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The Increase in Maternal Expression of *axin1* and *axin2* Contribute to the Zebrafish Mutant *Ichabod* Ventralized Phenotype

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ABSTRACT

β-catenin is a central effector of the Wnt pathway and one of the players in Ca^{+}-dependent cell-cell adhesion. While many wnts are present and expressed in vertebrates, only one β-*catenin* exists in the majority of the organisms. One intriguing exception is zebrafish that carries two genes for β-*catenin*. The maternal recessive mutation ichabod presents very low levels of β-catenin2 that in turn affects dorsal axis formation, suggesting that β-catenin1 is incapable to compensate for β-catenin2 loss and raising the question of whether these two β-catenins may have differential roles during early axis specification. Here we identify a specific antibody that can discriminate selectively for β-catenin1. By confocal co-immunofluorescent analysis and low concentration gain-of-function experiments, we show that β-catenin1 and 2 behave in similar modes in dorsal axis induction and cellular localization. Surprisingly, we also found that in the ich embryo the mRNAs of the components of β-catenin regulatory pathway, including β-catenin1, are more abundant than in the Wt embryo. Increased levels of β-catenin1 are found at the membrane level but not in the nuclei till high stage. Finally, we present evidence that β-catenin1 cannot revert the ich phenotype because it may be under the control of a GSK3β-independent mechanism that required Axin′s RGS domain function. J. Cell. Biochem. 116: 418–430, 2015.

KEY WORDS: BETA-CATENIN; Wnt SIGNALING; Axin2-RGS DOMAIN; AXIS FORMATION; ZEBRAFISH

β-catenin is a central player in Ca^{+}-dependent cell-cell adhesion and in transduction of canonical-Wnt signaling. At the plasma membrane β-catenin mediates cell-cell adherence junction adhesion by interacting with α-catenin and type I Cadherins [Stepniak et al., 2009; Valenta et al., 2012]. In response to canonical-Wnt signaling, it is also cytoplasmically stabilized and translocates into the nucleus where it binds to DNA binding factors of the lef/tcf family to regulate transcription of a battery of canonical-Wnt-target genes [Cadigan and Waterman, 2012]. Due to its dual role in linking the adherence junctions to the cytoskeleton and mediating canonical-Wnt signaling, β-catenin plays a central role in many aspects of embryonic development, stem cell renewal and differentiation [Langdon and

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Mullins, 2011; Valenta et al., 2011]. It is further well known that aberrant β-catenin activity can produce disruptive effects in the embryogenesis and in adult tissues, resulting in developmental defects and cancer [MacDonald et al., 2009; Anastas and Moon, 2013].

In absence of canonical-Wnt signaling, the cytoplasmic accumulation of β-catenin is limited by binding of β-catenin to a protein complex termed the "destruction complex," which include Axin, APC, GSK3 and Casein Kinase1 proteins. In this complex, β-catenin is dually phosphorylated by the priming kinase CK1α and subsequently by GSK3-β [Aberle et al., 1997]. This phosphorylated β-catenin is recognized by β-TRCP, an F box protein that is a subunit of an ubiquitin ligase complex and targets it for degradation by the proteosome [Westfall et al., 2003]. Molecular and genetic evidence have suggested that intracellular Ca^{2+} levels lead to an alternate mechanism for regulation of β-catenin stability [Lyman Gingerich et al., 2005; Ma et al., 2009]. High Ca^{2+} levels in the cytoplasm can lead to the activation of a GSK3-independent degradation of β-catenin that includes the activity of APC and p53 [Liu et al., 2001; Matsuzawa and Reed, 2001]. Recently, has been shown chemokine GPCR signaling can modulate Ca^{2+} levels and inhibits β-catenin during zebrafish axis formation [Wu et al., 2012]. The current model suggest that this pathway limits β-catenin nuclear localization during pre-MBT stages and helps to shape the localized transcriptional activity of β-catenin in the cells of the prospective dorsal axis [Wu et al., 2012].

In human and mouse, there is a single gene encoding for β-*catenin,* but in zebrafish there are two different genes, with a high sequence identity [Bellipanni et al., 2006]. In zebrafish, the β-*cat2* gene is down-regulated in the embryos produced by female homozygous mutants in the line called *ichabod* [Bellipanni et al., 2006]. Beside the genetic role of β-cat2 in the development of zebrafish embryos, we do not know much on the regulation or function of the two independent β-catenins.

The first biological event in vertebrates controlled by nuclear localized β-catenin is the specification of the dorsal side of the embryo, which happens in the early blastula stage of *Xenopus*, zebrafish embryos and in the mouse primitive streak at gastrula stage [Kelly et al., 1995; Valenta et al., 2011]. In *Wild type* (*Wt*) zebrafish embryos at 128/256-cell stage, a small group of nuclei become positive for β−catenin and cells in this region induce the shield/organizer, which is required for subsequent pattern formation. Zebrafish females homozygous for the maternal mutation *ichabod* (*ich*) produce embryos, which lack a shield/organizer because β-catenin fails to accumulate in the nucleus of the prospective dorsal cells and this yields a severely ventralized phenotype [Kelly et al., 2000; Bellipanni et al., 2006]. Analysis of the *ich* mutants identified a mutation within the 5′ region of the second β-*catenin* gene (β-*cat2*, or *ctnnb2*–Zebrafish Information Network) and this mutation results in lower levels of maternal β-*cat2* expression in the egg. The two β-*catenin* genes code for highly similar proteins (92.9 identical, 96.4 similar) with only the C-terminal 92 amino acids show less similarity (70.2%). Despite the high homology between the two β-catenins, endogenous β-catenin1 (β-cat1, *ctnnb1*–Zebrafish Information Network) function is not able to compensate for β-cat2 loss in the *ich* embryo during early blastula stage suggesting that β-cat1 is not necessary for early dorsal induction [Bellipanni et al., 2006]. Hence, it is possible that at this early stage, the two zebrafish β-catenins may be differentially regulated.

It has been difficult to directly address questions on the function and localization of these two zebrafish β-catenin proteins during early blastula stage because there is a lack of specific antibodies for each β-catenin. However, we can pool together a series of indirect observations: over-expression of either one of the β-*catenin* gene of zebrafish, Xenopus β-*catenin* or Drosophila *armadillo* are able to completely rescue *ich* embryos [Kelly et al., 2000] suggesting that a reduction of the endogenous levels of β-catenin protein is responsible for the phenotype. However, these rescues do not tell much on the specificity of either one of the two β-*catenin* genes of zebrafish because they may result from non-physiological concentration of β-catenin, which in turn may affect the stoichiometry of other molecules of the canonical Wnt pathway [Kelly et al., 2000].

However, later in development, during gastrulation, in the margins of the ventro-lateral region of the embryo, Wnt8b/β-catenin signaling induces the expression of *vox* and *vent* that in turn repress *chordin* expression [Ramel and Lekven, 2004]. In *ich* Wnt8b is still able to repress *chd* via *vox* and *vent* because at this stage, both β-catenins respond to canonical-Wnt signaling. Thus, during gastrulation, in the margins of the ventro-lateral region of the embryo, both zebrafish β-catenins cooperate to induce meso-endoderm tissues [Bellipanni et al., 2006; Varga et al., 2007].

These results suggest two working hypothesis for the β-catenins activities during zebrafish early blastula stage: (i) is that the stability of the two zebrafish β-catenins may be differentially regulated for the dorsal specification of the embryo. So that only β-cat2 goes in the nucleus; or (ii) both β-catenins can enter the nucleus at this early stage but β-cat1 requires β-cat2 presence to be nuclear localized via an unknown mechanism. A third hypothesis, not mutually exclusive with the first two, is that something else in the regulation of canonical-Wnt pathway is changed in *ich*. In this work, we decided to further investigate these hypotheses by molecular and biochemical characterization of embryos produced by *ich* homozygous female (*ich* embryos).

We have identified a monoclonal antibody against mouse β-catenin C-terminal domain, which cross-reacts specifically with β-cat1. We used this antibody to determine that both β-catenins are nuclear localized at 128/256 cell stages, and that their nuclear localization mostly overlaps. In addition, both β-catenins present identical ability to rescue *ich* phenotype at the minimal expression dosage. However, β-cat2 seems to be the first and/ or more abundant β-cat variant that localizes in the nucleus. Moreover, we were surprise to found that β-cat1 protein and transcripts are more abundant in *ich* than in *Wt* pre-MBT embryo, that results in similar protein concentration of total β-catenins in *Wt* and *ich* embryos. To explain this unexpected result, we determined that one possible reason why β-cat1 is not able to compensate for the maternal loss of β-cat2 in *ich* embryo is because β-cat1 cytoplasmic stability is highly controlled by a large up-regulation of transcripts of the multi-protein complex composed of Axin1, Axin2, GSK3β and APC. It is still unclear if β-cat1 cytoplasmic stability is dependent to this complex or to some of its components, in fact, LiCl treatments, which block GSK3 activity, were not able to revert *ich* phenotype while

Ca^{2+} depletion or chemokine-GPCR signaling down-regulation can significantly rescue *ich* phenotype [Wu et al., 2012]. Interestingly, we show that over-expression of a Dominant Negative (DN) form of Axin2 in *ich* resulted in milder ventralized phenotypes or complete reversion into dorsalized phenotypes. This latest result may suggest that Axin2 is cooperating in a GSK3-independent pathway to control stability and localization of β-cat1 in the membrane.

MATERIALS AND METHODS

FISH HUSBANDRY

Embryos of the *AB* or *brass* strain of zebrafish (originally obtained from EkkWill Waterlife Resources, Gibbonston, FL), chosen because of delayed onset of pigmentation, were used as *wild type* (*Wt*) embryos in these experiments. *ichabod p1* (*ich*) embryos were obtained by breeding homozygous *ichabod* females with *Wt* males. The severity of *ich* phenotype was assessed in each experiment by growing at 24 hpf a portion of the laid embryos. Throughout this report, we will use the expression '*ich* s' to mean those embryos derived from homozygous *ichabod* mothers. All procedures involving zebrafish were conduct in accordance with Institutional Animal Care and Use Committee (IACUC) policies.

WESTERN BLOT

Western blot for anti myc antibody was conduct as in [Bellipanni et al., 2000]. We used as primary antibodies anti-pan-β-catenin (Sigma) 1/500 dilution, mAb-Cterminal-β-catenin (clone 14) (BD Transduction Laboratories) 1/500 dilution, mouse anti Flag (Rockland) 1/500 dilution, anti Myc (Santa Cruz) 1/500 dilution, mouse anti-α-Tubulin (SIGMA) 1/1000 dilution. Anti-mouse and anti-rabbit peroxidase-conjugated (Amersham) as secondary antibodies. Membranes were incubated with ECL plus (Amersham) for detection.

IMMUNOHYSTOCHEMISTRY

Immunohystochemistry with DAB detection for myc- or flag- tagged proteins was conducted as in [Bellipanni et al., 2010]. For Fluorescent Co-immunofluorescence was conducted as in [Macaluso et al., 2012]. Primary antibodies were diluted 1/50 for mAb-Cterminal-β-catenin(BD Transduction Laboratories) and 1/100 for anti-pan-β-catenin (Sigma). Secondary antibodies were diluted 1/200.

CONFOCAL IMAGING

For the confocal analysis, we used a laser-scanning confocal microscope (Leica DMRE, Leica Microsystems, Inc., Exton, PA). Both primary antibodies were detected using either a Cy5- or Alexa Fluor 488-conjugated secondary antibody. We used always the same parameters for both secondary antibodies for imagines detections.

LICL TREATMENT

Pools of 40–50 embryos laid in a time range of 15 min were raised to 2–4; 16–32 or 64/128 cells stages and then exposed to 0.3 M LiCl in embryo medium for 10 min followed by three washes in fresh embryo medium and finally moved to a new dish with fresh embryo medium. Embryos were analyzed for phenotype at 24 hpf or protein extracted at sphere stage to be processed for Western blot. Ventralized phenotypes were classified as in [Bellipanni et al., 2006], dorsalized phenotypes were classified as in [Kishimoto et al., 1997] with D5 as the most severe dorsalized phenotype and D1 as the least severe dorsalized phenotype.

CDNA CONSTRUCTS: β-CAT1CTERM-B-2; β-CAT2CTERM-B-1; MYC-DN-AXIN2

The *pCS2 + myc*-β *cat1* and *pCS2 + myc*-β-*cat2* were constructed by a two-step PCR approach. The *pCS2 + myc*-β-*cat1*-Cterm-β-*cat2* and *pCS2+myc*-β-*cat2*-Cterm-β *cat1* were prepared by cloning in frame the NcoI/XmaI fragments from one clone into the other clone such that the last 38 amino acids of the C-term domain of β-cat1 were in β-cat2 and vice versa. By the same two-step PCR approach, we prepared *pCSSN-DN-axin2*, in which a stop codon has been inserted in position 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 *axin* clones were obtained with a multi-step reaction: at the beginning the first 700 bp of the ORF of *axin1* and *axin2* were cloned in the correct frame in pCS2 + 2xFlag by PCR amplification of *pCSNC-axin1* or *-axin2* using a 5′ oligo, which introduced the EcoRI digestion site few nucleotides 5′ of the ATG of each cDNA. The obtained *pCS2 + 2xFlag-axin1* was cut with BamH1/NruI, and *pCS2 + 2xFlag-axin2* was cut with HindIII. The digested fragments of ~700 bp containing the 5′ of *axin1* or *axin2* fused in frame with the Flag were cloned using the same digestion sites in *pCSSN-axin1*, *pCSSN-axin2*, *pCSSN-DN-axin2*. The correct sequence and orientation of the clones was confirmed by sequencing and Western blot of in vitro translated and transcribed proteins.

INJECTIONS

mRNAs were synthesized in vitro using mMessage mMachine transcription kit (Ambion), stored in aliquots at −80°C. One to 2 nl of mRNA at either 20, 50 or 200 ng/μl mRNAs was injected at the one cell stage or at the 4–8 cell stage into a single blastomere. Embryos were analyze for phenotype at 24 hpf or fixed at the appropriate stages to be processed for immunohystochemistry.

REAL-TIME RT-PCR (QRT-PCR)

Samples were lysed with cold TRIzol (Invitrogen) and RNA was isolated according to the manufacturer's instructions. cDNA from one microgram RNA was obtained with SuperScript III First-Strand Synthesis System (lifetechnologies) for RT-PCR, according to the manufacturer's instruction. The cDNA was diluted to a final concentration of 100 ng/μl, and then used for the subsequent experiments. Real-Time PCR was carried out using a Lightcycler 480 II (Roche) with the 2x SYBR Green I master (Roche). Amplification conditions, after an initial pre-incubation step for 5 min at 95°C, consist of: 45 cycles of 95°C 25 s for denaturation, 55°C 25 s, 55°C 25 s and 72°C 25 s for annealing and elongation. Melting curve analysis was performed to check for a single amplicon. Lightcycler 480 II analysis software was used for determining crossing points. Data were analyzed by the $2^{\Delta\Delta CT}$ method, and presented as percent gene expression compared to Brass strain (*Wt*) embryos. Data were obtained and mediated from real-time experiments performed using

TABLE I. Primer Sequences for qRT-PCR Analysis

Gene	Forward primer	Reverse primer
β-*actin*	TCACCACCACAGCCGAAAG	AGAGGCAGCGGTTCCCAT
axin1	ACGGCATCCACTTGTTTAGG	AGCATCTTCTCGTTGCCATC
axin2	CAACCAAGCACATCCATCAC	TGCGAATGTAAGGAGCAGTG
β-*catenin1*	TTGTGAGGACCATGCAGAAC	AAGATGGCAAGCAGTCCTTC
gsk3-β	TGGTGGCCATTAAGAAGGTT	GACGAACAATGTTGCAGTGG
apc	ACCATGAAAGTCCCACCAAG	ATGGGCCTTGTCAGAGATTG

two cDNA preparations and all the samples reaction were in triplicate in each Real-Time PCR. β-actin was used as reference gene for calculation [Malafoglia et al., 2014]. In the case of *axin1* and *axin2,* a second biological replica was tested. Primer sequences are listed in Table I.

RESULTS

β-CATENIN1 LOCALIZATION IN EARLY BLASTULA STAGE ZEBRAFISH EMBRYOS

To determine if both β-catenins are nuclear localized at blastula stage, we searched for antibodies specific for each β-catenin. We identified a β-catenin monoclonal antibody clone 14 Mab, BD Biosciences, raised against the C-terminal domain of mouse β-catenin. This antibody recognizes an epitope region, in zebrafish β-cat1, with the highest divergence between the zebrafish β-catenins. We tested this antibody specificity in Western blots against β-cat1, β-cat2 in vitro transcribed, translated, in zebrafish embryo protein extracts, where either myc-β-cat1 RNA or myc-β-cat2 RNA have been injected at one cell stage. In both conditions, the C-term- β-catenin antibody selectively recognized β-cat1 Figure 1A and B.

To determine the sub-localization of β-cat1 in early blastula and gastrula zebrafish embryos, we used this antibody in whole mount immunohistochemistry assays in zebrafish embryos at different embryonic stages (Fig. 1C). At 256-cell stage both the anti C-terminal β-catenin antibody and an anti pan-β-catenin antibody (SIGMA) detected zebrafish β-catenins in a few nuclei of the embryo. In embryos at 256-cell stage, only about one third were positive for either one of the two antibodies (Table II). This is probably a technical limitation, also seen by others, where 15% of the analyzed embryos at 128-cell stage showed nuclear staining [Dougan et al., 2003]. In the two older stages, we did observe the percentage of embryos having at least one positively stained nucleus increased steadily, at high stage 70–90% of the embryos showed nuclear staining, while at

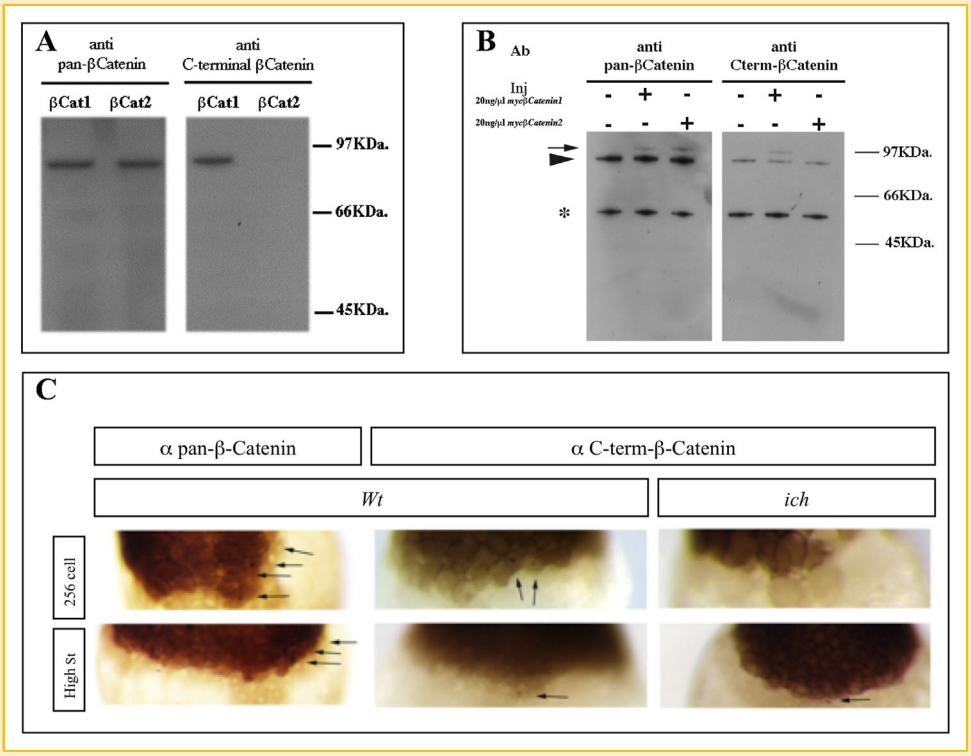


Fig. 1. Identification of a β-cat1 specific antibody. We have identified a mAb-Cterminal-β-catenin that was made by using the C-term region of mouse-β-cat as antigene. The C-term region of mouse-β-cat is much more similar to β-cat1 than β-cat2 of zebrafish. A. We have tested the ability of this antibody to recognize selectively β-cat1 but not β-cat2 in Western blot assay in which in vitro transcribed and translated β-cat1 and β-cat2 were loaded. The left panel shows the Western blot using a pan-β-catenin antibody in which both β-catenins have been detected, the right panel shows an identical blot but reacting with the C-terminal β-catenin antibody in which only β-cat1 is reacting. In B, we tested the ability of the C-Term β-catenin antibody to recognize selectively β-cat1 translated in vivo, to do so we injected 40 ng/μL myc-tagged *β-cat1* or *β-cat2* mRNAs in *Wt* embryos at one cell stage and extract the proteins from 128 cell stage and sphere stage embryos. The arrow indicates the myc-tagged β-catenins, the arrowhead indicates the endogenous β-catenins and the asterisk the alpha tubulin for loading control. C. Immunohistochemistry of *Wt* and *ich* embryos at 256 cell stage and high stage using the C-terminal β-catenin antibody or the pan-β-catenin antibody. Arrows indicate positive nuclei.

TABLE II. Localization of Total β-catenin Population or β-cat1 in *Wt* or *ich* Mutant at Different Developmental Stages

	α-pan-β-catenin		α-C-term-β-catenin			
	Wt		*Wt*		*ich*	
	nuclear	membrane	nuclear	membrane	nuclear	membrane
256 cell st.	10/29	29/29	8/32	32/32	0/68	68/68
High st.	9/11	11/11	6/9	9/9	3/30	30/30
50% epiboly	11/11	11/11	9/10	10/10	14/14	14/14

50% epiboly all the embryos tested had at least 1 nucleus positive (Table II). The anti C-terminal β-catenin antibody was able to detect β-cat1 at the cell membrane of *ich* embryos at 256-cell stage, while it did not detect β-cat1 in the nuclei. At high stage, 10% of *ich* embryos had nuclei positive for β-cat1 and at 50% epiboly all *ich* embryos analyzed showed β-cat1 in the nuclei (Table II). These data show, we have identified a commercial monoclonal antibody, which cross-reacts specifically with β-cat1 but not with β-cat2. This antibody allows us to specifically distinguish the β-cat1 pool and compare it with the total zebrafish β-catenin pool identified by the anti-pan-β-catenin antibody. Using this anti C-term- β-catenin antibody, we showed that β-cat1 is nuclear localized in the prospective dorsal side of *Wt* zebrafish embryos from the beginning of mid-blastula transition, while in *ich* mutant embryos β-cat1 begins to be nuclear localized only at high stage, in time to respond to ventro-lateral signal of Wnt8b and induce the expression of *vox* and *vent* in order to repress the expression of the dorsal gene *chordin* [Bellipanni et al., 2006; Ramel and Lekven, 2004; Varga et al., 2007].

β-CAT1 AND β-CAT2 NUCLEAR LOCALIZE IN THE SAME CELLS

In order to test if the nuclei positive for β-cat1 and β-cat 2 overlap, and to determine if both β-catenins translocate synchronously into the nuclei, we analyzed whole mount zebrafish embryos at 256-cell stage, high and sphere stages by confocal microscopy after co-immunofluorescence (Co-IF) (Fig. 2). In these studies, we used the anti C-term- β-catenin antibody conjugated with a goat anti-mouse- Cy5 antibody and the anti-pan-β-catenin antibody conjugated with a goat anti-rabbit-FITC antibody. Thus, any green stained nuclei and membranes would be recorded as positive for β-cat2, any yellow staining would indicate either β-cat1-only localization or β-cat1 and β-cat2 co-localization, while we expected to detect virtually no red stained membrane or nucleus.

Interestingly, the Co-IF assay analyzed by fluorescent confocal microscopy was shown to be much more sensitive than DAB staining, with 85% of the embryos presenting at least 1 nucleus positive for the β-catenins (Tables II and IV). About 65% of the embryo at 256 cell stage had 1–3 nuclei positive with the anti-pan-β-catenin antibody and 35% had 4–6 nuclei positive. At high and sphere the area of cells that had nuclei positive for anti-pan-β-catenin antibody was increasing up to nine cells (Table IV). At 256-cell stage, 55% of the embryos show more nuclei positive for the anti-pan-β-catenin antibody (green) than double staining (yellow). The percentage of embryos demonstrating nuclei positive for only the anti-pan-β-catenin antibody was reduced to 28% and 18% in the high and sphere stages, respectively. As expected, no red-only nuclei were identified in these assays. These studies together show that at early stages of development both β-catenin proteins are nuclear localized, but β-cat2 either may be more abundant of β-cat1 or may have a faster response to canonical-Wnt signaling and hence localize more abundantly in the prospective dorsal cell nuclei as

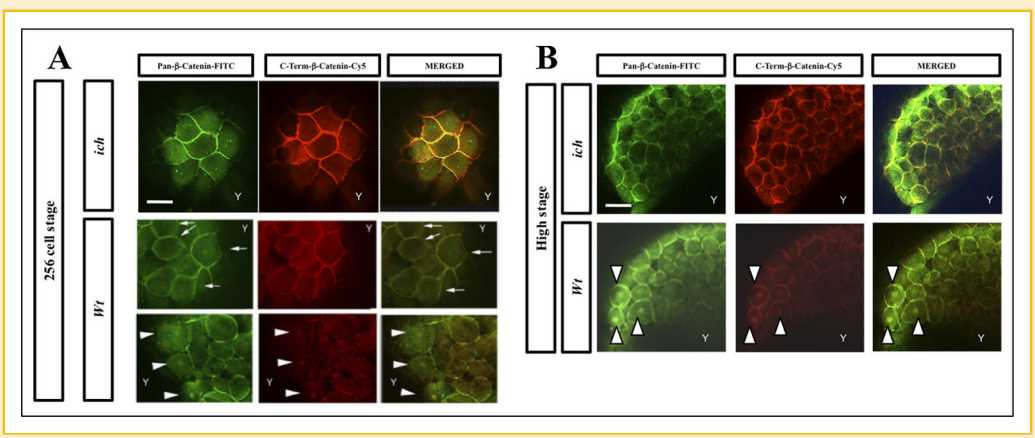


Fig. 2. Confocal co-immunohistochemistry analysis of β-catenin localization. *Wt* and *ich* embryos at 256 cell stage (A) and high stage (B) developmental stages with the two fluorescent-antibodies pan-β-catenin-FITC, C-term-β-catenin-CY5 and the two images merged. Arrows mark the FITC-only positive cells, arrowheads mark the double positive cells. Scale bar = 40 microM.

TABLE III. Distribution of all β-catenins Population and β-cat1 Positive Nuclei in *Wt* Embryos at Different Developmental Stages

256 Cell Stage		*High Stage*		*Sphere Stage*	
Nuclei + for α-pan β-cat/α-C-term-β-cat1 Staining	Number of embryos with the same ratio	Nuclei + for α-pan β-cat/α-C-term-β-cat1 Staining	Number of embryos with the same ratio	Nuclei + for α-pan β-cat/α-C-term-β-cat1 Staining	Number of embryos with the same ratio
1:1	7/17	1:1	2/15	1:1	1/21
2:0	3/17	2:1	1/15	2:2	3/21
3:1	1/17	2:2	1/15	3:2	2/21
3:3	1/17	3:0	2/15	3:3	4/21
4:2	1/17	3:3	2/15	4:3	1/21
5:2	2/17	4:0	1/15	4:4	3/21
6:3	1/17	4:3	1/15	5:4	1/21
6:6	1/17	4:4	1/15	6:6	2/21
		5:4	1/15	7:7	1/21
		5:5	1/15	8:8	2/21
		6:6	1/15	9:9	1/21
		7:7	1/15		

compared to β-cat1 (Fig. 2). Later, in post-MBT stages, the percentage of embryos containing nuclei positive for both antibodies increased significantly (Fig. 2; Tables III and IV). These results are consistent with our previous reports that both β-catenins act redundantly during late blastula stages to induce meso-endoderm fates in the margins of ventro-lateral territories of the embryo [Bellipanni et al., 2006; Ramel and Lekven, 2004]. While, the possible synchronous co-localization of both β-catenins at 256 cell stage rises the question of why β-cat1 is not able to compensate for β-cat2 loss in the *ich* embryo.

TOTAL β-CAT LEVELS ARE SIMILAR IN WT AND IN *ICHABOD* EMBRYO

In our initial immunohistochemistry analysis with the anti-β-cat1 and anti C-term-β-catenin antibody (Figs. 1C and 3 C), we noticed faster and stronger DAB staining at the membrane level in *ich* with respect to *Wt* embryos. To determine if the RNA levels of β-*cat1* are increased in the *ich* mutant embryo, we analyzed the relative quantity of β-*cat1* RNA using Real Time-PCR (qRT-PCR) at distinct developmental stages including a pre-MBT stage (2–8 cell stage), a post-MBT stage (256–512 cell stage) and two later stages (sphere and shield stages). Results show that the maternal β-*cat1* RNA is approximately 15 times more abundant in *ich* as compared to *Wt* embryos. Just after MBT at 256–512 cell stage, there is approximately eightfold more β-*cat1* RNA in *ich* as compared to *Wt* embryos. While at sphere and shield stages, the levels of β-*cat1* RNA in *ich* are approximately 11–13 fold higher than that within *Wt* embryos (Fig. 3A). As there was an increase in β-*cat1* RNA in *ich,* we tested if this was reflected as an increase of β-cat1 protein level. Protein extracts from *Wt* and *ich* embryos at 128-cell stage and sphere stage were assayed by Western blot analysis using the anti C-term-β-catenin antibody and the anti pan-β-catenin antibody (Fig. 3B). Several dilutions of each sample were loaded in the same gel to determine the linear range of intensity of each band. Results from densitometric scans of the Western blot probed with the anti C-term-β-catenin antibody (Fig. 3B) indicated that β-cat1 level in *ich* embryos at 128 cell stage is approximately 140% more than that in *Wt* embryo, and at the sphere stage the β-cat1 level is approximately 43% more abundant than in *ich* embryo. Densitometric analysis of the Western blot probed with the anti pan-β-catenin antibody show that total β-catenin level in *ich* embryos at 128 cell stage is approximately the same of that in *Wt* embryo, while at the sphere stage the total β-catenin level is approximately 200% more abundant than in *ich* embryo. Figure 3C shows an animal view of *ich* and *Wt* embryos at 256-cell stage after immunohistochemistry using the anti C-term-β-catenin antibody (using identical exposure times for staining) and these studies show that the increased β-cat1 protein was observed accumulated at the plasma membrane.

Taken together, these experiments show that both β-*cat1* RNA and protein levels are substantially increased in *ich* embryos and that total β-catenin levels are similar between *ich* and *Wt* embryos. However, the higher concentration of β-cat1 results in its localization at the membrane, but not in the nuclei, and hence this increased level is not sufficient to rescue the resulting mutant phenotype.

TABLE IV. Confocal Analysis of Co-localization of Total β-catenin Population and β-cat1 in *Wt* Embryos or Ich Mutant Embryos at Different Developmental Stages

	Number of embryos with any nuclear localized β-cat		Percentage of embryos with overlapping nuclear α-pan β-cat/α-C-term-β-cat1 Staining		Percentage of embryos with more nuclei positive for α-pan β-cat then α-C-term-β-cat1 Staining	
	Wt	*ich*	*Wt*	*ich*	*Wt*	*ich*
256 Cell St.	17/20	0/10	45%	0%	55%	0%
High St.	16/21	0/10	72%	0%	28%	0%
Sphere St.	21/25	4/9	82%	100%	18%	0%

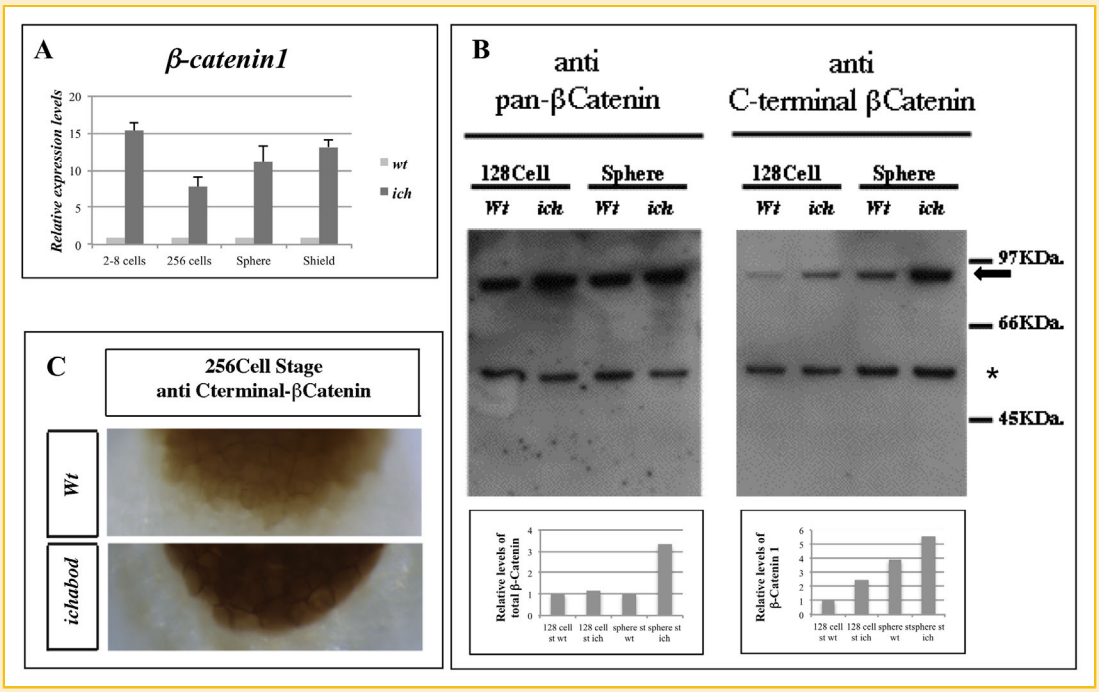


Fig. 3. β-catenin1 levels are increased in the *ich* embryo. A. Relative levels of ***β-catenin1*** mRNA in four developmental stages in *Wt* and *ich* embryos, analyzed by Real-time qRT-PCR. We used the relative quantification of ***β-catenin1*** mRNA at each stage in the *Wt* embryo to express as fold change its expression in the *ich* embryo, data are expressed as mean ± SEM. B. Western-blot of total protein extracts from *Wt* and *ich* embryos at 128 cells and Sphere developmental stages with the two antibodies pan-**β**-catenin and c-term- **β**-catenin (arrowhead). In the bottom, there are the relative quantification obtained using **β**-actin (*) for normalization. C. Animal view of 256 cell stage embryo stained with c-term-**β**-catenin antibody in *Wt* and *ich* embryos. Embryos were stained for the same time and show an increase of **β**-cat1 localization at the plasma membrane.

β-CAT1 AND β-CAT2 ARE FUNCTIONALLY EQUIVALENT IN DORSAL-INDUCING MECHANISMS DURING EARLY EMBRYOGENESIS

We reasoned that the failure of β-cat1 to rescue *ich* embryos may be due to two possible non-mutually exclusive mechanisms: (i) β-cat2 may play a functional role that cannot be compensated for by β-cat1; or (ii) cytoplasmic stability/membrane localization of β-cat1 is more stringently regulated than that for β-cat2. We explored each of these possibilities to try and understand why the increased level of endogenous β-cat1 levels could not rescue the *ich* phenotype. The first hypothesis implies that β-cat2 may play additional and/or unique roles in the induction of the dorsal side during early zebrafish embryogenesis. Therefore, one would expect that only β-cat2 could rescue *ich* phenotype. However, *ich* embryos can be completely rescued by injection of both zebrafish β-*catenins* RNAs, *Xenopus* β-*catenin* and Drosophila *armadillo* mRNAs [Kelly et al., 2000]. Since it is possible that over-expression of any β-*catenin* mRNAs and subsequent higher protein concentrations in the cells could have the artificial effect to titrate out some of the components involved in β-catenin cytoplasm stability, regardless the affinity of these different β-catenins to the factors they interact, we decided to determine the lowest concentration of mRNAs needed to rescue *ich* phenotype. We determined that injection of 1–2 pL of 20 ng/μl of either of these RNAs into one-cell stage *ich* embryos was the lowest concentration producing a shift of *ich* strong ventralized phenotype (V1) to milder ventralized/dorsalized phenotypes or to complete rescue (Table V). Then, we injected the same quantity of myc-tagged β-*cat1*, β-*cat2* RNAs and chimeric mRNAs obtained by C-terminal swap of the two β-catenins (myc-β-*cat1-C*β-*cat2* or myc-β-*cat2-C*β-*cat1*). β-cat1 and β-cat2 differ in small region at the N-term and in a larger domain within the C-terminus, and the C-terminus region has been correlated, using in vitro assays, with higher cytoplasmic stability of β-cat2 versus β-cat1 [Mo et al., 2009]. For each construct, all of injected embryos were divided in two batches, one was harvested at the 256 cell and sphere stages to be assayed with an anti-myc antibody to assess where the protein products of our injections were localized, while, the other batch was evaluated for phenotype at 24 hpf (Table V). Membrane and nuclear localization were observed to be very similar for each of these constructs, as well as the rescue effect with eventually β-*cat1* RNA being more efficient either in the rescue or nuclear localization. Immunohistochemistry assay showed membrane staining in 80–100% of the embryos and nuclear localization in about 15–29%. All mRNAs injections shifted the severe ventralized phenotype of *ich* (V1 and V1a) versus the milder V4 and V5 or even mild dorsalized phenotypes (Table V).

These experiments together suggest that β-*catenin1* RNA has the same ability as β-*catenin2* RNA to rescue *ich* phenotype and that the relative proteins similarly localize at the level of the membrane and nuclei. This further suggests that both β-catenins are able to regulate the same down-stream mechanisms.

TABLE V. Low dose β–*catenins* mRNAs over-expression in *ich* embryos

	ich	20 ng/μl β–*cat1* mRNA	20 ng/μl β-*cat2* mRNA	20 ng/μl *myc*-β–*cat1* mRNA		20 ng/μl *myc*-β–*cat2* mRNA		20 ng/μl *myc*-β–*cat1*-Cβ–*cat2* mRNA		20 ng/μl *myc*-β–*cat2*-Cβ–*cat1* mRNA	
V1	25/29	4/17	5/15	–		–		1/14		–	
V1a	3/29	2/17	4/15	–		–		–		–	
V2	1/29	3/17	2/15	–		2/14		2/14		–	
V3	–	1/17	–	–		–		1/14		2/15	
V4	–	–	–	2/19		–		–		3/15	
V5	–	7/17	4/15	11/19		10/14		7/14		8/15	
Dorsalized	–	–	–	6/19		2/14		3/14		2/15	
256 cell Stage	–	–	–	Nuclear 2/8	Membrane 8/8	Nuclear 1/10	Membrane 10/10	Nuclear 3/20	Membrane 16/20	Nuclear 5/17	Membrane 17/17
Sphere Stage	–	–	–	Nuclear 2/10	Membrane 8/10	Nuclear 3/10	Membrane 7/10	Nuclear 2/20	Membrane 19/20	Nuclear 4/17	Membrane 17/17

FACTORS RELATED TO β-CATENIN DEGRADATION SIGNALING ARE UP-REGULATED IN *ICH* EMBRYOS

To explore the mechanism that leads to increased concentration and membrane localization of β-cat1 in *ich* embryos, we analyzed using qRT-PCR, the relative RNA levels of factors known to be involved in this mechanism. We tested the RNA levels of *axin1*, *axin2/conductin*, *apc*, *gsk3*-β in *ich* with respect to *Wt* embryos in four stages of development, 2–8 cell, 256–512 cell, sphere and shield stages.

qRT-PCR analysis showed that the relative levels of both *axin1* and *axin2* RNAs were 15–17 fold more abundant in *ich* as compared to their levels in the *Wt* embryo at 2–8 cell stage. However, at 256–512 cell stage levels ranged only approximately 10 fold more in *ich* than in *Wt* (Fig. 4A and B). Maternal levels of *apc* RNA was 10 times higher in *ich* than in *Wt* and at the 256–512 cell stage, this difference decreased to five fold higher (Fig. 4C). *gsk3*-β mRNA was 20 fold higher more in 2–8 cell stage in *ich* than in *Wt* embryos (Fig. 4D) and this level was maintained in all the subsequent stages analyzed.

Similar as we did for β-cat1, we tried to ascertain if the increase in RNA levels observed was translated to the protein level. However, we were unable to identify specific antibodies that recognize Axin1, Axin2 and GSK3-β in either *ich* or *Wt* embryo extracts.

These studies suggest that the maternal contribution to the mechanism regulating β-catenin cytoplasmic stability is in place and actually more abundant in *ich* than in *Wt* embryo. It is therefore possible that this could be responsible for the β-cat1 failing to localize in the nucleus. However, it does not affect the ability of β-cat1 to localize at the membrane.

LICL TREATMENT STABILIZES β-CAT2 BUT NOT β-CAT1 AND IS NOT ABLE TO RESCUE *ICH* PHENOTYPE

It has been shown that a specific region in the C-terminal tail of β-catenin is involved in the regulation of the protein stability and that in-vitro β-cat1 rate of turn-over is faster than that of β-cat2. This suggests that β-cat1 is more sensitive to degradation than

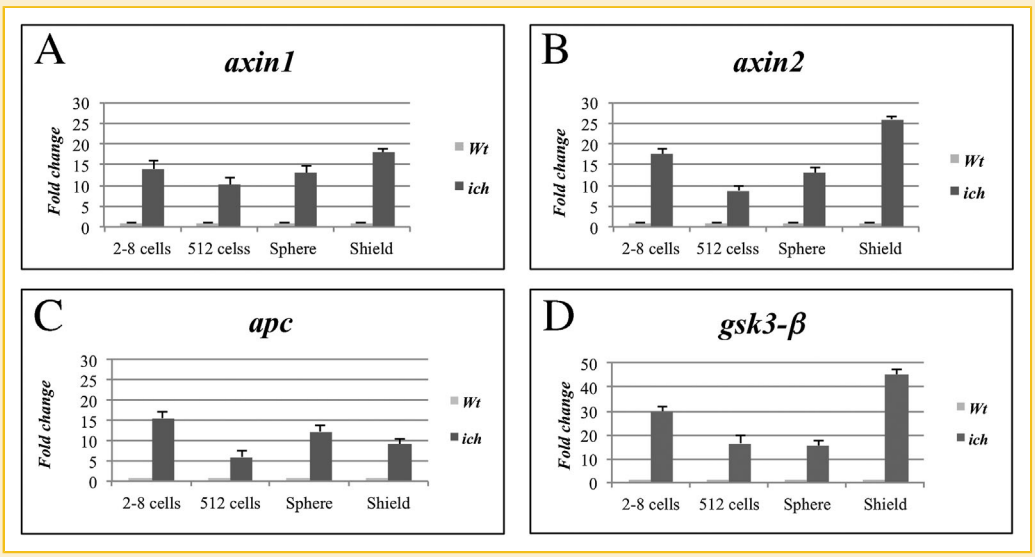


Fig. 4. Quantitative analysis of *axin1*, *axin2*, *apc* and *GSK3-β* mRNAs expression. Relative levels of *axin1*, *axin2*, *apc* and *gsk3-β* mRNAs in four developmental stages in the *ich* embryo compared to the *Wt* embryo, analyzed by qRT-PCR. β-actin was used for normalization within each experiment. We used the relative quantification of each mRNAs at each stage in the *Wt* embryo to express as fold change the expression of the same gene in the *ich* embryo, data are expressed as mean ± SEM.

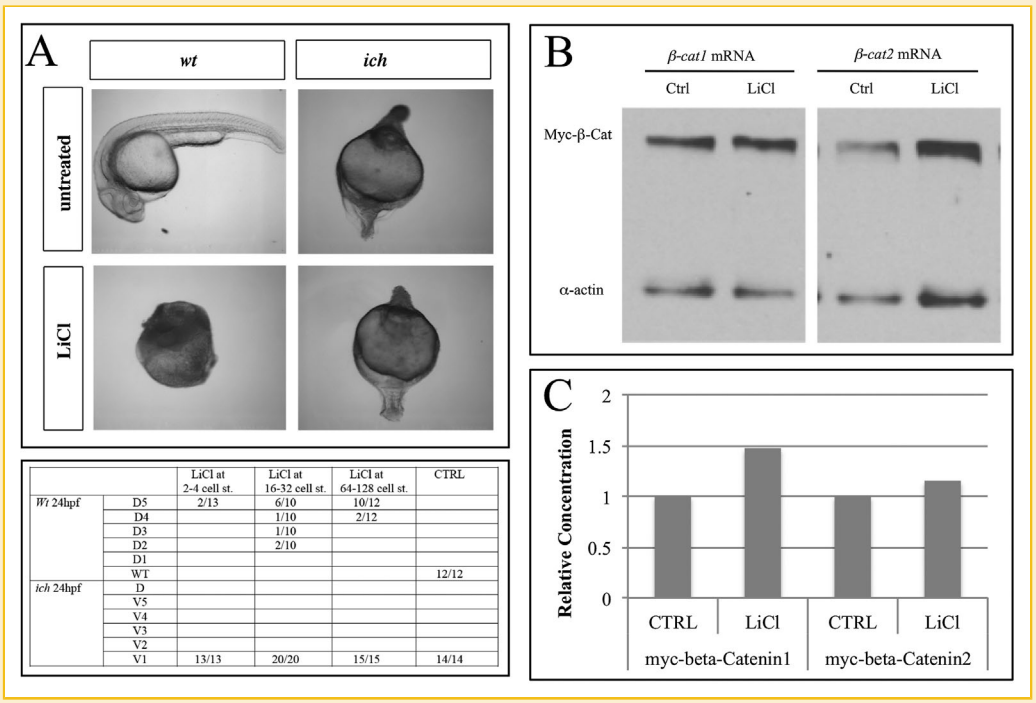
β-cat2 [Mo et al., 2009]. To test if this is also the case in vivo, we treated *Wt* and *ich* embryos at various pre-MBT stages with 0.3 M LiCl for 10 min using conditions that were previously published [Stachel et al., 1993]. Lithium is a known inhibitor of the protein kinase, GSK3 [Klein and Melton, 1996] and exposure to LiCl inhibits β-catenin phosphorylation by GSK3 that targets β-catenin for degradation, resulting in an increase in β-catenin cytoplasmic concentration. For this analysis, we used a batch of *ich* with a severe ventralized phenotype (all V1a V1b phenotypes) and with LiCl exposure, we expected to have some rescue of the severe *ich* phenotype. Control *Wt* embryos treated with LiCl at different embryonic stages from 2–4 to 128–256 cell stages were always strongly dorsalized by the treatment, while none of the *ich* batches showed sign of reversion of the phenotype (Fig. 5A).

To follow the effect in *Wt* embryos of LiCl treatment in β-catenin stability, we conducted a Western blot analysis using proteins extracted from 256 cell stage *Wt* embryo pools (∼20 embryos each, 2 experiments and 2 west-blot per experiment) that were injected at one cell stage with 20 ng/μl of *myc-β-cat1* or *myc-β-cat1* RNAs and treated with 0.3 M LiCl for 10 min at 32–64 cell stage. The resulting Western blot and densitometric analysis revealed that Myc-β-cat1 from LiCl treated embryos resulted in 48% increase in abundance of Myc-β-cat1 compared to untreated embryos. Myc-β-cat2, however, had a lower response to LiCl treatments being ∼18% more abundant than in untreated embryos. Thus in a *Wild type* context, the stability of both β-catenins is enhanced by LiCl treatments while in *ich* embryo, the inhibition of GSK3 activity do not produce any effect at the phenotypic level. These studies suggest that it is not the relative levels of cytoplasmic accumulation of β-catenin by GSK3 that is affected.

OVER-EXPRESSION OF A DOMINANT NEGATIVE FORM OF AXIN2/CONDUCTIN IS ABLE TO PARTIALLY RESCUE *ICH* PHENOTYPE

We next focused on an alternative way to affect membrane bound β-catenin by employing a mutant form of the zebrafish *axin2* gene that cannot dimerize [Chia and Costantini, 2005]. We generated a construct in which a stop codon (TGG>TGA; Trp635stop) was been inserted in position 1905 of the ORF of *axin2*, thus resulting in the complete elimination of the dimerization domain of zebrafish Axin2, the DIX domain, while the RSG (Regulator of G-protein Signaling) and GID domains are unaffected (Fig. 6A). This construct is substantially different from the *axin* construct used previously [Kelly et al., 2000] that contained only the GID2 domain and was not able to rescue *ich* embryo. This new construct contains the RGS domain that has been shown for Axin1 to be involved in the regulation of Wnt signaling in anteroposterior nervous system patterning but not during axis induction in Xenopus and zebrafish [Schneider et al., 2012].

We injected two different concentrations of this RNA (200 ng/μl and 50 ng/μl) in *ich* embryos either at the one cell stage or in one



		LiCl at 2-4 cell st.	LiCl at 16-32 cell st.	LiCl at 64-128 cell st.	CTRL
Wt 24hpf	D5	2/13	6/10	10/12	
	D4		1/10	2/12	
	D3		1/10		
	D2		2/10		
	D1				
	WT				12/12
ich 24hpf	D				
	V5				
	V4				
	V3				
	V2				
	V1	13/13	20/20	15/15	14/14

Fig. 5. LiCl treatments of *Wt* and *ich* embryos. Embryos were treated with 0.3 M LiCl for 10 min. at 2–4, 16–32 or 64–128 cell stages, washed with embryo medium, raised to 24 hpf and then used for phenotypic analysis. A. Pictures of 24 hpf LiCl treated and untrated *Wt* and *ich* embryos. In the bottom table, statistical analysis of the LiCl-treated embryos. Most of the *Wt* embryos became dorsalized after LiCl treatment. The ich embryos, instead, remained all ventralized. B. Western blot of protein extract from *Wt* embryos injected with *myc-β-catenin1* or *myc-β-catenin2* mRNAs and treated or not with LiCl, α-actin for normalization. C. Quantification of the Western blot in B.

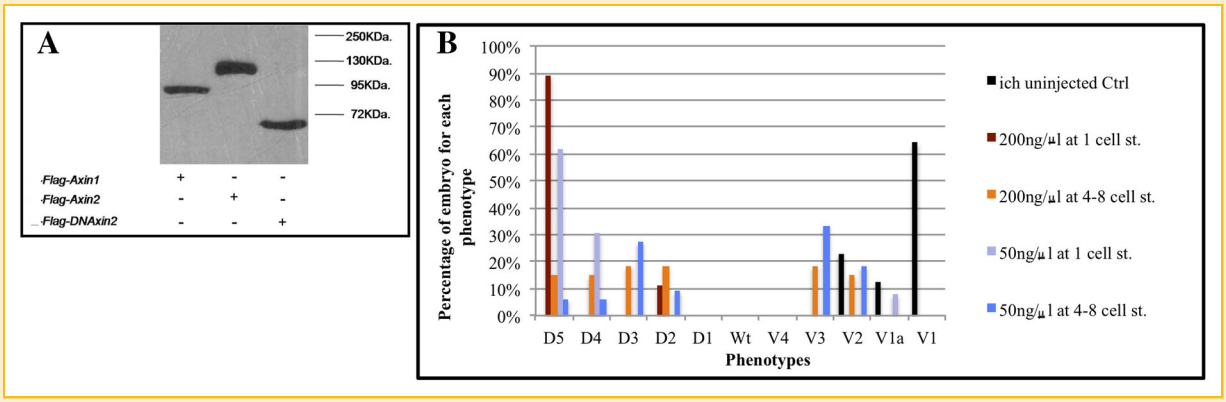


Fig. 6. Rescue of *ich* embryos by injection of *flag-DN-axin2* mRNA. A. Western blot of *Wt* embryos injected with the flag-tagged in vitro produced selected proteins, using an anti-flag antibody. The Flag-DN-axin2 protein is smaller than the *Wt*-flagged Axin2, as expected. B. Percentage of embryos in the ventralized, *Wt* or dorsalized phenotypes after injection of 50 or 200 ng/µl of *flag-DN-axin2* at 1 or 4–8 cell stage *ich* embryos. The higher dose of construct injected at one cell stage produce the most-severe dorsalized phenotype. Uninjected control n = 39 in black; embryos injected with 200 ng/µl in one cell stage, n = 9, in red; embryos injected with 200 ng/µl in4/8 cell stage, n = 27, orange. Embryos injected with 50 ng/µl in one cell stage, n = 13, violet color; embryos injected with 50 ng/µl in4/8 cell stage, n = 33, blue color.

blastomere at the 4–8-cell stage and scored for rescued phenotypes at 24 hpf. The batch of *ich* used for these experiments produced 77% of V1 phenotypes (light blue columns) and 23% of V3 phenotype embryos. Injections in one-cell stage always resulted in a complete reversion of the phenotype into very dorsalized embryos (red and green columns). The targeted injections of the RNA into a subset of cells by injections in one cell at 4–8 cell stage or the injection of lower concentrated mRNAs at one cell stage resulted in a more distributed range of phenotypes including more mild ventralized and dorsalized phenotypes (V2 and C2) in which a clear dorsal axis is formed (Fig. 6B), but we never recovered any *Wt* phenotypes in this studies.

The recovery of dorsal structures, more visible in the majority of the embryos injected at low doses of mRNA or in a subset of blastomers at later stages, suggest that this form of Axin2, that lacks the dimerization ability, can ameliorate the ventralized phenotype of the *ich* embryo acting during the first weave of Wnt/β-catenin activity during blastula stages.

DISCUSSION

β-catenin activity plays crucial roles during embryonic development and adult tissues homeostasis and deregulation of its function are frequently linked to birth defect disorders and tumorigenesis. The zebrafish embryo possesses two β-catenin genes as compared to other vertebrate model organisms and it is not fully understood what the roles of either of genes are. It is further possible that they may have independent roles similar to those of the Sys1 and Wrm1 genes of nematodes [Kidd et al., 2005]. In these studies, we sought to examine this crucial question and took advantage of the zebrafish model and the *ichabod* mutant, which harbors a mutation in the upstream regulatory region of the β-*cat2* gene and affects its levels of expression. These studies provided intriguing insights into differential regulation of the function of the two β-catenin proteins and we further discovered that *ich* mutant embryo is substantially different from a *Wt* embryo in the expression levels of other factors involved in Wnt/β-catenin signaling.

We first determined if there were any differences of either forms of β-catenin proteins to translocate into the nucleus and to temporally define this translocation at specific stages of development. To determine this nuclear translocation of both β-catenins, we were able to identify and took advantage of a specific commercial antibody for β-cat1 (α-C-term-β-catenin) and used it alone or in combination with an antibody that recognizes both β-catenins (α-pan-β-catenin). Using these two antibodies, we found that by the 256-cell stage both β-cat1 and β-cat2 are nuclear localized in *Wt* embryos. However, the staining from the α-pan-β-catenin antibody is visible in more nuclei than with α-C-term-β-catenin antibody, suggesting that β-cat2 is entering earlier into the nucleus though we cannot rule out whether there was more β-cat2 protein endogenously present. Confocal analysis showed that both β-catenins nuclear localize in the same side of the 256-cell stage embryo and confirmed that β-cat2 nuclear localization is present in more nuclei as compared to β-cat1. In later developmental stages (high and sphere), the number of nuclei positive for both antibodies increased.

Taken together, these data suggest that both β-catenins enter in the nucleus by the 256-cell stage and that β-cat2 might be the first and/or the most abundant protein to nuclear localize in the cells of the future dorsal side. More direct analysis of the β-cat2 localization awaits the availability specific antibody for β-cat2. Nevertheless, ours results indicate that β-cat1 nuclear localization is happening in temporal and spatial manner similar to that of β-cat2.

To further characterize *ich* embryos, we conducted Western blot analysis to examine the total β-catenin pool and the β-cat1 pool in both *Wt* and *ich* embryos. These assays unexpectedly revealed that the levels of total β-catenins in *ich* embryos are similar to that in *Wt* embryos due to a substantial increase in the maternal expression of β-*cat1*. Further immunohistochemistry analysis showed that in the 256-cell stage *ich embryo,* β-cat1 localizes exclusively in the plasma

membrane. We believe this may be the primary reason that even though β-cat1 levels were increased, the phenotype displayed by the *ich* embryos were not rescued. The surprising results encouraged us to investigate in more detail possible functional differences between the two β-catenin proteins. It was previously known that over-expression of β-*catenin* mRNAs from frog or fly as well as β-*cat1* were able to rescue *ich*. However, over-expression experiments hold the caveat that too high levels of proteins could mask subtle differences in substrate affinity and rescue capability and therefore we determined the minimal requirement in over-expression assays of β-*cat1* and β-*cat2* mRNAs for the rescue of *ich* phenotype. We found that the minimal concentration of mRNAs sufficient to rescue *ich* embryos were comparable for both β-*catenin* mRNAs. We also generated 2 chimeric constructs in which the 3′-ends of each β-*cat* ORF were swapped and using these constructs, the optimal rescue was obtained at the same concentration of mRNAs than the previous tests. All these constructs were tagged and immunohistochemistry of the injected embryos demonstrated that all the constructs had comparable ability to localize in the nucleus and at the membrane. These results further indicate that both β-catenins are functionally equivalent.

To understand if the regulation of β-catenin localization is impaired in *ich* mutants, we focused on known protein factors involved in β-catenin stability. We found that in the *ich* embryo during early embryogenesis the relative levels of mRNAs for the genes coding for the multi-protein complex that regulates β-catenins stability ("destruction complex" proteins), *gsk3*-β, *axin1* and *axin2* and *apc* are higher respect the same stages analyzed in *Wt* embryo. We were not able to confirm at the protein level the increase of expression of these factors as we did for β-cat1 probably because of the inability of the available commercial antibodies to recognize the endogenous proteins.

From our studies, it appears that within the *ich* embryo, not only β-cat1 protein and mRNA levels increased, but levels of mRNA for components of the destruction complex are also up-regulated. It is possible that the β-cat1 protein and mRNA levels are increased as a compensatory mechanism that requires an optimal amount of β-catenin for maintaining cell-cell adhesion in the developing embryo. Interestingly, the total protein levels of β-catenins are indeed very similar between *ich* and *Wt* embryo, however, this increase does not lead to a correct transduction of canonical-Wnt signaling, nuclear localization of β-*cat1* and formation of proper dorso-ventral axis in the mutant. The increased levels of mRNA for these factors involved in β-catenin stability in theory could result in higher phosphorylation levels of cytoplasmic β-catenin and therefore its degradation. It has been shown that the destruction complex can also co-localize at level of cell contact with β-catenin and it may be possible that β-cat1 membrane localization in *ich* is linked to increased levels of membrane-associated destruction complex proteins [Maher et al., 2009]. However, the lack of specific antibodies did not allow us to determine if the increased level of β-cat1 at the membrane in the *ich* embryo was due to a co-localization with the destruction complex proteins. Indeed additional detailed studies examining the phosphorylation status of β-cat1 in *ich* would be a part of future studies to address this important point.

What may cause these higher levels of expression of the destruction complex components? We know that at least one of the factors studied in our qRT-PCR, *axin2*, is a target of Wnt/β-catenin signaling [Jho et al., 2002; Leung et al., 2002]. A possible explanation could be that during oogenesis, high levels of β-*cat1* may allow for β-cat1 nuclear localization and therefore in induction of downstream gene expression.

To further understand the role of the Axin/Gsk3 complex in *ich*, we first used LiCl to inhibit GSK3 activity and this resulted in increased levels of both β-catenin proteins in *Wt* embryos but this had no effect on rescuing the *ich* phenotype. This is consistent with the findings of a previous report [Kelly et al., 2000], that showed that blockage of GSK3 by injection of zebrafish GBP RNA, thus depleting GSK3 in the embryo [Dominguez and Green, 2000], and injection of the RNA coding for the GID2 domain (domain of interaction with GSK3) of Axin1 of Xenopus, which binds and depletes GSK3 [Hedgepeth et al., 1999], had no effect in *ich* phenotype. However was observed that, similar to our LiCl treatment, over-expression of the same mRNAs in the *Wt* embryo produced strong dorsalized phenotypes [Kelly et al., 2000].

There is an increasing amount of evidence from *Drosophila* to *Xenopus* that indicate putative GSK3–independent pathways for the control of β-catenin levels in the cytoplasm [Topol et al., 2003; Tolwinski, 2009]. This fostered us to create a dominant negative form of Axin2, lacking the DIX domain [Chia and Costantini, 2005], thus unable to dimerize, DN-Axin2. We were able to partially revert the *ich* phenotype by injecting in vitro synthesized mRNA of this construct. This construct is different from that used previously [Kelly et al., 2000] because it contains not only the GID but also the RGS domain. The partial rescue obtained by the injection of this dominant negative construct reverts dorso-ventral asymmetry in the *ich* embryo when injecting low dose of mRNAs in one cell stage embryo or high dose in one blastomere of the 8-cell stage embryo. However, at high injection concentration and at the one-cell stage, we obtained highly dorsalized embryos because the nature of the construct in theory should interfere with the zygotic canonical Wnt/GSK3β pathway and produces high levels of β-catenin in the nucleus during sphere and 30% epiboly stages when the second wave of Wnt/β-catenin signaling represses the dorsal region therefore causing the very dorsalized phenotypes.

These and the previous results suggest that the effect of DN-Axin2 is not linked to a reduced GSK3β activity, instead the RGS (Regulator of G-protein Signaling) domain may be responsive of the partial rescue of *ich* embryo [Egger-Adam and Katanaev, 2010]. However, previous reports have shown that RGS domain of Axin1 is involved in mediating Wnt signaling in developmental post-zygotic steps but it is not affecting maternal stages of Wnt induced axis formation [Schneider et al., 2012]. We do not think that these results and ours are in contradiction because the changes we have seen in the *ich* embryo may have created a different molecular landscape in this mutant phenotype such that RGS domain of Axin may play a predominant role also at maternal stages. Moreover, the ability of DN-axin2 to partially rescue *ich* phenotype are reminding of with the findings of Wu et al., [2012] who were able to partially rescue *ich* by repressing the chemokine-GPCR pathway that induces in the cytoplasm high Ca^{2+} levels that in turn increase β-catenin

degradation in a GSK3 independent fashion, further suggest that the Axins may be also part of this GSK3-independent mechanism.

In summary, we have shown that both β-catenins play similar roles during blastula stages in the zebrafish embryo and that the embryo of the maternal mutation *ichabod*, depleted of maternal β-*cat2*, present other and intriguing changes at a molecular level. The *ich* embryo shows higher levels of the cytoplasmic components of the Wnt/β-catenin such that the total β-catenin levels in this mutant are comparable to the *Wt* embryo. Our studies put also in evidence a putative role of Axin in the GSK3-independent pathway of the regulation of β-catenin nuclear localization.

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