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In Vivo Study of Epithelial Adhesion *via* E-Cadherin-β- and α- Catenin-small GTP-Binding Proteins under T3 Regulation

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Abstract

Since the middle of last century there have been a plethora of studies trying to comprehend the fine control of adhesive contacts involved in establishment, maintainance and remodeling of epithelial architecture during animal development and adulthood. Depending on the experimental model, surprising and even contradictory data have been obtained. Among these, the *in vivo* systems outperform *in vitro* models in terms of understanding the animal biology.

It is known that thyroid hormones (THs) modulate energy metabolism, growth and development by independent mechanisms. Thyroid calorigenesis is influenced predominantly *via* thyroid hormone receptors (TRs) that mediate synthesis of mitochondrial respiratory complexes and cell membrane sodium-potassium ATPase; whereas it is debate matter if many of the TH effects over epithelial development are principally mediated *via* growth factors, TRs or transmembrane proteins.

Present work presents molecular evidences that T3 modulates the epithelial adhesive potential during gut remodeling in *X. laevis* development, differentially activating E-cadherin, β-catenin and α-catenin genes, and downstream, modulating small GTP-binding proteins involved in adhesive epithelial properties.

Keywords: E-cadherin; β-catenin; α-catenin; p120-catenin; Small GTPases; Epithelia; Cell-cell junction

Introduction

Cell adhesion molecules have critical roles in the establishment, maintainance and remodeling of epithelial architecture during animal development and adulthood. Their localization and concentration on the cell surface are deeply regulated by a delicate interplay of extracellular signals, cell membrane receptors and intracellular signaling messengers [1-4]. Adherens junctions (AJs) mediated by E-cadherin calcium-dependent glycoprotein and β- and α-catenin, as cytoskeletal linker molecules and its active turnovers, are particularly crucial for cell-cell contact survival and epithelial function [2]. These dynamic adhesion complexes capture and integrate signals from both extracellular and intracellular environment [2,5-7]. Indeed, there is a widespread knowledge over the THs control in animal growth and development. However, there is scarce information explaining the molecular mechanism(s) involved in cell shape control, tissue organization and remodeling *via* TH_S *in vivo*.

The routing of E-cadherin and catenins (β-, α- and p120) to the basolateral cell surface are critical to the establishment and maintenance of cell polarity and epithelial function [8-14]. Phosphorylation level of epithelial AJs molecules also causes deep changes in the expression pattern of E-cadherin and β-catenin during epithelial development [2]. Interesting, cell-cell contacts mediated by E-cadherin, β- and α-catenin change in response to extracellular TH levels through epithelia development, conducting tissular remodeling and adult cell contact strengthening [2,4,15-17].

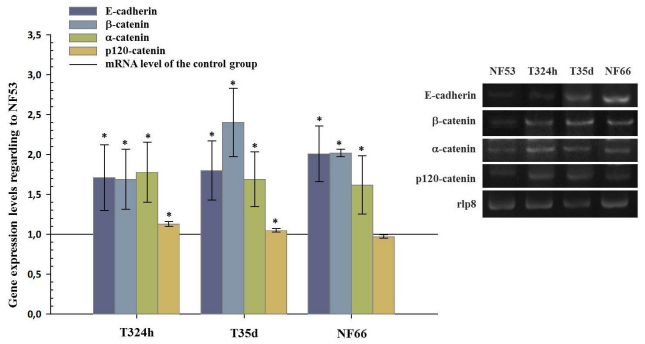

Figure 1: Gene transcription of E-cadherin and β- and α-catenin is highly and early upregulated by T3 in metamorphic *Xenopus laevis* gut. In contrast, p120-catenin remain unchanged. Bar diagram represent mRNA expression ratio respect to NF53-mRNA (n=5) from sqRT–PCR. NF66 (n=5) control and T3-treated (24 h (n=5) and 5 days (n=5) tadpoles). Rlp8 was used for normalization, mRNA=1 is the value at NF53; *Increases ≥ 50% were considered physiologically significant.

Whereas β and α-catenin facilitate interactions between classic cadherins and the actin cytoskeleton at AJs *in vivo*, p120-catenin

subfamily members induce the lateral (cis) clustering of cadherins [18]. P-120-catenin is a multisite substrate for both kinases and phosphatases [19], and it inhibits the cadherin endocytosis and thereby its degradation or surface back-recycling [20-24]. Nowadays, p120-catenin is considered as a master regulator of cadherin stability; [25] as well as an important modulator of Rho-GTPase activities-RhoA, Rac1 and Cdc42 [26,27] and gene transcription [28-31].

Small GTP-bound-activated proteins (G-proteins) are involved in cytoskeleton rearrangements, which impact on the coupled processes, such as cell adhesion, polarity, motility and gene regulation. Additionally, small GTPases in active (GTP-bound) versus inactive (GDP-bound) states are influenced by activating (e.g. GEFs) or inactivating (e.g. GDIs or GAPs) factors. Small-GTPase roles are often complex, since the crosstalk between players and cellular context remain not very well understood. Some studies suggest that the activation of Rac1 and Cdc42 regulate the cytoskeletal dynamic, promoting cell motility and invasion, while the RhoA stimulation increases cell adhesiveness [32]. However, Terry et al. [33] sustain that the signaling by the RhoAGTPase, a key regulator of epithelial cell behaviour, can stimulate opposing processes in the same cell at a given time. This is, while Rho promotes apical epithelial junction formation and constriction, it reduces basal cell adhesion and promotes cell spreading. Authors propose that such molecular mechanisms are a specific RhoA activation processes and spatially restricted. P114Rho-GEF, also named GEF 18, is required for RhoA activation at cell-cell contact sites, driving junction assembly and epithelial morphogenesis. Conversely, p114Rho-depletion stimulates non-junctional Rho signaling and induction of myosin phosphorylation along the basal domain [33,34].

Therefore, the goal of this work is focoused on an *in vivo* analysis of the T3-signaling pathway in cell-cell junctions mediated by complexes of E-cadherin-catenins-small GTP-binding proteins, and their influence in amphibian gut epithelia morphogenesis.

Materials and Methods

Animals

Xenopus laevis tadpoles were purchased from Liquanfish Aquarium, Buenos Aires, Argentina, bred in our laboratory and staged according to Niewkoop and Faber (1956).

3,5,3'-Triiodothyronine (T3) Bioassay

Premetamorphic tadpoles (stage NF53) were placed in 2l containers (18.4 cm diameter and 14.7 cm height) at a population density of 10 larvae/l at 20ºC ± 2ºC and in 12:12 h light-dark photoperiod, and were maintained in artificial pond water (pH 6.86, 149 μohms/cm-1 conductivity, 66.6 mg/l $CaCO_3$ hardness, 4.8 mg/l Ca2+, 7.2 mg/l Mg2+ and 5.5 mg/l dissolved oxygen) up to final metamorphosis (NF66).

Premetamorphic tadpoles NF53 were immersed in 7.5 nM T3-solutions (Sigma-Aldrich Co. St. Louis, MO, USA) for 24 h (T3 24 h) and 5 days (NFT35d), and T3-effects compared with those in control groups pre- and post-metamorphic stages (NF53 and NF66). Solutions were daily renewed and animals maintained without feeding. Bioassays were performed by quintuplicate, according to preliminary assayed conditions and previous reports [35].

Morphological and morphometric analysis

Control (NF53 and NF66) and T3-treated (24 h and 5d) tadpoles were fixed in a solution of 3% glutaraldehyde, 3% formaldehyde, satured solution of 1% picric acid in 0.1 M phosphate buffered saline (PBS), pH 7.4 at room temperature (RT). Under a stereomicroscope, digestive tracts were carefully dissected in esophagus, fore-, medium- and posterior-gut, and immersed in fresh fixative for 2 h at RT. Following, tissues were extensively washed in PBS and post-fixed in aqueous solution of 1% OsO_4 for 2 h, washed in PBS, dehydrated in increasing concentrations of acetone and embedded in araldite resin (Ladd Research Industries, Williston, VT, USA), at RT. After polymerization at 60ºC, tissues were sagitally sectioned at 0.5 μm with a Reichert Ultracut-S and stained with toluidine blue. Thin sections of 70 nm for electron microscopy analysis were done from pre-selected areas of each tissue under study.

Ultrastructural morphometric analysis of *X. laevis* larval stomach/fundus was made to analyze the AJs dynamics during both spontaneous and T3-induced metamorphosis—larval stomach or foregut for NF53 tadpoles, and fundus for NF66 and T3-treated (24 h and 5d) tadpoles. Quantitaive analysis was done from selected areas of the fundus with single columnar surface epithelium, and using sections from two regions. Images were registered with a TEM Philips EM300, systematically sampling the whole epithelial surface to imagining all cell-cell junction complexes. From each depth level 10 images were registered at 20,000X. Length and intercellular distance of each junction-type were measured by using the ImageJ 1.47a software (National Institutes of Health, USA).

Gene expression quantification by sqRT-PCR

Digestive tracts were dissected, their contents were carefully and completely removed and immediately used for RNA extraction. Nine tadpole digestive tracts were pooled for each of five replicates per treatment. Total RNAs were isolated using TRIzol reagent® (Invitrogen, Carlsbad, CA, USA). RNAs samples were purified in column by using a GeneJET kit (Thermo Scientific, Vilnus, Lithuania). Following, samples were resuspended in 50 μl diethyl pyrocarbonate treatedwater and kept at -20°C until use. RNAs quality was checked in 1.5% agarose gel and concentration were measured by UV-spectrometry at 260 and 280 nm.

First strand cDNAs were obtained from 5 μg of total RNAs, using a retrotranscription (RT) kit (ThermoScientific Inc., Maryland, USA) according to the manufacturer's instructions. The PCR reactions were developed from 5 μl of cDNA, 1 μM each of the gene-specific primers (Table 1), 0.8 mM dNTPs and DNA polymerase buffer (Fermentas International Inc., Vilnus, Lithuania) up to final volume 25 μl. The PCRs were carried out initializing the reaction heating to 95°C for 3 min. After that, 30 cycles were carried out under the following conditions: denaturation heating to 95°C for 30 s, annealing to 55–58°C (depending on primers) for 35 s, elongation to 72°C for 1 min, and final elongation to 72°C for 5 min. As internal control the ribosomal protein L8 (rlp8) constitutive gene was used.

Molecule	Primer	Sequence (5`→3`)	Amplicon size
E-cadherin	Fo	CGGATACACCTCCAGAAGGA	225
	Rv	GCACAGAGCCTTCAAAGACC	
β-catenin	Fo	AGATGCAGCAACTAAACAGGA	290

	Rv	GTACTGCATTTTGAGCCATCT	
α-catenin	Fo	CAGAGGATGACCAGCTTATTG	390
	Rv	GGCAGTACAAGGCAATTCT	
p120-catenin	Fo	CACTCGCTGATCACTTAACC	322
	Rv	GCAGCACGTATCTCTCTTTC	
occludin	Fo	GTGTGTGGCTTCAACTCTAC	319
	Rv	GAAGACCAGGCCTCCTATAA	
ZO1	Fo	GGGAAACCTCTATTGTCATCTC	263
	Rv	AGGCACATCCTCCTCATAA	
RhoA	Fo	GACATGGCAAACCGTATCT	200
	Rv	CCCAGAGGAGTATGGGAATA	
Rac1	Fo	GTCCCAACACTCCCATTATC	258
	Rv	GCCGACATCTTCTCTTTCTC	
Rap1	Fo	CTAGCCAGACAGTGGAATAAC	394
	Rv	CAGGAAGCATTGACACAATATG	
Cdc42	Fo	CTATCCACAGACAGACGTGTT	359
	Rv	GCAGCATACACTTGCGTTT	
TIAM	Fo	GGCGAAGACCACTCTTATTT	266
	Rv	GAGTTCAGCGTGGAGTTATT	
C3G	Fo	TGTCACCACTCACTACATACA	305
	Rv	GTCGTCGCCTTCACATAAC	
ARHGEF18	Fo	CCATTGTTCGGTCCCTAAAT	293
	Rv	GAAGCCATCTGCGTCATAG	
FRG	Fo	TCAGCCCAAACCGAAATC	376
	Rv	CCTGCTCACTACTCAAGAAAG	
p190RhoAGAP	Fo	CCGAAGGACCACTAAGAAAG	322
	Rv	CGGTAGCTCAGAGAAGAAAC	
ARHGAP12	Fo	CTTTGCCGTCTCGTTTCT	289
	Rv	GAGTGAGTAGAAGTCCGTTTG	
SPA1	Fo	GCTCGTGACCATGTTACTAC	474
	Rv	GTCTCACAACCACCAGATAC	
Rich	Fo	CCCAACATACAGAAGCAGAG	440
	Rv	GTCTCCAGCAGCATCATTAC	
PGGT1	Fo	GCAAACTGGAGGAGGTATTT	310
	Rv	ATGCCAGGCTCTCCTATAA	
Arp2	Fo	GCAATACGGGAATAGGGAAG	253
	Rv	GGGTTGGACACATGATACAG	
RT beta	Fo	GGACATTGGACAAGCTCCCA	197
	Rv	ACATGATCTCCATACAACAGCCTTT	
IFABP	Fo	CTTGGAACTTTCACCAGGAA	306
	Rv	TTGACATTTCAGGAACCACA	
rpl8	Fo	CGTGGTGCTCCTCTTGCCAAG	577
	Rv	GACGACCAGTACGACGAGCAG	

Table 1: Forward and reverse primers (Primer list).

Forward and reverse primers were designed based on sequences from indicated GenBank accessions and following, were checked to corroborate that there were no amplicons on the entire gene (Table 1).

The mRNAs levels were established by co-amplification of each interest gene with rlp8, using as template the RNA extracted from larval digestive tracts of NF53, NF66, T324h and T35d, and visualized in ethidium bromide-stained 2.5% agarose gel. The cDNA bands were visualized by a UV transiluminator Spectroline TE-312S (Spectronics Corporation, Westbury, NY) and registred using a digital camera. Bands intensity of each row, internal control and problem gene, were quantified by ImageJ software and intensities ratios were calculated. The control group value was considered as 1, whereas the treatment-group values were analysed regarding to control and plotted on a barr diagram.

The thyroid hormone receptor beta (RTβ) mRNA quantification was used as positive control of a T3-direct response gene, whereas the intestinal fatty acid binding protein (IFABP) mRNA was used as a positive control of T3 inhibitory-response gene [36].

Statistical analyses

Five biological replicates were analyzed, each consisting of nine pooled digestive tracts from control (NF53, NF66) and T3-treatment (T324h, T35d) groups.

The differences greater than 50% (between control and treated animals) were considered physiologically significant, according [37,38]. Difference between means was evaluated according by the [38] method. Raw transcript abundance values were used to calculate 95% confidence intervals (CIs) for each transcript-mRNA/NF53-mRNA ratio and P values for those ratios of transcript levels. Data are shown as confidence intervals (CIs) of target gene expression in treatment versus NF53 control (each expression level in turn relative to rlp8 reference gene).

In addition, since some genes under evaluation are undetectable during premetamorphic stages, the CIs were plotted relative at one (1) arbitrary value from the start point of the bioassays.

Results

T3 influence on epithelial cell-cell adhesion

Before evaluating the effect of T3 on genes of the junctional complexes molecules (AJs and TJs), two genes known to respond to T3 in intestinal tissue were examined. While T3 directly upregulates RTβ gene transcription [37,39,40] this hormone indirectly and negatively modulates the IFAPB gene [36].

Premetamorphic tadpoles treated for 24 h with T3 (7.5 nM) exhibit an increasing of 0.6-fold of RTβ mRNA levels, regarding untreated

NF53 tadpoles. These values reach more than 0.7-fold during the whole gut remodeling, after 5 days of treatment (Table 2). In contrast, the IFAPB-mRNAs levels remains unchanged at 24 h, decreasing approximately 40% at 5 days of T3-tratment.

In this study, we have analyzed the levels of mRNA expression of both AJs (E-cadherin, β -, α-and p120-catenins) and TJs (occludin and ZO1) molecules, under T3-exogenous hormonal treatment (Figure 1). Gene transcription of E-cadherin, β- and α-catenin was early (24 h) ~0.7-fold upregulated by T3. While E-cadherin and α-catenin reach a plateau at 5 days T3-post induction, β-catenin continues increasing, reaching 1.4-fold levels. Low levels of p120-catenin were detected in NF53 and remain virtually unchanged during T3-treatment as well as during spontaneous metamorphosis (NF66) (Figure 1). The responses of E-cadherin, β- and α-catenin genes were similar than the positive T3-responsive control gene (RTβ) at 24 h.

mRNA	$T3_{24h}$	$T3_{5d}$	NF_{66}
RTβ	1.6 ± 0.02*	1.74 ± 0.001*	1.42 ± 0.09*
IFABP	1.14 ± 0.185	0.66 ± 0.07*	1.03 ± 0.07

Table 2: T3-responsive intestinal gene expression index. Note: *Statiscally significant data ($p<0.05$).

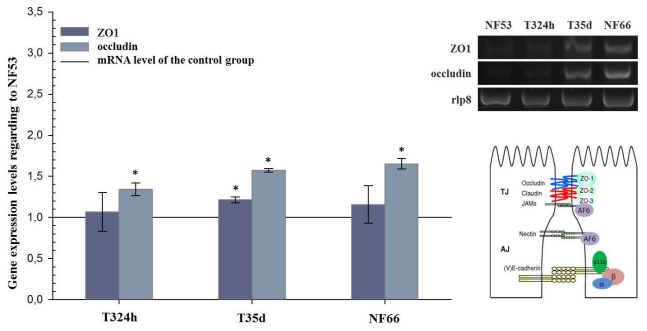

Figure 2: Gene transcription of occludin is highly upregulated in metamorphic *Xenopus laevis* gut at 5 days T3-treatment, whereas ZO1 only shows a small increase (0.2-fold) at this time. Bar diagram represent mRNA expression ratio respect to NF53-mRNA (n=5) from sqRT–PCR. NF66 (n=5) control and T3-treated (24 h (n=5) and 5 days (n=5) tadpoles). Rlp8 was used for normalization, mRNA=1 is the value at NF53; *Increases ≥ 50% were considered physiologically significant. Schematic comparative view showing the main molecules involved in two components of the apical adhesive complex of mature digestive tract (modified from Kooistra et al., 2007).

During the non-induced remodeling of the digestive tract (from NF53 to NF66), E-cadherin, β-catenin and α-catenin mRNAs were upregulated, increasing between 0.7 to 1-fold, being the anuran metamorphosis fully T3-depent. In contrast, mRNA levels of p120-catenin (Figure 1) and Arp2 actin-nucleation protein (not shown) remain practicaly unchanged at 24 h and 5 days' post T3-treatment, as well as during natural metamorphosis.

Unlike E-cadherin, β- and α-catenin genes involved in AJs formation, the transcription levels of some genes involved in TJs establishment were not significantly affected by T3-treatment. Thus, occludin mRNAs levels exhibit a light 0.3-fold increase at 24 h of T3-treatment, whereas ZO1 practically remain unchanged [41-46]. Notably, occludin expression becomes more significant at day 5, reaching a 0.6-fold increase, like the spontaneous metamorphosis. In contrast, ZO1 shows small increase (0.2-fold) under T3-treatment at 24 h and remain constant at 5 days, showing a similar behavior than in natural metamorphosis (Figure 2).

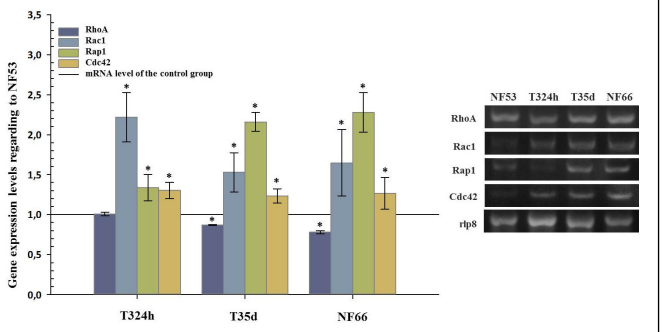

Figure 3: T3-responsive small GTP-binding proteins guide the reorganization of the actin cytoskeleton and the apical adhesive complexes. Transcription of Rac1 and Rap1 genes is highly upregulated by T3 during gut metamorphic remodeling of *Xenopus laevis*. While Rac1 principally mediates actin reorganization at 24 h of exogenous T3-treatment, Rap1 works at T3-5 days, coinciding with metamorphic climax. Bar diagram represent mRNA expression ratio respect to NF53-mRNA (n=5) from sqRT–PCR. NF66 (n=5) control and T3-treated (24 h (n=5) and 5 days (n=5) tadpoles). Rlp8 was used for normalization, mRNA=1 is the value at NF53; *Increases ≥ 50% were considered physiologically significant.

Assembly-disassembly of the apical adhesive complexes require reorganization of the actin cytoskeleton, mediated by small GTP-binding proteins (small GTPases and receptor tyrosine kinases, RTKs). The analysis of small GTPase-mRNA levels and those of their GEF and GAP partners shows a differential and tuned control during epithelial remodeling. Rac1 was 1.2-fold upregulated at 24 h post T3-induction whereas Rap1 and Cdc42 were only 0.3-fold upregulated, and RhoA stays invariable regarding to NF53 tadpoles. At 5 days of T3-treatment, RhoA was deeply decreased, whereas Rac1 fall up to 0.5-fold over the control and Rap1 significantly increased 1.2-fold regarding to the control level (Figure 3). In contrast, Cdc42 remains constant both during exogenous T3-induced and uninduced metamorphosis. All analysed GTPases exhibited similar behaviours both at exogenous 5-days T3-treatment and the spontaneous metamorphosis end (NF66).

In spontaneous metamorphosis, most the GTPase-GEF expression patterns showed similar behaviour-RhoA-GEF18; Rac1-TIAM and Rap-1C3G- reaching the highest levels after 5 days of T3-treatment, 0.5; 0.6 and 0.3-fold respectively (Figure 4). Cdc42-FRG expression was not detected under the different experimental conditions. Regarding the expression levels of the evaluated GTPase-GAPs, RhoA-p190 and Rac1-GAP12 displayed notable and significant differences to 5 days of T3-treatment [47-55]. While p190 decreased 0.2-fold, GAP12 increased 5.5-fold. In contrast, Rap1-SPA1 expression was not detected in the different experimental conditions, whereas Cdc42-Rich1 reached 1.5 level at NF66 (Figure 4).

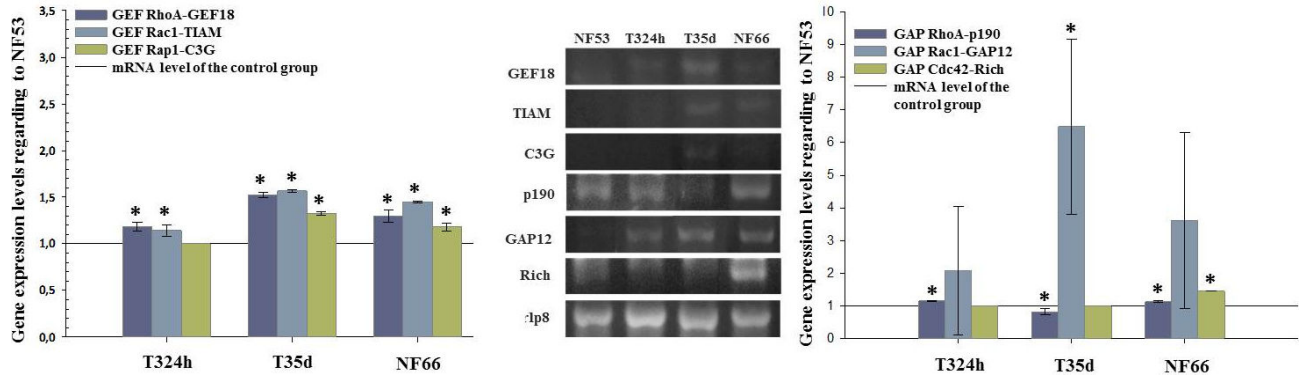

Figure 4: GEFs and GAPs T3-response of GTP-binding proteins. While gene transcription of the Rac1-TIAM/GAP12 pair significantly increases at T3-5 days, the RhoA-GEF18/p190-GAP pair shows opposite levels (GEF18 increase and p190-GAP decrease). Notice also that, the Rap1-C3G/SPA1 pair shows different behavior. C3G increases just at T3-5 days whereas SPA1 was not detected. For to Cdc42-FRG/Rich pair, only Rich lightly increased at the end of spontaneous metamorphosis. Bar diagram represent mRNA expression ratio respect to NF53-mRNA (n=5) from sqRT–PCR. NF66 (n=5) control and T3-treated (24 h (n=5) and 5 days (n=5) tadpoles). Rlp8 was used for normalization, mRNA=1 is the value at NF53; 0.5 fold-change.

Interesting, (Figure 5) clearly depicts the differential and complex response of each small-GTPase and its GEF/GAP pair through digestive tract remodeling of *Xenopus laevis* mediated by T3.

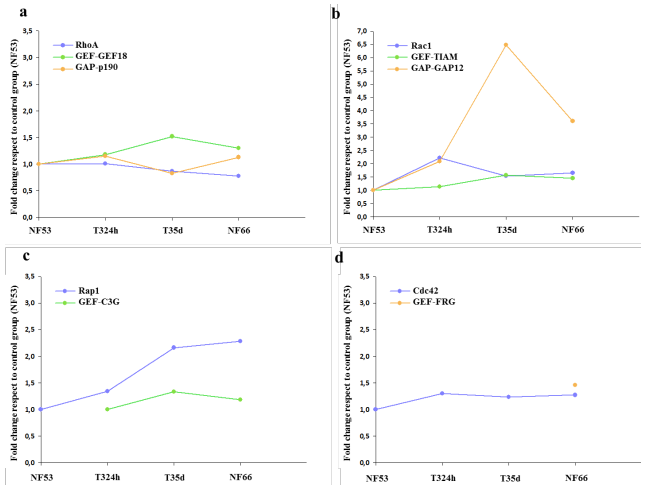

Figure 5: Gut mRNAs profile of GTP-binding proteins and their GEFs and GAPs during *X. laevis* natural and T3-induced metamorphosis.

Morphological and Ultrastructural Analysis

Molecular changes leading up to the gut remodeling, are also reflected at cellular and tissular levels of the whole organ (Figures 6-9).

Foregut mucosa of NF53 tadpoles is lined by a pseudostratified epithelium of 70 to 100 µm thickness with alternate ciliated and mucus cells below which is located a glandular-like epithelium surrounded by connective tissue. The mucosa, rest on thin muscular and serosa layers of approximately 10 µm thickness between both (Figure 6). After the spontaneous metamorphosis (stage NF66), the superficial epithelium of the mucosa becomes columnar (50 µm thickness) and beneath of it can be visualized as a folded glandular epithelium of variable thickness (60-120 µm). In this stage, the muscular layer of the mucosa separates the very well developed non-glandular sub mucosa from mucosa. Underneath, the muscular layer achieves higher development (20 µm) and longitudinal and circular layers are easily distinguished. Serosa layer develops to 30 µm thickness. This organ remodelling also is produced during exogenous T3-induction from 24 h of treatment, although non-mucosa muscular and folding were detected. At 5 days of T3-tratment, fundus morphology is very similar that those of NF66 (Figure 6).

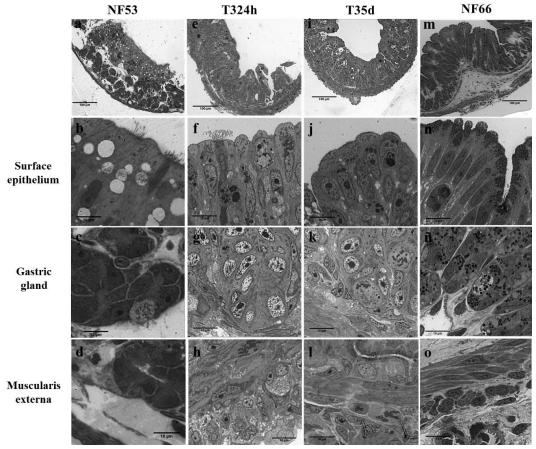

Figure 6: Sagital sections of *Xenopus laevis* larval fundus through both spontaneous (a-d; m-o) and T3-exogenous induced (e-l) metamorphosis. Toluidine blue, bar = 10 µm.

At ultrastructural level, epithelial cells of NF53 tadpoles are binded apico-basally by tight junctions (TJ), adherens junction (AJ) and desmosomes (Dms) (Figure 7). While TJs were indentified in 100% of

tissue sections, AJs and Dms only were registered in 77% and 54% of studied regions respectively (Figure 8).

The classical tripartite apical (TJ-AJ-D) (or apical junctional) complexes (AJC) are beneath 10%, because desmosomes localize more basally away from AJs (Figures 7-8). These AJCs increase to 77% in NF66, and frequently additional desmosomes appear towards the basal membrane. This AJCs-increase is a hallmark in epithelial barrier maturation. After 24 h of T3-treatment, although the Dms-number decreases, the AJCs lightly increase. In addition, the inter membrane space both AJs and Dms significantly increase, and numerous endocytic vesicles and membrane protrusions are observed (Figures 7-9).

After 5-days of T3-treatment the AJCs number continues increasing (~20%), whereas the AJs suddenly are reduced. However, the intermembrane distance has decreased, reaching average values of NF53 and NF66. Again, numerous endocytic vesicles (Figures 7-9), fagosomes and apoptotic cells are visualized (not shown).

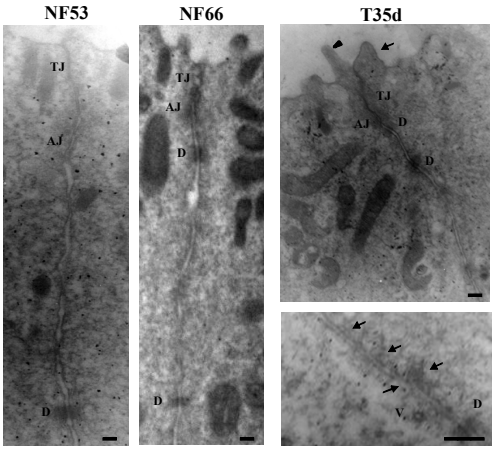

Figure 7: Apical Junction Complexes (AJCs) distribution during gut metamorphic remodeling. While the desmosomes (Dms) are located away from the apical tight (TJs) and adherens junctions (AJs) in NF53 stage (a), they are immediately beneath at NF66 stage (b). Bar: 100 nm. Gut remodeling induced by T3 shows membrane protrusion numerous and endocytic vesicles at 5 days of treatment (c-d).

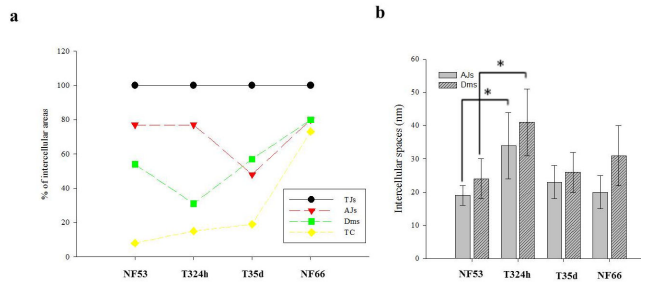

Figure 8: Establishment of the epithelial barrier in *Xenopus laevis* larval fundus. Curve and histogram represent the distribution of cell-cell junction types and the variations in intercellular space during metamorphosis natural and by exogenous T3 (a and b). a. Changes in frequency of the intercellular junction-types correlate with epithelial barrier remodeling mediated by T3. Desmosomes and apical junction complexes (AJCs) increase towars 5 days of T3-treatment and NF66 metamorphic tadpoles. b. Early, at 24 h of treatment, T3 significantly affects cell-cell distance of AJs and Dms. TJ: tight junction; AJ: adherens junction; Dm: desmosome. *p ≤ 0,05.

Agreeing with the epithelial barrier maturation, as ocurrs in the spontaneous metamorphosis end (NF66), a remarcable increase of AJCs is registered at 5 days of T3-treatment. This epithelial barrier remodeling can be correlated with changes in frequency and structural configuration of cell-cell junction types (Figures 6-9).

Discussion

Digestive tract remodeling during anuran metamorphosis is an excellent *in vivo* model to study tissue-architecture, cell differentiation regulation, and the transitions from a well organized larval mature epithelium, to a structural reorganization during metamorphosis until reaching the final adult mature epithelia. Anuran metamorphosis is developmentally similar to the postembryonic organogenesis in mammals, and it can be easily controlled by regulating the availability of a single factor, the thyroid hormone [35].

	Spontaneous metamorphosis				**T3-induced metamorphosis**			
	NF53		NF66		T324h		T35d	
	Length nm	Distance nm	Length nm	Distance nm	Length nm	Distance nm	Length nm	Distance nm
TJs	711 ± 22	-	576 ± 17	-	1,546 ± 66	-	768 ± 28	-
AJs	236 ± 14	19 ± 3	244 ± 65	20 ± 5	422 ± 28	34 ± 10	207 ± 99	23 ± 5
Dms	118 ± 37	24 ± 6	121 ± 61	30 ± 9	225 ± 82	40 ± 10	123 ± 29	26 ± 6

Table 3: Changes in length and cell-cell distance of apical junctions in the *Xenopus laevis* fundus during spontaneous and T3-induced metamorphosis. During spontaneous metamorphosis (NF53-to-NF66) AJs length and cell-cell distance remain constant.

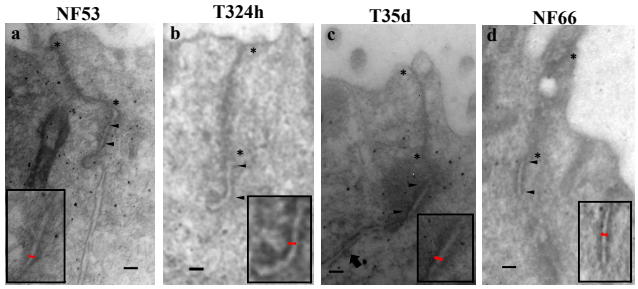

Figure 9: Changes in length and cell-cell distance of apical junctions in the *Xenopus laevis* fundus during spontaneous and T3-induced metamorphosis. During spontaneous metamorphosis (NF53-to-NF66) AJs length and cell-cell distance remain constant. In contrast, the T3-exogenous stimulation promotes significant increases in length and cell-cell distance of AJs at 24 h, decreasing at 5 days of T3-treatment, emulating AJs behavior at the end of spontaneous metamorphosis. At 24 h of T3-treatment the TJs length signifincantly increases, decreasing at 5 days of T3-tratment like the natural end of metamorphosis. Bar=100 nm. TJs: demarcated between asterisks; AJs demarcated between small arrowhead; Dms: demarcated between large arrowhead; red line: cell-cell distance; arrow: endocytic vesicle.

Since the pioneering works of the nineteenth century to the present, studies on timing and mechanisms involved on digestive tract functionalization has been the subject of a controversial debate matter [35,41-56]. This is because there are numerous processes involved in the amphibian-digestive tract morphogenesis. Juvenile anuran gut results from the tissue disruption and larval cell apoptosis, as well as stem cell proliferation and differentiation to replace larval epithelial, connective and muscle tissues. As expected, these events involve deep disassembly-reassembly of cell-cell and cell-extracellular matrix contacts [2,35,54,56,57]. Thus, the study of the TH-dependence of these events, is critical for vertebrates, including humans. Additionally, to this well known role for orchestrating amphibian metamorphosis, some observations suggest now that THs and their metabolites are not restricted to the vertebrates but instead are widely distributed in the animal and plant kingdoms [58,59]. In fact, Heyland and coworkers have recently shown that these hormones can act *via* exogenous routes as environmental messengers in echinoderm larvae [60,61], in turn suggesting a possibility of cross-kingdom interaction.

The available data suggest that postembryonic remodeling, governed by thyroid hormones, is an ancestral feature of chordates, being anuran metamorphosis an extreme example of a widespread life history transition [62]. Taking as own the Laudet's words, we believe the metamorphosis analysis may rejuvenate the research on vertebrate's post-embryonic development, a relatively neglected ontogenetic period. Therefore, the post-embryonic development maybe an additional source of information to explain, in turn, the origins of species diversity [62]. This developmental period is fully open to variations controlled by the environment and genetic cascades, taking up a central role the thyroid hormones. In addition to these facts, the study of metamorphosis is useful to investigate intestinal physiology and pathology, not only in anurans but also in mammals [56,63].

The results of the current work confirm our own assumptions and those reported by other authors, and allow to deepen the molecular mechanisms involved in dynamic regulation of amphibian gastrointestinal epithelial cell-cell adhesive contacts in physiological conditions [17,2] allowing to understand some pathological behaviors [56].Thus, as early as within 24 h post T3-treatment, E-cadherin, β- and α-catenin significantlly increase their expression. While intercellular distances of AJs and Dms increase, just the Dms number decrease.

Notably, β-catenin continues significantly increasing at day 5 of T3-treatment, suggesting that it participates not only in epithelial AJs, but also in muscle AJs and in cell proliferation through nuclear signal pathway [62-64]. At day 5 of T3-treatment, the occluding expression significantly rises closely with those of E-cadherin, β-and α-catenin, supporting the inducer role of E-cadherin on the formation of other junction complexes [65], triggering the epithelial barrier strengthening, such as it occurs in spontaneous metamorphosis. *In vivo* studies have showed that the epidermal E-cadherin loss results in mice perinatal death, owing to defective epidermal barrier function as consequence of faults in tight junction formation [66]. Even when p120-catenin would be a master regulator of the cadherin stability [25], during *X. laevis* gut remodelling conduced by T3, the levels of p120-catenin-mRNAs suggest control of E-cadherin-mediated junctions mainly through its role in back-recycling. Aside from the transcriptional regulation of these molecules, the endocytosis and recycling of junction proteins are alternative mechanisms that allow cells undergoing rapid changes in morphology in response to extracellular stimuli.

Another important issue to be considered is the cytoskeleton reorganization during epithelial remodeling [57-66]. In our *in vivo* model, the notable and significant increase of gastrointestinal Rac1 expression during the first 24 h of T3-induction results in heightened membrane protrusions (lamellipodia), which does increase the cell contact areas. In contrast, the Cdc42-expression remained practically unalterated, wich correlated at ultrastructural level, where filopodia were not detected, suggesting a scarce migratory cell-behavior [67].

There are several studies suggesting that the E-cadherin-mediated junctional complexes formation triggers the activation of the phosphatidylinositol-3-kinase (PI3K)–Akt–protein kinase B pathway [67,68], whose p85 subunit is directly binded to AJ-β-catenin [126]. This PI3K recruitment generates phosphatidylinositol-(3,4,5)-triphosphate (PIP3) and the membrane recruitment of guanine nucleotide exchange factors–Tiam and Vav2–, which possess PIP3-binding pleckstrin homology domains, and act as both Cdc42 or Rac1 activators [69-72]. The small GTPase Rac1 translocates to the membrane leading edge where it promotes lamellipodial extension and intestinal epithelial cell restitution to induce the intestinal wound closure [14]. In our model, in contrast to Rac1-mRNA both the Rac1-TIAM-GEF and GAP12 RNAms significantly increased at day 5 of T3-treatment, suggesting a key role of Rac1 during early gut remodeling. It has been detected that inositol phospholipid-dependent activation of Tiam1 occurs preferentially at AJs, and its phosphorylation in residue Y384 by Src kinase triggers its degradation, leading to AJ disruption and increased cell migration. Tiam1 is a key regulator of Rac1 activity at epithelial junctions since it is required for the efficient formation of TJ, and it is inhibited in confluent cells by Par3 [15]. Therefore, it is postulated a negative feedback mechanism upon junction maturation. In confluent cells, in turn, junctionally localized merlin-protein relieves the inhibition of AMOT over Rich-1, thereby allowing Rich-1 to inhibit Rac1, and thus inhibit downstream MAPK and PAK signaling [16]. Thus, merlin functions to block mitogenic signaling, by

inhibiting Rac1 activity at TJ. Striking, in our animal model Rich1 significantly increase at spontaneous metamorphosis (NF66) coinciding with gut epithelial barrier maturation.

Interesting, *in vitro* Arhgap12 inactivates Rac1 by increasing the rate of GTP hydrolysis, and when constitutively expressed, it negatively regulates extracellular matrix-cell adhesion, scattering and invasion, processes all dependent on the Rac1 activity. In addition, these authors found that Arhgap12 is selectively suppressed at transcriptional level by hepatocyte growth factor (HGF), a promoter of cell invasion. In our study, conversely, Arhgap12 would be activated at transcriptional level by T3, promoting cell-cell adhesion and cell differentiation at the final phase of the metamorohosis.

The Rac-GEF Vav2 and Tiam, both promote Rac1 activation whichin turns stimulates membrane actin dynamics adjacent to the initial site of contact, and thus increases new E-cadherin engagements. Rac1 would facilitate α-catenin homodimerization to be released of the cadherin-catenin complexes to bind at actin and antagonize Arp2/3 function promoting the belt formation of unbranched actin filaments. Meanwhile, PtdIns(3,4,5)P3 accumulation in the membrane signals the formation and expansion of the basolateral surface and Rac1 promotes the polarity orientation and lumen formation, and downstream signals cell cycle arrest and survival of confluent polarized epithelial cells. In addition, other actin-binding proteins such as vinculin, afadin and α-actinin may provide a link with actin cytoskeleton, and microtubule-binding proteins such as β- and p120-catenin, and link with tubulin cytoskeleton to route vesicles of E-cadherin-catenin complexes to the cell-cell contact sites [72]. Recently, *in vivo* [73] and quantitative studies using 3D superresolution microscopy [74] suggest that E-cadherin clustering depends on key cortical regulators, which provide tunable and local control over E-cadherin organization, thereby might regulate adhesion force transmission *in vivo*. These studies suggest that E-cadherin differentially packed nanoscale clusters are distributed along the cell-cell interface. Adhesive interactions between neighboring cells would promote the precursor cluster compaction to adhesive clusters *via* cis and trans interactions highly enriched at adherens junctions [74]. Therefore, the hierarchical and modular organization of E-cadherin could contribute to the adaptive plasticity of living animal AJs [74].

It is accepted that F-actin geometry is dinamycally controlled for the small Rho GTPases (RhoA, Rac1 and Cdc42) family members, which localize to E-cadherin-based cell-cell contacts and mediate the necessary cytoskeletal rearrangements and dynamic of surface E-cadherin during AJ assembly on cell-cell adhesion and compactacion [75,76]. In contrast to Rac1-activation, the Rho-activation is reduced by homophilic engagement of E-cadherin [77-79]. Coincidently, in our animal model RhoA-mRNAs decrease mainly at day 5 of T3-treatment.

In addition to Rac, Cdc42 and RhoA, Rap1 –a member of the Ras-like small G-proteins, which can reverse the Ras oncogenic potential by competition for Ras effectors [80] also controls the cell polarity signaling network [81] and strengthening of cell attachment to both extracellular matrix and neighbouring cells [82]. In our studies, Rap1 significantly increases at day 5 of T3-treatment, and thus, reinforce this last hypothesis and is coincident with epithelia functional maturation at the end of *X. laevis* metamorphic climax.

We now believe that the correlation between morphometric, ultrastructural and mRNA-expression studies of the main signaling pathway player´s at adherens junctions using hormone-dependent tissue remodeling, constitutes a significant contribution to elucidate the *in vivo* epithelial morphogenesis.

From pioneering studies of epithelial cell-cell junctions [83], numerous AJ-subtypes have been charactized dependig of cell-type and development stage [73,84-87]. However, even when some authors argue that the ultrastructural morphology of AJs is similar across different animal species [85-88], there are scarce ultrastructural studies analyzing its remodeling mechanism.

Therefore, the study of structural and functional changes of AJs during spontaneous anuran metamorphosis and under exogenous T3-influence is a relevant experimental model. T3-treatment produces disappearance of ciliated epithelial cells in *X. laevis* foregut, as occurs in spontaneous metamorphosis. As in other vertebrates, the fore-intestine surface epithelium of NF53 *X. laevis*, places within the lateral surface domain, strand- and belt-forming cell-cell junctions, such as tight and adherens junctions respectively. Even though these junctions display its fixed apico-basal relative localization, frequently two or three "AJs" or spot-like cell-cell junctions [74,84-86] are placed between TJs and Dms, reaching only 10% of AJCs. After 5-days of T3-treatment, apical-basal junction pattern changes as in the classical AJCs: TJ-AJ-Dm and notably increasing the complexes number. In addition, higher cell protrusion number like lamellipodia, but not filopodia were detected, suggesting increasing of membrane interactions but non-migrating phenotype [67].

Even thougth, numerous efforts were made to understand the molecular programs that govern the epithelial-mesenchymal transition (EMT) in various developmental contexts [89], most of them only have explored early developmental stages, being the organ remodeling, such as vertebrate metamorphosis set out, a major challenge.

During development-EMT, epithelial cells lose apico-basal polarity and intercellular junctions. These changes in cell polarity and adhesion, disrupt the epithelial basement membrane and allow cellular invasion into extracellular matrix (ECM)-rich compartment, a process referred as delamination. Fortunately, EMT is reversible, and cells can suffer mesenchymal-epithelial transition (MET) [89]. The role of THs and TRs on cell proliferation and differentiation is not homogeneous. Cell response to THs, strongly depends on cellular context, that is, on the cell-type, ontogeny (progenitor or differentiated cell) and physiological state (normal or tumoral cell) [90]. Thus, the amphibian gastrointestinal remodeling comprises a first phase of cell apoptosis and then a burst in cell proliferation and differentiation. Under the actions of increasing levels of THs and TRβ at the metamorphic climax, the lamina propria becomes thicker and permeable. This permeability promotes that both the differentiated and a few proliferating larval epithelial cells lose their contact at basal lamina, and that large numbers of cells undergo apoptosis [90]. Surprising, not only macrophages removal the apoptotic larval epithelial cells but also itself participate in the removal of their apoptotic neighbors even though they themselves are destinated to eventually die [35]. Indeed, concomitant EMT/MET processes are produced, probably not only for different cell types but also for reversible transitions of some individual cells. Thus, the permeability of the lamina propria allows a few epithelial cells to establish contact with sub-epithelial fibroblasts, evade apoptosis and form primordia from which the adult epithelial cells will be derived [53,90,91]. These islets of primordial cells localized between the residual epithelia and the connective tissue, proliferate very quickly and then invaginate into the connective tissue so that the mature adult epithelium starts to be functionally organized and becomes folded [53]. From this stage onward, the epithelium is constantly renewed

along the crest-axis [53], which strongly resembles the mammalian intestine. Intriguing, these cells arise from dedifferentiated larval epithelial cells under the influence of the THs, making intestinal metamorphosis a unique model to study development of adult organ-specific stem cells [53]. The cascade of events of the larval-to-adult epithelial transition is entirely regulated by THs; being the TRβ gene expression ubiquitously upregulated by the increasing levels of circulating THs, which signals the beginning of metamorphosis [40]. TRβ has been implicated in both apoptosis and proliferation during metamorphosis, because TRβ is highly expressed in larval epithelial cells just before the onset of apoptosis, and then its level decreases. TRβ is also expressed in the primordial proliferative epithelial cells and decreases when they start to differentiate [40]. Besides epithelium, the lamina propria of larval intestine suffers drastic restructuration during metamorphosis, possibly trougth the stromelysin 3 (ST3) matrix metalloprotease (MMP), target of THs [92] and TRβ in the intestine. This larval-to-adult epithelium transition depends on contact between epithelial and mesenchymal cells [57,53]. The dialogue between these cell types should be mediated by sonic Hedgehog (Shh) and BMP4 [93]. Shh is an early induced target of THs (Stolow and Shi, 1995), and is highly expressed in adult epithelial primordia, decreasing when differentiation markers, such as IFABP, are reelevated at primordial level [36]. Shh induces cell proliferation of both adult epithelial primordia and sub-epithelial fibroblasts [94]. Shh, in turn, activates the expression of BMP4 in sub-epithelial fibroblasts, which represses fibroblast proliferation through an autocrine loop, and induces differentiation of adult epithelial cells *via* paracrine action [93,94]. Recently has been identified that the Hedgehog interacting protein (Hip), Shh-inhibitor, mediates this complex epithelial–mesenchymal regulatory loop [95]. Also, a functional interaction between Shh and BMPs of epithelial–mesenchymal interaction in mammalian intestine has been described [96].

In this complex scenario, we have analyzed the T3 influence on epithelial cell-cell junctions mediated by E-cadherin, and some of their partner proteins –β-, α-, p120-catenins, Arp2, sGTPases, GEFs and GAPs–, in two experimental stages both spontaneous and T3 exogenous-induced metamorphosis. Notably, at 24 h, digestive tract remodeling is globally dominated by the effector action of Rac1 signal, which promotes the initiation and expansion of epithelial cell-cell junctions [97], and the establishment of apico-basal complexes mediated by E-cadherin–β-,–α-catenin, and later by occludin–ZO-1. In contrast, at day 5, the tissue scenario changes and Rap1 seems to command the E-cadherin-E-cadherin junctions and the tissue fate, because participate in the maturing and maintaining of cell-cell junctions [98] and preserving the cellular architecture. In human skeletal muscle, up-regulation also was observed for β- and α-catenins mRNA post-treatment with T3 during 14 days [99]. It has been described that C3G binds to the cadherin cytoplasmic tail, enabling to E-cadherin to locally activate Rap1 [98], in addition of their role downstream of the nectin-nectin interactions, previously established [100]. Rap1 is activated by extracellular signals, particularly growth hormone-dependent, through several regulatory proteins to modulate the cell proliferation and differentiation, the endocytosis and exocytosis, the integrin-mediated cell adhesion, the epithelial invagination and thereby the morphogenesis [101-103]. In addition, Rap1 activity should be necessary for the proper targeting of E-cadherin to maturing cell-cell contacts, and downstream the Cdc42 activation [98]. Supporting the present report, recient studies in mammals show that Rap1 acts *in vivo* epithelial barrier strengthening [104]. Opposite, *in vitro* investigations suggest that the cadherin-dependent activation of Rap1 promotes AJ-disassembly and integrin-mediated focal adhesion formation [105], indicating a Rap1-mediated cadherin-integrin crosstalk.

Most of studies on AJ-remodeling and formation find opposite levels of activated-Rac1 and Cdc42 versus RhoA [32,106,107]. In the present study, RhoA-mRNA levels became constant at 24 h and lightly decrease at day 5 of T3-treatment, as in spontaneous metamorphosis (NF66). It has been postulated that cadherin trans-binding triggers, through p120-catenin, local signaling at the contact, thus activating Rac1, inhibiting RhoA and Arp2/3 actin-branching function. This signaling is thought to disrupt the contractile actomyosin cortex at the contact, thereby lowering cell-cell interfacial tension and expanding the contact. Finally, mechanical coupling of contacting cells helps to stabilize the expanded cell-cell contacts at intracellular region [107].

Obviously, the coordinate modulation of cadherin and integrin functions plays an essential role in organ reshaping, such as gastrointestinal metamorphosis. The *in vitro* studies by Balzac et al. [105] found that a strong activation of Rap1 occurs upon AJ-disassembly triggered by E-cadherin internalization and trafficking along the endocytic pathway, however, Rap1 activity is not influenced by integrin outside-in signaling. Interesting, *in vivo* induction of Rap1 activity strengthen the mammal retinal pigment epithelial-barrier against the pathological choroidal endothelial cell invasion that occurs in macular degeneration [104]. Similar results were verified in *X. laevis* gut remodeling induced by 5-days T3-treatment, in which, coincident with the epithelial barrier maturation, the Rap1-mRNAs levels, are remarkably increased. Even though not significant differences in length and thickness of AJs were detected at 5-days of T3-treatment regarding to NF53 tadpoles, the junction pattern is notably modified, increasing the number of AJCs. At present, just few studies have morphometrically analyzed the cell-cell junctions during development. Among these, it is interesting to note that, the differentiation of the ependymal-glial cell contacts seems to respond to thyroid hormone stimulus [108]. He found that, as early as 16-20 h after a DL-thyroxine injection, expansion of gap cell-cell contact and extracellular space reduction were detected.

Interesting, Li et al. [109] demonstrated that functional interactions between Rap1 and E-cadherin regulate the self-renewal of human embryonic stem cells (hESCs). Rap1 indirectly influences the stem cell pluripotency through influencing the endocytic recycling pathway involved in the formation and maintenance of E-cadherin-mediated cell-cell cohesion, which is essential for the colony formation and self-renewal of hESCs. Conversely, disruption of E-cadherin adhesions induces lysosome delivery and degradation of Rap1, which in turn, leads to a further downregulation of E-cadherin function and a subsequent reduction in hESC clonogenic capacity. Thus, coincident with the beginning of T3-mediated intestine remodeling, larval primary epithelium degenerate and primordia of the secondary epithelium are detected at the epithelial-connective interface as small islets consisting of undifferentiated epithelial cells. These islets actively proliferate and differentiate to form the secondary epithelium, replacing the degenerating primary epithelium [35,54]. Even though, E-cadherin mRNA expression is significantly upregulated by T3 at 24 h post induction, their mRNA levels at NF66 suggest membrane Rap1-dependent E-cadherin recycling at the metamorphic climax end [110-126].

Forthcoming studies, will be focused on physical detecting of gene thyroid response elements involved in the epithelial-AJ establishing

and maintaning, as well as on searching TH-antagonists that help us to understand how is controled the epithelial physiopatology.

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