Università degli Studi di Palermo

Facoltà di Medicina e Chirurgia

Dottorato di Ricerca in Oncopatologia cellulare e Molecolare

(SSD Med 06)

XXIII Ciclo

Coordinatore : ***Prof.***

Study on β-Catenins *mechanisms of regulation in zebrafish blastula embryo*

PhD Thesis by:

Dr. Fabio Valenti

Tutor: ***Prof. Antonio Russo, MD***

Co-Tutors: ***Prof. Antonio Giordano, MD***

Prof. Gianfranco Bellipanni, PhD

Course Coordinator

Prof. E. Fiorentino

A.A. 2009 – 2011

Abstract

Background: *β-catenin* is a central component of the cadherin cell adhesion complex but also it plays an essential role in the canonical-*Wingless*/*Wnt* signaling pathway.
In vertebrates, one of the initial steps for the establishment of the correct dorso-ventral (D/V) pattern in the embryo is the cytoplasmic accumulation followed by nuclear localization of *β-catenin* in the cells of the prospective dorsal side of the embryo. In zebrafish there are two *β-catenins*, 92,7% identical. The mutant fish line *Ichabod (ich)*, with a mutation in the region of the β-catenin2 promoter that causes a decrease in the maternal accumulation of *β-catenin2* protein in the embryos, fail to nuclear localize *β-catenins* and to form a dorsal organizer, so the embryos become ventralized.

Aims: Taking advantage of the zebrafish model and in particular of this fish line, we investigated the regulation of *β-catenins* nucleus-cytoplasm translocation and in particular why in *ich* β-catenin1 cannot compensate for the loss of *β-catenin2*.

Materials and Methods: We analyzed by real-time PCR the levels of six genes involved in the canonical Wnt pathway: *axin1* and *axin2*, *pygopus1* and *pygopus2*, *bcl9* and *bcl9-2*.

Results: Unexpectedly, they are all up-regulated in *ich* embryos before and after mid-blastula-transition (MBT). Thus, *ich* embryos may have an overactive destruction complex, resulting in an increased degradation of β-*catenin1*. This is consistent with our finding that microinjection of a dominant negative *Axin2* (it destroys the degradation complex) in *ich* embryo partially rescue *ich* phenotype.

Conclusions:
Our results confirm *in vivo*, previous *in vitro* work showing that the two zebrafish β-catenins C-terminal domain are important for the stability of the protein, probably because shielding it from the β-catenin destruction complex. This, results in higher stability of β-Catenin2 than β-Catenin1. These data are the first *in vivo* indication that differences in the β-catenins CTD result in different stability of these proteins

Introduction

Wnt molecules

Wnt genes are defined by sequence homology to the original members Wnt-1 in the mouse (first called int-1; *Nusse and Varmus 1982;Van Ooyen and Nusse 1984*) and wingless (wg) in Drosophila (*Cabrera et al.1987; Rijsewijk et al. 1987*). They encode secreted glycoproteins, usually 350–400 aminoacids in length. Homologous genes have been found in increasing numbers in organisms ranging from mammals to the nematode *C. elegans* (Table 1). Wnt signaling is present in all phyla of the animal kingdom. The sequence identity in Wnt proteins is minimally 18%, including a conserved pattern of 23–24 cysteine residues, in addition to other invariant aminoacids (*Cardigan et al, 1997*).

Wnt signaling controls cell proliferation, stem cell maintenance and cell fate decisions, as well as organized cell movements and the establishment of tissue polarity. It is also frequently deregulated in human cancers and has been implicated in degenerative diseases (*Nusse, 2005; Carlson et al, 2008; Moon et al, 2004; Zhu et al, 2009*).

Wnt genes in Vertebrates					
gene	Mouse	Human	Xenopus	Chicken	Zebrafish
Wnt1	♦	♦	♦	♦	♦
Wnt2	♦	♦	♦		♦
Wnt2B	♦	♦	♦	♦	
Wnt3	♦	♦	♦		♦
Wnt3A	♦	♦	♦	♦	
Wnt4	♦	♦	♦	♦	♦
Wnt4B					♦
Wnt5A	♦	♦	♦	♦	♦
Wnt5B	♦	♦	♦		
Wnt6	♦	♦	♦		
Wnt7A	♦	♦	♦	♦	♦
Wnt7B	♦	♦	♦	♦	
Wnt7C			♦		

Wnt genes in Vertebrates					
gene	Mouse	Human	Xenopus	Chicken	Zebrafish
Wnt8A	♦	♦	♦	♦	♦
Wnt8B	♦	♦	♦	♦	♦
Wnt9A	♦	♦		♦	
Wnt9B	♦	♦			
Wnt10A	♦	♦	♦		♦
Wnt10B	♦	♦			♦
Wnt11	♦	♦	♦	♦	♦
Wnt-16	♦	♦			

Table.1 Wnt genes in vertebrates (Wnt homepage).

Wnt molecules are secreted in the extracellular space, where they can reach surrounding cells creating a gradient. In the membrane of the cells, wnt molecules bind receptors that transmit a signal inside of the cell. There are three different pathways activated by wnt (Fig. 1): Canonical, Planar Cell Polarity and *Wnt*/Ca^{2+} pathway. The canonical pathway activates a cascade of events leading to the activation of transcription of different genes.

The other two pathways are called non-canonical pathways and are involved in cellular adhesion, motility and cytoskeletal changes through small GTPase and heterotrimeric G proteins (*Onizuka et al, 2011; Qui et al, 2011; De A., 2011; Lamonica et al, 2012; Pryor et al, 2012*).

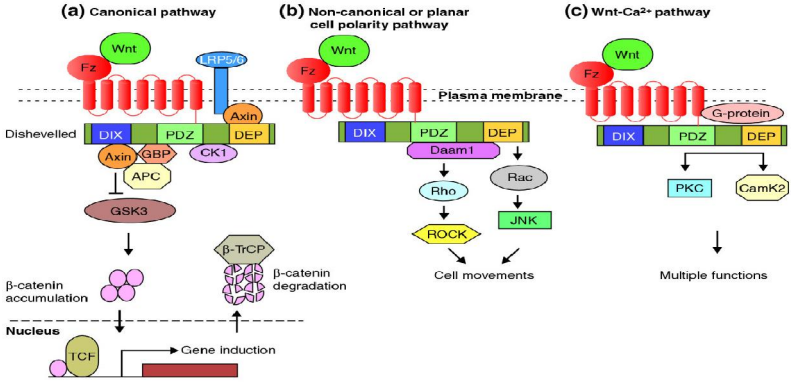


Fig.1 The three different Wnt pathways (*Mosimann C. et al, 2009)*

Wnt Canonical pathway

Wnt canonical pathway start in the membrane, where wnt molecules binds and activate the frizzled seven-pass transmembrane class of receptor and the low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6) receptor (*Pinson et al, 2000*; *Tolwinski et al, 2003; Clevers, 2006*).
Outside the cell, wnt signaling could be antagonized by wnt inhibitors, like *Wnt* Inhibitory Factor (WIFs), *Dickkopf* (Dkk) and secreted Frizzled-related proteins (sFRP) (*Bafico et al, 1999; Bafico et al, 2001; Pannone et al, 2010*).
Dishevelled (DSH) is a key component of the membrane-associated Wnt receptor complex that is assembled in the citosplasmic side of the membrane, near the frizzled receptor. Close to this first complex is assembled a second complex, called *Wnt* destruction complex (Fig. 2) .

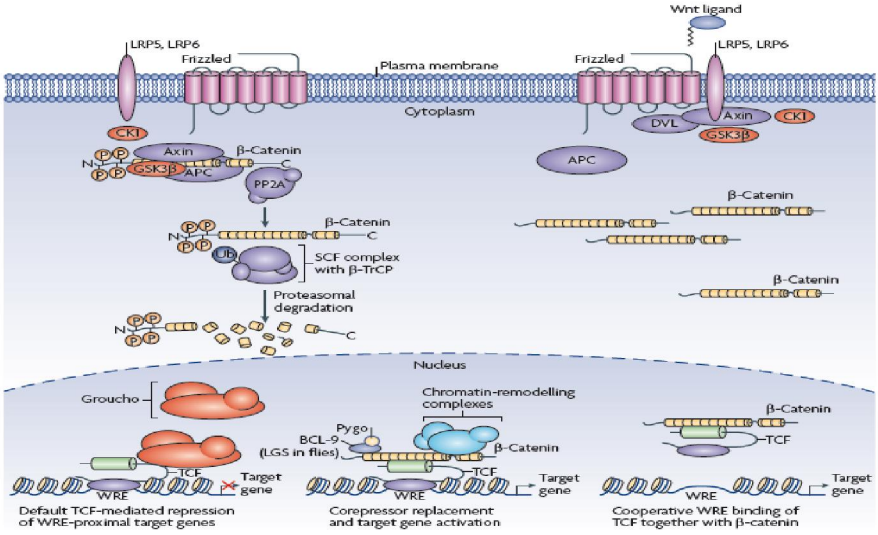


Fig.2 Wnt canonical pathway (Mosimann C. et al, 2009)

Members of this complex are: *Axin*, *Casein kinase 1* (CK1), *glycogen syntetase kinase 3 alpha and beta* (GSK-3α/β), *Adenomatus polyposis coli* (APC).
This complex binds and phosphorilate *β-catenin* in some conserved aminoacids of the protein's amino terminus, this mechanism will be thoroughly discussed later in this introduction. The phosphorilated *β-catenin* could be then recognized by *beta-TrCP* component of the ubiquitin ligase complex, polyubiquitinated and sent to proteasome-mediated degradation (*Aberle et al, 1997; Hart et al, 1999; Liu et al, 1999; Kitagawa et al, 1999*).

During wnt signaling, the membrane-associated Wnt receptor complex activate Dsh protein, which in turn bind and disassemble the *Wnt* destruction complex. So, *β-catenin* is not recognized from *beta-TrCP* and could accumulate in the cytoplasm.

As a result of its cytoplasmic accumulation, *β-catenin* is able to go inside of the nucleus of the cell apparently through non-mediated transport (*Fagotto et al, 1998*), where interact with the *T cell factor* (TCF)*/ lymphoid enhancer factor* (LEF) family of transcription factors to promote specific gene expression. In the absence of a Wnt signal *TCF/LEF* family members interact with transcriptional inhibitors such as Groucho (*Daniels et al, 2005*), which serve to repress *Wnt* signaling. The repressing effect of *Groucho* is mediated by interactions with Histone DeACetylases (HDAC) which are thought to make DNA refractory to transcriptional activation (*Arce et al, 2009*).

β-catenin

β-Catenin belongs to the armadillo family of proteins, which are characterized by a central domain consisting of a repeating 42 amino acid motif termed the “arm repeat.” These repeats were originally identified in the *Drosophila* segment polarity gene product and β-catenin orthologue *Armadillo* (*Riggleman B et al, 1989*). The *β-catenin* protein was initially discovered for its role in cell adhesion (*Morin PJ, 1999*). As a component of adherens junctions, it promotes cell adhesion by binding to the intracellular domain of the transmembrane protein *cadherin*, a Ca2+-dependent homotypic adhesion molecule, and linking *cadherin* to the actin cytoskeleton through the adaptor protein α-*catenin* (Fig. 3).

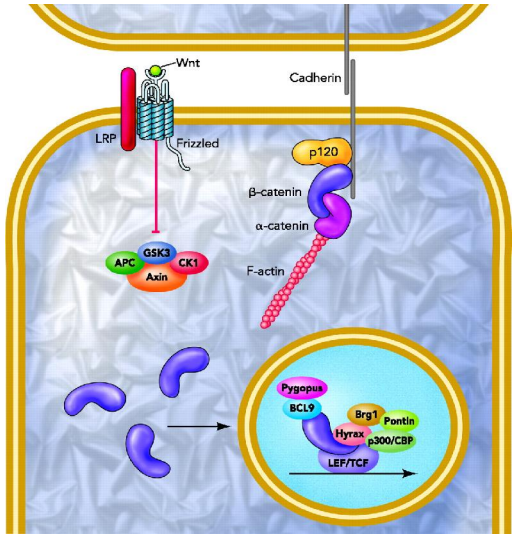


Fig.3 *β-catenin* in wnt canonical pathway Daugherty et al, 2007

This adhesion function is based on a subcellular pool of *β-catenin* that is membrane-associated and stable. Membrane associate *β-catenin* is phosphorylated in specific residues, and this phosphorilation is responsible for it localization.

As discussed previously, *β-catenin* is also a key component of the canonical Wnt pathway. It could be phosphorylated in different aminoacids, and these different phosphorylation are responsible for the decision of *β-catenin* adhesion or signaling role, other that for the stability of the protein itself.

β-catenin stability and localization

β-catenin stability is regulated by the interaction with different partner, which in turn affect the phosphorylation status of *β-catenin*. The kinases responsible for *β-catenin* phosphorylation are *Casein kinase 1* and 2 (CK1 and CK2), *glycogen syntetase kinase 3 alpha and beta* (*GSK-3α/β*), *src* and *EGFR* family kinases.

CK1 family members (including α, δ, and ε) phosphorylate *β-catenin* at serine 45. This priming phosphorylation is required for subsequent phosphorylations by *GSK3* at residues 41, 37, and 33 (*Verheyen at al, 2010*). The *β-catenin* that is phosphorylated at residues 37 and 33 is ultimately recognized by the *β-TrCP* E3 ubiquitin–ligase complex, ubiquitinylated, and rapidly degraded by the 26S proteasome (*Hart et al., 1999*).

During Wnt signaling, the phosphorylated *LRP6* co-receptor could directly inhibits *β-catenin* phosphorylation by *GSK3* at S33, S37, and T41, preventing its interaction with the *β-TrCP* E3 ubiquitin–ligase complex and so the degradation.

Phosphorylation of S675 by *PKA* may enhance *β-catenin* transcriptional activity by promoting *β-catenin* stability (*Hino et al., 2005*) and association with Creb Binding Protein (*CBP*) (*Taurin et al., 2006*).

Phosphorylation of *β-catenin* at S552 by AKT has also been found to enhance *β-catenin* protein levels and nuclear signaling by standard reporter assays (*Tian et al., 2004; Fang et al., 2007*), although the precise mechanism remains unclear. Lastly, serines 191 and 605 were recently identified as *Rac*-activated *JNK2* sites, and mutations of these residues appears to reduce the nuclear accumulation of *β-catenin* in a murine bone marrow-derived stromal cell line, ST2 (*Wu et al., 2008*).

β-catenin role is strictly dependent from its localization. Membrane localization is necessary for its adhesive function; cytoplasm localization lead to its rapid degradation; the signaling role needs nuclear localization.

Without *Wnt* signaling, *β-catenin* is phosphorylated and then degraded by the “destruction complex”, resulting in a low level of cytoplasmic β-catenin.

Instead in presence of *Wnt* signaling, there is an accumulation of cytoplasmic *β-catenin*, that can move to the nucleus and act as a transcription factor.

A lot of factors are moved to the nucleus using an importing pathway: they possess an NLS (Nuclear Localization Signal) (comprising one or two

clusters of basic amino acids), and these sequences are recognized by soluble receptors. These receptors, generically called “importins/exportins” or “karyopherins,” interact directly with the Nuclear Pore complex (NPC) and shuttle between the cytoplasm and the nucleus. The export work in a similar manner. There are Nuclear Export Signals (NES) recognized by exportins receptors that, interacting with the NPC, provides the nuclear export of the factor. These are energy-dependent process involving the small soluble GTPase *Ran*.

β-catenin protein share a strong sequence similarity with importins, they contain similar periodic 42 amino acid repeats, called arm/HEAT repeats. The arm repeats are necessary and sufficient for β-catenin nuclear localization (*Funayama N. et al, 1995*). *β-catenin* have no NLS and it was demonstrated that can move to the nucleus in an importins independent manner, interacting directly with the NPC (*Fagotto et al, 1998*).

The current model is that *β-catenin* is maintained in the nucleus by retention by interaction with many factors that block *β-catenin* in the nucleus where it act as a transcription factor. Several reports (*Sustmann et al, 2007; Kennedy et al, 2010; Nakamura et al, 2007*) show that *β-catenin* can interact with a factors, *BCL9/Legless*, and this in turn with *Pygopus*. *Pygopus* is nuclear localized, instead *BCL9/Legless* can shuttle between cytoplasm and nucleus, and both these factors have NLS. *β-catenin* remain in the nucleus for a small time, blocked by this factors and then is exported to the cytoplasm where could be degraded (*Takemaru et al, 2009; Neufeld, 2009*). Others have shown how *β-catenin* in the nucleus interact also with a lot of factors like *chibby/14-3-3* (*Feng-Quian Li et al, 2010*), *APC* (*Henderson, 2000*), *axins*, that can be responsible for the nuclear export of *β-catenin*.

Zebrafish as a model

The zebrafish, *Danio rerio*, is a small tropical fish of the *Cyprinidae* family, native to the streams of South-eastern Himalayan region (India, Pakistan, Bangladesh) (Mayden et al., 2007).

There are several important features of zebrafish making it an ideal experimental animal. Differences in the appearance between male and female make zebrafish easily distinguishable. In the laboratory, a couple of zebrafish can produce up to 400 embryos per spawning, throughout the whole year depending on the level of maturity.

Zebrafish eggs are transparent and relative large (~0.7 mm in diameter) compared to other teleost of a similar size. Embryogenesis is rapid and all major organs develop within 24 hours. The generation time is also relatively short requiring 3-4 months.

The zebrafish has become one of the most important model organisms for vertebrate developmental biology, genetic, neurology and cancer, used at the beginning from George Streisinger at the University of Oregon, in 1981.

In the last years the resources available for this system are increasing with the number of laboratories that work with it (Table 2).

Year	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Genes													
Genes	220	608	974	1384	1715	12205	16785	20332	23049	27490	28948	30796	31135
Genes on Assembly					0	94	2130	3277	5664	10454	10422	15344	15935
Transcripts								0	8116	16457	22524	25913	26166
EST/cDNAs	0	97	5845	9169	10493	11847	19233	23847	26494	28603	34286	34865	34865
Full length cDNA clones (ZGC)						0	6057	8348	9626	11784	16808	17192	17191
Genetics													
Features	1896	1951	2018	2088	2278	2343	2828	3217	4485	5007	5674	6111	6730
Transgenic Features	3	3	6	12	122	137	512	613	694	985	1460	1662	2046
Transgenic Constructs									0	320	458	522	652
Transgenic Genotypes									0	1276	2355	2821	3630
Genotypes									0	5645	7512	8416	10048
Genes with GO annotations						0	4783	10421	12173	13581	14925	15076	15450
Genes with IEA GO annotations						0	4530	6753	7565	9600	11001	11250	11562
Genes with Non-IEA GO annotations						0	684	6490	7688	8060	8584	8637	8819
Total GO annotations						0	20741	46981	64576	81470	91078	105672	107969
Reagents													
Morpholinos						0	73	552	1018	1614	2259	2871	3928
Antibodies										0	217	424	660
Expression & Phenotypes													
Gene expression patterns	0	0	0	83	739	1295	6821	14282	21667	29339	38671	46431	52488
Images	1851	1858	1866	2598	8473	16874	21980	38339	53074	60263	69677	75423	78616
Anatomical structures	0	0	0	769	1134	1163	1131	1394	1665	2125	2203	2384	2671
Genomics													
Mapped markers	3138	2942	10509	14891	25483	26858	35578	36679	36983	35465	37708	38019	38139
Links to other databases	0	8621	31589	46522	60417	99118	65886	84701	114943	228689	157123	447176	522249
Community information													
Publications	1913	2415	2856	3517	4246	4986	5789	6739	7797	8860	10143	12441	13730
Researchers	1403	1640	2092	2431	2683	2916	3170	3624	4104	4368	4642	4977	5469
Laboratories	190	228	280	324	329	372	419	441	493	539	583	626	667
Companies	35	41	49	61	45	49	53	58	61	62	93	120	132
Orthology													
Genes w/Human Orthology					0	1051	1640	2987	3905	5264	6657	8025	9882
Genes w/Mouse Orthology					0	819	1163	1480	1722	2312	3519	4528	6655

(Table. 2 http://zfin.org/; Zebrafish Information Network (ZFIN) 2011)

Zebrafish development

Zebrafish are photoperiodic in their breeding, and produce embryos every morning, shortly after sunrise. The fertilization is external, the eggs are protected from a chorion membrane that cover the embryo from the zygotic stage to the hatching stage (third day). The chorion swells and lifts away from the newly fertilized egg. Fertilization also activates cytoplasmic movements, easily evident within about 10 minutes. Non-yolky cytoplasm begins to stream towards the animal pole, segregating the blastodisc from the clearer yolk granule-rich vegetal cytoplasm. The cells start dividing every 30 minutes, the blastula stage is after 2 ¼ hours, the gastrulation start at 5 ¼ h (Fig. 4).

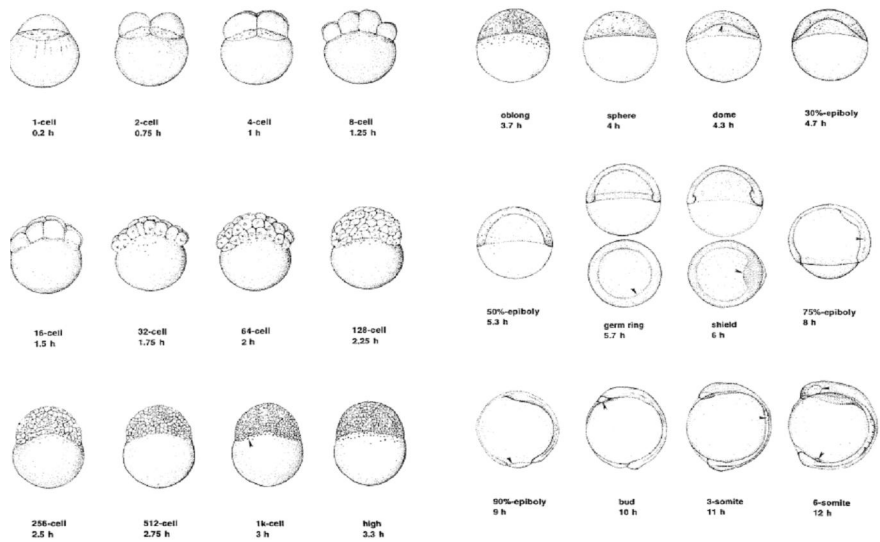


Fig. 4 Zebrafish development (*Kimmel at al, 1995*)

Between 10 and 24h there is the segmentation period in which the somites develop, the rudiments of the primary organs become visible, the tail bud becomes more prominent and the embryo elongates. The AP and DV axes are unambiguous. The first cells differentiate morphologically, and the first body movements appear. Between 24 and 48h the embryo is in the “Pharyngula” period, and between 48 and 72h instead there is the “Hatching” period (Fig. 5), with the embryo that hatch from the corion and start to swim, becoming a larva.

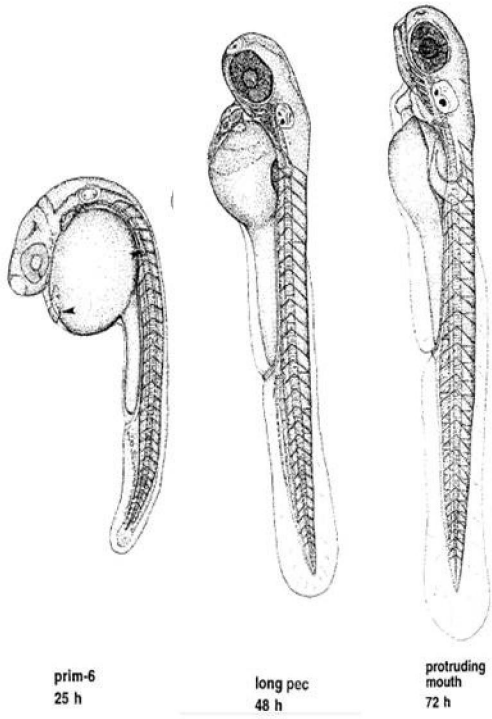


Fig. 5 (*Kimmel at al, 1995*)

A *Wnt* canonical pathway's mutant in zebrafish

The *wnt* canonical pathway is involved in the formation of the major dorsal signaling centers in vertebrate embryos, like Nieuwkoop center and Spemann organizer (*Sokol, 1999; Tao et al., 2005; Schier and Talbot, 2005*) and the posteriorizing and ventralizing signals that derive from more lateral and ventral embryonic regions (*Erter et al., 2001; Lekven et al., 2001*). In zebrafish, this pathway and its component are well conserved.
Zebrafish embryos obtained from females homozygous for the maternal recessive mutation called *ichabod* are ventralized and fail to develop the organizer region.
This mutation was mapped in the region LG19 (*Bellipanni et al, 2006*), in the same region in which was mapped the gene for a second *β-catenin*, called *β-catenin*2 (*Bellipanni et al, 2006; Woods et al., 2005*).
This mutation show variable expressivity and the phenotypes have been classified into four groups (Fig. 6)(*Kelly et al, 2000*).

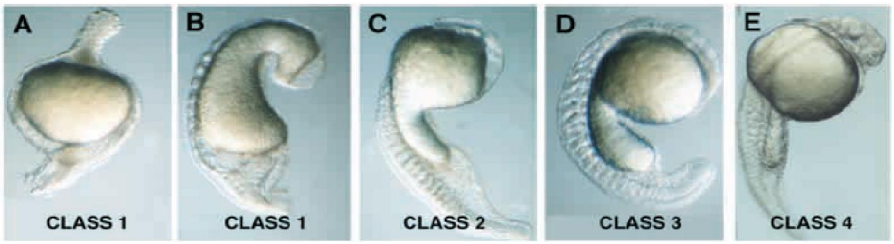


Fig. 6 *ich* phenotypes (*Kelly et al, 2000*)

Different experiments showed a reduction in the total quantity of *β-catenin*2 and the lack of nuclear localization of *β-catenins* into the nuclei of the future dorsal organizer (*Bellipanni et al, 2006*; *Kelly et al, 2000)*. Moreover, injection of mRNAs coding for *Xenopous β-Catenin* or for zebrafish *β-Catenin1 or β-Catenin2*, or injections of mRNAs coding for factors downstream the Wnt/β-Catenin pathway (znr-2, Bozozok) were able to revert the *ich* mutant phenotype. On the other hand, injection of mRNAs coding for factors that control *β-Catenin* stability like z-*Wnt8*; *zFzA*; kinase dead X-DN-*GSK3*, *GBP*; a fragment of *Xenopus Axin* that bind and inhibits GSK3, GID2; a dominant negative form of the F-box/ WD40 repeat that recruits phosphoriyated *β-Catenin* for degradation by the ubiquitination-proteosome pathway, β-Trcp-ΔF, were not able to rescue, even partially, *ich* phenotype (Kelly et al. 2000).

β-Catenins localization in developing *zebrafish*

To determine if both z-*β-Catenins* enter in the nucleus and when this is happening Dr. Bellipanni took advantage of the specific antibody for zb-Catenin1 () and used it in combination with an antibody that recognizes both z-*β-Catenin*s (Sigma). Using the two z-*β-Catenin* antibodies (α-pan-*β-Catenin* FITC coniugated and α-*c-term-β-Catenin* Cy5 coniugated) was done an immunohistochemistry on embryos at three developmental stages, a pre-Mid Blastula Transition (pre-MBT) stage (256 cells) and two post-MBT (High and Sphere) stages. Were used ich embryos at 256 cell stage as a control. The embryos were visualized at a confocal microscope to study the localization of the two z-*β-Catenin* proteins.

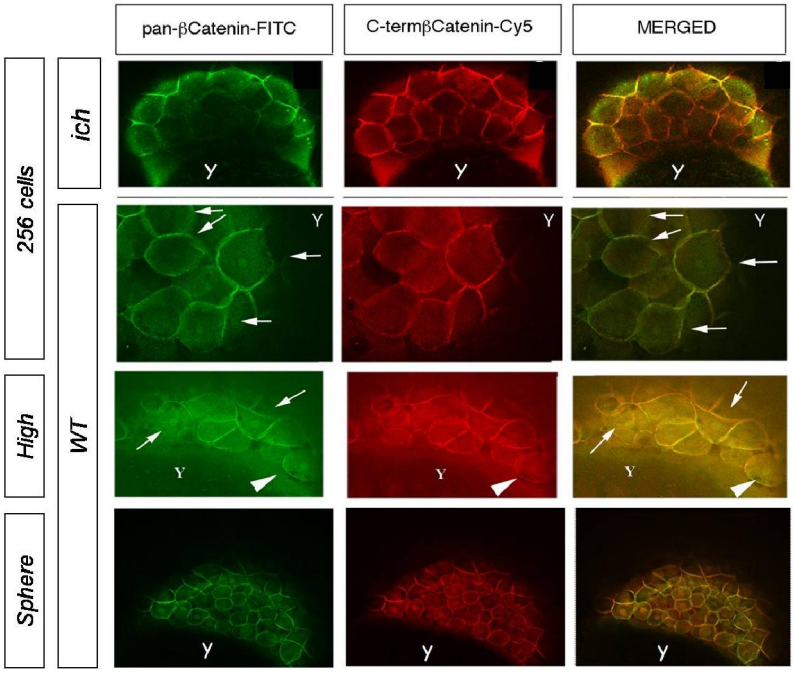


Fig. 7 Immunohistochemistry of *WT* and *ich* embryos at 256 cell stage, *Wt* embryos at high stage, *Wt* embryos at sphere stage. (*Bellipanni and Weimber*, unpublished).

At 256 cell stage, in 55% *Wt* embryos is visible nuclear accumulation of the α-pan-β-Catenin FITC coniugated antibody in more nuclei than the of α-c-term-β-Catenin Cy5 coniugated antibody. Both z-*β-Catenins* nuclear localize in the prospective dorsal side of the 256 cell stage embryo, but also that z-*β-Catenin*2 seem to preceeds z-*β-Catenin*1 nuclear localization. In *ich*

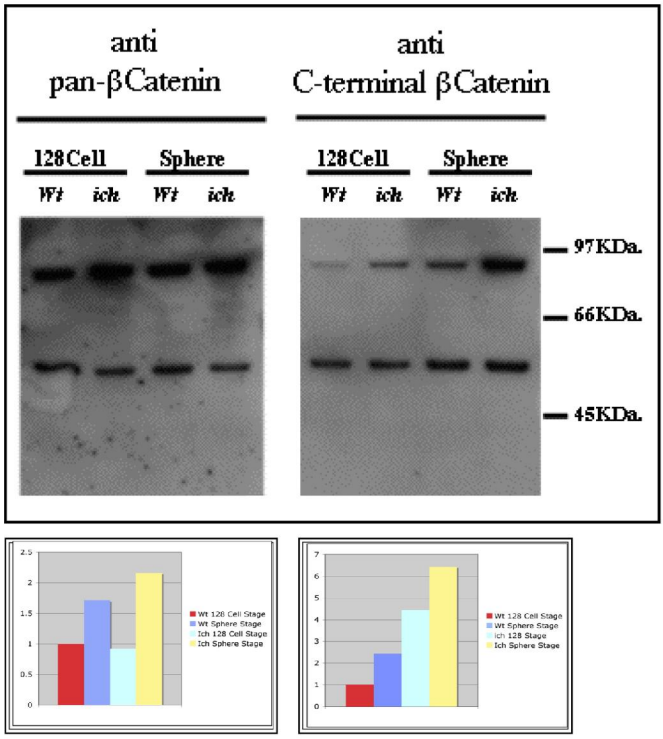
embryos, at the same stage, as expected, there is no nuclear localization at any z-*β-Catenin*s. In later developmental (High and Sphere) stages in Wt embryos, the number of cells positive for both nuclear localized z-*β-Catenin* increase.

These data clearly show that both z-*β-Catenin*s enter in the nucleous at these early embryonic stages and that z-*β-Catenin*2 might be the first protein to nuclear localize in the cells of the future dorsal side but that in later stages both *z-β-Catenins* or only z-*β-Catenin*1 is nuclear localized. These results need to be confirmed by more direct analysis of the z-*β-Catenin*2 localization by the use of not yet available specific antibody for z-*β-Catenin*2, nevertheless they suggest that z-*β-Catenin*1 nuclear localization mechanism may require first the nuclear localization of z-*β-Catenin*2.

Quantification of the total z-*β-Catenin* pool and z-*β-Catenin*1 pool in *wild-type* and *ich* embryos at 128 cell stage and High stage. was conduct via Western blot analysis. When the α-pan-*β-Catenin* antibody was used, it revealed that the total pool of z-*β-Catenin*s in *ich* embryos is not lower of that in *Wt* embryos. Further analysis using the antibody specific for z-*β-Catenin*1 showed that *z-β-Catenin*1 is much more abundant in *ich* embryos than in the *Wt*.

An immunohistochemistry with the only α-c-term-*β-catenin* antibody on 256 cell stage *Wt* and *ich* embryos shows how in *ich* there is a bigger quantity of z-*β-Catenin*1 respect to *Wt*, but z-*β-Catenin*1 localize in the membrane. These analysis foster us to further describe the molecular landscape of the *ich* embryos during early embryogenesis respect the Wt molecular landscape to understand how regulation of z-*β-Catenin*s localization in *ich* embryos is impaired.

A



B

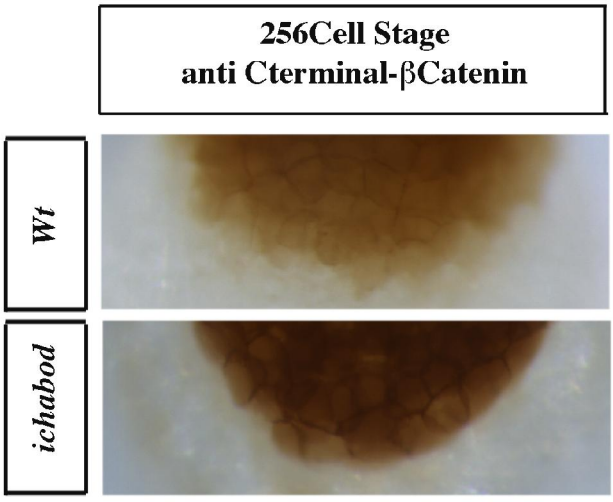


Fig. 8 A: Western blotting using α-c-term-*β-Catenin* antibody and pan β-Catenin antibody in *Wt* and *ich* embryos at 256 cell stage; B: *ich* embryos at 256 cell stage. (*Bellipanni and Weimber*g, unpublished).

Pygopus and *BCL9/Legless*

Studying the wnt pathway in *Drosophila*, were identified two genes, called *Legless*, homologus to the human *BCL9*, and *Pygopus* (*Kramps et al, 2002*), required for the proper transmission of the wingless signal to the nucleus. Later work founds homologous of this genes in different organism, from mouse to *Xenopus*, included *Zebrafish*.

Pygopus is a protein containing a PHD domain in the c-terminal domain, while *BCL9/Legless* contain multiple repetition of Homology domains (HD). Of this domains, the HD1 mediate the bind of Pygopus, the HD2 the bind of *β-catenin*.

Both *Pygopus* and *BCL9* contain NLS, and seem that *Pygopus* is a nuclear protein (*Belenkaya et al,2002*) whereas *BCL9* seem shuttling between nucleus and cytoplasm, so it seems that *BCL9* have the role of adaptor between *β-catenin* and *Pygopus*, and that *Pygopus* is required to anchor *β-catenin* to the nucleus.

There are different models about the functions of *Pygopus* and *BCL9*.

One model is that Pygopus and BCL9 in the nucleus bind and anchor β-catenin in the nucleus, where it can act as transcription factor. Another model (*Carrera et al, 2008*) explain that nuclear *β-catenin* can bind *Pygopus* and this last one, binding the mediator complex, can activate the transcription of the wnt target genes.

Other studies found two proteins, *BCL9*-2 and *Pygopus2*, homologues respectively of *BCL9* and *Pygopus*. The switch between the adhesive and the transcriptional function of *β-catenin* depend on the phosphorilation of Tyr-142, and a work of 2010 show that this phosphorylated *β-catenin* favors *BCL9-2* binding, precluding the α-catenin binding (*Brembeck et al, 2010*).

Another work show that *Pygopus2* can bind the methylated tails of histone H3, and that this function require the binding of *BCL9/BCL9-2* (*Miller et al, 2010*). A model is that Pygopus and *BCL9* can bind the methylated tails of Histones, and open the chromatin in the Wnt target genes (*Mosimann, 2009*).

Other studies showed that *Pygopus* can work as an anti-repressor in facilitating *Wnt*-dependent transcription (*Mieszczanek et al, 2008*).

Aims of the thesis

In *Zebrafish* there are two *β-catenins*, 92,7% identical. The mutant fish line *Ichabod (ich)*, with a mutation in the region of the *β-catenin*2 promoter that causes a decrease in the maternal accumulation of *β-catenin2* protein in the embryos, fail to nuclear localize *β-catenin2* and to form a dorsal organizer, so the embryos become ventralized. Preliminary results of our laboratory have shown that both zebrafish *β-Catenin* are nuclear localized in the early zebrafish embryo (~256 cell stage), while in *ich* embryo both *β-Catenins* fail to nuclear localize. Moreover, looking at the protein levels in *Wt* and *ich* embryos at 128-256 cell stage and High stage we have indications that while *β-Catenin2* levels in *ich* are reduced due to the maternal mutation, the levels of *β-Catenin1* are increased. The increase *β-Catenin1* level results in a total *β-Catenins* protein level in *ich* slightly higher than in *Wt* embryos (fig.8A), however it appears to be enriched only at the membrane (fig.8B), suggesting that the cytoplasmic pool of β-Catenin is still under a strong control by the signalosome complex..

Taking advantage of the zebrafish model and in particular of this fish line, we investigated the regulation of *β-catenins* nucleus-cytoplasm translocation and in particular why in *ich β-catenin*1 cannot compensate for the loss of *β-catenin*2. In particular we investigated the role of factors involved in the nuclear localization and in the regulation of *β-catenins* stability.

Materials and methods

Zebrafish strains

Wt and *ichabod* (*ich*) fish strains were used during this work.

They were maintained in a closed system at 28°C, following standard husbandry procedures (*Westerfield M., 2000*).

Wt were selected as “wild type” because their delayed onset of pigmentation.

ichabod embryos were obtained by breeding homozygous *ichabod* females with heterozygous males. For this study were used only ichabod embryos obtained from homozygous females that reproducibly gave severely ventralized embryos.

Synthetic mRNA in vitro transcription

mRNA to be used for microinjection on zebrafish embryos was produced using as template plasmid DNA digested on the opposite side of the required polymerase promoter, present in the plasmid before the gene of interest (table 3)

The DNA was digested and then purified by phenol/chloroform purification, and precipitated with ammonium acetate and 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 spectofotometer and then stored at -80°C.

Plamid name	Restriction Enzyme used	Polymerase used
pCS2 *zβcatenin1+myc*	NotI	SP6
pCS2 *zβcatenin2+myc*	NotI	SP6
pCS2+2xFlag *zpygopus1*	NotI	SP6
pCS2+2xFlag *zpygopus2*ΔPHD	NotI	SP6
pCS2+2xFlag *zaxin1*	NotI	SP6
pCS2+2xFlag *zaxin2*	NotI	SP6
pCS2+2xFlag *DNzaxin2*	NotI	SP6

Table.3 Constructs used for mRNA synthesis.

Microinjections on zebrafish embryos

Microinjection in zebrafish embryos were conducted at 1-2 cell stage or at 4-16 cell stage in one blastomere. The mRNA for microinjection were mixed with an injection solutions prepared by adjusting the RNA concentration to twice that desired and then adding an equal volume of Dulbecco's modified phosphate-buffered saline (PBS) containing 0.5% Phenol Red (Sigma). All mRNAs were injected at 200 ng/µl, injecting 1-3 pL per embryo.

Total RNA isolation

RNA was isolated from four different stages of zebrafish embryos: 2-4 cell stage; 256-512cell stage; sphere stage; shield stage; The RNA was extracted from *Wt* and from *ichabod* embryos.

One hundred embryos raised to the selected stage were moved to an 1,5ml tube, washed two times with cold PBS, then smashed with a sterile pestle. Then was added 600 µl of cold TRIzol (Invitrogen), mixed and then added other 400 µl of Trizol. Then 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. Then recovered the aqueous phase, and added 200 µl of chloroform:isoamyl alcohol 24:1 (Acros), 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, centrifuged at 4°C for 15 min at max speed, removed the liquid phase, the pellet was washed with cold 70% ethanol and air dried. The pellet was then resuspended with autoclaved milliq sterile water; it was treated with DNase (promega) for one hour, and after this purified by classical acid phenol/chloroform extraction. The total RNA was tested by PCR for DNA contamination, and if pure, aliquoted and stored at -80°C.

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. This samples were called Rt- and were used as negative controls.

Real Time PCR

Quantitative real time PCR where done using a Lightcycler 480 II (Roche). cDNAs from two pre-Mid Blastula Transition (pre-MBT) stages (2-4 cells and

256-512 cells) and two post-MBT (Sphere and Shield) stages were used for these experiments. Every reaction was performed in 96well transparent plates (Roche) using 100 ng of cDNA, 0,25 µl of 10 µM Gene Specific-Primers and 2x SYBR Green I master (Roche) in a final volume of 10 µl.
For normalization were used primers for two housekeeping genes, ActinB and GAPDH.
The primers used are in table 4.
Cycling conditions:
pre-incubation: 95°C 5 min;
amplification: 95°C 25 sec; 55°C 25 sec; 72°C 25 sec;
melting curve: 95°C 5 sec; 67°C 1 min; 97°C continuous;
cooling
Cycling conditions for real time PCR of z-pygopus2 isoforms:
pre-incubation: 95°C 5 min;
amplification: 95°C 25 sec; 57°C 25 sec; 72°C 2 sec;
melting curve: 95°C 5 sec; 67°C 1 min; 97°C continuous;
cooling

Analysis with Second Derivate relative method. Data were obtained and mediated from real-time experiments done using cDNA preparations synthesized from two different total RNA extractions (two biological replica). One total RNA extract was used for two independent cDNA synthesis, the other total RNA extract was used for one cDNA synthesis, and all the samples reaction were in triplicate in each real time PCR.
Every reaction have the Rt- samples amplified with all the primers, at the same condition of the other samples, to ensure no DNA contamination. Melting curve analysis was performed to ensure no primer dimmers amplification.
For evaluation of PCR efficiencies of all primers sets, standard curves were generated using serial diluted cDNA samples.

Primer name	Primer sequence	product size
zActinB Forward	5' TCACCACCACAGCCGAAAG 3'	
zActinB reverse	5' AGAGGCAGCGGTTCCCAT 3'	98 bp
zGAPDH forward	5' GTGTAGGCGTGGACTGTGGT 3'	
zGAPDH reverse	5' TGGGAGTCAACCAGGACAAATA 3'	121 bp
zAxin1 Forward	5' ACGGCATCCACTTGTTTAGG 3'	
zAxin1 reverse	5' AGCATCTTCTCGTTGCCATC 3';	121 bp
zAxin2 forward	5' CAACCAAGCACATCCATCAC 3'	
zAxin2 reverse	5' TGCGAATGTAAGGAGCAGTG 3'	141 bp
zβcatenin1 Forward	5' TTGTGAGGACCATGCAGAAC 3'	
zβcatenin1 reverse	5' AAGATGGCAAGCAGTCCTTC 3'	103 bp
zβcatenin2 forward	5' TGTATTCCCCCATCGAGAAC 3'	
zβcatenin2 reverse	TGGTTTGTCCTCAGACATGC 3'	194 bp
zBCL9 forward	5' GAAGAAGCGCAAACAGGAAC 3'	
zBCL9 reverse	5' TTGGTCAGGGAAAGGTTCAG 3';	160 bp
zBCL9-2 forward	5' AGCCAACCAGATTTCACCTG 3'	
zBCL9-2 reverse	5' TAGCATCTGGCTCCAAAACC 3'	161 bp
zPygopus1 forward	5' AAAACGCGACTCAGGACAAC 3'	
zPygopus1 reverse	5' GAAAACAGGCTCGGATGAAG 3'	161 bp
zPygopus2 comm. forward	5' CAACTTTAGCCCTCCAATGC 3'	
zPygopus2 comm. Reverse	5' TTGCTGAGGCATACCAAACC 3'	124 bp
zPygopus2 ΔPHD forward	5' GCTCCAGGTTCCACACCTTA 3'	
zPygopus2 ΔPHD reverse	5' GCTGACAATGAATCGCAGAA 3	207 bp
zPygopus2 PHD forward	5' GTGTGGGCATGTGACTTTTG 3'	
zPygopus2 PHD reverse	5' ATCGCTCATCACCCATCTTC 3'	125 bp

Table 4 Primers used in real time pcr

Protein extraction

Total protein preparations were done from *Wt* and *ichabod* embryos in two pre-Mid Blastula Transition (pre-MBT) stages (2-8 cells and 256-512 cells) and two post-MBT (Sphere and Shield) stage.

100-150 embryos were dechorionated using Pronase (Sigma) for 5 min at RT, then washing twice with embryo medium (*Westerfield, 2000*) and twice with PBS. Then embryos were collected in an agarose-coated plate (with agarose dissolved in embryo medium + methylene blue) and deyolked manually with glass capillaries. Then moved with a glass pipette to a 1,5ml tube and extracted with RIPA buffer + protease inhibitors (Sigma), or 0,5% NP-40 extraction buffer + protease inhibitors (Sigma).

Protein concentration was measured using Bradford protein assay with a Biophotometer (eppendorf), then the protein samples were aliquoted and stored at -80°C.

SDS-Page and western blotting

Protein samples were melted on ice, mixed with 2x Laemmli loading buffer, boiled for 10 min and then loaded on polyacrilammide gel, using Pageruler prestained ladder (Fermentas) as a marker.

Run was at 100V constant for an hour and 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, shacking. After an hour, was incubated with the desired antibody dissolved in 3% milk on PBS-tween 0,1% overnight (ON) at 4°C shacking. We used as primary antybodies anti-pan-β-catenin (Sigma), mAb-Cterminal-β-catenin(BD Transduction Laboratories), mouse anti Flag (Rockland), mouse anti-α-Tubulin and anti-mouse and anti-rabbit peroxidase-conjugated (Amersham) as secondary antibodies.

The day after, was removed the primary antibody, three washes shaking with PBS-tween 0,1%, than incubated with the correct secondary antibody for an hour at RT, with shaking. After this, the membranes were washed three times with PBS-tween 0,1% and then incubated with ECL plus (Amersham). After 5 min of incubation, the ECL plus was removed and the film was exposed for the requested time.

Chemically-competent cells preparation

DH5alpha cells were pre-inoculated in 3 ml of LB sterile medium and let grown ON at 37°C shaking.

The day after, 100 µl of this pre-inoculation is transferred in 10 ml of LB medium and grown to obtain an absorbance value (measured in the Biophotometer at 600 OD) of 0.2 OD.

Then the 10 ml are inoculated a flask with 500 ml of pre-warmed LB and let grown at 37°C shaking to an A600 value of 0.4-0.5 OD.

Then the flask is chill in ice for 10 min, and then pelleted at 6000 RPM for 10 min at 4°C. After this, the medium is discarded, and the bacteria are resuspended in 200 ml of cold Transforming Buffer (TB: 50 mM $CaCl_2$; 10 mM MOPS; 15% glycerol; pH > 6.6 ; Autoclaved to sterilize) and left on ice for 20 min. Then the bacteria are pelleted at 6000 RPM for 10 min at 4°C, the medium discarded and the pellet resuspended in 20 ml of chilled TB. Then the bacteria are divided in 200 µl aliquots in pre-chilled 1,5 ml tubes, quickly frozen in dry ice and stored at -80°C.

The transforming efficiency is measured transforming an aliquot of cells with 10 pg of pUC plasmid and plating it in LB plates, incubate the plate ON at 37°C and counting the colonies obtained. The efficiency obtained was $\approx 10^6$.

Heat-shock transformation of DH5 alpha competent cells

An aliquot of DH5alpha cells is transferred in ice until is melted, then is added the desired amount of plasmid, incubated 30 min on ice, then moved to 42°C for 30 sec, then quickly transferred to ice for 2 min, then added 600 µl of LB and moved to a shacking incubator at 37°C for 1 hour. After an hour, the bacteria are spread in a plate of LB plus the selective antibiotic and let grow ON at 37°C.

Sub-cloning and constructs preparation

zpygopus 1 clone was ordered by imaGene (clone IRBOp991B0599D). The clone had zpygopus1 gene cloned in pExpress1. I did a PCR using it as a template, using the primers **z-pygopus1 EcorI** forward and **z-pygopus1 XhoI** reverse (Table 5), using a mix 0,125:1 respectively of cloned PFU DNA Polymerase (stratagene) and taq DNA polymerase recombinant (fermentas) with this conditions: pre-denaturation 94°C 5 min; amplification 94°C 30 sec, 58°C 30 sec, 72°C 2 min, repeated 30 cycles; final extension 72°C 7 min.

Primer name	Primer sequence
Z-pygopus1 EcoRI forward	5' GGAATTCCTTGTCCACAGAGC 3'
Z-pygopus1 XhoI reverse	5' CCGCTCGAGCGGAACTTTG 3'
Z-Pygopus2 ΔPHD EcorI forward	5' GGAATTCCTTGGCCGACGAG 3'
Z-Pygopus2 ΔPHD XhoI reverse	5' CCGCTCGAGCGGTCTACTT 3'

Table 5. Primers used in sub cloning

z-pygopus 2 clone was ordered from imagine (clone IRAKp961D09315Q). The clone had zpygopus2 ΔPHD gene cloned in pME18S-FL3. I did a PCR using it as a template, using the primers **z-pygopus2 ΔPHD EcorI** forward and **z-pygopus2 ΔPHD XhoI** reverse, and using a mix 0,125:1 respectively of cloned PFU DNA Polymerase (stratagene) and taq DNA polymerase recombinant (fermentas) with this conditions: pre-denaturation 94°C 5 min; amplification 94°C 30 sec, 58°C 30 sec, 72°C 2 min, repeated 30 cycles; final extension 72°C 7 min.

The PCR product was loaded in a 1% agarose gel, the band was cutted and the DNA recovered with Zymoclean Gel DNA Recovery Kit. The DNA was then double digested with EcoRI and XhoI (roche), purified by gel cleaning

and used for the ligase reaction. The same double digestion and purification was done for pCS2+2xFlag, that was also dephosphorylated with the rAPid alkaline phosphatase kit (Roche). The dephosphorylated pCS2+2xFlag was ligated with z-*pygopus1* and z-*pygopus2* with T4 DNA Ligase kit (Roche). All cut plasmid in this study were dephosphorylated and the ligated using these kits.
Our collaborator, Dr Daniele Castiglia from the IDI in Rome, created through PCR mutagenesis the pCSCN DN-*axin2* by modification of the nucleotide 1905 of the ORF from G **>** A of the pCSSN-*z-axin2*. This created an early Stop codon, which eliminates the dimerization domain, DIX from the translated protein.
Flagged z-*axin* clones were obtained with a multi-step reaction: at the beginning the first 700bp of the ORF of ***z-axin1*** and ***z-axin2*** were cloned in the correct frame in pCS2+2xFlag by PCR amplification of pCSNC zAxin1 or zAxin2 using a 5' oligo which introduced the EcoRI digestion site few nucleotides 5' of the ATG of the cDNAs. The obtained pCS2+2xFlag-**z-axin1** was cut with BamH1/NruI, and pCS2+2xFlag-**z-axin2** was cut with HindIII. The digested fragments of ~700bp containing the 5' of zAxin1 or 2 fused in frame with the Flag were cloned using the same digestion sites in pCSSN-**z-axin1**, pCSSN-**z-axin2**, pCSSN-**DN-z-axin2**. The correct sequence and orientation of the clones was confirmed by sequencing.

In vitro transcription/translation

Both pCS2+Myc+ *β-catenin*1 and 2 were digested with NotI (Promega), purified with phenol/chloroform extraction, precipitated a used for in vitro transcription/translation with TnT SP6 High-Yield Wheat Germ Protein Expression System (Promega). The obtained protein mix was stored at -80°C and then used in Western blotting experiments to test the specificity of new rabbit bleeds for the identification antibodies specific for *z-β-Catenin1*.

Plasmid preparation

A single colony from an LB plate in which was plated the desired transformed bacteria is inoculated in 5 ml of LB plus the correct antibiotic, and let grow ON at 37°C shacking. The day after, the plasmid is recovered using the High Pure Plasmid Isolation Kit (Roche).
The concentration was measured with NanoDrop ND-1000.

Results

Testing of *β-catenin1* specific antibodies

In previous studies (G. Bellipanni and E.S. Weinberg unpublished data, see Introduction) was used a α-pan-*β-catenin* antibody that recognize both z*-β-Catenin* proteins, and an antibody directed against the carboxy-terminal region of *z-β-Catenin1* (c-term-*β-catenin*). The antibody specificity was confirmed by western blotting on in vitro transcribed/translated protein and on protein extracts obtained from zebrafish embryos injected with the mRNAs for the myc-tagged version of the two z-*β-Catenins* (G. Bellipanni and E.S. Weinberg, data not shown).

The C-terminal β-Catenin antibody resulted sometime difficult to work with so we decided to generate a new antibody against a peptide of the C-terminal side of *z-β-catenin2* (amino acids GQDAMGMDPMMEHEMAGHHPGPDY-PVDGLPDLGHT), which retains the highest number of differences with the z-*β-catenin1*. The peptide was synthesized on site in the SBARRO proteomic facility and then sent to Rockland for rabbit immunizations. We obtained serum of 3 different rabbits. We tested the ability of these bleeds to recognize *β-Catenins in vitro* transcribed and translated with TnT SP6 High-Yield Wheat Germ Protein Expression System (Promega). After ~1 year from the first bleeding, we received two aliquots of serum obtained from two immunized rabbits (Bleed Rb86 and Rb87) and two aliquots of affinity-purified antibodies, AP16284 and AP16330, from the respective bleed. We tested these two bleeds and the two purified antibodies by western blotting, using protein extracts from zebrafish embryos at sphere stage, which were injected with either one of the two myc-tagged mRNAs for the 2 z-*β-catenins* forms.

Of the two antibodies, only the bleed and the AP16330 antibodies from rabbit87 recognized in a specific manner z-*β-catenin1* (Fig.9).

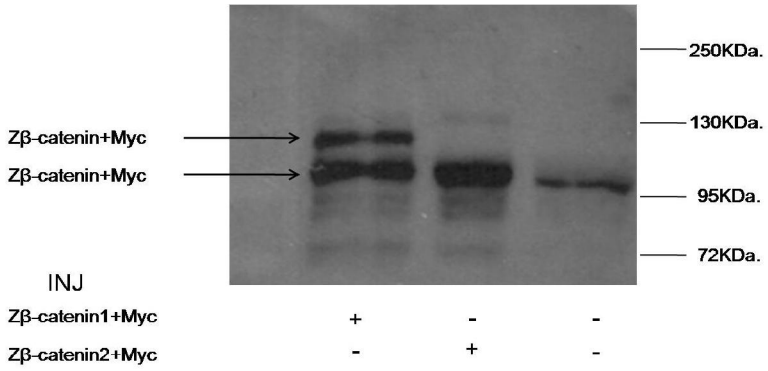


Fig. 9 Western blotting analysis with AP16339 antibody of protein extracts from Wt embryos microinjected with the indicated mRNA.

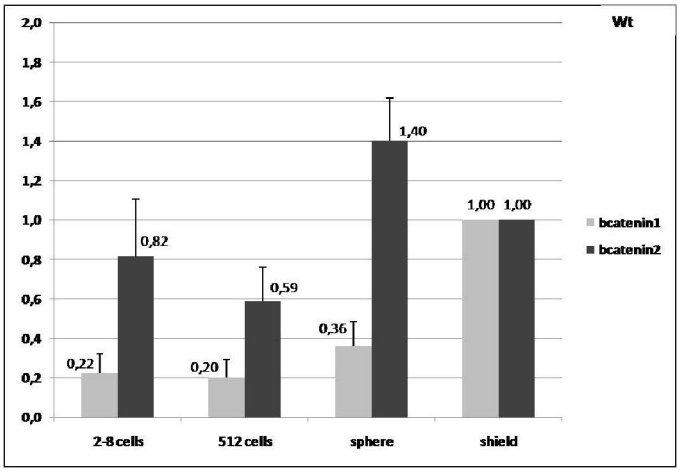
Variation of *z-β-catenins* mRNA expression levels in *ichabod* embryos

To confirm the down-regulation of z-*β-Catenin*2 and up-regulation z-*β-Catenin*1, we studied the expression levels of both the genes during four developmental stages, both in *Wt* and in *ich* embryos using relative real time PCR .

To choose the corrects housekeeping genes to be used in the analysis, we first tested by real time PCR three different genes, *zActinβ*, *zGapdh* and *zEf1α,* using cDNA from *WT* and *ich* embryos in the four developmental stages. The results were analyzed with three different programs used to validate genes used for normalization of real time PCR experiments: geNorm (*Vandesompele et al., 2002*), Normfinder (*Andersen et al, 2004*), BestKeeper (*Pfaffl et al, 2004*). Then, we analyzed the Ct data with the Comparative Ct method (*Livak et al, 2001*; *Shmittgen et Livak, 2008*). All these different method gave as a result that zEf1α is not usable as a normalizer gene, while *zActinβ* and *zGapdh* are optimal for this kind of study. So we choose to use both *zActinβ* and *zGapdh* as normalizers for the real time PCR analysis.

Then, we analyzed the two z-*β-catenins* genes expression by relative real time PCR using the Comparative Ct method.

First, we plotted our results to obtain a measure of the variations of the two genes expression levels in the four different stages (Fig. 10), using shield stage as a calibrator.



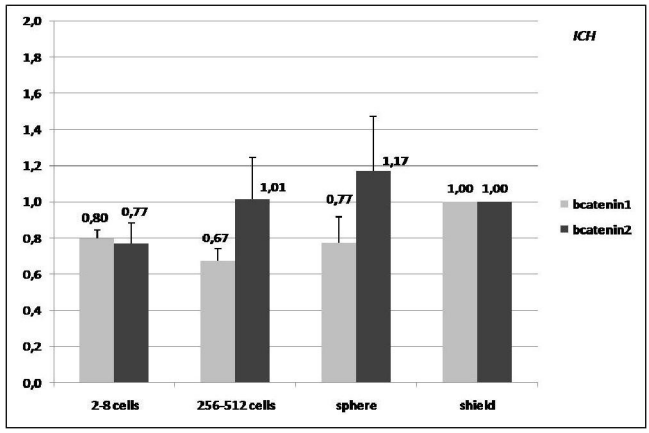


Fig. 10 Real time PCR of z-*β-catenin1* and 2 on *Wt* and *ich* during 4 developmental stages.

In this way, we found that the expression levels of z-*β-catenin1* and *2* change during development in *Wt* embryos and remains pretty stable in *ich*. Then we plotted our data to analyzed the expression levels of the two z-*β-catenin*s in *ich* respect to *Wt* (Fig. 11).

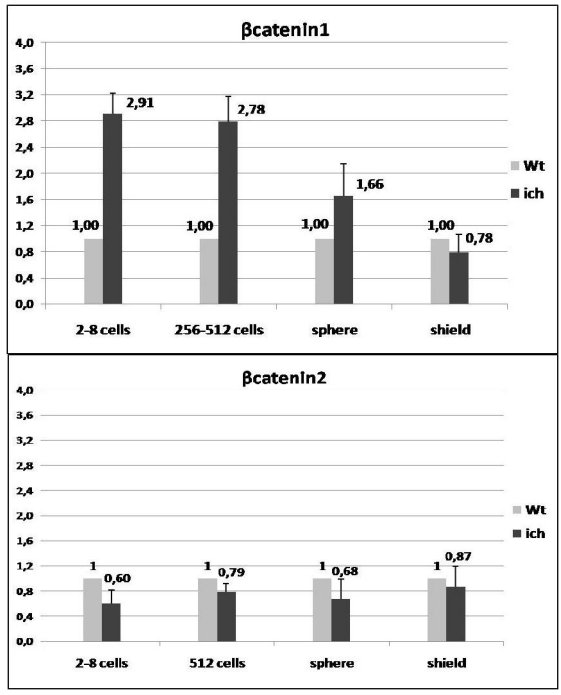


Fig. 11 Real time PCR of z-*β-catenin*1 and 2 in *Ich* respect to *Wt* during 4 developmental stages.

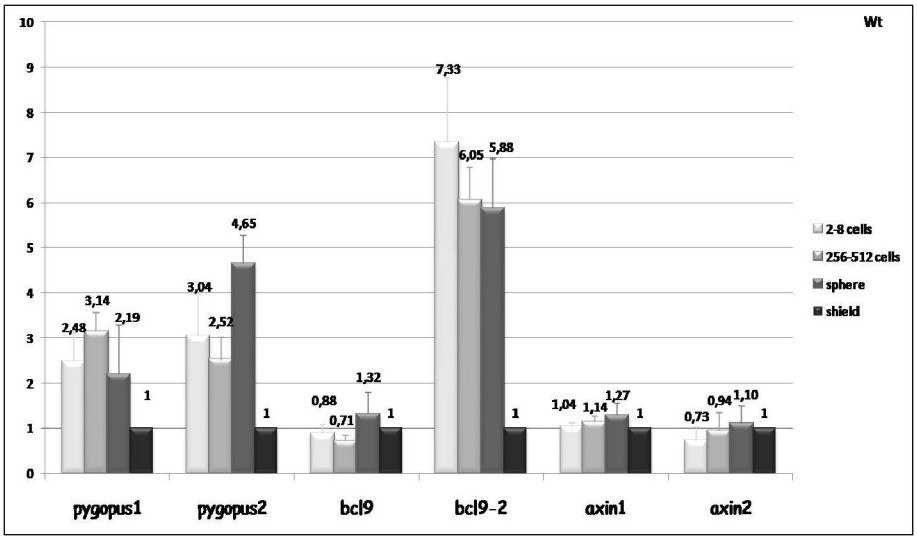
These data confirm at mRNA level that the expression of *z-β-catenin1* mRNA is increased in *ich* respect to *Wt* embryos, conversely expression levels of *z-β-catenin2* mRNA are lower in *ich*.

These results, taken together with that done at the protein level clearly show that *z-β-catenin1* is up-regulated at transcriptional and translational levels. Interesting, while the mRNA level of *zβ-catenin1* is up-regulated ~3 folds in 2-8 cell stage and 256-512 cell stage *ich* embryos the increase of *z-β-Catenin1* concentration in 128 cell stage embryos is increased of only ~2 folds respect the *Wt* suggesting either that not all the mRNA for *z-β-catenin1* is properly translated or that the z-*β-Catenin*1 stability is largely compromised in *ich* embryos. Finally mRNA and protein levels data suggest us a paradox that despite the increased level of *z-β-catenin1* expression and stability of the total *z-β-Catenin* proteins pool in *Wt* and *ich*, *z-β-Catenin1* is not able to compensate for the decrease in *z-β-Catenin2* levels in *ich* embryos.

Analysis of the expression levels of genes involved in *z-β-Catenins* stability and nuclear anchoring.

To better understand the involvement of other factors in the regulation of z-*β-Catenins* nuclear localization or mRNAs stability, we analyzed the relative expression levels of genes of the signalosome involved in z-*β-Catenin*s stability (*zAxin1 and zAxin2*) and the zebrafish homologues of the *Drosophila legless* and *pygopus* (*zBCL9, zBCL9-2, z-Pygopus 1 and 2*) able to respectively bind armadillo and anchor it in the nucleus. (Fig 3). We analyzed these genes expression levels by relative real time PCR using the Comparative Ct method.

First, we plotted our results to obtain a measure of the variations of the genes expression levels in the four different stages, using shield stage as a calibrator (Fig 12).



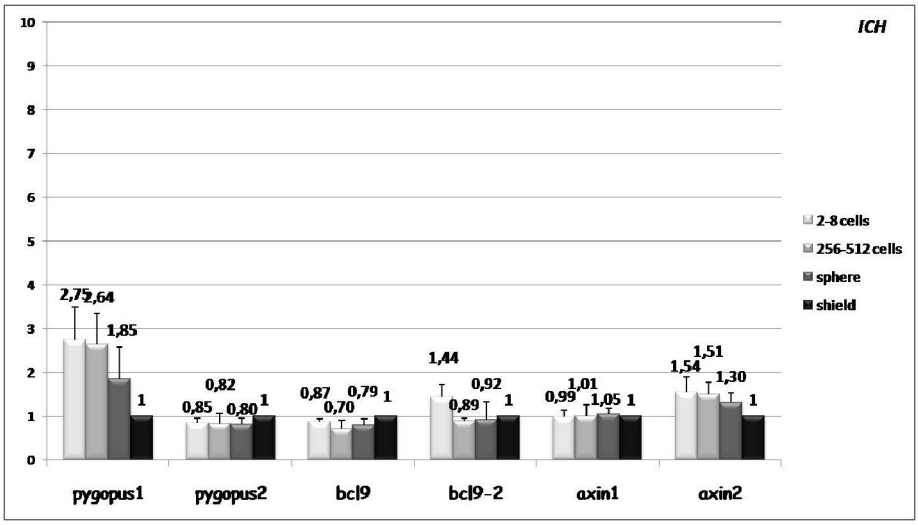
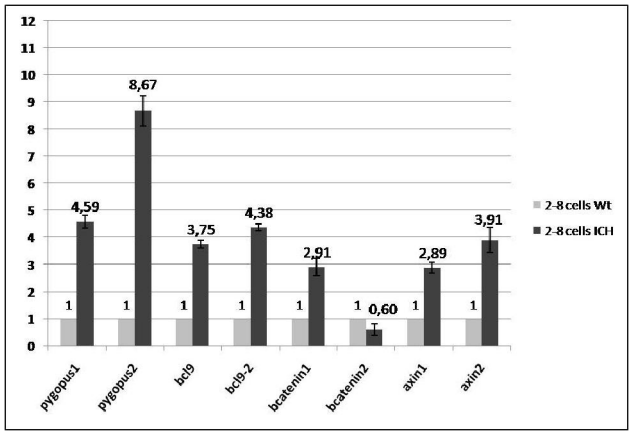


Fig. 12 Real time PCR of different genes on *Wt* and *ich* during 4 developmental stages.

The results show that in *Wt* embryos pygopous1 pygopous2 and bcl9-2 expression levels at 2-8 cell stage, 256-512 cell stage and High stage are much higher than in sphere stage. In *ich*, instead, the expression level of these genes but pygopus1 remain similar in all the four stages analyzed.

Then we plotted our data to analyzed the expression levels of all these genes in *ich* respect to *Wt* (Fig. 13).



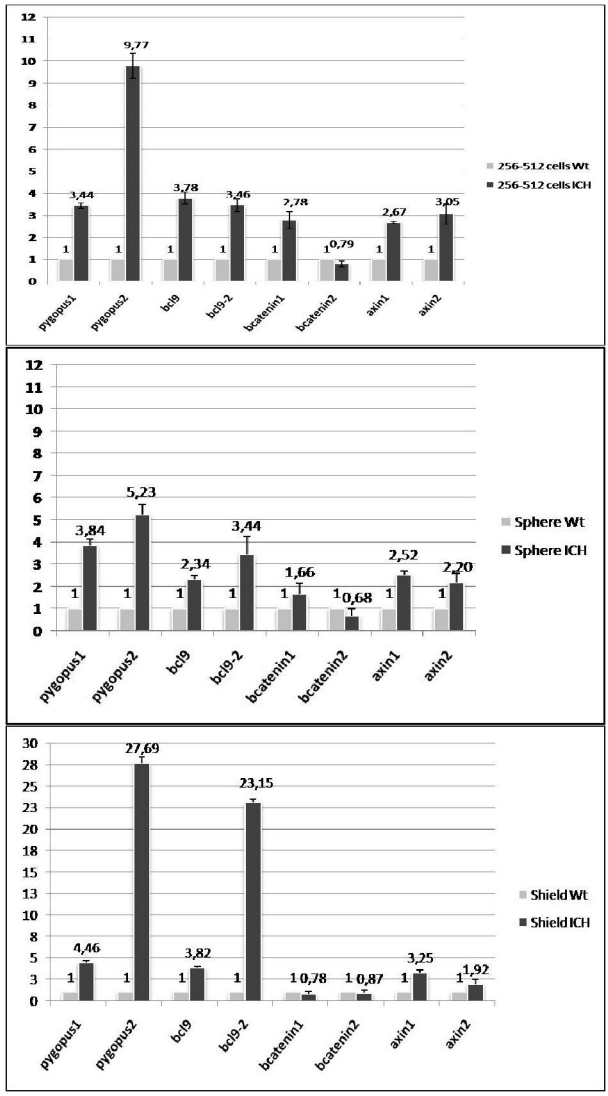


Fig. 13 Real time PCR of different genes in *ich* respect to *Wt* during 4 developmental stages.

We found that all these genes are up-regulated in *ich* respect to *Wt* in the analyzed developmental stages. Particularly strong appeared the up-regulation of z*pygopus2* mRNA, while we were very surprised to see *z-axin2* up-regulated in *ich* embryos because its expression is known to be induced directly by Wnt/z-*β-Catenin* signaling. Furthermore, even though we do not measured directly the level of concentration of the *z-Pygopous* and *z-BCL9* group we could infer from the results that the physiological levels of *z-Pygopus*/*z-BCL9*/*z-β-Catenin* were probably compromised.

zpygopus2 splicing variants

In our attempt to obtain the zebrafish homologues of the Drosophila *pygopus* and *legless,* we found on the web databanks pubmed and Ensembl five possible *pygopus2* mRNA sequences (Accession numbers: NM_001033111, BC159191, BC100039, ENSDART00000104731, ENSDART00000046225 and ENSDART00000129994). We used a PCR-based approach to confirm the real existence of these sequences. Of these five, we confirmed the existence of two sequences, NM_001033111, which represent a *bona-fide* homologues of Drosophila *pygopous* and Human *Pygopous2, thus we named it, z-pygopus2*, and BC159191. We also ordered a *z-pygopus2* clone from IMAGE (clone IRAKp961D09315Q), which resulted, after sequencing, the same as BC159191. This clone has a different 5' region, containing the ATG, and lacked the PDH domain.

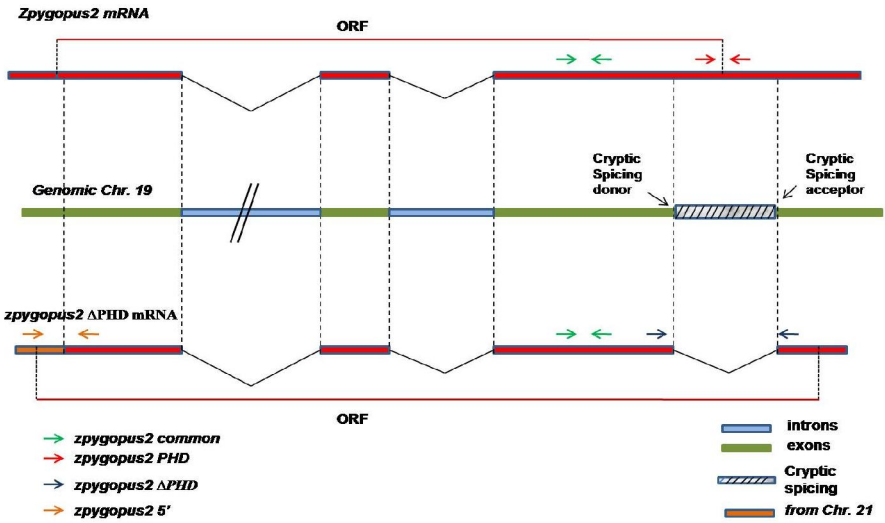


Fig. 14 Schematization of the two *z-pygopus* isoforms and the genomic, with the primers used.

To finally determine that this cDNA was really expressed in zebrafish embryos, we used a PCR based approach using the set of primers indicated with a color code in Fig14 and which sequences are listed in table 4. The fragments obtained with all the primer sets indicated in the figure, amplified by RT-PCR, were sequenced to confirm the identity with the sequence of BC159191. The sequence of BC159191 (which is identical to the sequence of the IMAGE clone IRAKp961D09315Q) results ~100% identical in the common regions with the sequence for the predicted *z-pygopus2*, thus

suggesting that the clone we bought was a splicing variant of the predicted *z-pygopus2*. We called this putative splicing variant *z-pygo2-ΔPHD* (Fig. 14).
The existence of these two isoforms of *z-pygopus2* could be really important, in fact the PHD domain is fundamental to interact with *zBcl9* and therefore to anchor the z-*β-Catenin* in the nucleus. Common regions between the two *z-pygopus2* isoforms contain the NLS and the domain fundamental for the interaction with the mediator complex, with *CBP*, and with chromatin remodeling proteins like histone deacetylases (HAT).
For real-time PCRs showed in fig 13 we used a set of primers for *zpygopous2* that would have recognized both isoforms. Thus, we decided to investigate the mRNA expression profile of *z-pygo2* and *z-pygo2-ΔPHD* by real-time PCR.
First, we ordered primers specific for *z-pygo2*, see fig 14 red primer set, and a set of primers that would amplify only *z-pygo2-ΔPHD* at a certain temperature (see material end methods for modifications). We run a real time PCR to analyze the differences in gene expression level between this two isoforms (Fig. 15).

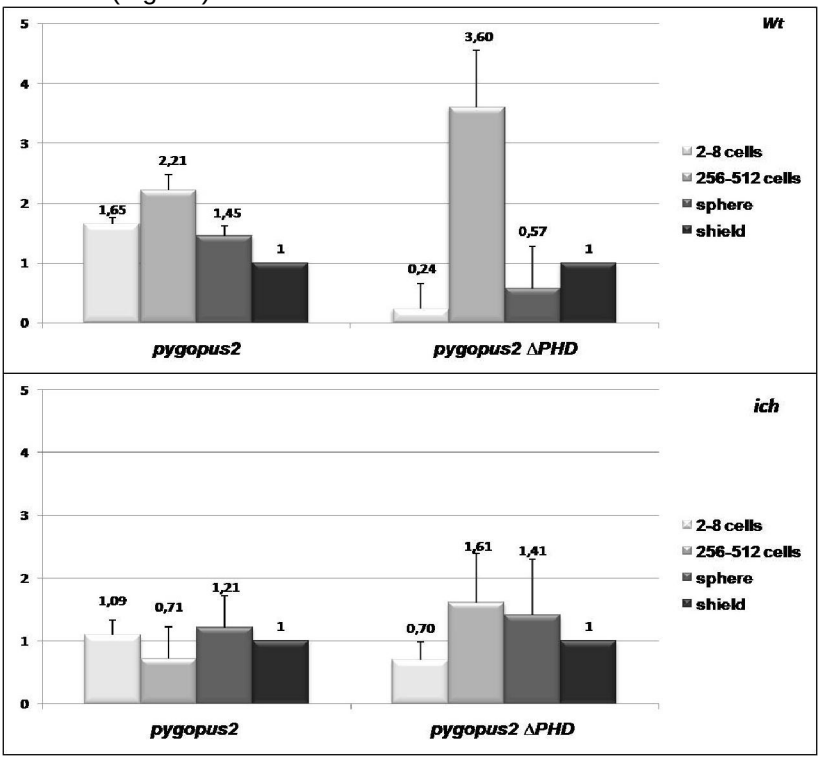
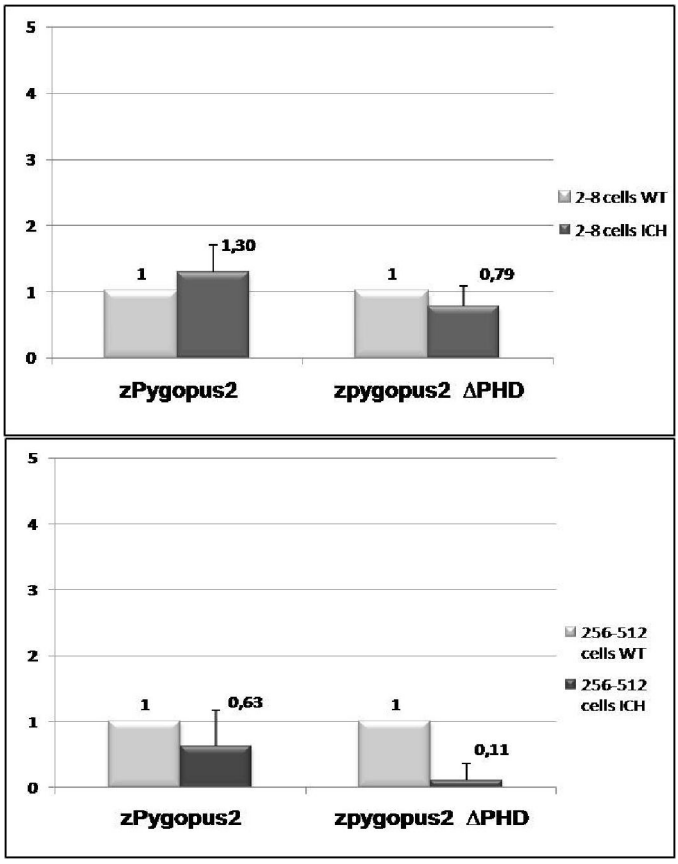


Fig. 15 Real time PCR of the two *zPygopus2* and *zPygopus2-ΔPHD* isoforms in *Wt* and in *ich* during 4 developmental stages.

These experiment show that in *Wt z-pygopus2-ΔPHD* isoform is expressed in a very dynamic way. At 2-8 cell stage embryos the *z-pygopus2-ΔPHD* isoform is expressed at very low levels (respect to shield stage), the following stage we analyzed ,256-512 cell stage, that is very close to the Mid-Blastula Transition, shows a pick of expression. At High stage the level of *z-pygopus2-ΔPHD* expression returns below the level at shield stage but more than 2-8 cell stage. The isoform with the PHD domain instead have less differences in levels, with shield stage the one with the lower level respect to the others.

The situation is different in *ich*, where the *z-pygopus2* isoform with PHD domain remain within a constant level of expression, and the increase of *z-pygopus2-ΔPHD* isoforms at the 256-512 cell stage instead is much more reduced.

To better understand the expression of these two isoforms between *ich* and *Wt*, we plotted our real-time PCR data in the four developmental stages of the two fish lines , using the data for the same stages in *Wt* as a calibrator (Fig. 16).



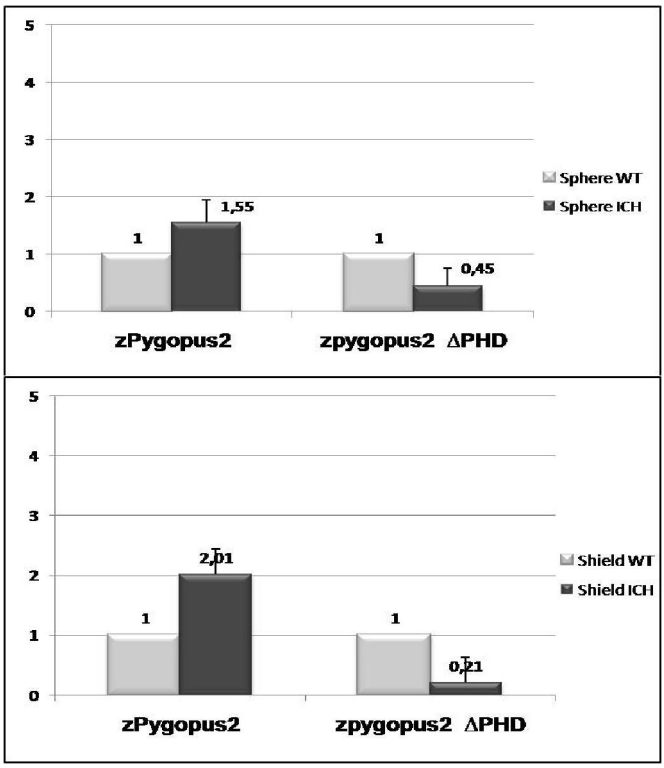


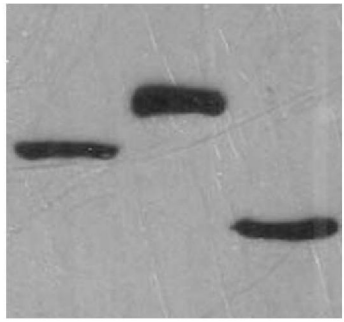
Fig. 16 Real time PCR of the two zPygopus2 splicing variants in *Wt* and in *ich* during the early developmental stages

In this way we determined that the isoform with PHD has slight high values in *ich*, but the differences are within the standard error bar for 2-8 cell stage, 256-512 cell stage and sphere stage. *zpygopus* levels in *ich* at shield stage are truly almost twice the level in Wt embryos at the same stage. Instead the ΔPHD isoforms is expressed in *ich* at very low level respect the same stages in *Wt* embryos. Taking together these data we may hypothesize that the ΔPHD isoform could have an early developmental role, and its absence in *ich* may be responsible for the inability of *z-β-Catenin1* to compensate for the reduced levels of *z-β-Catenin2*.

zAxin2 Dominant Negative form

Work of Mo et al. (*Mo et al 2009*) showed *in vitro,* using an heterologous system, that the two *z-β-Catenins* have different stability. As postulated previously a difference in stability, in this case having *z-β-Catenin1* less stable *of z-β-Catenin2*, could explain the differences between levels of *z-β-catenin1* mRNA measured at 2-8 cell stage and at 256-512 cell stage in *ich* (Fig. 10) and the concentration of *z-β-Catenin1* in *ich*. These results are in agreement with the hypothesis that excess of cytoplasmic *z-β-Catenin1* in *ich* may be kept to relatively low levels by the signalosome complex, while we see more *z-β-Catenin1* in *ich* only because highly localized at cells membrane (Fig9B). Thus, this would not allow *z-β-Catenin1* to compensate for the loss of *z-β-Catenin2* in *ich*.

To test our hypothesis and that regarding *z-pygopus2* Δ*PHD* we prepared Flag-tagged versions of *z-pygopus2* and *z-pygopus2* Δ*PHD*, *z-axin1*, *z-axin2* and the Dominant Negative form of *z-axin2* (*zDN-axin2*). The proper transcription of the Axins constructs was checked by western blots of the *in vitro* transcribed and translated proteins (Fig. 17).

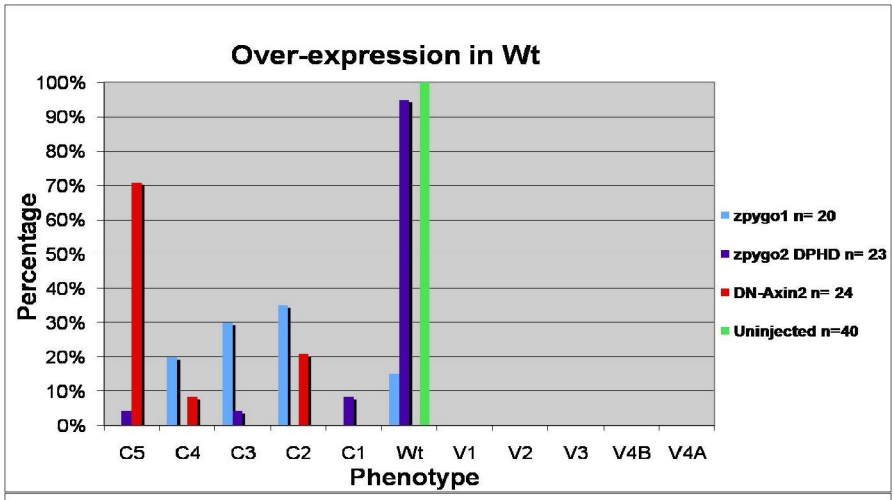


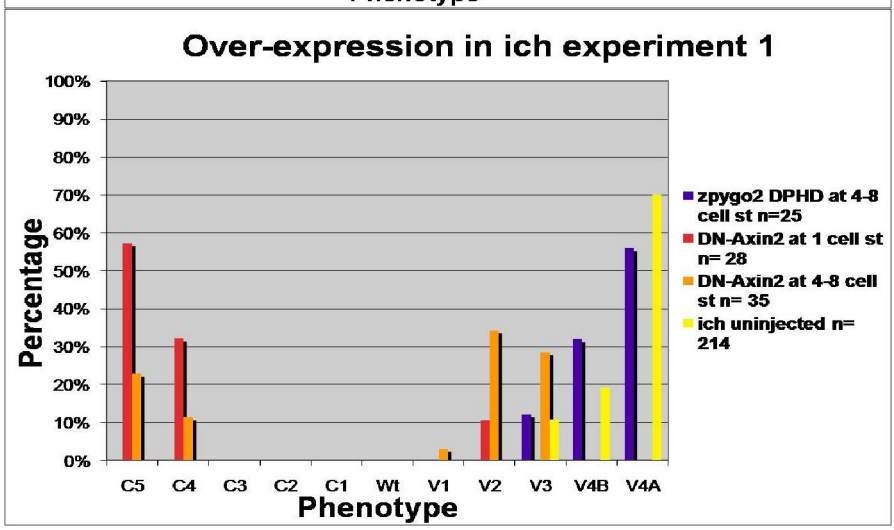
Z-Flag-Axin1	+	-	-
Z-Flag-Axin2	-	+	-
Z-Flag-DNAxin2	-	-	+

Fig. 17 Western blotting of the *in vitro* transcribed/translated *z-flag-axin* constructs.

We microinjected in 1 or at 4-8 cell stage *Wt* or *ich* embryos *in vitro* synthesized mRNA for *z-Flag-DN-axin-2*, for *z-pygopus2* Δ*PHD* and for *z-pygopus1*. The injection in 1 cell stage ich embryos of *z-pygopus2* Δ*PHD* did not affected the mutant phenotype even though the injection of the same

transcript in *Wt* embryos produced some dorsalized phenotype (Charts in fig.:18). Injection of *z-pygopus1*, as expected because ich embryos already have very high level of this transcript, did not affected *ich* phenotype, while it was inducing severe dorsalized phenotypes when injected in *wt* embryos For the *z-DN-axin2* over-expression experiment, we expected that this form of *zAxin2*, which cannot form a dimer, would interact with APC and GSK3 thus blocking the assembling of the destruction complex. Blocking the destruction complex should increase the levels of z-*β-Catenin1*, thus, this microinjection should recover the mutant phenotype.





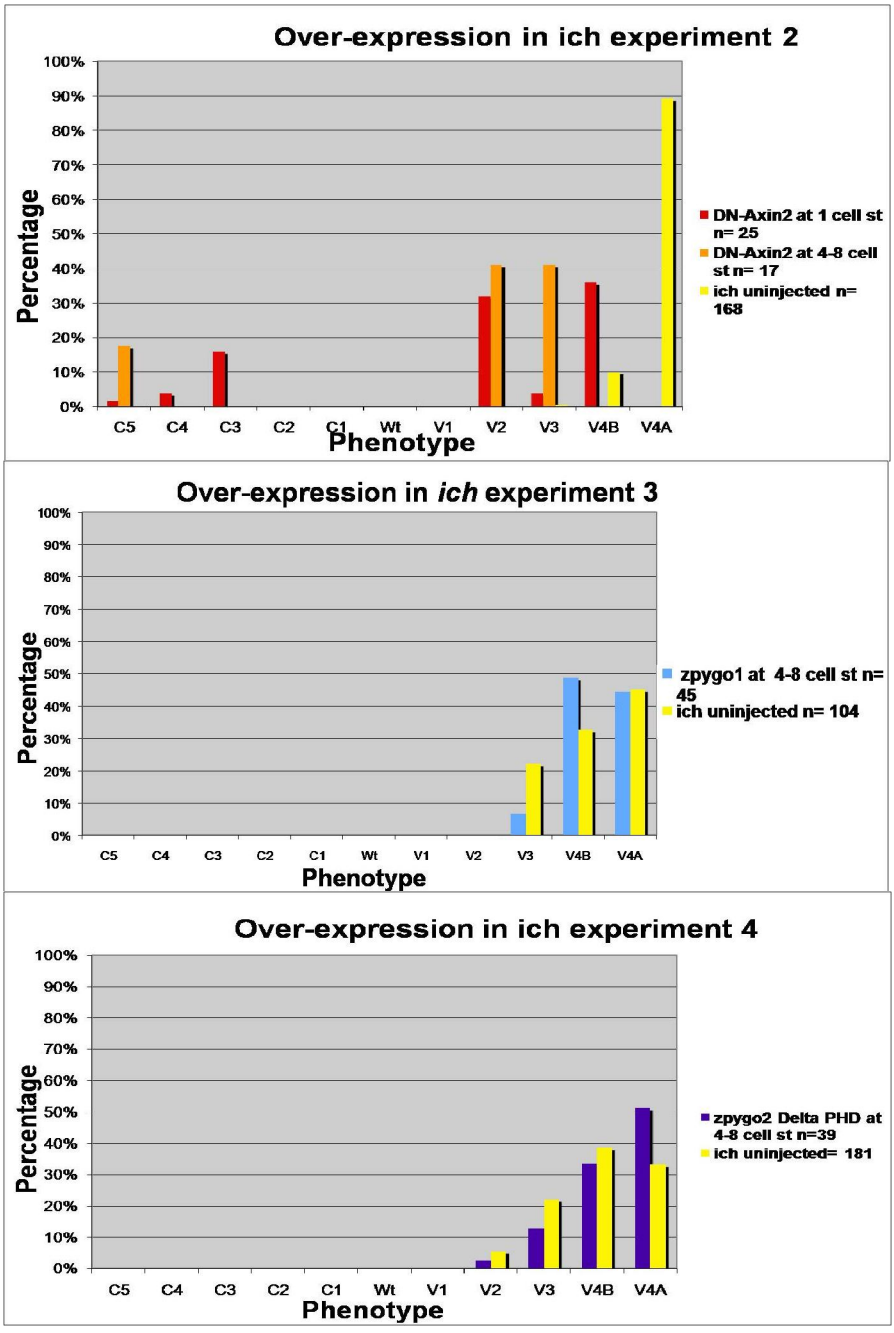


Fig. 18 Results of the over-expression experiments in Wt and ich embryos expressed as of embryos with a given phenotype. The phenotypes are referring to Kishimoto et al, 1997, with a small variation (V4A and B) to accommodate ichabod mutant phenotype characteristics.

Over-expression of *flag-DN-axin2* mRNA in *Wt* embryos produced the strongest phenotype with virtually no embryo injected having a normal phenotype. All the embryos injected were dorsalized from medium (C2 phenotype in 20% of the injected embryos) to a very severe extent (C5 phenotype in 70% of the injected embryos). Injection of *flag-pygo1* mRNA resulted in similar dorsalized phenotypes, but less severe than the expression of DN-Axin2 (30% C3, ~35% C2 and ~15% Wt). It is difficult to interpret the result of *flag-pygo2-ΔPHD* mRNA, perhaps due to the low number of sampled embryos, only 1 embryo in the injected batch had a C5 phenotype, 1 embryo had a C4 phenotype and 2 embryos with a C1 phenotype, while the remaining 19 embryos of this batch were *Wt*.

Injection of *flag-pygo2-ΔPHD* mRNA had no effect in *ich* as checked in 2 experiments (exp#1 and exp#4). In particular, referring to experiment #4 *flag-pygo2-ΔPHD* mRNA was over-expressed in a mild batch of *ich* embryos, but still it did not rescue the mutant phenotype.

Only the over-expression of DN-*Axin2* determined a reversion of the mutant phenotype. This appeared to be dependent on the severity of the mutant batch: injection at 1 cell stage of a relative mild batch of *ich* embryos (experiment #1) resulted into a complete reversion from the most ventralized phenotype in *ich* to the most dorsalized phenotype (C5) of ~55% of the embryos. When the *ich* batch was more severe (almost all V4A phenotype) only ~18% of the embryos injected had a C5 phenotype. Injections in one cell at 4-8 cell stage gave a more graded response with embryos falling either in the mild ventralized categories (V2 and V3) or in the mild dorsalized category (C3).

Interestingly we never obtained normal reversion to Wt, neither C1 or V1 phenotypes. One of the possible explanation for the absence of reversion to *Wt* is that the over-expression of DN-Axin2 would probably continue for hours after the stage in which we need to induce *β-Catenin* nuclear localization resulting in aberrant dorsalizing effect. Another explanation is that *DN-Axin2* interferes with other pathways like the Wnt non canonical pathway or the *Axin*/*JNK* pathway.

Discussion

Canonical-Wnt pathway have a central role in the regulation of transcription of many genes, in different tissues.
To transmit the Canonical-Wnt signaling from outside the cells to the nucleus, there are many steps that comprises the formation of a destruction complex with Axin1 and 2, APC, GSK3 and CK1, the differential phosphorilation of *β-Catenin*, the regulation of its stability and its movement to the nucleus. All these steps have to be tightly regulated, to permit gene transcription activation in particular moments and in a particular tissue/set of cells. Aberrant nuclear localization of β-Catenin during embryogenesis may produce severely deformed embryos, while in the adult mammals induces cancer. Therefore, the understanding of the mechanisms involved in the regulation of β-Catenin stability and localization could be relevant for bio-medical studies .
Due to a recent genome duplication event, in zebrafish are present two different genes coding for two almost identical *β-Catenins*, *β-Catenin1* and *2*. A region close to *β-catenins2* gene (we suppose is a maternal enhancer) was found mutated in zebrafish, and the line was called *ichabod*. The embryos produced by an homozygous *ich* mother fail to have a correct dorsalization, this because *β-Catenins* fail to nuclear localize in the nuclei of the prospective dorsal organizer, the region responsible for the correct induction of the dorsal tissues.
Looking at the protein levels, we found that in the cells of the prospective dorsal organizer, there is a decrease in the *β-Catenin2* levels and an increase in the *β-Catenin1* levels so that the total β-Catenins concentration do not change between *Wt* and *ich* embryos. We saw similar behaviour looking at the mRNA levels, with the expected decrease in the *β-catenin2* mRNA level, and an increase of *β-catenin1* level. However, even if there is an over-expression of *β-Catenin1*, this cannot compensate for the small reduction of *β-Catenin2*. To understand better this apparent paradox, we studied the levels of other factors involved in the regulation of *β-Catenin,* two factors involved in the regulation of *β-Catenin* stability, *Axin1* and *Axin2*, and four factors involved in the *β-Catenin* localization, *Pygopus1* and *2, Bcl9* and *Bcl9-2*.
In zebrafish all these genes are expressed during the four stages studied in this thesis, and their levels are up-regulated in *ich* embryos.
We identified another splicing isoform of *z-pygopus2*, that we called *z-pygopus2* Δ*PHD*, because this isoform lack of the PHD domain, essential for the interaction with Bcl9/Bcl9-2 and therefore with *β-Catenin*. Analyzing the transcription levels of these two isoforms we found that the isoform with PHD domain have an higher level both in *Wt* and in *ich*, respect to the Δ*PHD*, and

during the embryo development this level remain pretty constant. The isoform lacking the PHD domain is expressed in lower level, but in *Wt* this levels changes, with a peak of expression during the 256-512 cell stage, instead this level remain constant in *ich*, without any peak of expression. This let us hypothesize a role of the Δ*PHD* isoform during the development of zebrafish embryos. However, microinjection of in vitro transcribed mRNA for this isoform fail to revert the ich phenotype, even if in *Wt* it cause a small dorsalized phenotype. Thus, *z-pygopus2* Δ*PHD* must be playing some other role in the zebrafish embryo.

Then, we turned to look to factors involved in β-Catenin stability, we know from previous works that the two z-*β-Catenin*s *have* different stability. Moreover, the scenario seen through real-time PCR was particularly favorable because the levels of the two *axins* mRNAs were higher in *ich* , suggesting that the β-Catenins in the cytoplasm of *ich* blastomeres may be subjected to higher rates of degradation, while we see more *z-β-Catenin1* in *ich* only because highly localized at cells membrane (Fig9B). Thus, this would not allow *z-β-Catenin1* to compensate for the loss of *z-β-Catenin2* in *ich*.

To confirm this hypothesis we over-expressed a Dominant Negative form of z-Axin2 for microinjection in zebrafish embryos and we were able to partially revert the *ich* phenotype and cause dorsalization on *Wt* embryos.

From our results it seems that a different sensibility to *z-axins* protein could be responsible for maintaining a low cytosplasmic level of *β-Catenin1* in *ich* embryos, so even if over-expressed it could just accumulate in the membranes and not move to the nucleus. Coupling this with a decreased level of *β-Catenin2*, there is a fail of activate the transcription of the Wnt responsive genes in the prospective organizer, and therefore a fail to induct the dorsal tissues.

Understanding better the role of the pygopus2 Δ*PHD* isoform, and the different sensibility of the two *z-β-Catenin*s to the signalosome complex could be really important to unravel the mechanisms involved in the regulation of a correct Wnt canonical pathway.

Conclusions

The mutant zebrafish line *ichabod (ich)*, with a mutation in the region of the *zβ-catenin2* promoter that causes a decrease in the maternal accumulation of zβ-Catenin2 protein in the embryos, there is not nuclear localization of zβ-Catenins. So, the embryo fail to form a dorsal organizer and become ventralized. To understand why β-Catenins can not go to the nucleus, we analyzed by real-time PCR the levels of six genes involved in the canonical Wnt pathway: *zaxin1 and zaxin2, zpygopus1 and zpygopus2, zbcl9 and zbcl9-2.* Unexpectedly, they are all up-regulated in *ich* embryos before and after mid-blastula-transition (MBT). The finding of a second isoform of *zPygopus2* that in *Wt* embryos is up-regulated at 256-512 cell stage and in *ich* is not, let us hypothesize that this isoforms could be involved in the *ich* phenotype, however we failed to rescue *ich* by over-expressing *z-pygopus2* Δ*PHD* mRNA. Instead, microinjection of a dominant negative *zaxin2* mRNA can partially rescue the *ich* phenotype or revert it to very severe dorsalized phenotypes strongly suggesting that the regulation of stability of β-Catenin1 could be responsible for the *ich* phenotype. .

References

Aberle H, Bauer A, Stappert J, Kispert A, Kemler R. *beta-catenin is a target for the ubiquitin-proteasome pathway.* EMBO J. 1997 Jul 1;16(13):3797-804.

Arce L, Pate KT, Waterman ML. *Groucho binds two conserved regions of LEF-1 for HDAC-dependent repression*. BMC Cancer. 2009 May 21;9:159.

Bafico A, Gazit A, Pramila T, Finch PW, Yaniv A, Aaronson SA. *Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling.* J Biol Chem. 1999 Jun 4;274(23):16180-7.

Bafico A, Liu G, Yaniv A, Gazit A, Aaronson SA. *Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow*. Nat Cell Biol. 2001 Jul;3(7):683-6.

Belenkaya TY, Han C, Standley HJ, Lin X, Houston DW, Heasman J, Lin X. *pygopus Encodes a nuclear protein essential for wingless/Wnt signaling*. Development. 2002 Sep;129(17):4089-101.

Bellipanni G, Varga M, Maegawa S, Imai Y, Kelly C, Myers AP, Chu F, Talbot WS, Weinberg ES. *Essential and opposing roles of zebrafish beta-catenins in the formation of dorsal axial structures and neurectoderm.* Development. 2006 Apr;133(7):1299-309. Epub 2006 Mar 1.

Brembeck FH, Schwarz-Romond T, Bakkers J, Wilhelm S, Hammerschmidt M, Birchmeier W. *Essential role of BCL9-2 in the switch between beta-catenin's adhesive and transcriptional functions.* Genes Dev. 2004 Sep 15;18(18):2225-30. Epub 2004 Sep 1.

Cabrera CV, Alonso MC, Johnston P, Phillips RG, Lawrence PA. *Phenocopies induced with antisense RNA identify the wingless gene*. Cell. 1987; Aug 14;50(4):659-63.

Cardigan KM, Nusse R. *Wnt signaling: a common theme in animal development.* Genes Dev. 1997 Dec 15;11(24):3286-305.

Carlson ME, Silva HS, Conboy IM. *Aging of signal transduction pathways, and pathology*. Exp Cell Res. 2008 Jun 10;314(9):1951-61. Epub 2008 Apr 7.

Carrera I, Janody F, Leeds N, Duveau F, Treisman JE. *Pygopus activates Wingless target gene transcription through the mediator complex subunits Med12 and Med13*. Proc Natl Acad Sci U S A. 2008 May 6;105(18):6644-9. Epub 2008 May 1.

Clevers H. *Wnt/beta-catenin signaling in development and disease.* Cell. 2006 Nov 3;127(3):469-80.

De A. *Wnt/Ca2+ signaling pathway: a brief overview.* Acta Biochim Biophys Sin (Shanghai). 2011 Oct;43(10):745-56. Epub 2011 Sep 7.

Daniels DL, Weis WI. *Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation.* Nat Struct Mol Biol. 2005 Apr;12(4):364-71. Epub 2005 Mar 13.

Daugherty RL, Gottardi CJ. *Phospho-regulation of Beta-catenin adhesion and signaling functions*. Physiology (Bethesda). 2007 Oct;22:303-9.

Erter CE, Wilm TP, Basler N, Wright CV, Solnica-Krezel L. *Wnt8 is required in lateral mesendodermal precursors for neural posteriorization in vivo*. Development. 2001 Sep;128(18):3571-83.

Fagotto F, Glück U, Gumbiner BM. *Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of beta-catenin*. Curr Biol. 1998 Feb 12;8(4):181-90.

Fang D, Hawke D, Zheng Y, Xia Y, Meisenhelder J, Nika H, Mills GB, Kobayashi R, Hunter T, Lu Z. *Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity*. J Biol Chem. 2007 Apr 13;282(15):11221-9. Epub 2007 Feb 7.

Funayama N, Fagotto F, McCrea P, Gumbiner BM. *Embryonic axis induction by the armadillo repeat domain of beta-catenin:*

evidence for intracellular signaling. J Cell Biol. 1995 Mar;128(5):959-68.

Hart M, Concordet JP, Lassot I, Albert I, del los Santos R, Durand H, Perret C, Rubinfeld B, Margottin F, Benarous R, Polakis P. *The F-box protein beta-TrCP associates with phosphorylated beta-catenin and regulates its activity in the cell*. Curr Biol. 1999 Feb 25;9(4):207-10.

Henderson BR. *Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover*. Nat Cell Biol. 2000 Sep;2(9):653-60.

Hino S, Tanji C, Nakayama KI, Kikuchi A. *Phosphorylation of beta-catenin by cyclic AMP-dependent protein kinase stabilizes beta-catenin through inhibition of its ubiquitination*. Mol Cell Biol. 2005 Oct;25(20):9063-72.

Kramps T, Peter O, Brunner E, Nellen D, Froesch B, Chatterjee S, Murone M, Züllig S, Basler K. *Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex*. Cell. 2002 Apr 5;109(1):47-60.

Kelly C, Chin AJ, Leatherman JL, Kozlowski DJ, Weinberg ES. *Maternally controlled (beta)-catenin-mediated signaling is required for organizer formation in the zebrafish*. Development. 2000 Sep;127(18):3899-911.

Kennedy MW, Cha SW, Tadjuidje E, Andrews PG, Heasman J, Kao KR. *A co-dependent requirement of xBcl9 and Pygopus for embryonic body axis development in Xenopus.* Dev Dyn. 2010 Jan;239(1):271-83.

Kitagawa M, Hatakeyama S, Shirane M, Matsumoto M, Ishida N, Hattori K, Nakamichi I, Kikuchi A, Nakayama K, Nakayama K. *An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin.* EMBO J. 1999 May 4;18(9):2401-10.

Lamonica K, Grabel L. *The planar cell polarity pathway and parietal endoderm cell migration.* Methods Mol Biol. 2012;839:187-200.

Lekven AC, Thorpe CJ, Waxman JS, Moon RT. *Zebrafish wnt8 encodes two wnt8 proteins on a bicistronic transcript and is required for mesoderm and neurectoderm patterning*. Dev Cell. 2001 Jul;1(1):103-14.

Li FQ, Mofunanya A, Fischer V, Hall J, Takemaru K. *Nuclear-cytoplasmic shuttling of Chibby controls beta-catenin signaling*. Mol Biol Cell. 2010 Jan 15;21(2):311-22. Epub 2009 Nov 25.

Mayden RL, Tang KL, Conway KW, Freyhof J, Chamberlain S, Haskins M, Schneider L, Sudkamp M, Wood RM, Agnew M, Bufalino A, Sulaiman Z, Miya M, Saitoh K, He S. *Phylogenetic relationships of Danio within the order Cypriniformes: a framework for comparative and evolutionary studies of a model species*. J Exp Zool B Mol Dev Evol. 2007 Sep 15;308(5):642-54.

Mieszczanek J, de la Roche M, Bienz M. *A role of Pygopus as an anti-repressor in facilitating Wnt-dependent transcription*. Proc Natl Acad Sci U S A. 2008 Dec 9;105(49):19324-9. Epub 2008 Nov 26.

Miller TC, Rutherford TJ, Johnson CM, Fiedler M, Bienz M. *Allosteric remodelling of the histone H3 binding pocket in the Pygo2 PHD finger triggered by its binding to the B9L/BCL9 co-factor*. J Mol Biol. 2010 Sep 3;401(5):969-84. Epub 2010 Jul 14.

Moon RT, Kohn AD, De Ferrari GV, Kaykas A. *WNT and beta-catenin signalling: diseases and therapies.* Nat Rev Genet. 2004 Sep;5(9):691-701.

Mo R, Chew TL, Maher MT, Bellipanni G, Weinberg ES, Gottardi CJ. The terminal region of beta-catenin promotes stability by shielding the Armadillo repeats from the axin-scaffold destruction complex. J Biol Chem. 2009 Oct 9;284(41):28222-31. Epub 2009 Aug 25.

Morin PJ. *beta-catenin signaling and cancer*. Bioessays. 1999 Dec;21(12):1021-30.

Mosimann C, Hausmann G, Basler K. *Beta-catenin hits chromatin: regulation of Wnt target gene activation*. Nat Rev Mol Cell Biol. 2009 Apr;10(4):276-86.

Nakamura Y, Umehara T, Hamana H, Hayashizaki Y, Inoue M, Kigawa T, Shirouzu M, Terada T, Tanaka A, Padmanabhan B, Yokoyama S. *Crystal structure analysis of the PHD domain of the transcription co-activator Pygopus*. J Mol Biol. 2007 Jun 29;370(1):80-92. Epub 2007 Apr 20.

Neufeld KL. *Nuclear APC*. Adv Exp Med Biol. 2009;656:13-29.

Nusse R. *Wnt signaling in disease and in development*. Cell Res. 2005 Jan;15(1):28-32.

Nusse R, Varmus HE. *Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome.* Cell. 1982; Nov;31(1):99-109.

Onizuka T, Yuasa S, Kusumoto D, Shimoji K, Egashira T, Ohno Y, Kageyama T, Tanaka T, Hattori F, Fujita J, Ieda M, Kimura K, Makino S, Sano M, Kudo A, Fukuda K. *Wnt2 accelerates cardiac myocyte differentiation from ES-cell derived mesodermal cells via non-canonical pathway.* J Mol Cell Cardiol. 2011 Nov 29.

Pannone G, Bufo P, Santoro A, Franco R, Aquino G, Longo F, Botti G, Serpico R, Cafarelli B, Abbruzzese A, Caraglia M, Papagerakis S, Lo Muzio L. *WNT pathway in oral cancer: epigenetic inactivation of WNT-inhibitors*. Oncol Rep. 2010 Oct;24(4):1035-41.

Pinson KI, Brennan J, Monkley S, Avery BJ, Skarnes WC. *An LDL-receptor-related protein mediates Wnt signalling in mice.* Nature. 2000 Sep 28;407(6803):535-8.

Pryor SE, Massa V, Savery D, Greene ND, Copp AJ. *Convergent extension analysis in mouse whole embryo culture.* Methods Mol Biol. 2012;839:133-46.

Qiu W, Chen L, Kassem M. *Activation of non-canonical Wnt/JNK pathway by Wnt3a is associated with differentiation fate*

determination of human bone marrow stromal (mesenchymal) stem cells. Biochem Biophys Res Commun. 2011 Sep 16;413(1):98-104. Epub 2011 Aug 22.

Riggleman B, Wieschaus E, Schedl P. *Molecular analysis of the armadillo locus: uniformly distributed transcripts and a protein with novel internal repeats are associated with a Drosophila segment polarity gene.* Genes Dev. 1989 Jan;3(1):96-113.

Rijsewijk F, Schuermann M, Wagenaar E, Parren P, Weigel D, Nusse R. *The Drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless*. Cell. 1987 Aug 14;50(4):649-57.

Schier AF, Talbot WS. *Molecular genetics of axis formation in zebrafish*. Annu Rev Genet. 2005;39:561-613.

Sokol SY. *Wnt signaling and dorso-ventral axis specification in vertebrates.* Curr Opin Genet Dev. 1999 Aug;9(4):405-10.

Sustmann C, Flach H, Ebert H, Eastman Q, Grosschedl R. *Cell-type-specific function of BCL9 involves a transcriptional activation domain that synergizes with beta-catenin.* Mol Cell Biol. 2008 May;28(10):3526-37. Epub 2008 Mar 17.

Takemaru K, Fischer V, Li FQ. *Fine-tuning of nuclear-catenin by Chibby and 14-3-3.* Cell Cycle. 2009 Jan 15;8(2):210-3. Epub 2009 Jan 12.

Tao Q, Yokota C, Puck H, Kofron M, Birsoy B, Yan D, Asashima M, Wylie CC, Lin X, Heasman J. *Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in Xenopus embryos*. Cell. 2005 Mar 25;120(6):857-71.

Taurin S, Sandbo N, Qin Y, Browning D, Dulin NO. *Phosphorylation of beta-catenin by cyclic AMP-dependent protein kinase*. J Biol Chem. 2006 Apr 14;281(15):9971-6. Epub 2006 Feb 13.

Tian Q, Feetham MC, Tao WA, He XC, Li L, Aebersold R, Hood L. *Proteomic analysis identifies that 14-3-3zeta interacts with*

beta-catenin and facilitates its activation by Akt. Proc Natl Acad Sci U S A. 2004 Oct 26;101(43):15370-5. Epub 2004 Oct 18.

Tolwinski NS, Wehrli M, Rives A, Erdeniz N, DiNardo S, Wieschaus E. *Wg/Wnt signal can be transmitted through arrow/LRP5,6 and Axin independently of Zw3/Gsk3beta activity*. Dev Cell. 2003 Mar;4(3):407-18.

Van Ooyen A,Nusse R. *Structure and nucleotide sequence of the putative mammary oncogene int-1; proviral insertions leave the protein-encoding domain intact.* Cell. 1984; Nov;39(1):233-40.

Verheyen EM, Gottardi CJ. *Regulation of Wnt/beta-catenin signaling by protein kinases.* Dev Dyn. 2010 Jan;239(1):34-44.

Woods IG, Wilson C, Friedlander B, Chang P, Reyes DK, Nix R, Kelly PD, Chu F, Postlethwait JH, Talbot WS. *The zebrafish gene map defines ancestral vertebrate chromosomes*. Genome Res. 2005 Sep;15(9):1307-14. Epub 2005 Aug 18.

Wu X, Tu X, Joeng KS, Hilton MJ, Williams DA, Long F. *Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling*. Cell. 2008 Apr 18;133(2):340-53.

Zhu M, Tang D, Wu Q, Hao S, Chen M, Xie C, Rosier RN, O'Keefe RJ, Zuscik M, Chen D. *Activation of beta-catenin signaling in articular chondrocytes leads to osteoarthritis-like phenotype in adult beta-catenin conditional activation mice*. J Bone Miner Res. 2009 Jan;24(1):12-21.

LAST THREE YEARS PHD CURRICULUM VITAE

Name : Fabio Valenti
Date of Birth: 26/12/1982
Place of Birth: Palermo
Nationality: Italian
Fellowship:
3 years PhD fellowship sponsored by the Pennsylvania Department of Health.

Scientific Activities and Oral Presentations:
2011 Mid-Atlantic SDB Meeting 3-5 June 2011, University of Pennsylvania, Philadelphia, PA

Temple Developmental Biology Joint Lab Meetings, Neurolunch.12 febrary 2010- 19 november 2010.

Temple Developmental Biology Joint Lab Meetings, Evo- Devo.19 january 2011- 20 april 2011.

BOOKS, PAPERS AND ABSTRACTS PUBLISHED DURING THE PHD COURSE

Ciarcia R, Damiano S, Fiorito F, Granato G, Pagnini F, Mastellone V, Iovane V, Alfano L, **Valenti F**, Florio S, Giordano A. *Hydrocortisone attenuates cyclosporin A-induced nephrotoxicity in rats*.
J Cell Biochem. 2012 Mar;113(3):997-1004. doi: 10.1002/jcb.23429.