*) (Figure 7A,B, C,D). We determined that the phenotypes obtained with both morpholinos were similar and consistent with a role of Pygo2 downstram c-Wnt/β-Cat pathway. However, the phenotypes obtained with MOB*pygo2* were always stronger than that obtained with MOA*pygo2*, suggesting that the novel ATG region we have identified is indeed belonging to *pygo2* transcripts. It is not yet clear if the other ATG is used at all in *pygo2* transcripts.

The loss of function experiments with two different morpholinos for *pygo2* in *ich* embryos and the double knock-down MO*pygo1*+ MOB*pygo2* in *Wt* embryos support our hypothesis that Pygo1 and Pygo2 cooperate with the c-Wnt/β-Cat pathway during early embryogenesis. In both cases we obtained a distinctive phenotype, *ciuffo* (Figure 7A.D'-E',B). *Ciuffo* phenotype indicates that the downstream genes activation in the c-Wnt pathway is blocked (Figure 2A; Figure 3B). In fact, originally this phenotype was obtained by blocking the expression of *β-cat1* in *ich* and *β-cat1* and *β-cat2* in *Wt* embryos (Figure 3B). Therefore, the induction of *ciuffo* phenotype in *ich* embryos suggests that MOB*pygo2* alone is sufficient to mediate c-Wnt/β-Catenin signaling in the ventro-lateral margins of late blastula embryos. While the requirement of MO*pygo1* and MOB*pygo2* in *Wt* embryos to obtain the same *ciuffo* phenotype indicates that both Pygopus are necessary for additional events controlled by c-Wnt/β-Cat signaling in the early zebrafish blastula.

To confirm the initial phenotype analysis at 24hpf we analyzed the expression pattern of *chordin, vox*, *vent* and *goosecoid* in loss of function experiments. The expression of these genes resulted consistent with the phenotype analysis at 24hpf and with results shown in previous studies (Bellipanni et al., 2006; Varga et al., 2007) schematized in Figure 2B.

Morpholino experiments require a specific control that is the rescue of the morphant by co-injection of the morpholino with its target mRNA. However, rescue experiments did not provided for us the expected results. These experiments are particularly difficult because as mentioned Pygo works in complexes stoichiometrically tightly controlled and either an increase or decrease of this molecule negatively affects the pathway. We have shown that this could be the case also in the zebrafish because overexpression of *pygo1* mRNA and *pygo1* Knock-down result into similar dorsalized phenotypes (Figure 8).

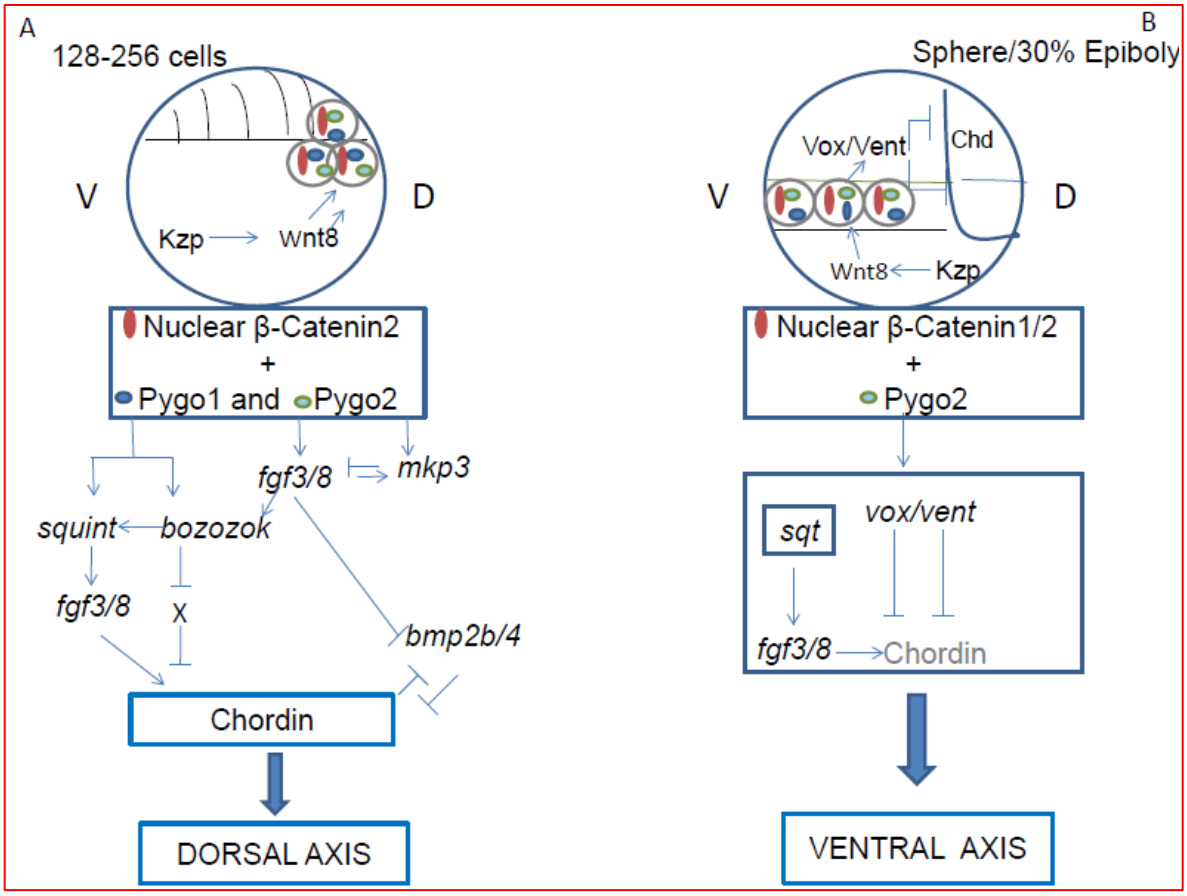

Figure 11. **Pygo1 and Pygo2 role in zebrafish embryos dorsovental specification.** Schematic of events consistent with our experimental data. Transcriptional factors Pygo1 and Pygo2 could mediate dorsal and ventral axis formation interacting with β-Catenin by BCL9 during 128-256 cells stage and sphere in zebrafish. (A) At blastula stage Pygo1 and Pygo2 activate cooperating with β-Catenin2 the transcription of c-Wnt downstream genes and indirectly regulate *chordin* expression. (B) At 30% di Epiboly, β-Cat is nuclear localized in the medio-ventral area and its interaction preferably with Pygo2 leads to the activation of ventralizing genes *vox* and *vent* that inhibit *chordin* expression restricting its patterning to the dorsal zone.

To obtain the *ciuffo* phenotype it is necessary to inhibit both the embryological events controlled by c-Wnt/β-Cat as reviewed in Figure 2 and Figure 11. We have demonstrated that Pygo1 and Pygo2 are also involved in mediating the first wave of activity of c-Wnt/β-Cat pathway (Figure 11A). In fact, MO*pygo1* and MO*pygo2* co-injection can stop the rescued expression of *mkp3* in *β-cat2* mRNA injected *ich* embryos (Figure 9).

Taken together our study indicates that Pygo1 and Pygo2 cooperate in enhancing c-Wnt/β-Cat pathway in early embryo development (Figure 11A,B). This is, to our knowledge, the first report in chordates showing a direct link of Pygopus with c-Wnt/β-Cat pathway during the early steps of dorso-ventral specification of the blastula embryo.

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