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A novel Yap/Taz zebrafish reporter reveals a role of Hippo pathway transducers in angiogenesis

Direttore della Scuola: Ch.mo Prof. Giuseppe Zanotti
Coordinatore d'indirizzo: Ch.mo Prof. Rodolfo Costa
Supervisore: Ch.mo Prof. Francesco Argenton

Dottorando: Matteo Astone

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1 ABSTRACT

YAP and TAZ, by orchestrating cell proliferation, cell death and cell-fate decisions, are key players of a complex network of signaling pathways acting during development. Deregulation of YAP/TAZ signaling causes robust organ overgrowth during organogenesis, which translates to loss of tissue homeostasis in the adult and consequent cancer development. YAP/TAZ are transcriptional co-activators that interact with TEAD transcription factors to promote cell proliferation and survival. Their transcriptional activity is regulated by nucleocytoplasmic shuttling and nuclear accumulation, which are controlled by the Hippo kinase cascade, but also by mechanical cues sensed by the cell and by other pathways. Among these, Wnt/β-catenin takes on a particular relevance, since it was recently shown to regulate YAP/TAZ activity through AXIN-mediated sequestration of YAP/TAZ in the β-catenin destruction complex.

Here, we describe the generation, validation and characterization of a novel biosensor zebrafish reporting the activity of Yap/Taz. It expresses nuclear mCherry, eGFP or the destabilized green fluorescent protein VenusPEST under the control of a promoter fragment of the human YAP/TAZ target gene *CTGF*, that contains 3 TEAD DNA-binding sites. Several independent founder fish transmitting the transgene to the germline were identified and used to establish the stable reporter lines. All stable transgenic fish shared a similar expression pattern, which was maintained in subsequent generations. Knockdown and overexpression approaches were used to validate the reporter. Co-injection of two morpholinos targeting Yap and Taz pre-mRNAs reduced the reporter signal, whereas injection of mRNAs coding for a constitutively active form of Yap, Taz and Tead (YAP-5SA, TAZ-4SA, TEAD-VP16) increased it. The *CTGF*-based transgenic lines represent therefore *bona fide* Yap/Taz reporters.

During development, strong reporter signal is visible mainly in the lens and otic vesicles, the pharyngeal arches, the heart, the pectoral fin and the vasculature, but the reporter protein expression is also detected in many other tissues and organs. The almost ubiquitous activation of Yap/Taz observed during early embryogenesis, consistent with the general role of YAP/TAZ in promoting cell proliferation and organ growth, is largely silenced in the adult fish, where the reporter signal is restricted to the lens, the ovary, the heart and the whole vasculature. We also showed that the *CTGF*-based biosensor zebrafish is able to report Yap/Taz activation during larval and adult fin

regeneration, as expected from the role that YAP/TAZ signaling plays in the regenerative processes.

The zebrafish *CTGF*-based reporter permitted to show in a living organism during development the regulation that the Wnt/β-catenin pathway exerts on Yap/Taz activity. Our results in terms of variations of the reporter signal, after both genetic and pharmacological modulation of the Wnt pathway activity, are in accordance with the model recently depicted *in vitro*.

The general and sustained reporter activity we observed in the endothelium during embryogenesis suggested a functional involvement of Yap/Taz signaling in developmental angiogenesis. Yap/Taz knockdown impaired the intersegmental vessels (ISVs) growth, while the overactivation of Yap/Taz-mediated transcription caused an aberrant sprouting from the ISVs. The vessel sprouting-promoting capacity of Yap/Taz is cell-autonomous, as the same phenomenon was observed by expressing TAZ-4SA under the control of an endothelium-specific promoter.

The *CTGF*-based zebrafish reporter is a new powerful tool to study *in vivo* Yap/Taz pathway activation, with possible applications in drug screening, regeneration and cancer biology. It permitted to confirm *in vivo* during development the crosstalk between Wnt/β-catenin and Yap/Taz pathways and to discover a novel role of Yap/Taz in vessel sprouting, suggesting a pro-angiogenic function of YAP/TAZ transcriptional activity.

1 SOMMARIO

YAP e TAZ, orchestrando la proliferazione, la morte e il differenziamento cellulari, rappresentano elementi chiave di una complessa rete di vie di segnalazione che agiscono durante lo sviluppo. L'alterazione della segnalazione YAP/TAZ causa una crescita fuori controllo degli organi durante l'organogenesi, che si traduce nella perdita dell'omeostasi tissutale nell'adulto e conseguente sviluppo tumorale. YAP/TAZ sono co-attivatori trascrizionali che interagiscono con i fattori di trascrizione TEAD per promuovere la proliferazione e la sopravvivenza cellulari. La loro attività trascrizionale è regolata dal trasporto nucleo-citoplasmatico e dall'accumulo nucleare, che sono controllati dalla cascata chinasica della via di Hippo, ma anche dagli stimoli meccanici percepiti dalla cellula e da altre vie. Fra queste, la via di Wnt/β-catenina assume una particolare rilevanza, dal momento che è stato recentemente dimostrato che essa regola l'attività di YAP/TAZ attraverso il loro sequestro nel complesso di degradazione della β-catenina mediato da AXIN.

In questa tesi vengono descritte la generazione, la validazione e la caratterizzazione di un nuovo zebrafish biosensore che riporta l'attività di Yap/Taz. Esso esprime le proteine mCherry nucleare, eGFP o la proteina verde fluorescente destabilizzata VenusPEST sotto il controllo di un frammento promotoriale del gene umano *CTGF* target di YAP/TAZ, contenente 3 siti di legame per TEAD. Sono stati identificati diversi pesci fondatori indipendenti in grado di trasmettere il transgene alla linea germinale, i quali sono stati utilizzati per instaurare le linee reporter stabili. Tutti i pesci transgenici condividevano un pattern di espressione similare, mantenuto nelle generazioni successive. Per validare il reporter sono stati usati approcci di downregolazione e overespressione. La co-iniezione di due morfolini diretti contro i pre-mRNA di Yap e Taz ha ridotto il segnale reporter, mentre l'iniezione di mRNA codificanti per una forma costitutivamente attiva di Yap, Taz o Tead (YAP-5SA, TAZ-4SA, TEAD-VP16) lo ha aumentato. Le linee transgeniche basate sul gene *CTGF* rappresentano perciò *bona fide* dei reporter dell'attività di Yap/Taz.

Durante lo sviluppo, un forte segnale reporter è visibile principalmente nella lente, la vescicola otica, gli archi faringei, il cuore, la pinna pettorale e la rete vascolare, ma l'espressione della proteina reporter è rilevabile in molti altri tessuti e organi. L'attivazione quasi ubiquitaria di Yap/Taz osservata durante l'embriogenesi precoce, consistente con il ruolo generale di YAP/TAZ nel promuovere la proliferazione

cellulare e la crescita degli organi, è ampiamente silenziata nel pesce adulto, dove il segnale reporter è ristretto a lente, ovario, cuore e intera rete vascolare. Lo zebrafish biosensore è anche in grado di riportare l'attivazione di Yap/Taz durante la rigenerazione della coda nella larva e nell'adulto, come atteso dal ruolo che riveste la segnalazione YAP/TAZ nei processi rigenerativi.

Lo zebrafish reporter basato sul gene *CTGF* ha permesso di mostrare in un organismo vivente durante lo sviluppo la regolazione che la via di Wnt/β-catenina esercita sull'attività di Yap/Taz. I nostri risultati in termini di variazione del segnale reporter, in seguito alla modulazione genetica e farmacologica dell'attività della via di Wnt, sono in linea con il modello disegnato di recente *in vitro*.

L'attività generale e sostenuta del reporter nell'endotelio durante l'embriogenesi ha suggerito un coinvolgimento funzionale della segnalazione Yap/Taz nell'angiogenesi precoce. La downregolazione di Yap/Taz è risultata in una compromissione della crescita dei vasi intersegmentali (ISVs), mentre l'attivazione spinta della trascrizione mediata da Yap/Taz ha causato un ramificarsi anomalo degli ISVs. La capacità di Yap/Taz di promuovere tale ramificazione vascolare è "cell-autonomous", dal momento che lo stesso fenomeno è stato osservato esprimendo TAZ-4SA sotto il controllo di un promotore endotelio-specifico.

Lo zebrafish reporter sviluppato è un nuovo potente strumento per studiare *in vivo* l'attivazione della via di Yap/Taz, con possibili applicazioni nello screening farmacologico e nella biologia della rigenerazione e del cancro. Ha permesso di confermare *in vivo* durante lo sviluppo l'interazione fra le vie di Wnt/β-catenina e Yap/Taz e di scoprire un nuovo ruolo di Yap/Taz nella ramificazione vascolare, suggerendo una funzione pro-angiogenica dell'attività trascrizionale di YAP/TAZ.

2 INTRODUCTION

2.1 Reporting *in vivo* a signaling pathway activity: transgenic zebrafish reporter lines

Zebrafish (*Danio rerio*) is a small teleost fish that, in the last decades, thanks to its biological characteristics, has gained great relevance in the biomedical research as an outstanding model organism. Zebrafish shares all features of vertebrates, its genome has recently been fully sequenced and the genes identified display high sequence similarity with the mammalian orthologues, as well as high synteny and conservation of gene function (Barbazuk et al., 2000). The ease of maintenance, its small size, the short generation time (2-3 months) and the prolific reproduction (about 200 eggs per female with a weekly frequency) make zebrafish an ideal model for large-scale genetic screening and analysis (Driever et al., 1996; Haffter et al., 1996). Moreover, the external fertilization and the embryonic semitransparency are probably the biggest advantages that zebrafish offers to the research in developmental biology, genetics and physiophatology. In fact, these characteristics allow *in vivo,* non-invasive observation of early development and organogenesis and open wide possibilities for genetic manipulation. Messenger RNAs, antisense oligonucleotides and transgenic constructs can be easily microinjected in the zebrafish embryo to study gene function through knockdown and overexpression experiments, as well as through the generation of mutant, transgenic and reporter lines. Being a vertebrate, zebrafish represents a good model organisms to phenocopy human diseases and many fish that reproduce different human disorders at the molecular, cellular, histological and etiological levels have been generated (Lieschke and Currie, 2007; Liu and Leach, 2011; Goldsmith and Jobin, 2012). New genetic manipulation technologies such as the CRISPR/Cas system are now available and, by permitting efficient and fast genome editing, will strengthen the ability of the zebrafish to model human diseases and to unravel cellular, molecular and genetic mechanisms (Hwang et al., 2013 a; Hwang et al., 2013 b; Jao et al., 2013).

One interesting use of zebrafish, that fully exploits its features and advantages, is represented by transgenic signaling pathway reporter lines, which enable the dynamic, *in vivo* monitoring of the activity of specific pathways during development and adulthood, in normal and pathological conditions. Embryonic and postnatal signaling pathways govern the basic biological processes, such as cell proliferation, survival,

death and differentiation, involved in animal development and tissue homeostasis and impaired in tumorigenesis and many other disorders. Therefore, the investigation of the molecular cascades of these signaling pathways, their upstream regulation, the complex network of interconnections and the downstream transcriptional and biological output takes on a crucial importance for the understanding of the biological mechanisms regulating physiological and pathological processes. Fundamental in regulating stemness, cell proliferation and differentiation are bone morphogenetic proteins (BMPs), Notch, wingless-related integration site (Wnt), sonic Hedgehog (Shh), fibroblast growth factor (FGF), signal transducer and activator of transcription (STAT), cAMP-response element-binding protein (CREB) and YAP/TAZ signaling (Perrimon et al., 2012; Aaronson and Horvath, 2002; Stork and Schmitt, 2002; Piccolo et al., 2014).

A signaling pathway reporter zebrafish is a transgenic biosensor fish expressing a fluorescent protein under the control of regulatory sequences responsive to the specific pathway. The result is a powerful tool reporting *in vivo* the spatio-temporal activation of the signaling pathway, with many possible applications, first in development biology but also for studies in adult physiology, human diseases and dynamic processes such as regeneration and tumorigenesis.

2.1.1 Generation of a signaling pathway reporter line

The first condition for the development of a signaling pathway reporter line is the identification of the pathway-specific responsive elements (REs). Each pathway consists of a signaling cascade that ultimately results in the activation of certain transcription factors, responsible for driving the expression of the pathway target genes. The pathway-specific REs are those regulatory sequences located in the promoters of these target genes and recognised by the transcription factors activated downstream of the signaling cascade. The REs sequences might be already available in the literature or can be identified by *in silico* analysis of conserved promoter regions of known target genes. To enhance the reporter responsiveness, these REs are multimerized in a variable number of repeats that need to be tested experimentally, as the final effect is hard to be predicted (Figure 2.1). Nevertheless, it has been shown that the cooperativity among responsive sequences and an higher number of REs repeats enhance the reporter protein expression level (Moro et al., 2012; Moro et al., 2013). Of note, higher responsiveness doesn’t mean higher sensitivity in all experimental contexts, since the potential signal

saturation could be a limit in reporting activity upregulations or subtle downregulations (Moro et al., 2013).

The second element a signaling pathway reporter construct needs is a minimal promoter to drive the expression of the reporter protein with the specificity instructed by the upstream multimerized REs. Minimal promoters can be taken from genes that are transcriptional targets of the specific pathway (Barolo, 2006; Dorsky et al., 2002; Moro et al., 2012); otherwise, artificial minimal promoter can be used, such as the pGL4 vector-derived promoter miniP, which should reduce potential unspecific signals due to sequences other than the TATA present in the natural promoters (Shimizu et al., 2012). As for the number of REs repeats, the suitable minimal promoter required to be chosen empirically for signal-to-noise ratio, responsiveness and specificity for the given pathway.

The reporter gene, placed downstream of the multimerized REs and the minimal promoter, is the last element to be considered for the generation of the signaling pathway reporter transgene. The choice of the appropriate fluorescent reporter protein depends on the biological processes that will be addressed, and takes into account the wide spectra of characteristics and properties of the different reporter proteins (Moro et al., 2013). Particularly important are the excitation/emission wavelengths, that influence the detection capacity of the specific protein fluorescence by the available optics and the possibility to generate double and multiple transgenics; the brightness, impacting on the reporter sensitivity; the half-life; the subcellular localization (e.g. cytoplasmic, nuclear, mitochondrial) and peculiar features of modified versions (e.g. photoconvertible and destabilized reporter proteins) (Shaner et al., 2005; Shaner et al., 2007; Müller-Taubenberger and Anderson, 2007; Miyawaki, 2011). Destabilized versions of reporter proteins might be ideal when addressing dynamic biological processes. Good examples are given by the d2eGFP, a destabilized version of the enhanced green fluorescent protein eGFP, and the Venus-PEST, a fusion protein containing the fluorescent protein Venus (a derivative of enhanced yellow fluorescent protein (eYFP)) and the PEST sequence from the mouse ornithine decarboxylase protein that confers fast degradation to the fusion protein (Nagai et al., 2002; Li et al., 1998). In any case, the design of more reporter constructs by using different reporter genes allow to create a versatile tool for the investigation of both rapid and slow phenomena, possibly in other fluorescent protein-expressing transgenic and reporter backgrounds at the same time, and to

overcame issues related to a too short or too long half-life, a too weak or saturated signal.

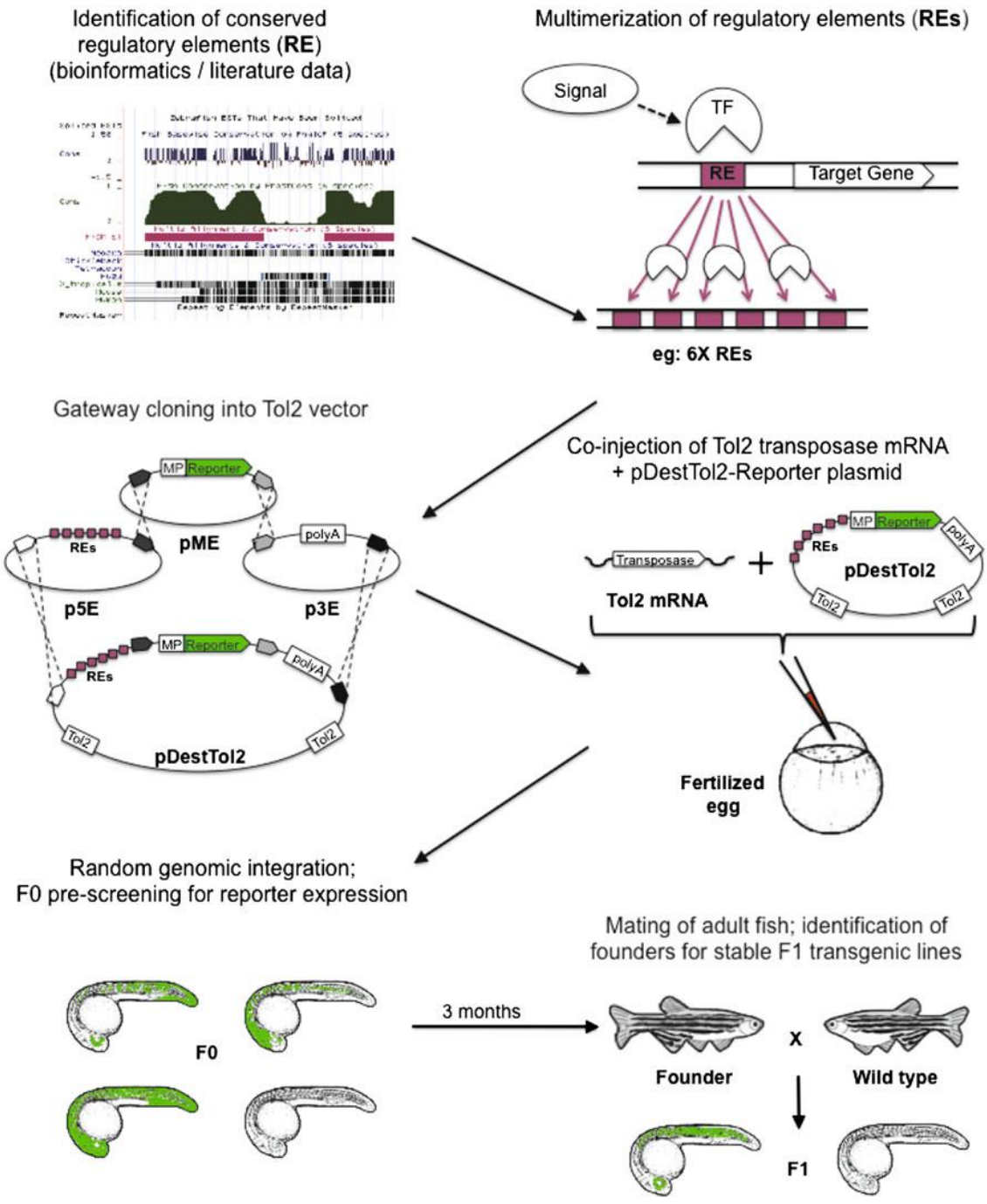

Figure 2.1. Generation of a zebrafish signaling pathway reporter line by the Tol2 strategy. See text for details (From Moro et al., 2013).

The multimerized REs, the minimal promoter, the reporter gene and a polyA sequence are assembled together in a final destination vector exploiting the Gateway-based Tol2 strategy, a site-specific recombination-based cloning method recently

emerged as a simple, quick and efficient technology for zebrafish transgenesis (Kwan et al., 2007). The destination vector obtained by the Gateway recombination reaction is a transposon-donor plasmid, whose Tol2 sequences are recognized by the transposase. When it is injected, together with the transposase mRNA, into one-cell stage zebrafish embryos, the transposase catalyzes the transgene excision and the recombination within the zebrafish genome. The microinjected embryos are screened for mosaic reporter fluorescent expression (if the reporter is expected to be active during development) and raised to adulthood. They are then outcrossed to wild-type fish and screened for germline transmission to finally establish the stable transgenic reporter line. A schematic representation of the whole procedure for the generation of a zebrafish reporter line by the Tol2 strategy is depicted in Figure 2.1.

When using the Tol2 transgenesis system, thanks to the high integration frequency into the host genome during early developmental stages, the rate of germline transmission to the offspring reaches around 70% of the microinjected fish. The high transgenesis rate can raise the question of multiple integration events occurring in the germline and consequently the presence of multiple copies of the transgene in single individuals of the offspring (Moro et al., 2013). Possible complications are the non-Mendelian inheritance of the transgene and the variability within the offspring from the same founder, both in terms of reporter expression level and in terms of reporter expression pattern, caused by the presence of a variable number of copies of the transgene and the influence that a different genomic environment could have on the transgene expression ("positional effect").

2.1.2 Validation of a signaling pathway reporter line

Before to use a zebrafish reporter line for any biomedical application, it is essential to ensure that the transgenic line is effectively reporting the activity of the desired pathway, i.e. is a *bona fide* reporter for a specific signaling pathway. The validation of a reporter line is the body of experimental observations and tests showing that the signal detected is a reliable readout of the signaling pathway activity. The major approaches adopted to validate a signaling pathway reporter line are represented by the comparison of multiple reporter lines, literature review, genetic and pharmacological perturbation of the pathway (Moro et al., 2013).

A first indication of the consistency of the reporter is given by the comparison of the spatio-temporal expression pattern of the reporter protein in the progenies from several

founder fish. The positional effect and the presence of multiple transgenic alleles in the same carrier, as mentioned before, can explain possible discrepancies in the reporter protein expression pattern and intensity. This issue can usually be attenuated by diluting the number of transgenic copies through subsequent generations and by selecting individuals with most shared signals (Moro et al., 2013). A stronger validation step in comparing multiple reporter lines is given by the sharing of a similar reporter expression pattern with transgenic lines reporting the same pathway but based on different designs of the REs and/or the minimal promoter components. These reporter lines might be already available in the literature (a clear example is given by the case of the Wnt reporter lines in Dorsky et al. (2002), Moro et al. (2012) and Shimizu et al. (2012)).

Literature review in the validation process consists in the comparison of the information acquired from the reporter line, about the anatomical districts and the time points in which the reporter is activated, with the data available in the current literature. These might be known profiles of target genes of the given signaling pathway and data relative to the activity and the function of that signaling during development, in adult tissues and organs or in particular biological processes (Moro et al., 2013).

The fundamental step required for the validation of a signaling pathway reporter line is the one that shows if the transgenic line is able to report variations in the activity of the pathway under investigation. Perturbations of the signaling can be achieved genetically or pharmacologically; with both approaches the aim is to monitor if the reporter protein expression level varies as expected from the known perturbation experimentally induced. Genetic methods to up- and downregulate the pathway activity include overexpression of mRNAs encoding for “natural” or dominant-active/negative versions of pathway components and knockdown by antisense morpholino oligos injection. Transient activity of mRNAs and morpholinos can be overcome by using stable transgenic lines or genetic mutants. To have a reporter line in a mutant background, reporter fish are first outcrossed to the genetic mutant and the heterozygous mutants obtained are then intercrossed. Heat shock inducible line may be available too and are particularly useful, as they permit to analyze the effect of the overexpression of pathway members skipping the possible early embryonic lethality of mutants and non-conditional systems. On the other hand, pharmacological methods entail the use of drugs represented by known chemical agonists or antagonists, possibly acting at different levels of the signaling cascade (Moro et al., 2013).

So far, the best way commonly recognised to conclude that a zebrafish transgenic line is a *bona fide* reporter for a specific signaling pathway remains the combined use of all the strategies described above (Moro et al., 2013).

2.1.3 Applications and limits of signaling pathway reporter lines

The first application of any zebrafish reporter line concerns the visualization and the analysis of the physiological activation of the pathway throughout vertebrate development and in adult tissues and organs (Dorsky et al., 2002; Molina et al., 2007; Schwend et al., 2010; Gorelick and Halpern, 2011; Moro et al., 2012). Thanks to the semitransparency of zebrafish during embryonic and early larval development, and the availability of unpigmented fish lines, reporter activation can be followed *in vivo* at single-cell resolution during gastrulation, segmentation and organogenesis, until juvenile stages. The signaling pathway activation can be dissected also in adulthood, but the *in vivo* analysis becomes limited to superficial structures, including interesting applications in regenerative biology. Good examples are given by the Yap/Taz reporter activity visualized *in vivo* in the endothelial wall of the adult fin vessels, reported in this thesis, and the activation of reporter signals during the epimorphic fin regeneration after amputation (this thesis and Moro et al., 2012). Reporter expression in internal tissues and organs is visualized by *post-mortem* dissection and subsequent direct fluorescence microscopy and/or immunostaining (Moro et al., 2013).

The possibility to treat living reporter fish with several chemical compounds and to monitor signal variations, potentially with automated systems, opens wide perspectives to the use of zebrafish reporter lines in the screening and validation of novel, therapeutically relevant drugs acting as agonists or antagonists of the considered signaling pathway (Weger et al., 2012; Moro et al., 2013).

The increasing number of zebrafish reporter lines available, together with the option of many different reporter proteins, makes wider and wider the variety of cross relationships among genes and signaling pathways that can be studied *in vivo* in zebrafish. The applications of double/multiple transgenics ranges from simple co-localization analysis to crosstalk/epistasis studies in physiological or pathological contexts (Moro et al., 2013).

One of the most important applications of the zebrafish reporter lines in the future will be their usage to explore signaling pathways activation and role in human diseases, taking advantage of the several zebrafish models of human disorders already developed

and that will be developed in the near future. The activity of a given signaling pathway can be followed during different stages or conditions of the disease progression, such as pre-cancerous lesions, carcinoma *in situ* and metastasis for an epithelial tumor (Moro et al., 2013; Schiavone et al., 2014).

Despite the plethora of opportunities offered by the zebrafish signaling pathway reporter lines, some limitations have also to be considered. A given reporter line, in fact, may not be able to fully recapitulate the activity of the signaling pathway. Anatomical districts effectively activating the pathway may not show the corresponding activation of the reporter, possibly due to the threshold level required to visualize the reporter protein, issue that could be partially overcame by *in situ* hybridization detection of the reporter transcript (Molina et al., 2007; Moro et al., 2013). Local chromatin effects at the level of the transgene insertion may also be responsible for an incomplete signaling recapitulation (Schwend et al., 2010). Another limit could be represented by the persistent expression of stable fluorescent reporter proteins (especially in those tissues with a limited protein turnover) also when the signaling activity has already been turned off (Moro et al., 2013). One limitation by which all zebrafish reporter lines are affected is given by the fact that REs recognised by specific transcription factors are used in the transgenic construct. This means that, if the signaling pathway under investigation somehow activates different transcription factors, for example less known or less represented, the reporter is not able to report this activity. On the contrary, if the transcription factors recognising the REs are activated also by pathways different from the one considered, the case can not be distinguished, as the reporter protein is anyhow expressed.

2.2 YAP/TAZ signaling

YAP and TAZ have been considered for years as simple downstream effectors of the Hippo pathway, a tumor suppressor pathway first discovered in *Drosophila* and defined as “Salvador-Warts-Hippo” pathway only twelve years ago (Wu et al., 2003). The most fascinating biological property of the Hippo pathway regards the still mysterious capacity to stop the growth of organs when they reach their correct size, basically by turning off Yorkie, the YAP/TAZ orthologue in *Drosophila*. From there, the field has incredibly evolved and YAP/TAZ are more and more appearing as key players among a

complex network of signalings during development, by orchestrating cell proliferation, cell death and cell-fate decisions. Deregulation of YAP/TAZ signaling is responsible for robust organ overgrowth during organogenesis (as first shown in *Drosophila* by Hippo mutants), which translates to loss of tissue homeostasis control in the adult and consequent cancer development. Thus, aberrant YAP/TAZ signaling is emerging as central determinant of malignancy in human cancer, as well as controlled YAP/TAZ signaling is essential for normal development, tissue homeostasis and regeneration (Piccolo et al., 2014).

YAP/TAZ are transcriptional co-activators and drive the transcription of a set of target genes by binding TEAD transcription factors. Important target genes are: *CTGF*, *CYR61*, *ANKRD1*, *BIRC5*, *AXL*, *InhA*, *Col8a1*. To be considered, TEAD transcription factors interact also with transcriptional co-activators other than YAP/TAZ, such as those belonging to the Vestigial family, as well as YAP/TAZ bind other nuclear proteins and transcription factors, such as RUNX, p73 and PPAR-γ (Piccolo et al., 2014).

YAP/TAZ field is still very young and many aspects of YAP/TAZ biology remain to be explored, starting from the poorly understood upstream regulators and downstream effectors of the pathway. Moreover, recent discoveries showing a variety of YAP/TAZ regulators independent of Hippo, such as biomechanical stimuli and a strict crosstalk with other pathways (the Wnt/β-catenin above all; see below), are opening the road to intriguing questions and possibly to new exciting horizons in YAP/TAZ biology (Piccolo et al., 2014).

Because of the large complexity of YAP/TAZ signaling, the following presentation of the pathway regulations and functions will take into consideration the most important known aspects of the field, far to claim to be comprehensive.

2.2.1 YAP/TAZ as nuclear transducers of the Hippo pathway

The Hippo pathway is an evolutionary-conserved tumor suppressor pathway, whose core is composed of a kinase cascade ultimately resulting in YAP/TAZ/Yorkie phosphorylation and inhibition. In mammals, the sterile 20-like kinases MST1/STK4 and MST2/STK3 (orthologues of the *Drosophila* Hippo kinase), when bound to their regulatory protein SAV1/WW45 and activated by upstream signals, phosphorylate and activate the LATS1/2 kinases (orthologues of Warts), together with their regulatory subunits MOB1A/B (Chan et al., 2005; Praskova et al., 2008). In turn,

LATS1/2/MOB1A/B complex phosphorylates YAP and TAZ (Dong et al., 2007; Zhao et al., 2007; Lei et al., 2008). LATS1/2 phosphorylate YAP at five and TAZ at four serine/threonine residues (Zhao et al., 2010). In particular, phosphorylation of S381 in YAP and S311 in TAZ is responsible for their polyubiquitylation and degradation. In fact, it is followed by additional phosphorylation by CK1 kinases, which generates a "phosphodegron" recognized by β-transducin repeat-containing protein β-TrCP, a key adaptor for the SCF E3 ubiquitin ligases (Liu et al., 2010; Zhao et al., 2010; Zhao et al., 2010). On the other hand, phosphorylation of S127 in YAP and S89 in TAZ creates a binding consensus for 14-3-3 proteins, responsible for YAP/TAZ sequestration in the cytoplasm (Dong et al., 2007; Zhao et al., 2007; Basu et al., 2003). YAP is mainly regulated through this mechanism, as it is a relatively stable protein, while TAZ is very unstable, with a half-life shorter than two hours, indicating that its major regulation is represented by protein degradation via polyubiquitylation (Piccolo et al., 2014). LATS-induced phosphorylation is reverted by phosphatases such as PP1 (Liu et al., 2011; Wang et al., 2011). Other post-translational modifications and protein interactions may also influence YAP/TAZ activity. Considering the different mechanisms of YAP/TAZ inhibition, including nuclear exclusion, sequestration in the cytoplasm and proteasomal degradation, YAP/TAZ transcriptional activity is overall regulated by their nucleocytoplasmic shuttling and nuclear accumulation. Essentially, focusing on the Hippo-dependent regulation, when the Hippo pathway is active, YAP/TAZ are transcriptionally inactive. Only when the Hippo cascade releases its inhibition, YAP/TAZ are translocated in the nucleus, where they drive the transcription of their target genes (Piccolo et al., 2014) (Figure 2.2).

Proteins involved in cell-cell adhesion and apical-basal polarity complexes are the main upstream regulators of the Hippo pathway. Merlin/NF2 is localized in close proximity to adherens and tight junctions and inhibits YAP/TAZ by activating the Hippo cascade through different possible mechanisms and protein interactions, not completely understood (Piccolo et al., 2014). Merlin is a member of the Expanded/Merlin/Kibra complex, which may function as an apical scaffold by recruiting the core Hippo pathway proteins at cell-cell junctions and promoting LATS activation (Genevet et al., 2010; Yu et al., 2010; Baumgartner et al., 2010; Xiao et al., 2011; Yin et al., 2013; Moleirinho et al., 2013) (Figure 2.2).

Scribble is a key cell polarity determinant localized at the plasma membrane, where serves as adaptor for the assembly of a complex containing MST, LATS and TAZ and

promoting MST-mediated activation of LATS. Loss of the epithelial architecture, such as during epithelial-to-mesenchymal transition in cancer, triggers Scribble delocalization and consequent TAZ and likely YAP activation, which is responsible for the acquisition of cancer stem cell like traits and the malignant properties of the tumor (Cordenonsi et al., 2011) (Figure 2.2). Similarly to Scribble, the Crumbs apical complex binds to YAP/TAZ and promotes their phosphorylation and cytoplasmic retention, through a not well known mechanism (Varelas et al., 2010).

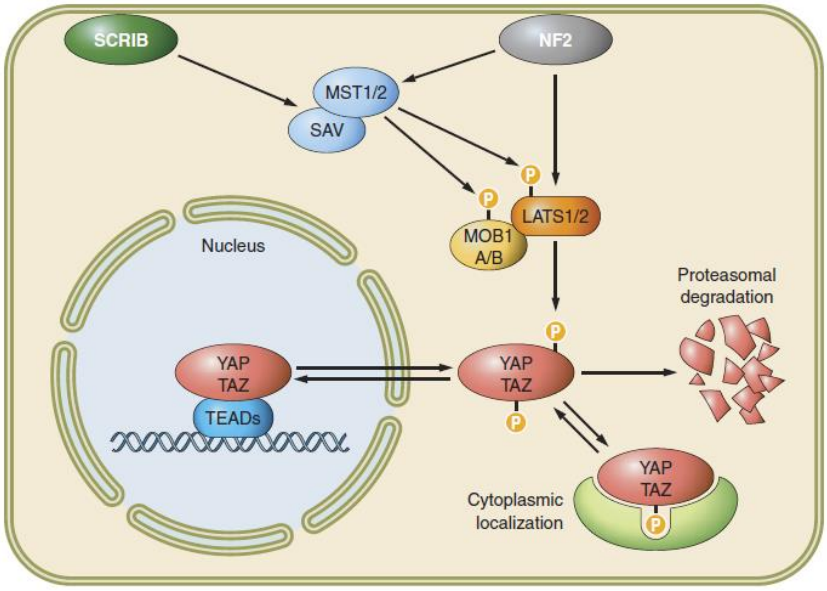

Figure 2.2. Schematic representation of the Hippo pathway-mediated regulation of YAP/TAZ. YAP/TAZ nucleocytoplasmic shuttling is depicted as the balance among nuclear translocation/exclusion, proteasomal degradation and cytoplasmic sequestration. The Hippo pathway upstream regulators Scribble and NF2 are also shown (From Piccolo et al., 2014).

E-cadherin/α-catenin complex, involved in cell adhesion and linked to the actin cytoskeleton, also inhibits YAP activity in normal conditions, probably by activating LATS or a yet unknown kinase phosphorylating YAP in the same residues targeted by LATS (Kim et al., 2011; Schlegelmilch et al., 2011). Again, perturbations of the cell adhesion, in this case disturbing the E-cadherin/α-catenin complex, impairs YAP inhibition and promotes its nuclear accumulation (Varelas et al., 2010). E-cadherin/α-catenin-mediated regulation of YAP/TAZ activity takes also part in the process by which cultured cells stop dividing when they become confluent, the historical knowledge of epithelial biology termed contact inhibition of proliferation (CIP). When cultured cells start making contacts with each other, the E-cadherin/α-catenin system activates LATS, leading to YAP/TAZ phosphorylation and proliferation arrest.

However, this mechanism accounts for only ~30% of YAP/TAZ-mediated CIP, that is mainly regulated by the YAP/TAZ mechanical pathway (see below) (Aragona et al., 2013).

2.2.2 YAP/TAZ as sensors and mediators of mechanical cues

Independently of the Hippo pathway, YAP/TAZ are strongly susceptible to signals coming from the physical properties of the microenvironment experienced by the cell. These include mechanical stimuli derived from the elasticity of the extracellular matrix (ECM), the forces exerted by neighbouring cells and the pressure experimented by the tissue at a more general level. YAP/TAZ are able to sense mechanical cues and to transduce them into a biological response of death/proliferation or differentiation decisions guided by YAP/TAZ-mediated target genes transcription. Specifically, the stretching of a single cell over the ECM and the consequent adaptation of the cytoskeleton cause YAP/TAZ nuclear accumulation, promoting cell proliferation and inhibiting differentiation. On the contrary, if cell adhesion is restricted to a very small ECM area and the cell acquires a round, compact shape, YAP/TAZ are inactivated and the biological response is reverted. YAP/TAZ are active mediators of the cell decisions induced by cell morphology change: YAP/TAZ artificial inactivation in stretched cells causes the cells to behave as if they were on small adhesive areas, while YAP/TAZ artificial activation in round cells cause the cells to behave as if they were stretched. Similarly, YAP/TAZ sense and transduce signals concerning ECM stiffness: a rigid ECM promotes their activity in the nucleus, whereas a soft ECM excludes them from the nucleus (Dupont et al., 2011) (Figure 2.3).

YAP/TAZ regulation by mechanical cues requires Rho GTPase activity and the tension of the actomyosin cytoskeleton. In fact, the remodeling of the F-actin cytoskeleton regulated by the Rho family of GTPases was shown to control YAP/TAZ activity. F-actin regulation is mediated by the F-actin capping and severing proteins Cofilin, CapZ and Gelsolin, but the mechanism linking the F-actin cytoskeleton with YAP/TAZ activity remains to be uncovered, ascertained the fact that is independent of LATS and of any phosphorylation of YAP/TAZ. This cytoskeletal/mechanical pathway explains the complete proliferation arrest occurring in CIP process after LATS activation by the E-cadherin/α-catenin system (see above). Independently of LATS, the remodeling of the F-actin cytoskeleton represents the second step in CIP. The mechanism is the same described for single cells plated on small ECM islands: after

reaching confluence, cell crowding in the monolayer restricts cells into smaller areas, causing the F-actin cytoskeleton remodelling responsible for YAP/TAZ nuclear exclusion and proliferation inhibition (Halder et al., 2012; Aragona et al., 2013).

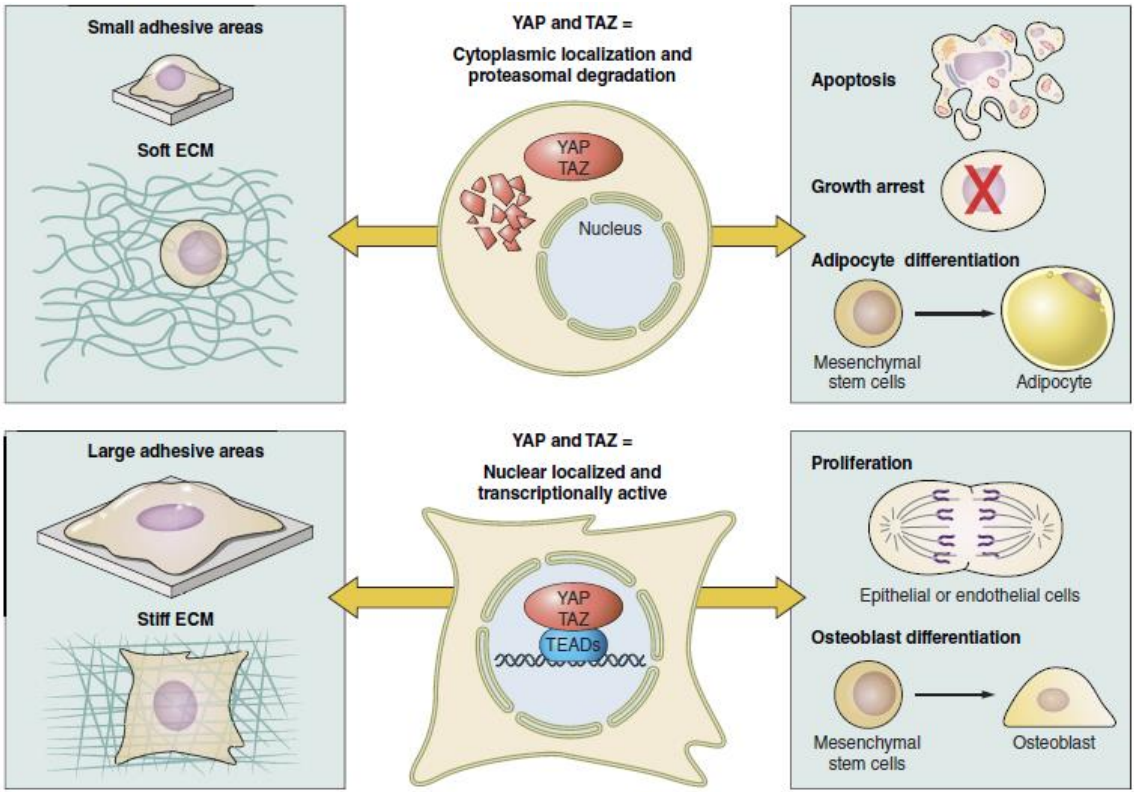

Figure 2.3. YAP/TAZ as sensors and mediators of mechanical cues. The area of ECM available for cell adhesion and the ECM stiffness regulate YAP/TAZ activity, leading to different biological response of death/proliferation and differentiation (From Piccolo et al., 2014).

2.2.3 YAP/TAZ in embryonic development

Yap/Taz double knockout embryos die at morula stages, *Yap* knockouts die shortly after gastrulation, while *Taz* knockouts, even if with an high rate of embryonic lethality, can complete development but die of polycystic kidney disease and pulmonary emphysema (Nishioka et al., 2009; Morin-Kensicki et al., 2006; Hossain et al., 2007; Makita et al., 2008). In the blastocyst an interesting patterning in YAP/TAZ subcellular localization is observed. The cells of the inner cell mass (ICM), nonpolar and expressing pluripotency transcription factors such as *Oct4* and *Sox2*, display YAP/TAZ enrichment in the cytoplasm, while in the outer cells of the trophoblast, with a strong apicobasal polarity and expressing *Cdx2* mastergene, YAP/TAZ are localized in the nucleus. Forced activation of YAP and TEAD is sufficient to induce Cdx2 expression in the ICM; conversely, LATS overexpression suppresses Cdx2 expression in the

trophoblast (Nishioka et al., 2009). Overall, these data indicate a crucial role of YAP/TAZ signaling in the first cell fate decision occurring during early embryonic development.

As mentioned before, the Hippo pathway is a key regulator of organ growth and organ size during development. Defects in components of the Hippo cascade, causing the loss of YAP/TAZ inhibition, as well as YAP/TAZ overexpression have been shown to determine a robust overgrowth phenotype in many organs and model organisms such as *Drosophila*, mice and zebrafish. As an example, YAP overexpression in developing mouse hearts increases the organ size in a TEAD-dependent manner, and the same phenotype was obtained after *Mst1/2* and *Lats2* inactivation (Heallen et al., 2011; Song et al., 2010). On the contrary, mice undergoing conditional deletion of *Yap* in embryonic cardiomyocytes display severe heart hypoplasia due to proliferation impairment, similar to what observed in *Tead1* knockouts (Chen et al., 1994; Sawada et al., 2008; von Gise et al., 2012; Xin et al., 2013).

A surprising phenotype appears when YAP and TAZ are inactivated in the developing kidney, representing also an example of possible distinct functions that YAP and TAZ may have in some districts or conditions. In fact, *Yap* conditional knockout within the nephrogenic lineage shows that YAP is essential for nephron induction and morphogenesis, in a manner independent of regulation of cell proliferation and apoptosis. On the other hand, *Taz* knockout causes polycystic kidney disease (Reginensi et al., 2013; Hossain et al., 2007; Makita et al., 2008).

2.2.4 Role of YAP/TAZ in the adult tissues and in regeneration

In the adult tissues such as brain, intestine, skin, and skeletal muscle YAP and/or TEAD expression was found to be enriched in anatomical compartments containing stem/progenitor cells (Ramos and Camargo, 2012). Consistently with the role of YAP/TAZ in prompting tissue proliferation during development until organs reach their proper size, a forced activation of YAP/TAZ signaling in adult organs leads in general to expansion of progenitor cells, aberrant proliferation and organ enlargement. Paradigmatic is the fourfold increase in liver mass in transgenic mice overexpressing YAP and the similar overgrowth phenotype observed in liver-specific *Mst1/2* knockout mice (Camargo et al., 2007; Dong et al., 2007; Lu et al., 2010). In the intestine, expression of endogenous YAP is restricted to the progenitor/stem cell compartment, and YAP overexpression expands the undifferentiated progenitor cells, without

affecting whole organ size (Camargo et al., 2007). Similarly, YAP overexpression in the basal layer of the epidermis increases keratinocytes proliferation, leading to defective stratification and reduced differentiation (Zhang et al., 2011). Apparently in contrast with these observations, *Mst1/2* conditional knockout in murine pancreas induces a reduction of organ size. Nevertheless, the increased YAP activity in the pancreas maintains ductal cells in an active proliferation status, but their overgrowth is likely balanced by tissue autodigestion due to defective formation of the pancreatic ducts (George et al., 2012; Gao et al., 2013).

Despite the tremendous impact that loss of YAP/TAZ signaling has on early development, YAP/TAZ inactivation in adult tissues may be inconsequential. The best example is given by the double conditional knockout in the intestinal epithelium, showing how YAP/TAZ are dispensable for normal tissue homeostasis (Azzolin et al., 2014). Liver homeostasis, instead, is disrupted by YAP/TAZ signaling inactivation. In particular, *Yap* conditional knockout in mouse liver leads to loss of hepatocytes and biliary epithelial cells due to decreased proliferation and increased apoptosis (Zhang et al., 2010). In the heart, *Yap* deletion after cardiomyocytes maturation results in dilated cardiomyopathy with thinning of the ventricular walls and possibly heart failure; the disease is accelerated by *Yap/Taz* double knockout, while *Taz* inactivation alone has no effect (Xin et al., 2013).

In the adult tissues, YAP/TAZ signaling plays a fundamental role in regeneration. YAP and possibly TAZ have been show to promote both the reprogramming of differentiated cells and the expansion of stem and progenitor cell populations, two key processes intervening during regeneration after different types of tissue injury. A number of studies highlighted how YAP/TAZ activation appears as a necessary and evolutionary-conserved step during regeneration of different organs (Johnson and Halder, 2014). In a mouse model of cholestatic injury, *Yap* liver knockout compromises bile duct and hepatocyte proliferation, but also enhances hepatocyte necrosis (Bai et al., 2012). After myocardial infarction, cardiac-specific deletion of *Yap* decreases cardiomyocyte proliferation and increases apoptosis and fibrosis. Conversely, YAP overactivation stimulates cardiac regeneration, which resulted to be correlated with YAP activation of embryonic and proliferative gene programs in cardiomyocytes (Xin et al., 2013; Del Re et al., 2013). Particularly interesting is the case of intestine regeneration, since lack of YAP/TAZ in this tissue has no consequences in normal conditions (see above). However, during intestinal regeneration, increased YAP

expression is registered and lack of YAP impairs the regenerative response (Cai et al., 2010), thus showing the potentiality of YAP/TAZ signaling reactivation for the regulation of tissue proliferative homeostasis.

2.2.5 Role of YAP/TAZ in cancer

YAP/TAZ signaling might be seen as a finely tunable system that tissues and organs use to keep under control the balance between cell proliferation and cell death, tissue growth and growth arrest. During development this balance is more shifted toward proliferation and growth, until the signaling is progressively silenced and restricted to the small stem/progenitor cells niches. Conditions such as different types of injury require the reactivation of YAP/TAZ signaling in order to trigger transient expansion of progenitors for the replacement of the damaged tissue. However, similarly to what happens when YAP/TAZ signaling remains overactive during development, leading to the growth of the organs beyond their physiological limit, an aberrant activation of the pathway in the adult tissues means the proliferative homeostasis out of control and thus cancer.

This vision is strongly supported by numerous studies showing how YAP and TAZ are activated with a very high frequency in a variety of human malignant tumors, such as ovarian, colon, gastric, liver, esophageal, lung, breast cancers. Moreover, YAP/TAZ aberrant activation is associated with metastasis and poor outcome. Functionally, YAP/TAZ signaling is able to sustain tumor progression toward malignancy in different ways, by exploiting the same biological properties that promote and regulate normal organ growth. These are represented by proliferation, cell survival and "stemness" (Piccolo et al., 2014). Cell survival is promoted for example by the YAP-mediated blocking of TNF-α and FAS-induced cell death, while TAZ is able to foster the resistance to the chemotherapeutic drug Taxol in human breast cancer (Dong et al., 2007; Lai et al., 2011). Stem cell properties of the so-called cancer stem cells (e.g. proliferative potential, self-renewal, chemotherapy resistance) are promoted in breast cancer by TAZ activity, which is required and sufficient to sustain these cancer stem cell features (Cordenonsi et al., 2011).

What really turns on YAP/TAZ signaling in cancer is poorly understood, since mutations in Hippo pathway components may account for an extremely scant number of cases. Altered tissue architecture and mechanical cues derived from changes in ECM rigidity may explain at least part of the general YAP/TAZ activation. Intense research is

still needed to unravel more complete mechanisms driving YAP/TAZ behaviour and their biological responses in cancer, and to design novel therapeutic strategies aimed at disrupting the malignant YAP/TAZ potential (Piccolo et al., 2014).

2.3 Crosstalk between YAP/TAZ and Wnt/β-catenin pathway

YAP/TAZ signaling has been shown to interact with a number of other signaling pathways, such as Wnt/β-catenin, TGF-β/BMP, Notch, Hedgehog, HIF/hypoxia, cAMP and Rho GTPases signalings, during development, regeneration and cancer. The most known and likely the strictest crosstalk is that between YAP/TAZ and Wnt/β-catenin pathways. Indeed, recent discoveries indicate how, more than distinct pathways speaking to each other, they are better described as a unique signaling network, regulated by a complex body of upstream players and regulating a wide panorama of instrumental biological responses.

2.3.1 The Wnt/β-catenin pathway

The Wnt/β-catenin pathway shares key biological functions with YAP/TAZ, since Wnt ligands activating the pathway belong to a family of growth factors involved in cell proliferation, stem cell expansion, regeneration and tumorigenesis. The Wnt/β-catenin or canonical pathway is one of the three different pathways activated upon Wnt receptor activation, together with the noncanonical planar cell polarity pathway and the Wnt/Ca^{2+} pathway (Clevers, 2006).

Canonical Wnt signaling relies on the regulation of the transcriptional co-activator β-catenin by a cytoplasmic destruction complex. Basically, in the absence of Wnt signal, the destruction complex keeps the levels of free β-catenin in the cytoplasm low by driving it to degradation. The arrival of a Wnt ligand stabilizes β-catenin, which then translocates into the nucleus leading to the pathway target genes transcription. β-catenin destruction complex is composed of the central scaffold protein AXIN, the adenomatous polyposis coli (APC) and the two kinases casein kinase 1 (CK1) and glycogen synthase kinase-3 (GSK3). AXIN and APC act as scaffolds for the kinases activity on β-catenin. When Wnt signaling is off, CK1 and GSK3 sequentially phosphorylate β-catenin on conserved Ser/Thr residues. Phosphorylated β-catenin is

recognised and ubiquitylated by β-TrCP, a component of a dedicated E3 ubiquitin ligase complex, that targets β-catenin to proteasomal degradation. Wnt signaling is initiated by the Wnt ligands, a family represented in mammals by roughly 20 cysteine-rich secreted glycoproteins acting as morphogens. Wnts bind to Frizzled (Fz) proteins, which are transmembrane receptors with an extracellular N-terminal cysteine-rich domain, and to low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6). Direct binding to LRP5/6 is used also by the secreted Dikkopf (Dkk) proteins, to conversely antagonize Wnt signaling. The formation of Wnt/Fz/LRP5/6 complex enables Fz-mediated interaction with the cytoplasmic protein Dishevelled (Dvl). Dvl and LRP5/6 cytoplasmic tail are phosphorylated by CK1 and GSK3, leading to the recruitment of AXIN from the destruction complex to the plasma membrane, where it directly binds LRP5/6. This is the key event causing the functional inactivation of the destruction complex, β-catenin accumulation and its nuclear translocation (Clevers, 2006; Konsavage and Yochum, 2013) (Figure 2.4).

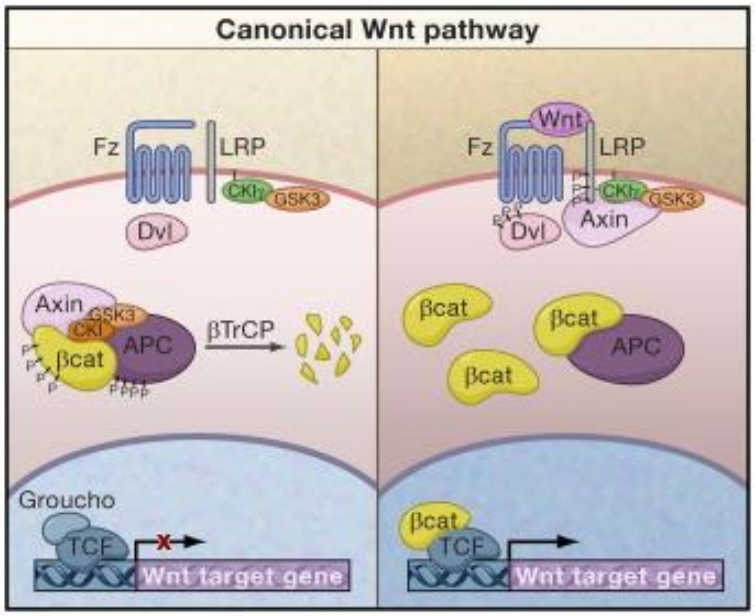

Figure 2.4. Schematic representation of the canonical Wnt/β-catenin pathway. See text for details (From Clevers, 2006. Reprinted with permission).

Within the nucleus, β-catenin binds TCF/Lef transcription factors. In the absence of Wnt signals, TCF acts as a transcriptional repressor bound to Groucho/TLE complex. However, β-catenin arrival displaces Groucho/TLE co-repressor, converting TCF/Lef into an activator. Other transcriptional co-activators are also recruited, including the histone acetylase CBP/p300 and the component of the SWI/SNF chromatin remodeling

complex BRG1. Altogether, they affect local chromatin status permitting the transcription of Wnt/β-catenin target genes. Numerous TCF/Lef target genes, such as *Axin*, *cMyc*, *CyclinD1*, have been identified, promoting cell proliferation but also controlling fate determination and terminal differentiation. The different transcriptional outputs is mainly ascribed to the developmental identity of the responding cell, resulting cell type-specific (Clevers, 2006; Konsavage and Yochum, 2013) (Figure 2.4).

2.3.2 Wnt signaling regulates YAP/TAZ activity

A recent discovery indissolubly linked YAP/TAZ activity to the Wnt signaling, as it demonstrated that YAP and TAZ are integral component of the β-catenin destruction complex. From here important functional consequences derive, impacting on both Wnt/β-catenin and YAP/TAZ biology. The destruction complex not only sequesters β-catenin, impeding its nuclear activity, but it also serves as cytoplasmic sink for YAP/TAZ, regulating their stability and nuclear availability. YAP/TAZ association to the destruction complex is mediated by AXIN. The binding requires the same domain of AXIN recognised by LRP5/6. Thus, Wnt signaling, by promoting LRP5/6-mediated AXIN recruitment at the plasma membrane, physically dislodges YAP/TAZ from the destruction complex. As a consequence, YAP/TAZ are free to enter the nucleus and activate transcription. These findings depict a scenario in which Wnt signaling emerges as a potent regulator of YAP/TAZ nuclear activity, in the same way as it regulates the nuclear activity of β-catenin. In the absence of a Wnt signal, both β-catenin and YAP/TAZ are sequestered in the cytoplasm and inactivated, while a Wnt signal causes the concomitant nuclear translocation and activation of β-catenin and YAP/TAZ (Azzolin et al., 2014). For TAZ, but not for YAP, besides the cytoplasmic sequestration the destruction complex represents also a means of degradation. In fact, within the complex, phosphorylated β-catenin bridges TAZ to its ubiquitin ligase β-TrCP, by a phosphorylated β-catenin/TAZ/β-TrCP association that leads to TAZ degradation (Azzolin et al., 2012) (Figure 2.5).

In such a context, YAP/TAZ appear as mediators of Wnt signaling and as a branch of the Wnt transcriptional effects. Therefore, YAP/TAZ enters the world of Wnt biological outcomes, so that a significant fraction of Wnt target genes and Wnt-induced biological responses are actually YAP/TAZ-dependent. Aberrant intestinal crypts proliferation upon loss of the APC tumor suppressor is a perfect example of this. APC inactivation in conditional knockout mice is indeed a model to study the effects of Wnt signaling on

the expansion of progenitor cells. YAP/TAZ were revealed to be essential for the APC loss-induced crypts expansion, since APC/YAP/TAZ triple knockout mice display a complete rescue of the intestinal phenotype, overcoming the rapid death provoked by the sole APC deficiency (Azzolin et al., 2012; Azzolin et al., 2014).

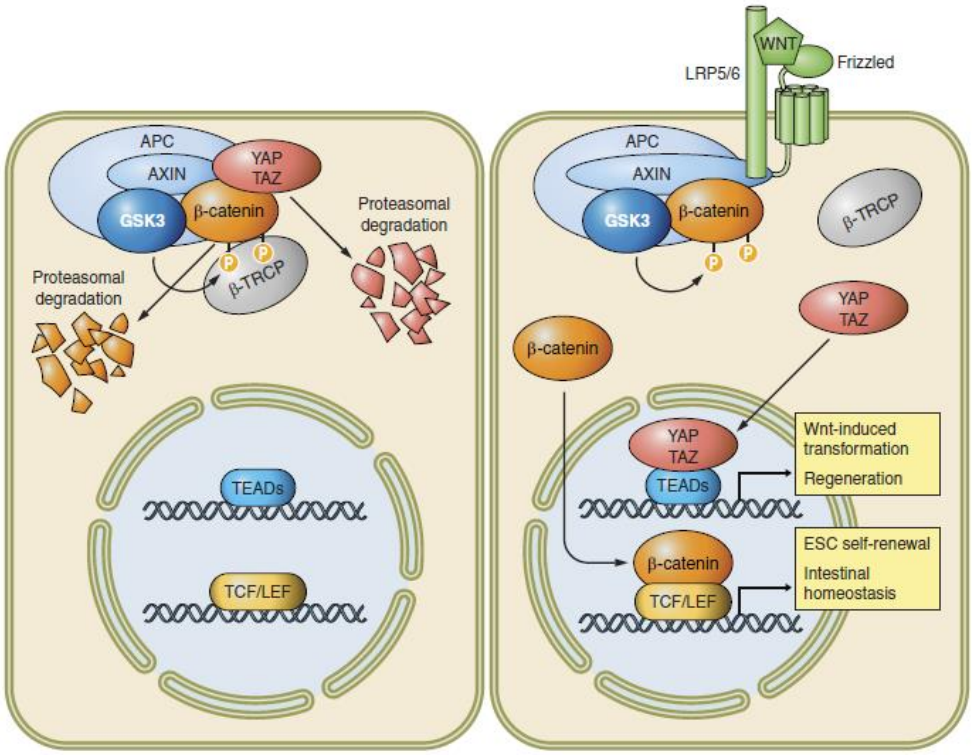

Figure 2.5. Crosstalk between YAP/TAZ and Wnt/β-catenin pathway. On the left, in the absence of Wnt signals, the β-catenin destruction complex sequesters and inactivates YAP/TAZ together with β-catenin. On the right, upon Wnt signaling, YAP/TAZ are dislodged from the destruction complex, leading to the concomitant nuclear translocation and activation of β-catenin and YAP/TAZ. See text for details (From Piccolo et al., 2014).

2.3.3 YAP/TAZ as antagonists of Wnt/β-catenin signaling in the cytoplasm

YAP/TAZ incorporation in β-catenin destruction complex has also a significance different from the regulation exerted by Wnt signals on YAP/TAZ activity, that explains the function of YAP/TAZ cytoplasmic proteins as antagonists of Wnt/β-catenin signaling. YAP/TAZ associated to AXIN within the destruction complex, in fact, are essential for β-TrCP recruitment to the complex. In the absence of Wnt signals, this means that YAP/TAZ are required for β-catenin degradation, and depletion of YAP/TAZ leads to the activation of β-catenin, without the need of a Wnt upstream signal (Azzolin et al., 2014) (Figure 2.5).

The biological meaning of this YAP/TAZ cytoplasmic role is apparent in mouse ES cells, where YAP/TAZ depletion can substitute GSK3 inhibition in maintaining ES cells in undifferentiated/self-renewing state. YAP/TAZ depletion has no effect for ES cells maintenance in the classic medium supplied with the GSK3 inhibitor, so YAP/TAZ nuclear activity is irrelevant in these conditions. But, in the absence of the GSK3 inhibitor, cytoplasmic YAP/TAZ depletion is able to block β-TrCP-mediated degradation of β-catenin, thus restoring the β-catenin-dependent transcriptional response necessary for ES self-renewal (Azzolin et al., 2014).

Another way by which cytoplasmic YAP/TAZ antagonizes Wnt signaling is offered by TAZ, that was shown to inhibit the CK1-mediated phosphorylation of Dvl. This blocks Wnt signal transduction from the plasma membrane to the destruction complex, impeding β-catenin release and nuclear activity (Varelas et al., 2010).

2.4 Hippo pathway and YAP/TAZ: is angiogenesis on the way too?

The angiogenesis is the process of new vessels formation upon sprouting of capillaries from pre-existing vessels. The results shown in this thesis suggest a role of YAP/TAZ-mediated transcription in vertebrate angiogenesis, come from observations of developmental angiogenesis in a YAP/TAZ zebrafish reporter line. No direct link between YAP/TAZ-mediated transcription and angiogenesis exists in the literature. However, very recent publications are unraveling novel interactions between signaling and metabolic pathways promoting angiogenesis and YAP/TAZ signaling. These findings open at least the intriguing possibility of a YAP/TAZ involvement in this process as mediators of these pathways in angiogenesis regulation.

Before to present these advances, a brief introduction follows, about the use of zebrafish as a powerful tool and model to study developmental, physiological and pathological angiogenesis.

2.4.1 Studying angiogenesis in the zebrafish model

The zebrafish vasculature and the basic vascular plan of the developing embryo are remarkably similar to those in the other vertebrates and in mammals (Roman and

Weinstein, 2000; Isogai et al., 2001; Lawson and Weinstein, 2002). Developmental vasculogenesis, angiogenesis and vascular remodelling involve the same vascular growth and differentiation factors involved in mammals (Lawson and Weinstein, 2002), and the physiophatology of the vascular system is also highly similar between zebrafish and mammals (Lambrechts and Carmeliet, 2004; Dejana et al., 2009).

In zebrafish development, primary vessels form in a process known as vasculogenesis: at 13 somite-stage, endothelial cells precursors migrate from the lateral mesoderm and originate the first single circulatory loop present at 24 hours post fertilization (hpf). Subsequent vasculature development occurs by angiogenic processes, in particular in the formation of the intersegmental vessels (ISVs) of the trunk, which start sprouting from the dorsal aorta at 20 hpf (Isogai et al., 2001). This primitive embryonic vasculature expands by angiogenesis during later development and will be remodelled into the mature vascular system of the adult fish. Zebrafish cardiovascular system develops considerably fast, since a beating heart, a complete circulation loop and circulating erythroblasts are found as soon as at 24 hpf (Jensen et al., 2011).

These features, coupled with the general advantages of the zebrafish model organism described before, such as the extra uterine development, the transparency and the genetic amenability, make zebrafish a powerful experimental platform to study physiological and pathological angiogenesis (Jensen et al., 2011; Santoro, 2014). Moreover, transgenic zebrafish are available, expressing fluorescent proteins in particular districts of the cardiovascular system, that enable *in vivo* monitoring of angiogenic processes at single-cell resolution in such a quick and versatile way impossible to realize in mammals (Herbert et al., 2009; Jensen et al., 2011). Commonly used transgenic lines are those based on the *fli1a* or *kdrl* promoters, labeling the whole vasculature, and on the *gata1* promoter, driving the transgene expression in the erythrocytes (Jensen et al., 2011).

Pharmacological screens and analysis of chemicals interfering with or promoting angiogenesis are also easily performed in the zebrafish model. Small, orally active drugs can be added directly to the water and are homogeneously and simultaneously deliver to a high number of embryos (Kaufman et al., 2009; Jensen et al., 2011).

The strong similarities between the zebrafish angiogenesis and the mammalian counterpart in physiological and pathological conditions has opened promising possibilities to model human diseases characterized by angiogenic impairment, and mutants resembling human vascular disorders have already been developed. Another

interesting application is represented by the modelling of tumor and metastasis processes in the zebrafish embryo, for instance after implantation of fluorescently labeled mammalian tumor cells in transgenic zebrafish. This permits to follow *in vivo* dynamic aspects of tumor biology such as metastatic cells dissemination and invasion (Lee et al., 2009; Rouhi et al., 2010; Jensen et al., 2011).

2.4.2 Wnt pathway, hypoxia, Angiomotins and mevalonate/Rho GTPases signalings: possible roads linking YAP/TAZ and angiogenesis

Wnt pathway

Several knockouts and mutants of Wnt signaling components in animal models evidence a role of Wnt pathway in vascular development. Consistently, expression of Wnt ligands and receptors, as well as TCF/Lef transcription factors has been observed in endothelial cells, and supported by the activation of mouse Wnt/β-catenin reporter in the vasculature during embryonic development. The activation of β-catenin in the adult vasculature is low, but it has been reported during pathological neovascularization, suggesting a function for Wnt/β-catenin signaling in actively growing vessels but not in healthy, resting vasculature. Different studies showed a co-operation of Wnt/β-catenin and Notch signaling pathways in endothelial cells specification and morphogenesis. Moreover, various angiogenic factors, such as VEGF and IL-8, are transcriptionally induced by the Wnt/β-catenin signaling (Choi et al., 2012).

Nevertheless, some contrasting results have also been found, since the Wnt antagonists in some cases promoted, in others inhibited angiogenesis. The discrepancy is likely due to the fact that different levels of expression of these antagonists have a concentration-dependent Wnt regulatory activity (Choi et al., 2012).

Hypoxia

Hypoxia is an undeniable driving force of angiogenesis in many contexts, ranging from developmental angiogenesis to angiogenesis occurring during cutaneous wound healing and tumor progression (Jensen et al., 2011; Semenza, 2012; Liao and Johnson, 2007).

A very recent work strongly connected hypoxia to the Hippo pathway and YAP activity. Specifically, a direct binding between the E3 ubiquitin ligase SIAH2, an essential component of the hypoxia response pathway, and LATS1/2 kinases of the Hippo cascade was evidenced. Under hypoxia, SIAH2 promoted LATS1/2

ubiquitylation and degradation, leading to reduced LATS1/2-mediated YAP phosphorylation and increased YAP transcriptional activity. In a breast cancer xenograft mouse model, SIAH2 knockdown suppressed tumorigenesis by this mechanism. Furthermore, YAP was required for HIF1α accumulation and supported the transcription of HIF1α target genes, such as *VEGF*. The two proteins physically interacted in the nucleus and YAP/HIF1α was found at the HRE (hypoxia responsive element) site within the *VEGF* promoter region in response to hypoxia. Consistently, YAP knockdown in the same xenograft mouse model impaired HIF1α accumulation and reduced microvessel density (Ma et al., 2014) (Figure 2.6 A).

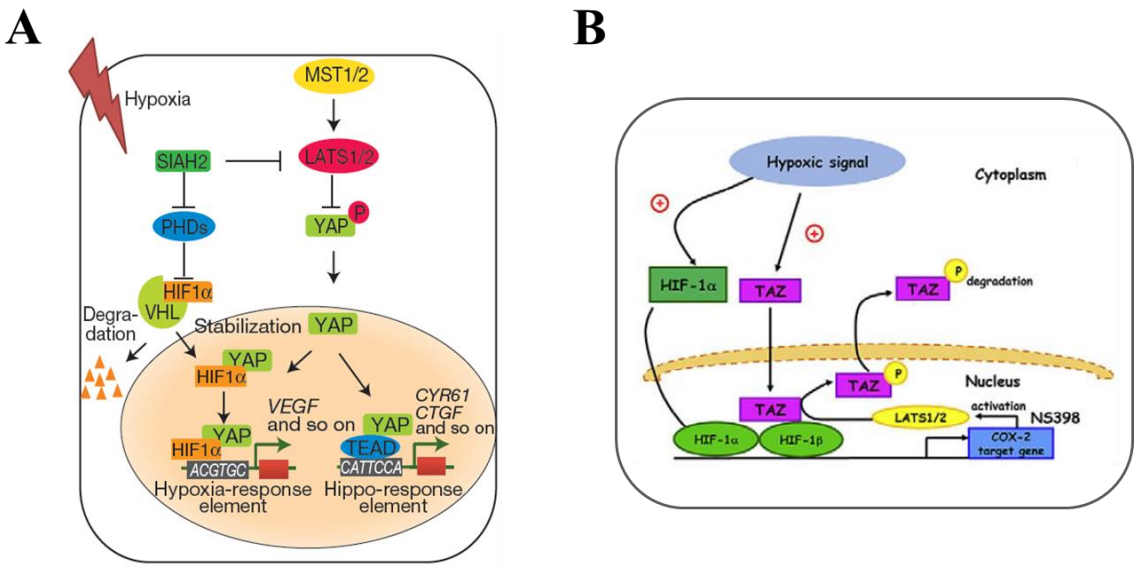

Figure 2.6. Crosstalk between the Hippo/YAP/TAZ and the hypoxia response pathways. (A) Proposed model of crosstalk between Hippo/YAP signaling and hypoxia response. Hypoxic signals promote YAP nuclear activity via SIAH2-mediated LATS1/2 inhibition. Within the nucleus, YAP binds HIF1α and supports its transcriptional activity (From Ma et al., 2014. Reprinted with permission). (B) Proposed model of crosstalk between Hippo/TAZ signaling and hypoxia response. As for YAP, hypoxia signals promote TAZ nuclear activity (through an unknown mechanism) leading to HIF1α/TAZ binding in the nucleus and HIF1α-driven transcription. COX-2 blockade by NS398 inhibitor stimulates LATS-mediated TAZ phosphorylation and subsequent degradation (From Bendinelli et al., 2013. Reprinted with permission).

A similar crosstalk has been reported also between hypoxia and TAZ in bone metastasis from breast cancer, although it is not known if the same SIAH2-mediated mechanism is involved too. HIF1α and TAZ were found to co-localize in bone metastasis specimen. In 1833 bone metastatic cells under hypoxia, HIF1α and TAZ levels increased in the nuclei and the two proteins directly interacted, enhancing HIF1α-

DNA binding. TAZ was found in the specific complex and was able to modulate the HRELuc hypoxia reporter expression. Moreover, the inhibition of COX-2 (encoded by a HIF1α target gene) prevented HIF1α induction and stimulated LATS-mediated TAZ degradation in both 1833 cells and xenograft model, where also a reduction in tumor growth was observed (Bendinelli et al., 2013) (Figure 2.6 B).

The mechanisms and crosstalks described here, however, appear far to be universal or unique in the hypoxia response. In fact, another recent work showed how hypoxia reduced YAP phosphorylation also in epithelial ovarian cancer cells, consistently with the SIAH2-mediated LATS1/2 degradation model, but had an opposite effect on TAZ, at least *in vitro*. Specifically, a potent S69-phosphorylation of TAZ was reported in hypoxic conditions in epithelial ovarian cancer, prostate cancer, colon adenocarcinoma and breast cancer cell lines, with a mechanism that seamed independent of LATS (Yan et al., 2014).

Angiomotins

Angiomotins were originally identified as angiostatin binding proteins, found predominantly expressed in the endothelium and implicated in the regulation of endothelial cell migration. *Amot* knockout mice and Amot knockdown zebrafish exhibit impairments of vascular development and of endothelial cells migration (Aase et al., 2007). Mechanistically, AMOT interacts with a polarity complex regulating cell junctions and consisting of RhoA, Patj/Mupp1 and Syx, a RhoA GTPase exchange factor (RhoGEF). The complex translocates to the leading edge of migrating cells by membrane trafficking, targeting Syx and RhoA to phosphorylated VEGFR2, for directional migration guided by EGF and PDGF/VEGF receptor tyrosine kinases (Garnaas et al., 2008; Ernkvist et al., 2009; Wu et al., 2011).

The interconnections between the Angiomotins and YAP/TAZ signaling reported in the last years delineate a complex and for some aspects controversial picture. It is clear that the Angiomotins directly interact with YAP, TAZ, LATS1/2, MST2, Merlin and Kibra proteins (Zhao et al., 2011; Chan et al., 2011; Wang et al., 2011; Oka et al., 2012; Hirate et al., 2014; Chan et al., 2013; Adler et al., 2013; Dai et al., 2013; Paramasivam et al., 2011; Yi et al., 2011). The first studies depicted the Angiomotins as inhibitors of YAP/TAZ activity: AMOTL2 (Angiomotin-like 1) knockdown resulted in YAP activation and YAP/TAZ-dependent loss of cell contact inhibition (Zhao et al., 2011); overexpression of AMOT and AMOTL1 caused TAZ cytoplasmic retention and AMOT

knockdown increased YAP/TAZ target genes expression (Chan et al., 2011); AMOTL1 and AMOTL2 (Angiomotin-like 2) overexpression favoured YAP cytoplasmic localization and AMOTL2 downregulation promoted epithelial-to-mesenchymal transition similar to YAP overexpression (Wang et al., 2011; Oka et al., 2012). Moreover, AMOT is phosphorylated by LATS downstream of signals such as cell adhesion, cytoskeleton remodelling and G-protein-coupled receptors (GPCRs) signaling and such phosphorylation promotes AMOT-YAP association and subsequent inhibition of YAP biological outcomes. Interestingly, AMOT directly binds F-actin and LATS-mediated AMOT phosphorylation disrupts this contact. As a consequence, YAP activity is inhibited, while, due to the loss of AMOT, F-actin stress fibers and focal adhesions decrease leading to inhibition of endothelial cell migration and angiogenesis (Hirate et al., 2014; Chan et al., 2013; Adler et al., 2013; Dai et al., 2013) (Figure 2.7 A).

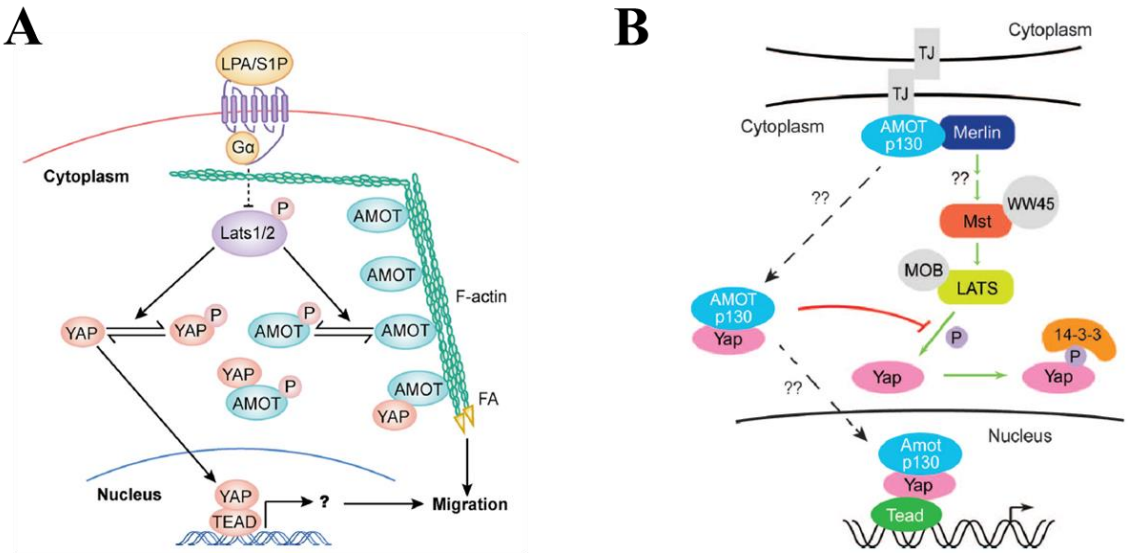

Figure 2.7. Crosstalk between AMOT and the Hippo/YAP pathway. (A) Proposed model of YAP negative regulation by AMOT. AMOT phosphorylation by LATS leads to the promotion of AMOT-YAP association, with consequent inhibition of YAP activity, and concomitant disruption of AMOT-F-actin interaction, with consequent inhibition of cell migration (From Dai et al., 2013). (B) Proposed model of YAP positive regulation by AMOT. AMOT binds to YAP, thus preventing LATS-mediated YAP phosphorylation, and within the nucleus associates with YAP-TEAD transcriptional complex in regulating YAP target genes expression (From Yi et al., 2013. Reprinted with permission from AAAS). TJ, tight junction.

This mechanism has been shown to explain the activation and inhibition of the Hippo pathway at the early blastocyst stage respectively in the nonpolar inner cells and in the polar outer cells (see above). In the adherens junctions of the inner cells AMOT is

phosphorylated by LATS, which inhibits the actin-binding activity, thereby activating the Hippo pathway. In the outer cells, the cell polarity sequesters AMOT from basolateral adherens junctions to apical domains, suppressing the Hippo pathway (Hirate et al., 2014).

A new but contrasting light on AMOT/YAP crosstalk has been shed by a recent work. Mice with a liver-specific *Amot* knockout displayed reduced proliferation and tumorigenesis in cancer contexts. Furthermore, AMOT binding to YAP prevented YAP phosphorylation by LATS, promoting YAP nuclear translocation. Within the nucleus, AMOT was associated with YAP-TEAD transcriptional complex and contributed to the regulation of a subset of YAP target genes, suggesting a positive role of AMOT for YAP/TAZ function *in vivo* (Yi et al., 2013) (Figure 2.7 B).

Mevalonate/Rho GTPases signaling

The mevalonate pathway is a metabolic pathway responsible for the synthesis of isoprenoid products of mevalonate, which are cholesterol precursors and substrates for protein prenylation, a posttranslational modification essential for multiple cellular processes, including cell migration, proliferation and angiogenesis (Figure 2.8). These isoprenoids, farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), provide lipophilic anchors essential for cytoplasm-to-membrane translocation of respectively Ras and Rho of the GTPase family. This translocation is necessary for Ras and Rho to exert their role in cell signaling transduction and in angiogenesis. In particular, the translocation of Ras activates the mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-kB) pathways, which play a fundamental role in angiogenesis. On the other hand, activated Rho associates with cortical actin and stress fibers at the membrane periphery and at the lamellipodia extensions, where it is crucial for the organization of the actin cytoskeleton and consequently for cell locomotion occurring in processes such as angiogenesis. Treatment with cerivastatin, which inhibits the HMG-CoA reductase, an enzyme upstream in the mevalonate pathway, has been show to inhibit angiogenesis *in vivo*. The effect, reversed by GGPP, is reached by inducing a delocalization of Rho from cell membrane to the cytoplasm, leading to the disruption of skeleton actin stress fibers (Vincent et al., 2001; Vincent, 2002; Choi et al., 2011).

A first regulation of YAP/TAZ activity by Rho GTPases was reported in the context of YAP/TAZ-mediated transduction of mechanical cues and equated to the effects of

Rho over the F-actin cytoskeleton, independently of YAP/TAZ phosphorylation (see above) (Aragona et al., 2013; Dupont et al., 2011). Only very recently a regulation of YAP/TAZ activity by the mevalonate pathway was reported, which is also Rho-dependent, but requires the phosphorylation of YAP/TAZ by an unknown kinase different from LATS. Mechanistically, the mevalonate pathway provides the GGPP essential for Rho GTPases membrane localization and activation. In turn, Rho GTPases activate YAP/TAZ signaling by inhibiting their phosphorylation, thus promoting their nuclear accumulation and biological activities. Statins like cerivastatin, by delocalizing Rho GTPases from the cell membrane, cause YAP phosphorylation and TAZ instability, even at doses unable to affect F-actin polymerization (Sorrentino et al., 2014).

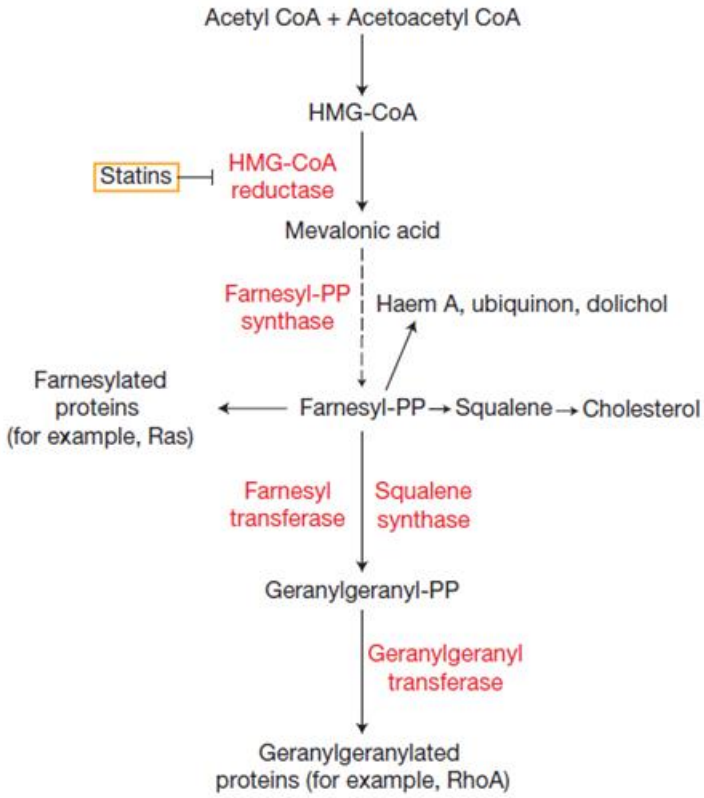

Figure 2.8. Schematic representation of the mevalonate pathway. The mevalonate pathway enzymatic cascade produces the isoprenoid derivatives of mevalonate farnesyl-PP (FPP) and geranylgeranyl-PP (GGPP), required for Ras farnesylation and Rho geranygeranylation (Modified from Sorrentino et al., 2014. Reprinted with permission).

3 MATERIALS AND METHODS

3.1 Animals

The fish were staged and fed according to the methods described by Kimmel et al., 1995, and maintained in large scale acquaria systems (Aquarienbau Schwarz, Göttingen-Germany; ZebTech, TECNIPLAST; Müller-Pfleger). A constant high-quality water exchange is guaranteed by recirculation systems with biological filters and a reverse osmosis supply. Water temperature (28,5 °C), pH (neutral) and saline composition (conductivity between 200 and 400 µS) are maintained constant. Before distribution to the tanks, water is sterilized by UV radiation to reduce the infectious agents spreading.

Depending on the number of individuals, the fish are kept in tanks with different volumes, ranging from 1 liter for single animals, to 5-10 liters for up to 30 fish. The dark-light cycle is based on a photoperiod of 12 hours of light and 12 hours of dark. The fish are fed once a day with dry food (TetraMin) and twice with living *Artemia salina* nauplia. *Artemia salina* is a primitive aquatic arthropod, bought as cysts and raised to nauplia stage into inverted cone hatcheries, in appropriate salinity, oxygen and light conditions.

The fish are mated in the late afternoon into 1 liter thanks holding an internal removable container with a grid on the bottom. This avoids fish to eat their own eggs and permits to collect the eggs from the bottom of the external tank. Male and female are kept apart by a plastic transparent separator, which is removed in the morning after the light turns on. The eggs are collected with a sieve and transferred in Petri dishes containing embryo medium (fish water), where they are incubated at 28,5 °C (Westerfield, 2000). At 5-8 hpf unfertilized eggs and dead embryos are selected under the dissecting microscope and removed. If they are not fixed or used in some experiments, at 6 days post fertilization (dpf) the larvae are transferred into 1 liter thanks, with the same medium and temperature, and maintained until 2-3 weeks of age, when they are moved to the ZebTech, TECNIPLAST acquaria. From 6 dpf to 2-3 weeks zebrafish larvae are fed once a day with dry Artemia powder (Novotom, JBL).

3.2 Generation of *Tg(Hsa.CTGF:nlsmCherry)*, *Tg(Hsa.CTGF:eGFP)* and *Tg(Hsa.CTGF:VenusPEST)* lines

The -200/+27-*CTGF* promoter fragment was amplified by PCR from a human genomic DNA using the following oligonucleotides, containing the suitable restriction sites for the subsequent clonings: Hsa.CTGF-for (5'-TCTAGAAGATCTTCTGTGAGCTGGAGTGTGC-3') and Hsa.CTGF-rev (5'-AAGCTTCCATGGAGCGGGGAAGAGTTGTTGT-3'). The amplified product was cloned in the pCR™-Blunt II-TOPO® plasmid (Invitrogen), according to the manufacturer's instructions. The *CTGF* promoter fragment was then subcloned in the pGL3-basic Luciferase Reporter Vector (Promega), for the luciferase assays (see below), using the BglII and HindIII restriction enzymes. The same restriction sites were also used to finally subclone the fragment into the Gateway 5' entry vector pME-MCS (Invitrogen). The resulting p5E-Hsa.CTGF entry vector was recombined with the nlsmCherry, eGFP and VenusPEST-carrying middle entry vectors and the p3E-polyA entry clone containing the SV40 late polyA signal (Invitrogen). Entry plasmids were recombined into the Tol2 destination vector pDestTol2pA2 (Invitrogen) through a MultiSite Gateway LR recombination reaction as previously described (Kwan et al., 2007). 30 pg of the recombined Tol2 destination vectors with 25 pg of *in vitro* synthesized Tol2 transposase mRNA (Kawakami et al., 2004) were co-injected into one-cell stage wild-type zebrafish embryos.

Microinjected embryos were selected for mosaic transgenic expression at 24 and 72 hpf using an epifluorescent microscope, raised to adulthood and outcrossed to wild-type fish. Overall, 13 out of 17 screened fish were identified as founders. Founder fish for each reporter line, *Tg(Hsa.CTGF:nlsmCherry)*, *Tg(Hsa.CTGF:eGFP)* and *Tg(Hsa.CTGF:VenusPEST)*, were selected for fluorescence level and the number of transgenic insertions (i.e. for the proportion of the progeny positive for the transgene) in their genome or in that of the fish of the following generations, in order to establish stable single allele transgenic lines.

3.3 Transient Yap/Taz overactivation in the endothelium

Flag-mTAZ-4SA was subcloned in pME-MCS (Invitrogen) from a pCS2 vector

(Azzolin et al., 2012). MultiSite Gateway LR recombination reaction was performed as described above, to recombine the obtained pME-TAZ-4SA, the p5E-fli1a and the p3E-polyA (Invitrogen) into the Tol2 destination vector pDestTol2CG2 (Invitrogen). 20 to 40 pg of the recombined Tol2 destination vectors with 25 pg of *in vitro* synthesized Tol2 transposase mRNA (Kawakami et al., 2004) were co-injected into one-cell stage *Tg(kdrl:GFP)* zebrafish embryos. The effect of Yap/Taz transient overactivation in the endothelium by TAZ-4SA expression was analyzed at 32 hpf at the confocal microscope.

3.4 Zebrafish transgenic lines

The following fish lines were used and outcrossed either to wild-type fish or to the *Tg(Hsa.CTGF:nlsmCherry)* Yap/Taz reporter line: Tg(7xTCF-Xla.Siam:GFP)^{ia4} Wnt/β-catenin reporter (Moro et al., 2012), *Tg(hsp70:dkk1-GFP)* heat shock inducible Dkk overexpressing line (Stoick-Cooper et al., 2007), *apc*^{hu745} mutant line (Hurlstone et al., 2003), *Tg(cmlc2:GFP)* (Burns et al., 2005), *Tg(gutGFP)*^{s854} (Field et al., 2003), and *Tg(kdrl:GFP)* (Beis et al., 2005).

3.5 Genotyping of APC mutants

apc^{hu745} mutant carriers were genotyped by PCR amplification followed by sequencing for the identification of the point mutation (cytosine to timine). Genomic DNA was extracted from the tail of adult fish and PCR amplified with the following primers: apc-for (5'-CTACCCAACTTTACCTATATCAG-3') and apc-rev (5'-GACTCTCAAAACTGTCAAGGG-3'). The PCR product was sequenced using apc-for primer and the sequence obtained was analyzed at position 33 downstream of the sequencing primer.

apc^{hu745} mutant carriers were outcrossed to the *Tg(Hsa.CTGF:nlsmCherry)* Yap/Taz reporter line, the progeny was screened for the reporter transgene expression and raised to adulthood. The reporter fish were screened for the *apc* mutation as described and incrossed to obtain Yap/Taz reporter embryos in *apc* homozygous mutant background.

Homozygous mutants were selected by the phenotype under the dissecting microscope. Wild-type and heterozygous embryos were used as control.

3.6 Luciferase assays

Luciferase assays were performed in MDA-MB-231 cells with the established YAP/TAZ-responsive reporters *4xGTIIC*-Lux and *8xGTIIC*-Lux (Dupont et al., 2011) and with the novel YAP/TAZ-responsive reporter *Hsa.CTGF*-Lux, generated in this study by cloning the -200/+27-*CTGF* promoter fragment into the pGL3-basic Luciferase Reporter Vector (Promega) (see above). Cell lysates were analyzed using the Dual-Luciferase Reporter Assay System (Promega, cod. E1910). Luciferase reporters (50 ng/cm^2) were transfected together with CMV-β-gal (75 ng/cm^2) to normalize for transfection efficiency with CPRG (Roche) colorimetric assay. NF2 plasmid was co-transfected at 50 ng/cm^2. DNA content in all samples was kept uniform by adding pBluescript plasmid up to 250 ng/cm^2. Cells were plated at 25% confluence (day 0) and the next day (day 1) were transfected with DNA. At day 2 cells were washed from DNA-transfection medium and harvested after 24 hours (day 3). For luciferase assays in siRNA-transfected cells, cells were plated at 20% confluence (day 0) and the next day (day 1) were transfected with the indicated siRNAs for no more than 8 hours before changing medium. At day 2 cells were transfected with DNA, at day 3 medium was changed and cells were harvested at day 4. Where indicated, cells were treated with the F-actin inhibitor latrunculin A (Lat.A, 0.5 μM). Each sample was transfected in duplicate and each experiment was repeated at least three times independently.

NF2 is Addgene #19701. Latrunculin A was from Santa Cruz. The sequences of the siRNAs used in this study are as follows (sense strand sequences are indicated):

- YAP 1: GACAUCUUCUGGUCAGAGA dTdT;
- YAP 2: CUGGUCAGAGAUACUUCUU dTdT;
- TAZ 1: ACGUUGACUUAGGAACUUU dTdT;
- TAZ 2: AGGUACUUCCUCAAUCACA dTdT;
- control: UUCUCCGAACGUGUCACGU dTdT.

YAP/TAZ siRNA 1 refers to the mix composed of oligos YAP 1 and TAZ 1; YAP/TAZ siRNA 2 refers to the mix composed of oligos YAP 2 and TAZ 2.

3.7 Morpholinos injections

The antisense morpholino oligos were obtained from Gene Tools, LLV (U.S.). The following splice blocking (MO-Yap and MO-Taz) and control morpholinos were used:

- MO-Yap: 5′-GCAACATTAACAACTCACTTTAGGA-3’;
- MO-Taz: 5’-GTATGTGTTTCACACTCACCCAGGT-3’;
- MO-ctrl: 5’-AGAACATAATCAGTAGTGTTCGA-3’.

For the validation of the Yap/Taz reporter line, MO-Yap and MO-Taz were co-injected into one-cell stage *Tg(Hsa.CTGF:nlsmCherry)* embryos, 0,7-2 ng each per embryo, and the effect on the reporter signal was analyzed at 24 hpf at the epifluorescent microscope. For the evaluation of the reporter responsiveness in the endothelium, 4-6 ng of MO-Yap per embryo were injected into *Tg(Hsa.CTGF:nlsmCherry)/Tg(kdrl:GFP)* embryos, and the effect on the reporter signal was analyzed at 32 hpf at the confocal microscope. To study the effect of Yap/Taz knockdown on the developing vascular system, MO-Yap and MO-Taz were co-injected into *Tg(kdrl:GFP)* embryos, 1-3 ng each per embryo, and the vascular phenotype was evaluated at 32 hpf at the confocal microscope.

3.8 mRNAs injections

pCS2 plasmids containing Flag-TAZ-4SA and TEAD-VP16, and pCSP1 plasmid containing Flag-YAP-5SA were a kind gift of Sirio Dupont. Each plasmid was digested with a specific restriction enzyme (NotI for Flag-TAZ-4SA and TEAD-VP16, AscI for Flag-YAP-5SA) and the genes were transcribed using the SP6 polymerase (AM1340, Lifetechnology). 0,2-1,5 pg of TAZ-4SA and TEAD-VP16 mRNAs and 50-100 pg of YAP-5SA mRNA per embryo were injected into one-cell stage *Tg(Hsa.CTGF:nlsmCherry)* embryos, *Tg(kdrl:GFP)* embryos or *Tg(Hsa.CTGF:nlsmCherry)/Tg(kdrl:GFP)* double transgenic embryos.

3.9 Protein extraction and western blot analysis

For protein extraction the embryos were deyolked (deyolking buffer: 55 mM NaCl,

1,8 mM KCl, 1,15 mM $NaHCO_3$), washed (washing buffer: 110 mM NaCl, 3,5 mM KCl, 2,7 mM $CaCl_2$, 10 mM Tris HCl pH 8,5) and lysed in RIPA buffer (Sigma) supplemented with protease and phosphatase inhibitor cocktail (Sigma). Protein concentrations were determined using the Bradford assay (BioRad) and western blot was performed as in Azzolin et al. (2014).

Anti-YAP (63.7) monoclonal antibody was from Santa Cruz. Anti-TAZ (560235) monoclonal antibody was from BD Bioscience.

3.10 Chemical treatments of zebrafish embryos

The following chemical compounds were used:

- IWR-1 (I0161, Sigma);
- XAV939 (X3004, Sigma);
- BIO (B1686, Sigma).

Tg(Hsa.CTGF:nlsmCherry) embryos were decorionated and exposed to the drugs from 24 to 48 hpf in fish water with 2 mM 1-phenyl-2-thiourea (PTU) to inhibit pigmentation. The drugs were used at 10 µM for IWR-1 and 5 µM for XAV939 and BIO, and the corresponding volume of DMSO, where the three chemicals are solubilized, was used in the controls.

After the treatments, the embryos were either analyzed under the epifluorescent microscope or fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4 °C and then stored in pure methanol at -20 °C for *in situ* hybridization.

3.11 *In situ* hybridization and quantification

Whole mount RNA *in situ* hybridization was performed on embryos pre-fixed in 4% PFA in PBS overnight at 4 °C and stored in pure methanol at -20 °C as previously described (Thisse et al., 1993). mCherry probe was synthetized from the pME-nlsmCherry supplied by the Tol2 kit (Invitrogen), which was linearized and transcribed with the T7 RNA polymerase using DIG-labeled ribonucleotides.

Variations in the intensity of *in situ* hybridization signal were quantified by subdividing the embryos of the control and the experimental group on the basis of the

signal intensity observed under the dissecting microscope. Three subgroups were made (strong, medium and weak), so that the "strong embryos" of the control pool displayed a signal intensity comparable to the "strong embryos" of the experimental pool. The embryos in each subgroup were counted and the data were plotted in a histogram. Contingency analysis were carried out with Prism software (GraphPad).

3.12 Alcian blue staining

For cartilage staining, larvae were fixed overnight in 4% PFA in PBS at 4 °C, washed in PBS and stained overnight at room temperature in Alcian blue solution (70% EtOH, 1% HCl, and 0.1% Alcian blue). Larvae were then cleared in 3% hydrogen peroxide and 1% KOH, rinsed in 70% EtOH and whole mounted in 85% glycerol.

3.13 Heat-shock treatments

Dkk overexpression in Yap/Taz reporter embryos was obtained by outcrossing the *Tg(Hsa.CTGF:nlsmCherry)* reporter line to the *Tg(hsp70:dkk1-GFP)* heat shock inducible Dkk overexpressing line. The offspring was heat-shocked every 12 hours, from 12 to 72 hpf, by replacing the fish water with water preheated to 40 °C and incubating the embryos in an air incubator at 37 °C for 1 hour. The embryos were then sorted by GFP fluorescence and GFP negative siblings were used as controls. The embryos were analyzed under the epifluorescent microscope for mCherry reporter fluorescence and then fixed in 4% PFA in PBS overnight at 4 °C and stored in pure methanol at -20 °C for *in situ* hybridization.

3.14 Organ dissection and fin surgeries

To analyze the reporter signal in the internal organs of the adult fish, *Tg(Hsa.CTGF:nlsmCherry)* fish were sacrificed and dissected under the epifluorescence microscope. The organs were imaged immediately after, either *in situ* or isolated, with conventional fluorescence or confocal microscopy. Imaging of *Hsa.CTGF*:nlsmCherry

signal in the vessels of the caudal fin was performed *in vivo* in anesthetized fish under the epifluorescence microscope.

For fin regeneration experiments, both *Tg(Hsa.CTGF:nlsmCherry)* larvae and adult fish were anesthetized with tricaine, the tail was amputated using a razor blade and the animals were returned to 28,5 °C fish water. Reporter larvae underwent fin primordia amputation at 72 hpf and amputated individuals were fixed for i*n situ* hybridization at 24 and 48 hours post amputation (hpa), together with age-matched uncut controls. Regenerating caudal fins of reporter adults were photographed *in vivo* after anesthesia at 24 and 48 hpa using brightfield and epifluorescence.

3.15 Image acquisition and analysis

Fluorescence was visualized and acquired using the conventional fluorescence dissecting microscope Leica M165FC and the confocal microscope Nikon C2 H600L. For brightfield and fluorescence imaging with the dissecting microscope, embryos and larvae were anesthetized with tricaine and mounted in 2% metilcellulose. For confocal imaging, anesthetized embryos and larvae and anatomical samples of adult fish were embedded in 1% low melting agarose. The fluorescence was visualized using 488 nm (for GFP and VenusPEST) and 561 nm (for mCherry) lasers and 20x or 40x immersion objectives (Nikon).

Fluorescence quantification of the images acquired either with the conventional fluorescence or the confocal microscope was carried out using Fiji software. Each image, saved as TIFF file and visualized as a RGB color image, was splitted in the three color components and only the channel corresponding to the fluorophore of interest was kept (red for mCherry, green for GFP and VenusPEST). A threshold was set to have a good signal-to-noise ratio and maintained for all the images of the experiment. Fluorescence was quantified as integrated density. For fluorescence quantification of whole embryos in images acquired at the conventional fluorescence microscope, the yolk and yolk extension areas were excluded from the analysis. For the quantification of the reporter signal specific of the endothelium, *Hsa.CTGF*:nlsmCherry reporter fluorescence was acquired with the confocal microscope together with the *kdrl*:GFP fluorescence in 32 hpf double transgenic embryos. Fiji software was then asked to filter the mCherry signal using the GFP as a mask, to isolate the reporter expression in the

endothelium. For each embryo, a confocal Z-stack projection was realized with the obtained filtered images. All the projections were finally analyzed as described before, by quantifying the fluorescent signal as integrated density.

Statistical analysis were carried out with Prism software (GraphPad).

3.16 Assessment of vascular changes

The defective ISVs sprouting angiogenesis in *Tg(kdrl:GFP)* Yap/Taz double morphants was evaluated at 32 hpf. Only embryos displaying a mild phenotype after brightfield observation at the dissecting microscope (i.e. in which the morpholinos are working but without a strong alteration of the phenotype) were imaged with the confocal microscope, *in vivo* or after fixation in PFA 1%. Three different parameters were then analyzed: the ISVs length, the percentage of complete ISVs, both measured at the midtrunk level, and the number of ISVs sprouts, measured at the caudal vein plexus (CVP) level. At the midtrunk level we looked at the 9 ISVs pairs corresponding to the yolk extension, but only one lane of them (9 ISVs), easily visible in the confocal acquisition, was considered for all the measurements. The length of each ISV was measured using Fiji software and expressed as percentage of the average ISVs length of the controls. The percentage of complete ISVs was calculated as the ratio, multiplied by 100, between the number of ISVs that at 32 hpf had already reached the roof level, starting to form the dorsal longitudinal anastomosing vessel (DLAV), and the total number of ISVs taken into consideration. The number of ISVs sprouts at the CVP level was obtained by counting all the ISVs (complete or not) sprouting from the dorsal aorta caudally with respect to the end of the yolk extension. For each parameter, 7-10 control and morphant embryos from two independent experiments were analyzed.

For the aberrant sprouting phenotype induced by Yap/Taz activity upregulation, again only *Tg(kdrl:GFP)* embryos with a general mild phenotype were considered and imaged at 32 hpf with the confocal microscope. The analysis focused on the same midtrunk region described before, but took into consideration both the lanes of ISVs. For each embryo, all anomalous vessel sprouts from the ISVs were counted, and divided into non-anastomosed and anastomosed sprouts, where anastomosed sprouting indicates a complete anastomosis between two adjacent ISVs. Data were expressed as number of abnormalities per embryo. A total of 51 control, 42 TAZ-4SA, 31 TEAD-VP16, 34

YAP-5SA and 16 *pDestTg(fli1a:TAZ-4SA)*-injected embryos from at least two independent experiments each were analyzed. Embryos injected with the *pDestTg(fli1a:TAZ-4SA)* construct were previously screened for *cmlc2*:eGFP expression.

All statistical analysis were carried out with Prism software (GraphPad).

4 RESULTS

4.1 Generation of Yap/Taz zebrafish reporter lines

4.1.1 ctgf *promoter contains three Yap/Taz REs and is conserved between zebrafish and humans*

A Yap/Taz zebrafish reporter line is a transgenic biosensor fish expressing a fluorescent protein under the control of regulatory sequences responsive to Yap/Taz signaling pathway. Specifically, Yap/Taz-responsive elements (REs) are represented by the DNA-binding sites recognized by Tead transcription factors and defined as MCAT sequences (AGGAATG) (Farrance et al., 1992). To drive the transcription of their target genes, the transcriptional co-activators Yap/Taz bind to Tead, which contacts its DNA-binding sites located in the regulatory regions of the target genes.

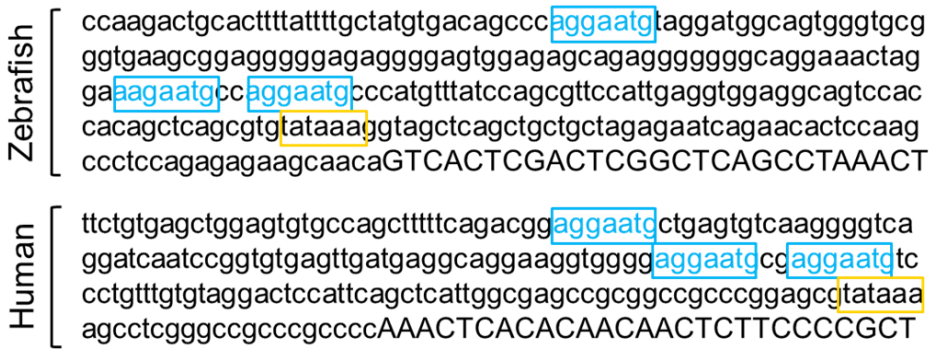

Figure 4.1. ***ctgf*** **promoter contains three Yap/Taz REs and is remarkably conserved between zebrafish and humans.** The promotorial region upstream of the 5'UTR (uppercase) of the zebrafish *ctgfa* and human *CTGF* genes both contain three TEAD DNA-binding sites (YAP/TAZ REs; in light blue) just before the TATA box (in yellow). Their sequences, orientation and distances are outstandingly conserved between zebrafish and humans.

To design a Yap/Taz-responsive construct, we therefore decided to look at the promoter of the most known Yap/Taz target gene: *ctgf*. By analyzing the promoter of the zebrafish *ctgfa* gene, we noticed that the 219 bp long sequence upstream of the 5'-UTR contains three MCAT sites just before the TATA box. We then compare this sequence with the one of the human *CTGF* gene promoter and, interestingly, we found that its corresponding region upstream of the 5'-UTR contains three MCAT sites too. Moreover, the REs sequences, their orientation and the distances among them are

remarkably conserved between zebrafish and humans, suggesting an evolutionary conserved and fundamental role of this short regulatory region in controlling *CTGF* gene expression in response to YAP/TAZ/TEAD activation (Figure 4.1).

Thus, we chose to use this YAP/TAZ REs-containing promotorial region of the *CTGF* gene as the YAP/TAZ-responsive element of the reporter transgenic construct. With the idea to filter the reporter construct from other different zebrafish-specific regulatory elements possibly present in the sequence among the desired MCAT sites, instead of the zebrafish one we used the human *CTGF* gene promoter fragment as starting point for the design of the reporter transgene.

4.1.2 Generation of CTGF*-based YAP/TAZ reporter constructs*

The transgenic construct on which a zebrafish signaling pathway reporter line is based is usually composed of three elements: the pathway-responsive fragment containing the repeated specific REs; a minimal promoter; a reporter gene. For the generation of the YAP/TAZ transgenic reporter construct we simply cloned, upstream of the reporter gene, the *CTGF* gene promoter fragment derived from the human genome. This because it contains both the YAP/TAZ REs and the TATA box, that in this case acts as a "natural" minimal promoter instead of an artificial one (Figure 4.2).

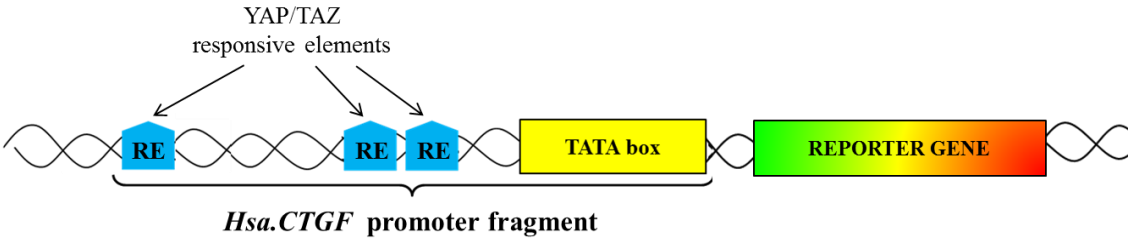

Figure 4.2. Schematic representation of the *Hsa.CTGF*-based YAP/TAZ reporter construct. The YAP/TAZ-responsive fragment of the construct is derived from the human *CTGF* promoter. It contains the pathway-specific REs MCAT and the TATA box, and regulates the expression of the downstream reporter gene.

The -200/+27-*CTGF* promoter fragment was amplified by PCR from a human genomic DNA and initially cloned in the pCR™-Blunt II-TOPO® plasmid. From this plasmid it was then cloned in different reporter vectors, first to test the responsiveness and specificity of the YAP/TAZ reporter construct *in vitro* and then to generate the transgenic biosensor fish reporting the activity of Yap/Taz *in vivo* with different fluorescent proteins.

4.1.3 The Hsa.CTGF*-based YAP/TAZ reporter construct specifically reports YAP/TAZ activity* in vitro

To assess the ability of the *Hsa.CTGF*-based YAP/TAZ reporter construct to specifically report YAP/TAZ activation, we performed a luciferase test *in vitro*. The *Hsa.CTGF* promoter fragment was cloned from the pCR™-Blunt II-TOPO® plasmid into the pGL3 luciferase reporter vector upstream of the luciferase gene, generating the YAP/TAZ-responsive reporter *Hsa.CTGF*-Lux.

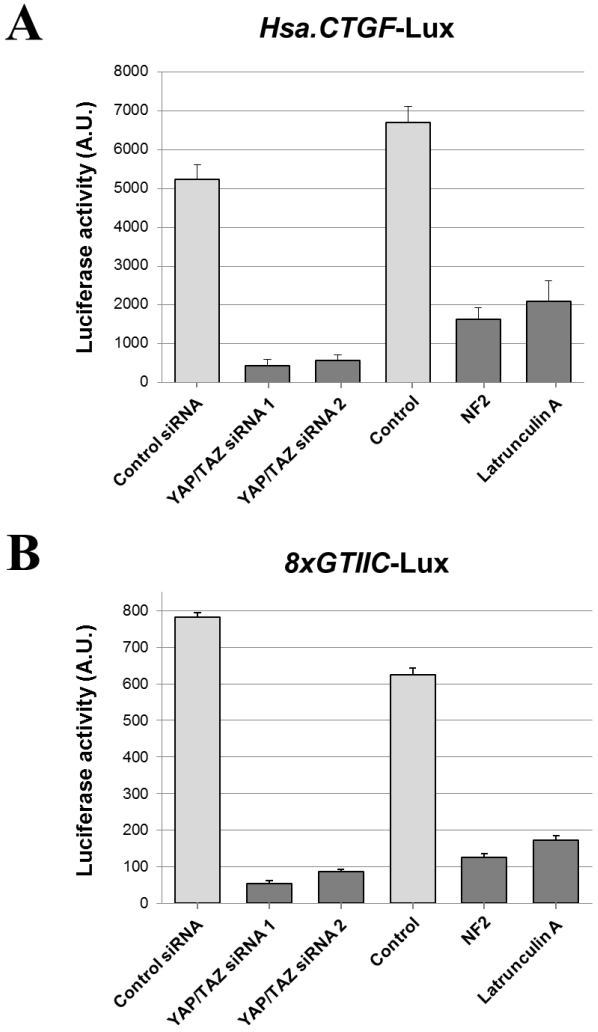

Figure 4.3. The *Hsa.CTGF*-Lux reporter is specific for YAP/TAZ activity. MDA-MB-231 cells, expressing high levels of TAZ, were transfected with the indicated siRNAs or plasmids (control is empty vector, NF2 is NF2 expression plasmid), or treated with latrunculin A. The panels display the results of luciferase tests with the *Hsa.CTGF*-Lux (A) or the *8xGTIIC*-Lux (B) reporters, recording YAP/TAZ-dependent transcriptional activity. YAP/TAZ siRNAs strongly downregulate the reporter with respect to control siRNA. Luciferase expression was also strongly reduced after both NF2-mediated Hippo pathway activation and latrunculin A-mediated mechanical pathway inhibition, when compared to transfection with empty vector. *4xGTIIC*-Lux is equivalent to *8xGTIIC*-Lux in reporting YAP/TAZ activity (data not shown).

When transfected in breast cancer cells expressing high levels of TAZ, the Hsa.*CTGF*-Lux plasmid gave a strong activation of the luciferase reporter gene. To demonstrate that the luciferase expression was specifically due to YAP/TAZ activation, we downregulated YAP/TAZ activity in different ways and monitored the reporter signal. Direct inhibition of YAP/TAZ by siRNA knockdown, activation of the Hippo pathway through NF2 and treatment with latrunculin A, an F-actin inhibitor opposing the mechanical pathway, were all able to cut down the reporter signal (Figure 4.3 A). These results clearly indicate that the *Hsa.CTGF*-based YAP/TAZ reporter construct is, at least *in vitro*, responsive to and specific for the activation of YAP/TAZ signaling.

Moreover, the *Hsa.CTGF*-Lux reporter behaves exactly as the *4x* and the *8xGTIIC*-Lux, the classic reporter plasmids commonly used to report *in vitro* YAP/TAZ activity (Mahoney et al., 2005) (Figure 4.3 B). The *4xGTIIC* element is also the YAP/TAZ-responsive fragment used for the generation of the Yap/Taz zebrafish reporters published by Miesfeld and Link, (2014). Hence, the *Hsa.CTGF*-based YAP/TAZ reporter construct represents a novel tool to effectively report YAP/TAZ activity, prompting its use *in vivo* for the development of a promising Yap/Taz zebrafish reporter.

4.1.4 Development of Tg(Hsa.CTGF:nlsmCherry), Tg(Hsa.CTGF:eGFP), Tg(Hsa.CTGF:VenusPEST) *Yap/Taz zebrafish reporter lines*

With the aim of generating transgenic Yap/Taz reporter constructs suitable for injection in the zebrafish embryo, the *Hsa.CTGF* promoter fragment formerly described was used to create the following Gateway destination vectors: *pDestTg(Hsa.CTGF:nlsmCherry), pDestTg(Hsa.CTGF:eGFP)* and *pDestTg(Hsa.CTGF:VenusPEST)*. To do this, the *Hsa.CTGF* promoter fragment was first cloned in a Gateway 5' entry clone (p5E-MCS). The p5E-Hsa.CTGF vector produced was recombined together with a middle entry clone, containing the reporter gene, and a 3' entry clone, containing the SV40 late polyA signal (p3E-polyA), in the Tol2 transposon backbone pDestTol2pA2 through a MultiSite Gateway LR recombination reaction. The reaction was repeated by using each time a middle entry clone containing a different reporter gene (pME-nlsmCherry, pME-eGFP and pME-VenusPEST). Three different Gateway destination vectors were obtained, in which the *Hsa.CTGF* promoter fragment controls the expression of the nlsmCherry, eGFP and VenusPEST fluorescent reporter genes, that codes respectively for the nuclear-localized

monomeric Cherry protein, the enhanced GFP and the destabilized green fluorescent protein Venus (Figure 4.4).

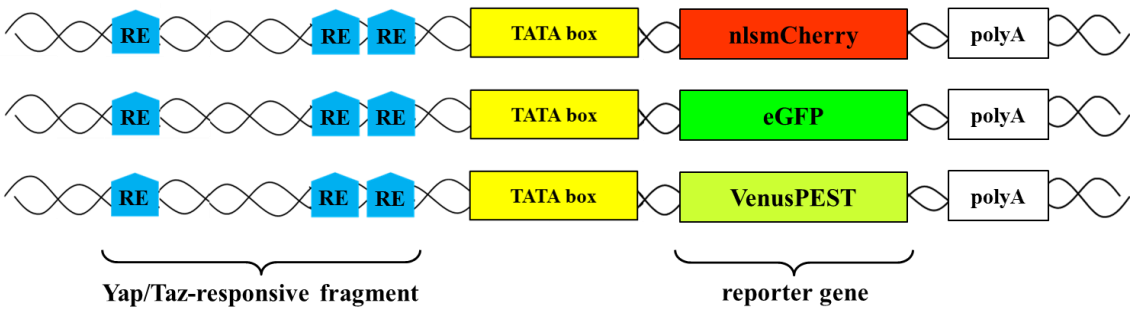

Figure 4.4. Schematic representation of the *Hsa.CTGF*-based YAP/TAZ reporter transgenes in the Gateway destination vectors. The human *CTGF* promoter fragment (Yap/Taz REs and TATA box) controls the expression of three different reporter genes (nlsmCherry, eGFP and VenusPEST).

One-cell stage zebrafish embryos were injected with each destination vector, together with the Tol2 transposase mRNA, that catalyzes the random integration of the transgenic construct into the zebrafish genome. Mosaic transgenic fish, displaying a strong transient fluorescence already at 24 hpf, were selected and raised to adulthood. Then, adult potential carriers were outcrossed to wild-type fish and the offspring was screened for the expression of the reporter protein. Averagely 75% of the injected fish prescreened for mosaic fluorescence were found to transmit the transgene to their germline. These identified founders were used to establish the stable reporter lines *Tg(Hsa.CTGF:nlsmCherry)*, *Tg(Hsa.CTGF:eGFP)* and *Tg(Hsa.CTGF:VenusPEST).*

All the offsprings from the different founder fish (injected with the same reporter transgene and injected with the different ones) shared a similar reporter protein expression pattern, displaying strong fluorescence in the same anatomical districts, such as the lens and otic vesicles, the heart, the pharyngeal arches, the vasculature. This consistency is important to exclude potential genomic positional effects due to the insertion of the transgene in different positions of the genome.

Differences among the three Yap/Taz reporter lines created are visible in terms of intensity of the fluorescent signal, more than in terms of pattern of expression. In fact, mCherry and eGFP proteins are more stable than the VenusPEST protein, which indeed resulted from the fusion of the fluorescent protein Venus with the PEST sequence, that confers fast degradation to the fusion protein. VenusPEST transgenic fish permit therefore to follow more dynamic changes of the reporter activation. Its expression

pattern, at 24 hpf, is similar to the mCherry and eGFP ones, even if the fluorescent signal is weaker, while at 48 hpf no Venus expression is easily detectable throughout the embryo, probably reflecting a general downregulation of Yap/Taz activity at this stage. This is not appreciable with the mCherry and eGFP lines likely because of the stability of the amount of fluorescent proteins produced before. At 72 hpf *Hsa.CTGF*:VenusPEST signal is almost exclusively restricted to the pharyngeal arches, which is consistently the most strongly activated region also in the mCherry and eGFP reporter lines.

4.2 *Hsa.CTGF*-based zebrafish transgenic lines are *bona fide* Yap/Taz reporters

The responsiveness and specificity of the *Hsa.CTGF*-based reporter construct for YAP/TAZ activity has been already shown *in vitro*. However, we needed to confirm that the zebrafish transgenic lines we developed, based on the same reporter construct, were able to report also *in vivo* variations of Yap/Taz activity. To validate the *Hsa.CTGF*-based Yap/Taz zebrafish reporter lines, we used different genetic approaches aimed at perturbing Yap/Taz signaling, in order to observe and report whether the fluorescent signal varied in a Yap/Taz activity-dependent manner as expected.

4.2.1 Morpholino-mediated Yap/Taz knockdown reduces Hsa.CTGF *reporter signal*

We first knocked down both Yap and Taz by co-injecting in one-cell stage *Tg(Hsa.CTGF:nlsmCherry)* embryos two splice morpholinos, targeting respectively Yap and Taz pre-mRNAs. The knockdown of endogenous Yap and Taz proteins was confirmed by Western blotting (Figure 4.5).

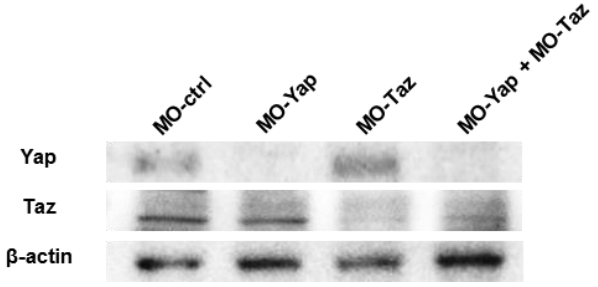

Figure 4.5. Western blot analysis of Yap and Taz morpholinos.

With the amount of morpholinos injected (0,7-2 ng each per embryo), only a slight phenotype of kinked notochord was visible at 24 hpf, while mCherry expression was significantly reduced throughout the entire embryo with respect to control morpholino injected embryos (Figure 4.6), showing that *Tg(Hsa.CTGF:nlsmCherry)* reporter line is able to report *in vivo* the downregulation of Yap/Taz activity.

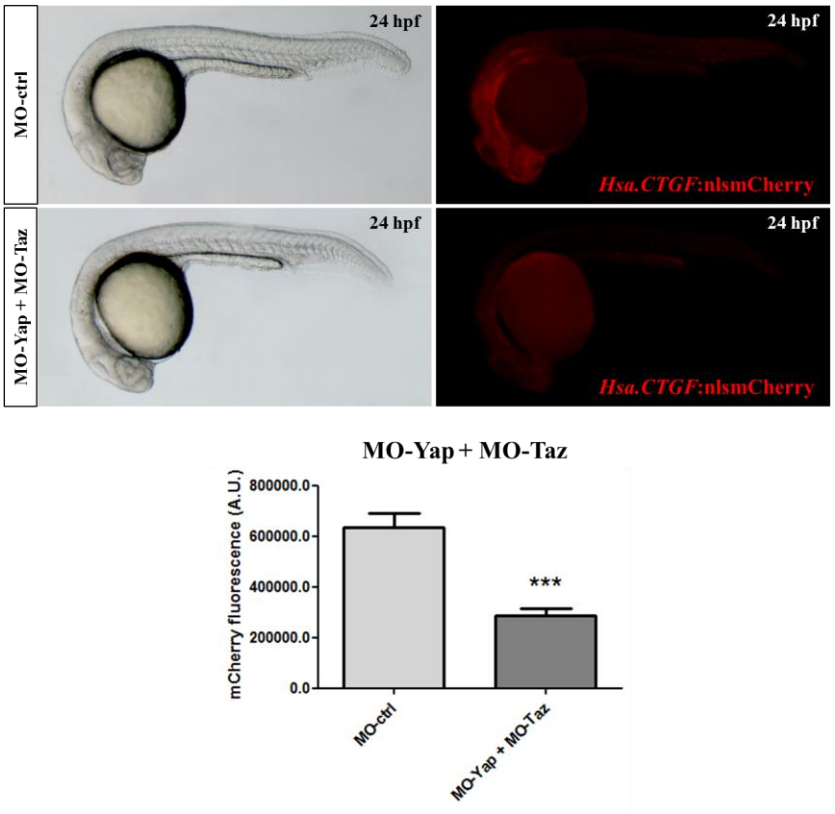

Figure 4.6. Morpholino-mediated Yap/Taz knockdown reduces *Tg(Hsa.CTGF:nlsmCherry)* reporter signal. Two splice morpholinos, targeting respectively Yap and Taz pre-mRNAs, were co-injected in one-cell stage *Tg(Hsa.CTGF:nlsmCherry)* embryos. The fluorescent reporter expression was documented and quantified at 24 hpf.

The same experiment was repeated with *Tg(Hsa.CTGF:eGFP)* and *Tg(Hsa.CTGF:VenusPEST)* lines, confirming that they are also able to report the morpholino-mediated knockdown of Yap/Taz.

4.2.2 Constitutive activation of Yap/Taz-mediated transcription increases Hsa.CTGF *reporter signal*

Three different mRNAs (coding for YAP-5SA, TAZ-4SA, TEAD-VP16 proteins) were injected in independent experiments in one-cell stage *Tg(Hsa.CTGF:nlsmCherry)* embryos to upregulate the reporter signal. YAP-5SA is a constitutively active version of

the human YAP protein, which has been mutated in its five key serine residues needed for the LATS1/2-dependent phosphorylation, that promotes binding to 14-3-3 proteins and subsequent YAP sequestration in the cytoplasm. YAP-5SA is therefore resistant to the Hippo pathway-mediated negative regulation, leading to YAP constitutive activation in the nucleus. Analogously, TAZ-4SA is the constitutively active version of the murine TAZ protein, mutated in its four serine residues recognized by LATS1/2 for the Hippo-dependent phosphorylation. Finally, TEAD-VP16 is a fusion protein of the N-terminal region of TEAD transcription factor and the activation domain of herpes simplex virus VP16. TEAD-VP16 does not need any transcriptional co-activator such as YAP or TAZ to work, leading again to a constitutive transcription of its target genes, independently of YAP or TAZ activation.

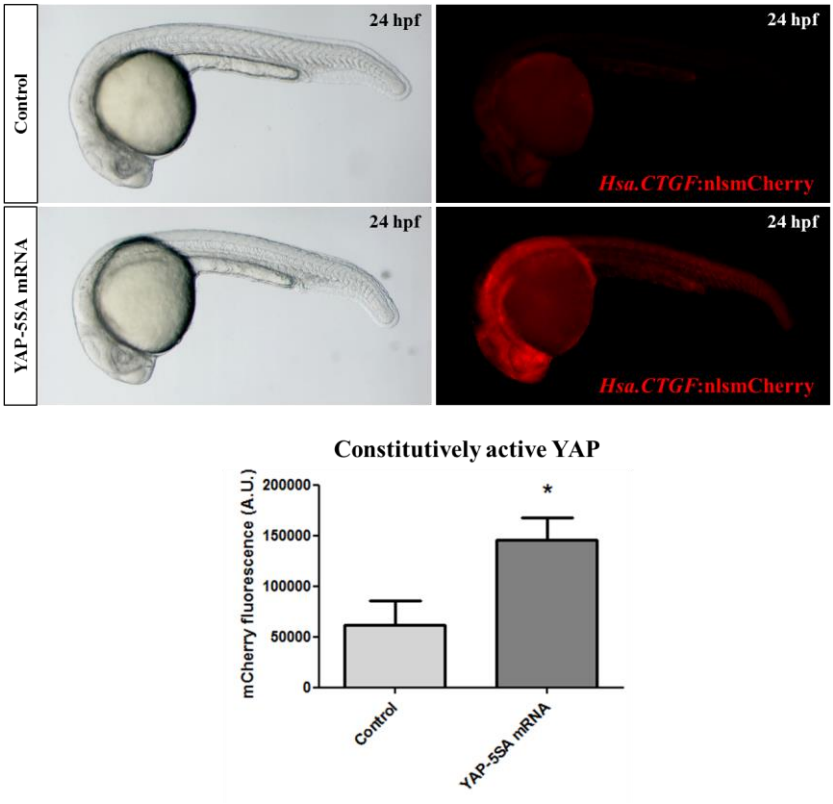

Figure 4.7. A constitutively active version of YAP increases *Tg(Hsa.CTGF:nlsmCherry)* reporter signal. YAP-5SA mRNA was injected in one-cell stage *Tg(Hsa.CTGF:nlsmCherry)* embryos. The fluorescent reporter expression was documented and quantified at 24 hpf.

When injecting YAP-5SA mRNA in one-cell stage reporter embryos, *Hsa.CTGF*:nlsmCherry expression was significantly increased in injected embryos compared to control embryos (Figure 4.7). This observation was confirmed by injecting the mRNA coding for the constitutively active version of TAZ. A strong increase of

reporter fluorescence was observed in TAZ-4SA injected embryos compared to controls (Figure 4.8). Finally, we injected also the mRNA coding for TEAD-VP16, acting downstream of Yap/Taz and directly on the activation of Tead target genes. Again, an increased mCherry expression was detected, obtaining in this case the strongest upregulation of the reporter fluorescence (Figure 4.9).

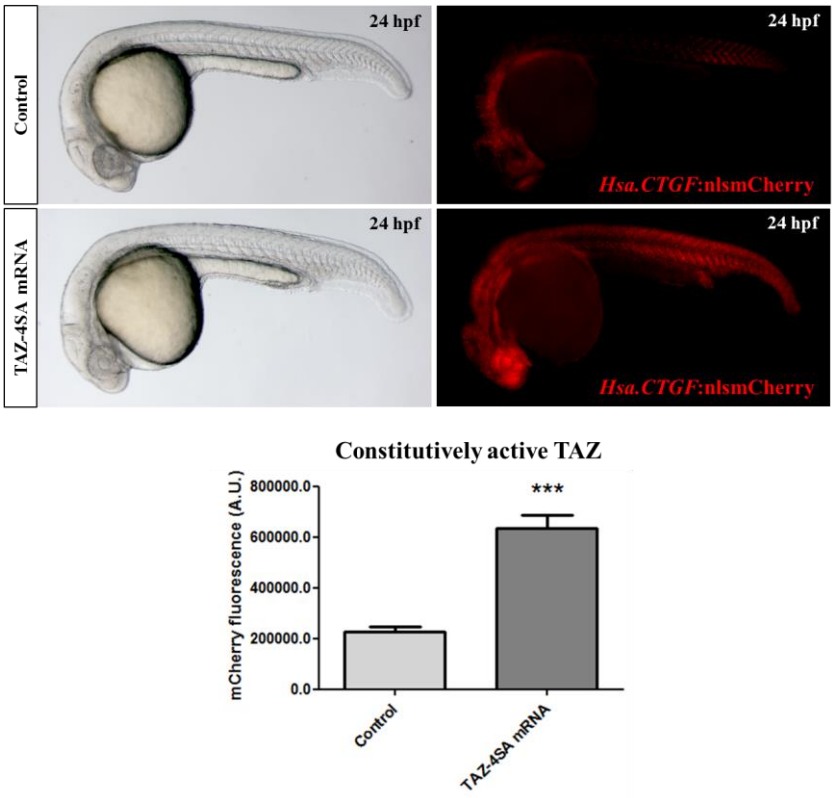

Figure 4.8. A constitutively active version of TAZ increases *Tg(Hsa.CTGF:nlsmCherry)* reporter signal. TAZ-4SA mRNA was injected in one-cell stage *Tg(Hsa.CTGF:nlsmCherry)* embryos. The fluorescent reporter expression was documented and quantified at 24 hpf.

The experiments performed with the constitutively active versions of YAP, TAZ and TEAD clearly indicate the ability of *Tg(Hsa.CTGF:nlsmCherry)* reporter line to signal *in vivo* an increased Yap/Taz activation.

A similar responsiveness to Yap/Taz activity upregulation, as observed for the downregulation, was found also for the other two Yap/Taz reporter lines produced, *Tg(Hsa.CTGF:eGFP)* and *Tg(Hsa.CTGF:VenusPEST).*

Taken together, all these Yap/Taz knockdown and upregulation experiments demonstrate that the *Tg(Hsa.CTGF:nlsmCherry), Tg(Hsa.CTGF:eGFP)* and *Tg(Hsa.CTGF:VenusPEST)* lines are responsive to Yap/Taz, being able to report *in vivo*

the variations of the signaling pathway nuclear activity. We can therefore conclude that these zebrafish transgenic lines are *bona fide* Yap/Taz reporters. Moreover, *Tg(Hsa.CTGF:nlsmCherry), Tg(Hsa.CTGF:eGFP)* and *Tg(Hsa.CTGF:VenusPEST)* lines revealed to have a good sensitivity in reporting Yap/Taz activity variations, since often the positive and negative regulation of the reporter expression has been observed in embryos treated with low reagents concentrations, at which they showed no evident morphological phenotype.

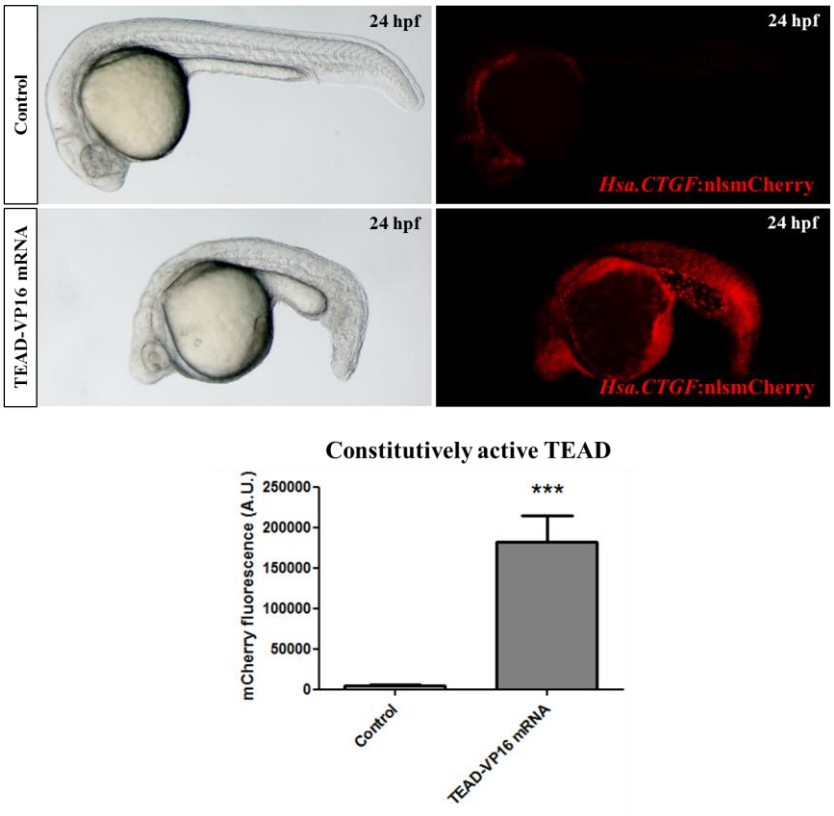

Figure 4.9. A constitutively active version of TEAD increases *Tg(Hsa.CTGF:nlsmCherry)* reporter signal. TEAD-VP16 mRNA was injected in one-cell stage *Tg(Hsa.CTGF:nlsmCherry)* embryos. The fluorescent reporter expression was documented and quantified at 24 hpf.

4.3 *Tg(Hsa.CTGF:nlsmCherry)* zebrafish reporter line reveals *in vivo* Yap/Taz activity domains

Hsa.CTGF-based zebrafish transgenic lines have been validated as Yap/Taz reporter and represent now a powerful tool to follow *in vivo*, during development and in adulthood, in physiological but also in pathological conditions, the spatio-temporal

activation of Yap/Taz/Tead-mediated transcription. Given the importance of Yap/Taz signaling in developmental as well as in tumor biology, this possibility could be very beneficial to study *in vivo* Yap/Taz involvement in these processes and it may open new insights into known or still undiscovered roles of Yap/Taz.

We started therefore the characterization of the zebrafish Yap/Taz reporter by analyzing the reporter protein expression pattern throughout embryonic and early larval development. We initially focused our attention on *Tg(Hsa.CTGF:nlsmCherry)* Yap/Taz reporter line.

4.3.1 Yap/Taz reporter expression is activated maternally

Hsa.CTGF:nlsmCherry fluorescence is ubiquitously detectable at dome stage and during epiboly in embryos derived from transgenic females crossed to wild-type fish (Figure 4.10). However, no signal is observed at these stages in the offspring of male carriers.

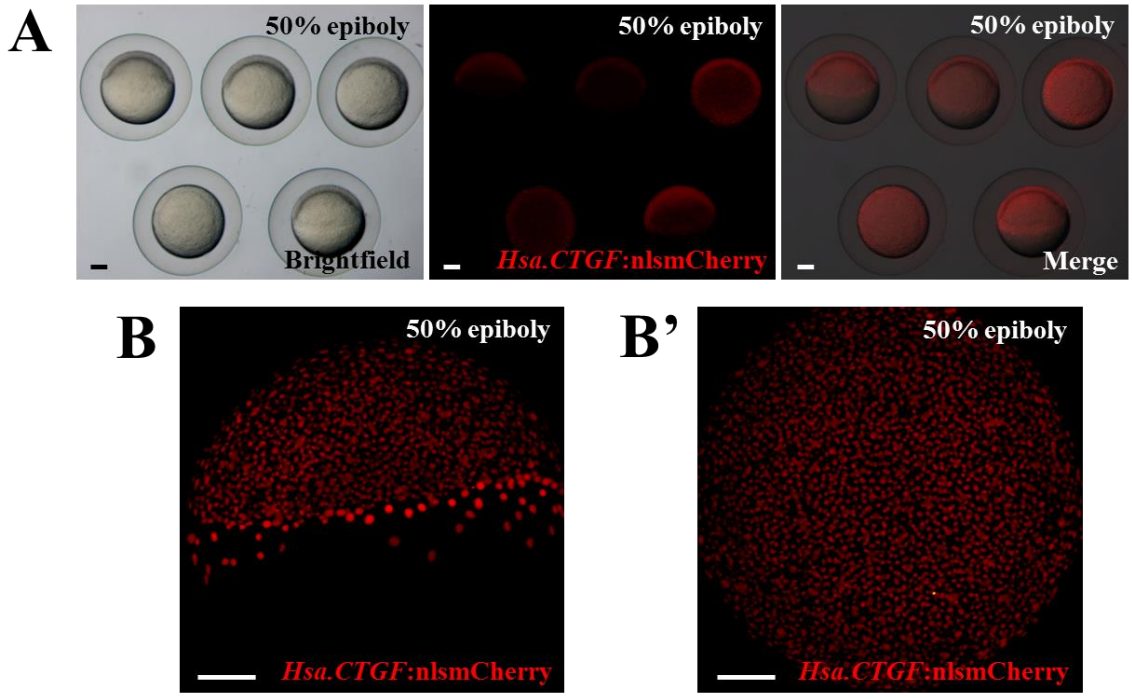

Figure 4.10. Yap/Taz reporter expression is activated maternally. (A) 50%-epiboly stage offspring of a *Tg(Hsa.CTGF:nlsmCherry)* female showing the ubiquitous maternally activated Yap/Taz reporter fluorescence. (B-B') Confocal images (lateral (B) and animal pole view (B')) of a 50%-epiboly stage embryo from the same offspring. Scale bar: 100 µm.

This is possibly due to maternal effect: before the midblastula transition at 512-cell

stage ($2^{3}/_{4}$ hours after egg fertilization), when zygotic transcription starts, all mRNAs, proteins and the other biomolecules in the embryo are maternal products deposited in the egg during oogenesis. Thus, the presence of the mCherry protein very early after fertilization indicates the Yap/Taz are transcriptionally active during oogenesis, driving the transcription of their target genes in the eggs.

Consistently with the maternal activation of the reporter, *Hsa.CTGF*:nlsmCherry expression is visible in the adult females ovary, which appears as the organ with the strongest reporter signal. In particular, mCherry protein is present in the nuclei of the oocytes and the accompanying follicle cells (Figure 4.11).

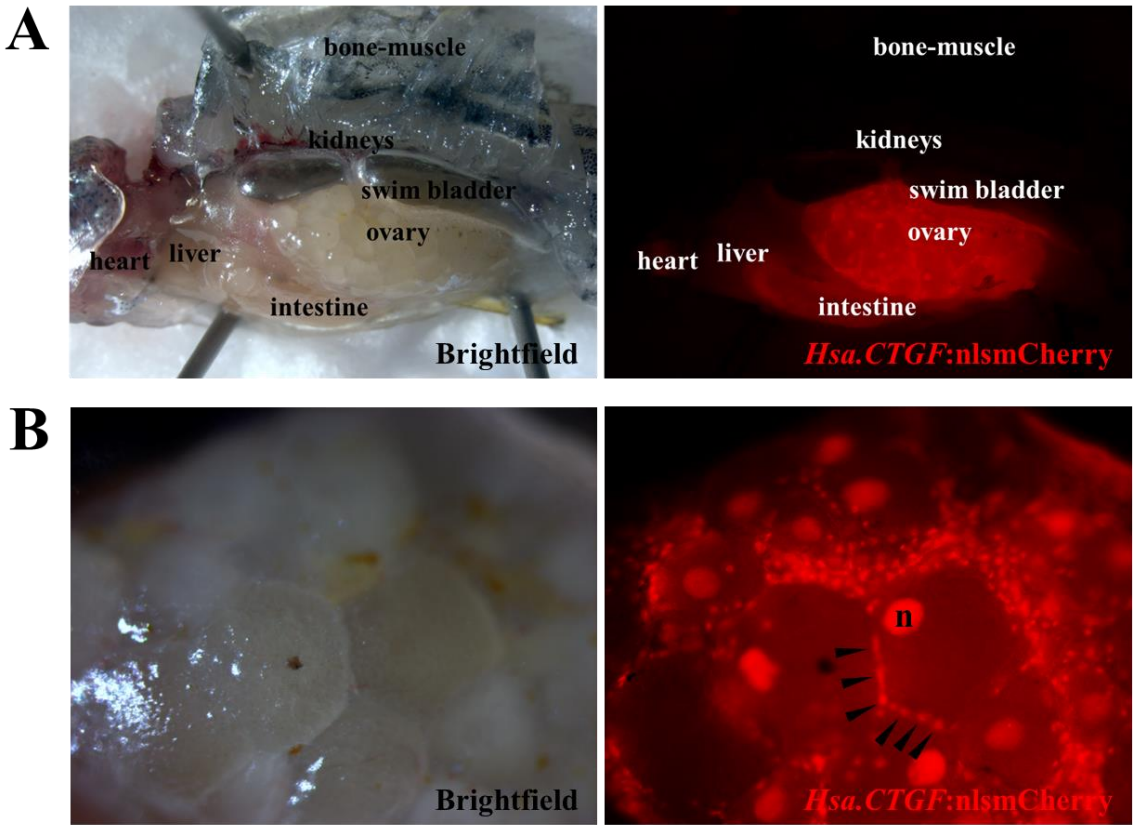

Figure 4.11. Yap/Taz reporter is active in the ovary of adult females. (A) *Tg(Hsa.CTGF:nlsmCherry)* adult female internal organs. A strong reporter expression is detected especially in the ovary. (B) Zoomed view of the ovary. mCherry signal is visible in the nuclei (n) of the oocytes at advanced maturation stages and in the accompanying follicle cells, arranged in a single layer adjacent to the oocytes (arrowheads).

4.3.2 Yap/Taz reporter signal is widely active in embryonic tissues and organs

Zygotic expression of the reporter protein appears at late somitogenesis. At 20 hpf *Hsa.CTGF*:nlsmCherry signal is widely active across the developing embryo, with the

strongest fluorescence localized in the highly proliferating and undifferentiated tail bud mesenchyme (Figure 4.12).

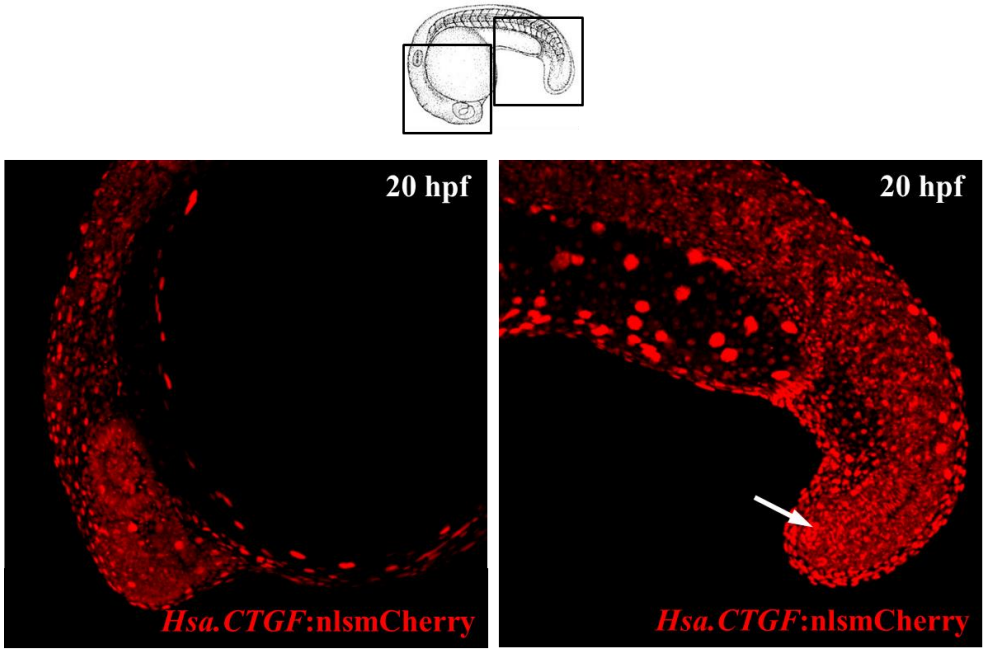

Figure 4.12. Yap/Taz reporter is widely active during early embryogenesis. *In vivo* confocal images of *Hsa.CTGF*:nlsmCherry fluorescence at 20 hpf. Yap/Taz reporter is activated early during embryogenesis and at 20 hpf is widely active across the embryo. The signal is stronger in the tail bud mesenchyme (arrow), which at this stage represents a highly proliferating and undifferentiated region of the developing embryo.

At 24 hpf mCherry expression is observed in many tissues and organs, such as the eye, the heart, the midbrain-hindbrain boundary (MHB), the rhombencephalon, the epidermis, the muscle, the neural tube, the notochord, the floorplate and the vasculature. A sustained expression is persistent in these districts even in the later developmental stages (Figure 4.13). In the eye the reporter signal is strong in the lens, mainly in the external layer of cells, but it is also present in the neural retina. Moreover, the lens remains strongly mCherry positive till adulthood (Figure 4.13 A-B). In the dorsal portion of the head, two districts are apparent from 24 hpf: the MHB and the rhombencephalon. In the rhombencephalic region, in particular, *Hsa.CTGF*:nlsmCherry expression pattern delineates six stripes of cells that seem to follow the metameric organization of the rhombomeres (Figure 4.13 C). At 72 hpf, besides the lens, the reporter signal is particularly strong in the pharyngeal arches (primarily the mandibular one), the otic vesicle, the pectoral fin and the heart (Figure 4.13 H-I).

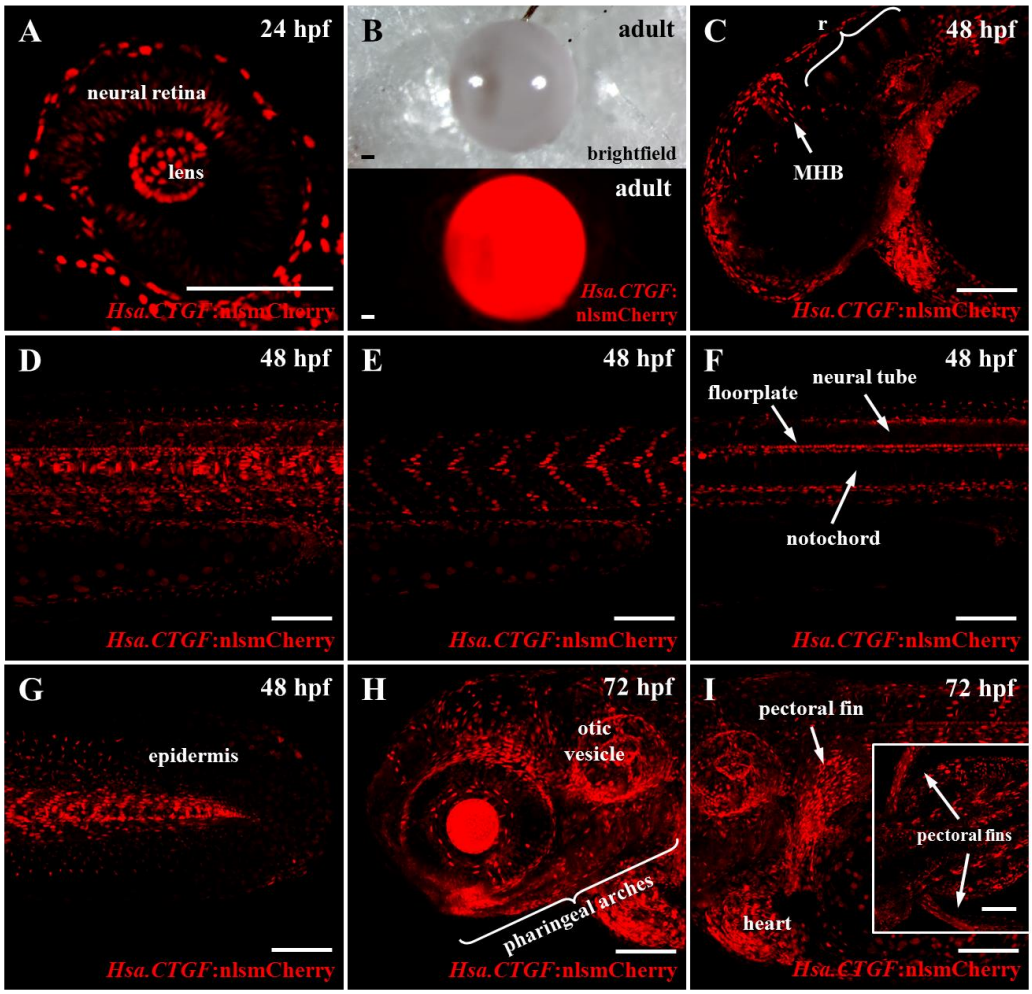

Figure 4.13. ***Tg(Hsa.CTGF:nlsmCherry)*** **expression domains.** (A) Confocal sagittal section of the eye at 24 hpf. Reporter signal is strong in the lens, mainly in the external layer of cells, but it is also present in the neural retina. (B) Lens of a *Tg(Hsa.CTGF:nlsmCherry)* adult fish. Reporter expression in the lens persists till adulthood. (C) Confocal Z-stack projection at 48 hpf showing the transgene expression in the rhombencephalon (r) and the midbrain-hindbrain boundary (MHB) regions. A pattern of six stripes of cells following the metameric organization of the rhombomeres is apparent. (D) Confocal Z-stack projection of the trunk at 48 hpf. A complex mCherry expression pattern is visible. (E-F) Sections of the Z-stack projection in (D) are shown to highlight reporter activation in the somatic muscle (E) and in the floorplate, the neural tube and the notochord (F). mCherry expression in the somatic muscle is visible as V-shaped rows of red nuclei. A strong signal is detected in a row of nuclei located between the neural tube and the notochord and representing the floorplate. The neural tube is only weakly mCherry positive. Below the floorplate, the notochord is expressing mCherry in the nuclei placed just dorsally and ventrally with respect to the dark strip of the notochord cells vacuoles. (G) Reporter fluorescence is visible in the epidermis along the whole embryo; here in a confocal Z-stack projection of the terminal portion of the tail at 48 hpf. (H-I) Confocal Z-stack projections of the rostral region of a 72 hpf reporter embryo. Besides the lens, the reporter signal is mostly strong in the pharyngeal arches, especially in the mandibular one, in the otic vesicle, in the pectoral fins and in the heart. In the inset, a dorsal view highlighting the mCherry expression in the pectoral fins is depicted. r: rhombencephalon; MHB: midbrain-hindbrain boundary. Scale bar: 100 μm.

mCherry expression in the heart is visible since the heart itself begins to develop and persists all lifelong. To confirm the expression in the cardiac progenitors and specifically in the cardiomyocytes, we outcrossed the *Tg(Hsa.CTGF:nlsmCherry)* line to the *Tg(cmlc2:GFP)* line (Burns et al., 2005), whose promoter (cardiac myosin light chain 2) drives eGFP expression in these cell types. As indicated by the co-localization between mCherry and eGFP at 22 hpf, just prior to heart formation, and later, Yap/Taz reporter is active in the cardiac progenitors as well as in differentiated cardiomyocytes (Figure 4.14 A-C). *Hsa.CTGF*:nlsmCherry fluorescence is visible also in the intestinal epithelium along the whole intestine. Specific expression in the epithelium of the intestine was demonstrated by the co-localization of the *Tg(Hsa.CTGF:nlsmCherry)* and the *Tg(gutGFP)*^{s854} signals in the double transgenic embryos, where *Tg(gutGFP)*^{s854} is a transgenic line known to label the intestinal epithelium (Field et al., 2003) (Figure 4.14 D-F).

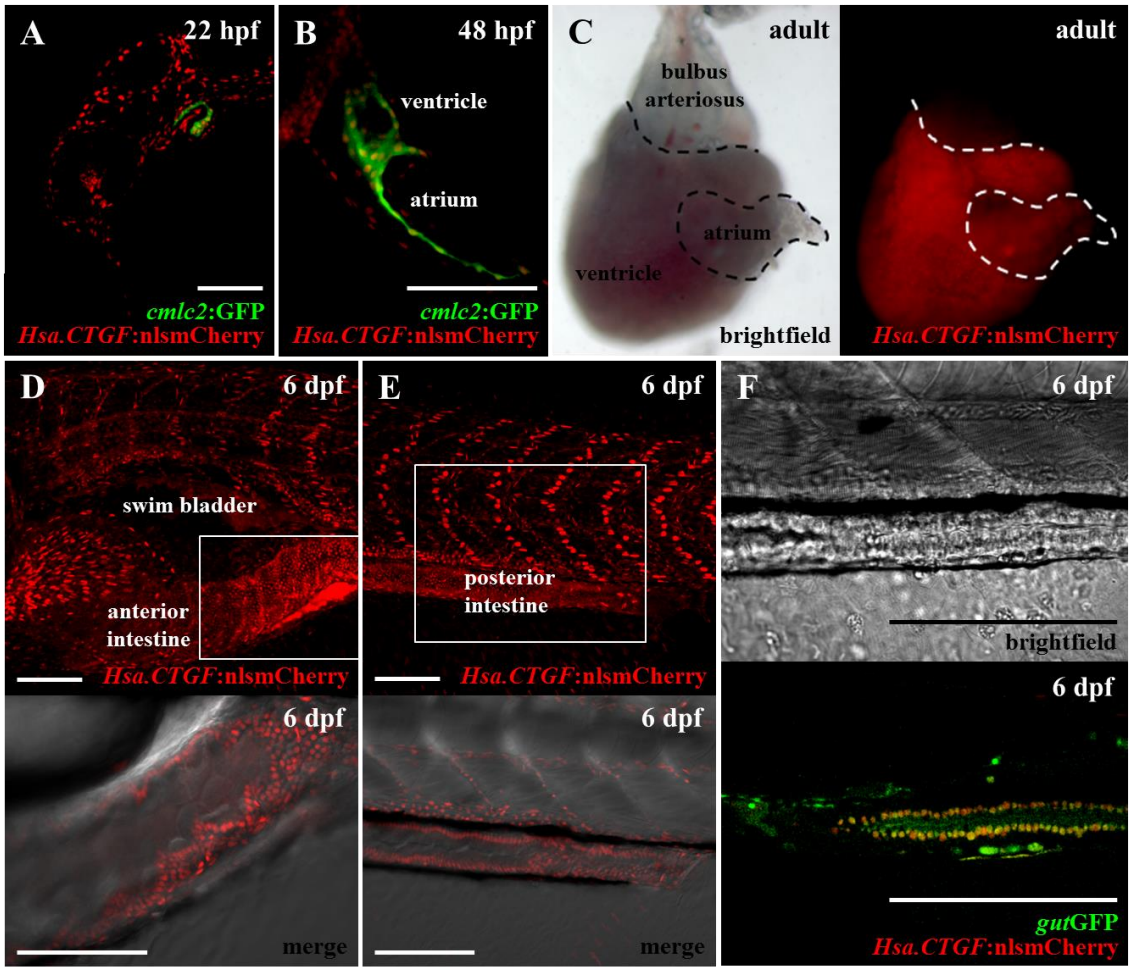

Figure 4.14. Analysis of *Tg(Hsa.CTGF:nlsmCherry)* reporter activation in the heart and in the intestine. (A) Confocal sagittal section of the heart region at 22 hpf. Yap/Taz are active in cardiac progenitors, as indicated by the co-localization between *Hsa*.CTGF:nlsmCherry and *cmlc2*:GFP just prior

to heart formation. (B) Confocal sagittal section of the heart at 48 hpf. mCherry reporter signal is still active in differentiated cardiomyocytes (*cmlc2*:GFP positive cells). (C) Heart of a *Tg(Hsa.CTGF:nlsmCherry)* adult fish. mCherry expression persists in the heart till adulthood, with a strong fluorescence detected in the ventricle, a little weaker in the atrium. (D-E) Confocal Z-stack projections at 6 days post fertilization (dpf), showing the strong transgene expression in the whole intestine. In the merge images below, a single confocal sagittal section is depicted, focusing on the reporter expression in the anterior (D) and the mid-posterior intestine (E). (F) Confocal sagittal section of the mid-posterior intestine of a 6 dpf *Tg(Hsa.CTGF:nlsmCherry)/Tg(gutGFP)*^{s854} double transgenic larva. The co-localization highlights the reporter activation in the entire intestinal epithelium (GFP positive cells). Scale bar: 100 μm.

4.3.3 Yap/Taz reporter signal is active in the endothelium

One of the most interesting body districts in which Yap/Taz reporter is active resulted to be the cardiovascular system, since the activation of *Hsa.CTGF*:nlsmCherry signal in the heart, as already shown, as well as in the vascular network, appears when these organs start to develop and it is persistent till adulthood. To analyze in more detail the reporter expression in the vasculature, *Tg(Hsa.CTGF:nlsmCherry)* line was outcrossed to the *Tg(kdrl:GFP)* line (Beis et al., 2005), which expresses the GFP in all endothelial cells from embryogenesis till adulthood. The co-localization between the two signals indicates that Yap/Taz are active in the endothelial cells in the first developing vessels and during later development (Figure 4.15 A-D). We also explored Yap/Taz reporter activation in the adult vascular system of a *Tg(Hsa.CTGF:nlsmCherry)/Tg(kdrl:GFP)* double transgenic fish. The co-localization was found in the vascular network of all the organs and tissues we looked at, such as the brain, the liver, the intestine, the pancreas, the kidney, the trunk musculature and the caudal fin (Figure 4.15 E-H). These results indicate a general activation of Yap/Taz in the endothelium throughout the body, both during development and in adulthood.

Among the adult body districts, the caudal fin is surely the one in which the distinction between arteries and veins is more apparent, thanks to the transparency of the tissues, that makes easy to see *in vivo* the direction of the bloodstream. Specifically, the arteries run in the middle of the bony fin rays, while the veins run laterally and adjacent to the bony rays on both sides, with a complex network of smaller blood vessels forming the microvasculature of the inter-ray mesenchyme (Huang et al., 2013). We observed that the whole vasculature expresses *Hsa.CTGF*:nlsmCherry in the endothelial cells, but, intriguingly, reporter signal in the veins is much stronger than the signal in the arteries (Figure 4.15 G-H).

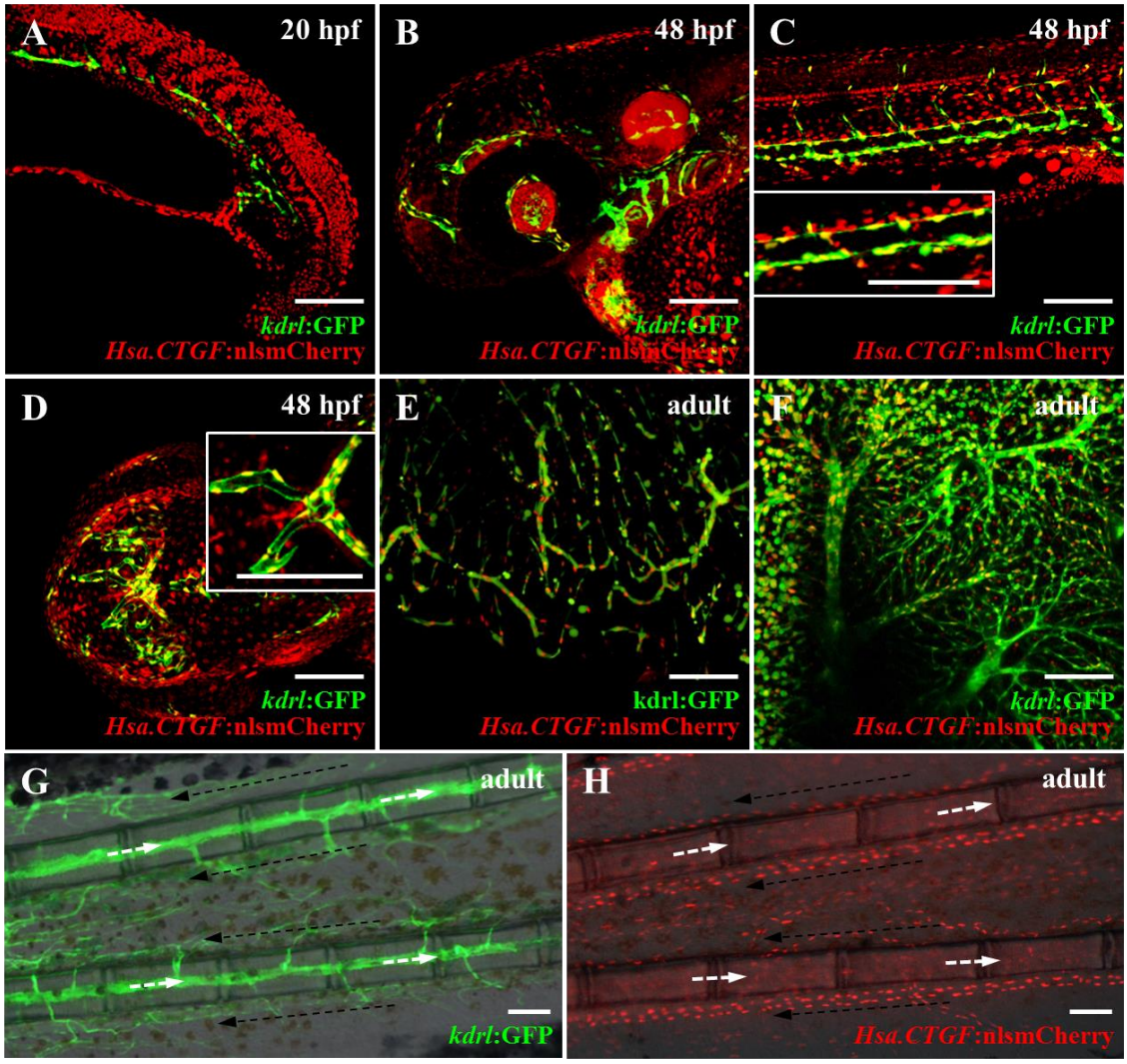

Figure 4.15. Analysis of *Tg(Hsa.CTGF:nlsmCherry)* reporter activation in the endothelium. (A-D) Reporter expression in the endothelium during development. (E-H) Reporter expression in the adult endothelium. (A) Confocal sagittal section of the trunk of a 20 hpf *Tg(Hsa.CTGF:nlsmCherry)/Tg(kdrl:GFP)* double transgenic embryo. Yap/Taz are active in the endothelial cells in the developing vessels, as shown by the co-localization between the two signals. (B-D) Confocal Z-stack projections of the head region (side view in (B), dorsal view in (D)) and the trunk (C) at 48 hpf. Reporter signal co-localizes with *kdrl*:GFP expression throughout the embryo. The insets represent zoomed views highlighting the co-localization between *Hsa.CTGF*:nlsmCherry and *kdrl*:GFP signals. (E-F) Confocal Z-stack projection of a section of a double transgenic adult brain (E) and liver (F), displaying Yap/Taz reporter activation in the endothelium of respectively the cerebral and epatic vascular networks. (G-H) Fluorescent microscope image of a *Tg(kdrl:GFP)* (G) and a *Tg(Hsa.CTGF:nlsmCherry)* adult caudal fin. Lateral view, anterior to the left, dorsal to the top. Arterial (white arrows) and venous (black arrows) bloodstream is indicated. Yap/Taz reporter activity is stronger in the veins running laterally to the bony fin rays with respect to arteries inside the bony rays. Scale bar: 100 μm.

4.4 *Tg(Hsa.CTGF:nlsmCherry)* line reports Yap/Taz activation during larval and adult fin regeneration

Together with the well-known role that YAP and TAZ play in organ growth control, stem cell and cancer biology, emerging evidences and interests are pointing toward their involvement in tissue repair and regeneration. Functional studies in mice and in *Drosophila* revealed that YAP/TAZ are essential in the regenerative processes of gut, liver, heart and potentially other tissues (Cai et al., 2010; Grusche et al., 2011; Staley and Irvine, 2010; Shaw et al., 2010; Karpowicz et al., 2010; Ren et al., 2010; Xin et al., 2013; Heallen et al., 2013; Lu et al., 2010). Ongoing experiments are demonstrating how Yap/Taz promote and are necessary for the regenerative proliferation also after adult fin amputation in the zebrafish model (Mateus et al., 2013).

In the last years, zebrafish has become a powerful model to dissect the genetic mechanisms that regulate vertebrate tissue regeneration (Gemberling et al., 2014). This is due to its high and diffuse regenerative ability, the accessibility and semitransparency of the caudal fin in particular and the growing panel of genetic tools available to label different cell populations or to conditionally manipulate gene expression. Moreover, recent evidences showed that larval fin regeneration shared similar cellular and molecular mechanisms with the adult, opening the road to an even easier and faster way to investigate *in vivo* the complex biology of the regeneration process (Kawakami et al., 2004; Mathew et al., 2007; Rojas-Muñoz et al., 2009). In such a context, it is easy to understand how useful signaling pathway reporter lines are to follow the spatio-temporal activation of specific pathways and their crosstalk during the regeneration processes (Wehner et al., 2014). Thus, we wondered whether the *Hsa.CTGF*-based zebrafish reporter we developed is able to report Yap/Taz activation during fin regeneration. Both larval and adult *Tg(Hsa.CTGF:nlsmCherry)* fin was amputated and the reporter signal was analyzed at 24 and 48 hpa. During normal larval development the strong mCherry expression in the tail epidermis makes difficult to see directly *in vivo* a possible upregulation of the reporter after fin amputation. Therefore we looked by *in situ* hybridization at the reporter expression in larval amputated fin and uncut controls. By 24 hpa we observed a clear signal at the tip of the tail, totally absent in the controls, which constitutes the blastema-like region of the regenerating fin. 24 hours later the reporter signal had extended also laterally in the mesenchymal cells underlying the stump, with a dynamic expression pattern resembling that of *raldh2* and *wnt5A*, key

players in the adult fin regeneration and components of retinoic acid and Wnt signaling pathways, respectively (Rojas-Muñoz et al., 2009) (Figure 4.16).

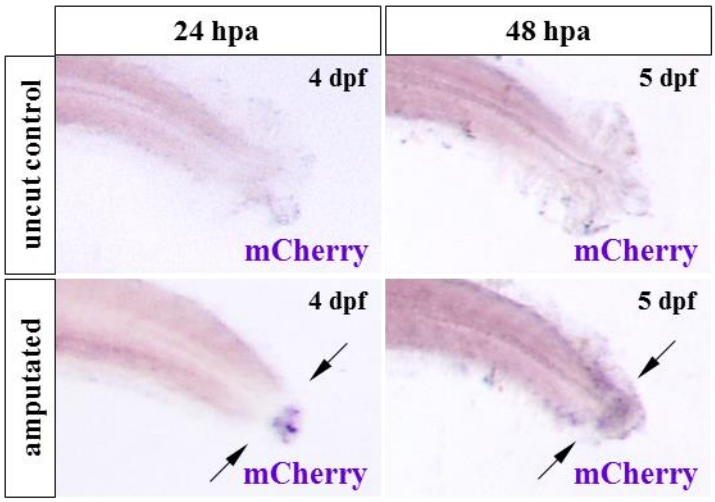

Figure 4.16. ***Tg(Hsa.CTGF:nlsmCherry)*** **reporter is activated during larval fin primordia regeneration.** *Tg(Hsa.CTGF:nlsmCherry)* larvae underwent fin primordia amputation at 72 hpf and i*n situ* hybridization for mCherry was performed at 24 and 48 hpa on amputated larvae and age-matched uncut controls. *In situ* signal is localized at the tip of the regenerating fin by 24 hpa, and extends laterally in the mesenchymal cells underlying the stump by 48 hpa. Arrows indicate the amputation plane.

As shown before, in the adult fin *Hsa.CTGF*:nlsmCherry expression is normally present in the endothelium of the whole vascular network (Figure 4.15 H). After amputation, some new mCherry positive cells appear in the stump within 24 hpa, and they are strongly increased in number by 48 hpa (Figure 4.17). At 24 hpa the few new mCherry positive cells could simply represent the initial vessel sprouts precursors to the anastomosis forming in the following 24 hours (Huang et al., 2013). Nevertheless, at 48 hpa the region and the time of the reporter activation correspond to those of the formation of the blastema, the homogeneous mass of de-differentiated and proliferative cells that forms soon after amputation and before the regenerative outgrowth. It was shown that the different cell lineages present in the adult caudal fin, including the vascular endothelium, retain fate restriction when passed through the regeneration blastema (Tu and Johnson, 2011). We did not investigate whether the *Hsa.CTGF*:nlsmCherry positive cells in the blastema represent the whole proliferative mass or only the subpopulation of endothelium-derived cells. However, consistently with our observations during regeneration of larval fin, Yap/Taz reporter resulted to be active in the de-differentiated proliferating cells of the blastema.

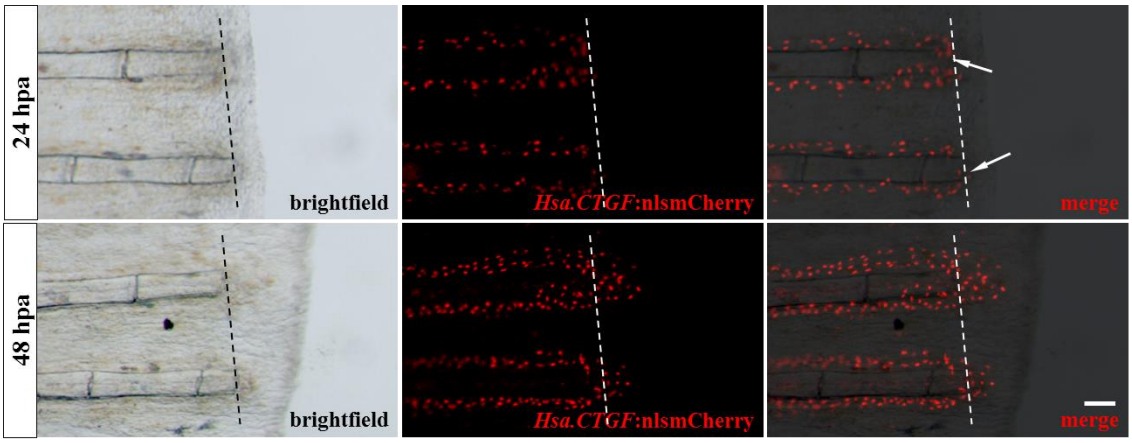

Figure 4.17. *Tg(Hsa.CTGF:nlsmCherry)* reporter is activated during adult fin regeneration. The fin of an adult *Tg(Hsa.CTGF:nlsmCherry)* fish was amputated and the reporter signal next to the amputation plane (dashed line) was analyzed 24 and 48 hours after the resection. Within 24 hpa, only a few mCherry positive cells appear along the amputation plane (arrows), while at 48 hpa the reporter is expressed in many cells of the regenerate (to the right of the dashed line), in the region of the regeneration blastema. Scale bar: 100 µm.

We conclude that the *Tg(Hsa.CTGF:nlsmCherry)* reporter is activated during fin regeneration. Furthermore, the *Hsa.CTGF*-based Yap/Taz zebrafish reporter proves to be a valuable tool to study Yap/Taz signaling during fin regeneration both in the adult fish and in the larval stage.

4.5 Yap/Taz reporter activity is regulated by the Wnt/β-catenin pathway

It was recently shown *in vitro* that YAP/TAZ activity is also regulated by the Wnt/β-catenin pathway, through AXIN-mediated sequestration of YAP/TAZ in the β-catenin destruction complex (Azzolin et al., 2012; Azzolin et al., 2014). Thus, we wondered whether the Wnt/β-catenin-mediated regulation of Yap/Taz activity is functional also *in vivo* during development, and whether the transgenic lines we developed are able to report this crosstalk. Hence, we designed a pharmacological/genetic strategy to inhibit and activate the Wnt pathway, using the *Hsa.CTGF*-based Yap/Taz zebrafish reporter as a readout of Yap/Taz activation.

4.5.1 Pharmacological inhibition of Wnt/β-catenin pathway reduces Yap/Taz reporter signal

Wnt/β-catenin pathway inhibition was achieved pharmacologically by using two different compound, IWR-1 and XAV939, whose action results in the increase of AXIN levels. In particular, they exerted their effects via inhibition of tankyrases 1 and 2 (TNKS1 and TNKS2). TNKS1/2 bind directly to AXIN proteins and regulate AXIN levels via poly-ADP-ribosylation and ubiquitylation (Fearon, 2009; Huang et al., 2009; Hsiao and Smith, 2008; Chen et al., 2009). Importantly, under physiological conditions AXIN represents the limiting factor in the β-catenin destruction complex (MacDonald et al., 2009). Therefore, AXIN upregulation by IWR-1 or XAV939 blocks β-catenin accumulation and the downstream effects of Wnt signaling (Figure 4.18 C).

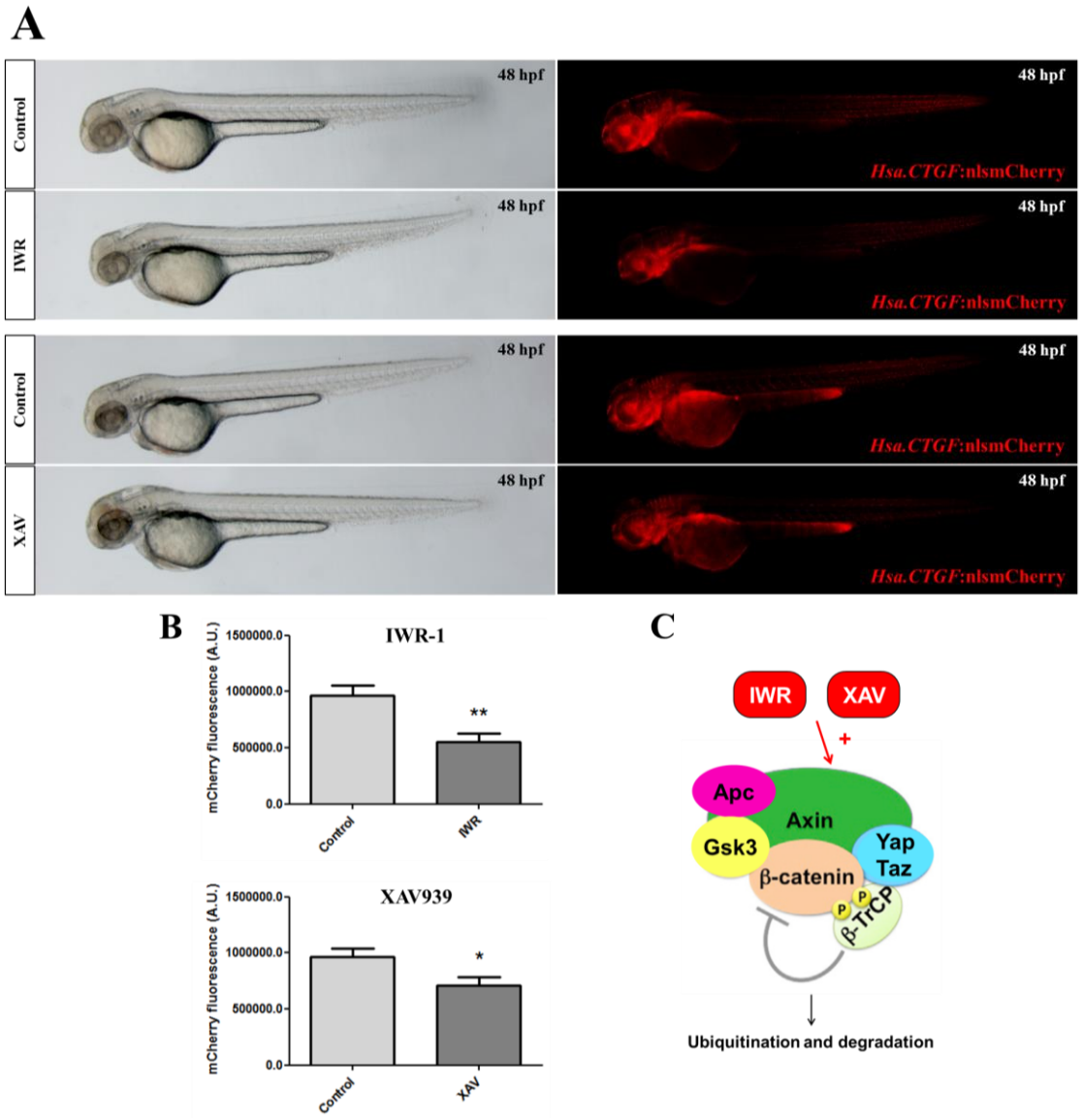

Figure 4.18. Wnt/β-catenin pathway inhibition via Axin upregulation reduces *Tg(Hsa.CTGF:nlsmCherry)* reporter signal. *Tg(Hsa.CTGF:nlsmCherry)* embryos were exposed to

IWR-1 or XAV939 from 24 to 48 hpf and the fluorescent reporter expression was documented (A) and quantified (B) at 48 hpf. (C) Schematic representation of IWR-1 and XAV939 action on Axin. Axin acts as scaffold for the retention, followed by ubiquitylation and degradation, of b-catenin and Yap/Taz in the destruction complex, as depicted by Azzolin et al. (2014).

We aimed to see whether IWR-1 and XAV939-mediated increase in Axin levels determines *in vivo* an effect on Yap/Taz transcriptional activity. To do this, we treated the *Tg(Hsa.CTGF:nlsmCherry)* line with the two compounds and looked for variations in Yap/Taz activity by evaluating mCherry reporter signal. In embryos exposed to IWR-1 from 24 to 48 hpf, as well as in embryos exposed to XAV939 for the same time, we observed a little but significant decrease in mCherry signal with respect to age-matched control embryos treated with DMSO (Figure 4.18). This indicates that inhibition of Wnt/β-catenin signaling pathway via Axin upregulation negatively influences Yap/Taz nuclear activity *in vivo*.

To confirm in our experimental setup the expected action of IWR-1 and XAV939 on Wnt/β-catenin pathway, in one of the experiments the drug treatment was performed on double transgenic embryos obtained by crossing the *Tg(Hsa.CTGF:nlsmCherry)* reporter line to the Wnt/β-catenin reporter line Tg(7xTCF-Xla.Siam:GFP)^{ia4} (Moro et al., 2012). The decrease of GFP fluorescence in IWR-1 and XAV939 treated embryos versus control embryos showed that the two compounds were properly working by inhibiting Wnt/β-catenin signaling (data not shown).

4.5.2 Genetic inhibition of Wnt/β-catenin pathway reduces Yap/Taz reporter signal

To confirm the result obtained with IWR-1 and XAV939 compounds, we also tried a different, genetic approach to inhibit the Wnt/β-catenin signaling pathway. We used the transgenic line *Tg(hsp70:dkk1-GFP)* (Stoick-Cooper et al., 2007), which expresses the upstream Wnt antagonist Dkk1, fused with the GFP, under the control of an ubiquitous heat shock inducible promoter (hsp70). By heat shocking the embryos at 42° C, the hsp70 promoter is activated, leading to the overexpression of Dkk1, visualized by the GFP which it is fused with. Dkk1 antagonizes Wnt pathway upstream with respect to IWR-1, XAV939 and the β-catenin destruction complex, acting directly at the level of the binding between the Wnt ligands and their receptor LRP6. As a consequence, the Wnt signal cannot reach the destruction complex and β-catenin cannot escape from ubiquitylation and degradation (Figure 4.19 E).

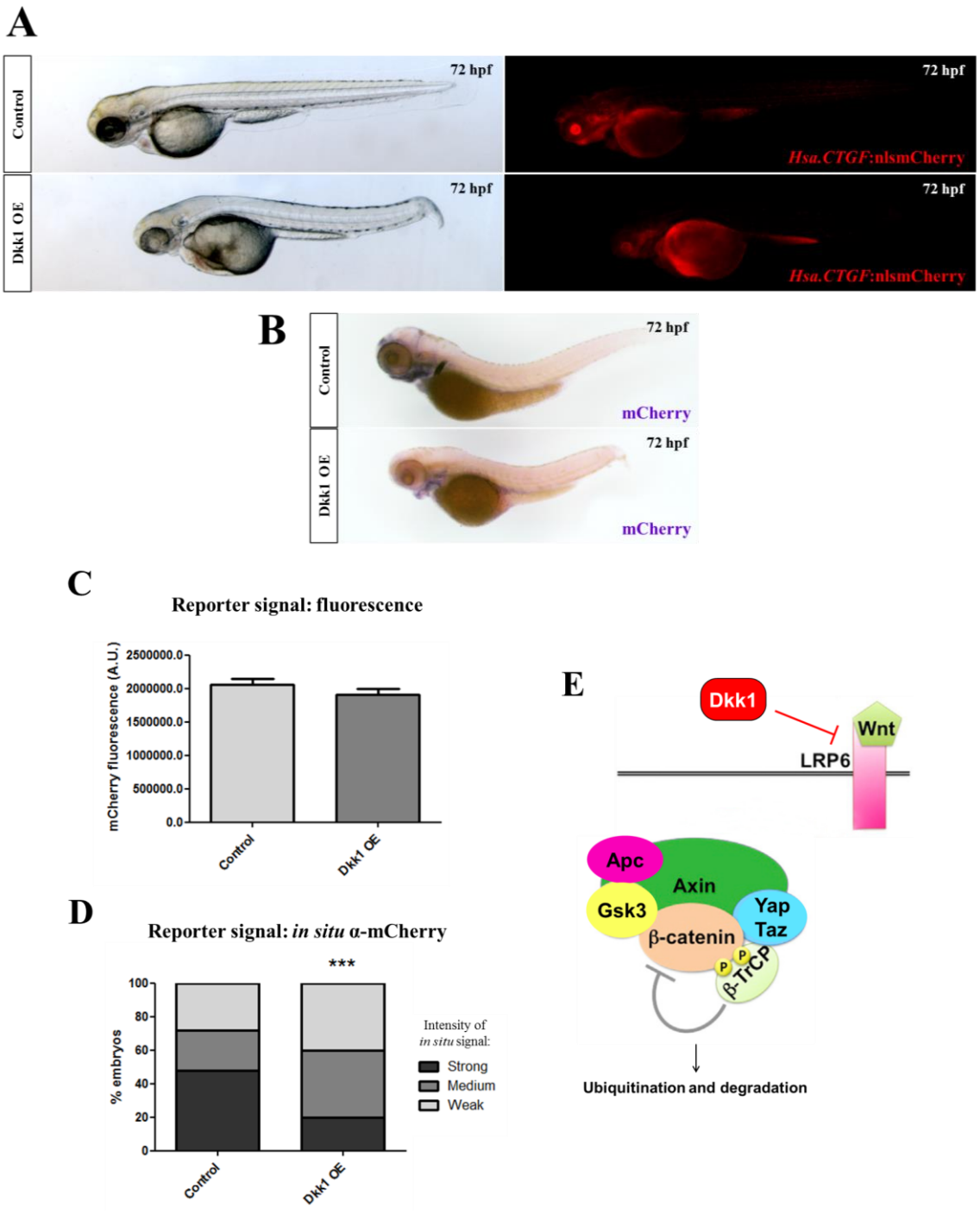

Figure 4.19. Wnt/β-catenin pathway inhibition via Dkk1 overexpression reduces *Tg(Hsa.CTGF:nlsmCherry)* reporter signal. *Tg(Hsa.CTGF:nlsmCherry)* fish were outcrossed to the *Tg(hsp70:dkk1-GFP)* line and the offspring was heat shocked, leading to the overexpression of the Wnt antagonist Dkk1. (A) Representative images of a double transgenic embryo at 72 hpf after Dkk1 overexpression compared to a sibling that doesn't carry the hsp70 transgene. Only a slight decrease in mCherry intensity was observed, as quantified in (C). (B) *In situ* hybridization for mCherry showing the reduction of mCherry mRNA level after Dkk1 overexpression. The result is quantified in (D). (E) Schematic representation of the Wnt/b-catenin pathway inhibition by Dkk1 overexpression. Dkk1 antagonizes the Wnt ligand binding to its receptor LRP6, blocking the Wnt signal transduction inside the cell.

We outcrossed the *Tg(Hsa.CTGF:nlsmCherry)* fish to the *Tg(hsp70:dkk1-GFP)* line and the offspring was heat shocked every 12 hours, from 12 to 72 hpf, in order to maintain Dkk1 overexpression over time. After global and sustained induction of Dkk1, mCherry intensity was only slightly decreased. We thus decided to look at the mCherry mRNA, which is more dynamic with respect to the protein, permitting to appreciate subtle variations. In fact, the *in situ* hybridization for mCherry showed a significant and general decrease of the mCherry mRNA level in the embryos that underwent Dkk1 overexpression when compared with the siblings that didn't carry the hsp70 transgene (Figure 4.19).

Together with the results obtained with the pharmacological approach, these data support the view that Yap/Taz activity is downregulated when the Wnt/β-catenin signaling pathway is inhibited upstream or at the level of the β-catenin destruction complex.

4.5.3 Pharmacological activation of Wnt/β-catenin pathway increases Yap/Taz reporter signal

We then asked whether Wnt/β-catenin signaling pathway is also able to positively regulate Yap/Taz reporter activity. To answer this question, we used a chemical activator of the pathway, BIO, that works by inhibiting the activity of GSK3, the kinase responsible for β-catenin phosphorylation, which drives β-catenin to degradation. By blocking β-catenin phosphorylation, BIO promotes its escape from the destruction complex and its subsequent nuclear translocation. It was also shown that phosphorylated β-catenin bridges TAZ to its ubiquitin ligase for degradation (Azzolin et al., 2012). Hence, BIO is expected to promote TAZ escape from the complex too (Figure 4.20 A).

Tg(Hsa.CTGF:nlsmCherry) Yap/Taz reporter embryos were exposed to BIO from 24 to 48 hpf. The effect on mCherry expression couldn't be observed as fluorescence levels variations *in vivo* due to the fact that BIO compound is also emitting fluorescence in red. To overcome this problem, we analyzed mCherry expression by *in situ* hybridization with the same probe for mCherry mRNA used for the heat shocked Dkk1 overexpressing embryos. The level of mCherry mRNA was significantly increased in embryos treated with the drug compared to DMSO treated control embryos (Figure 4.20). We can conclude that, also *in vivo* during development, β-catenin phosphorylation has an impact on Yap/Taz transcriptional activity, as revealed by the

modulation of Yap/Taz zebrafish reporter signal after inhibition of GSK3-mediated phosphorylation of β-catenin.

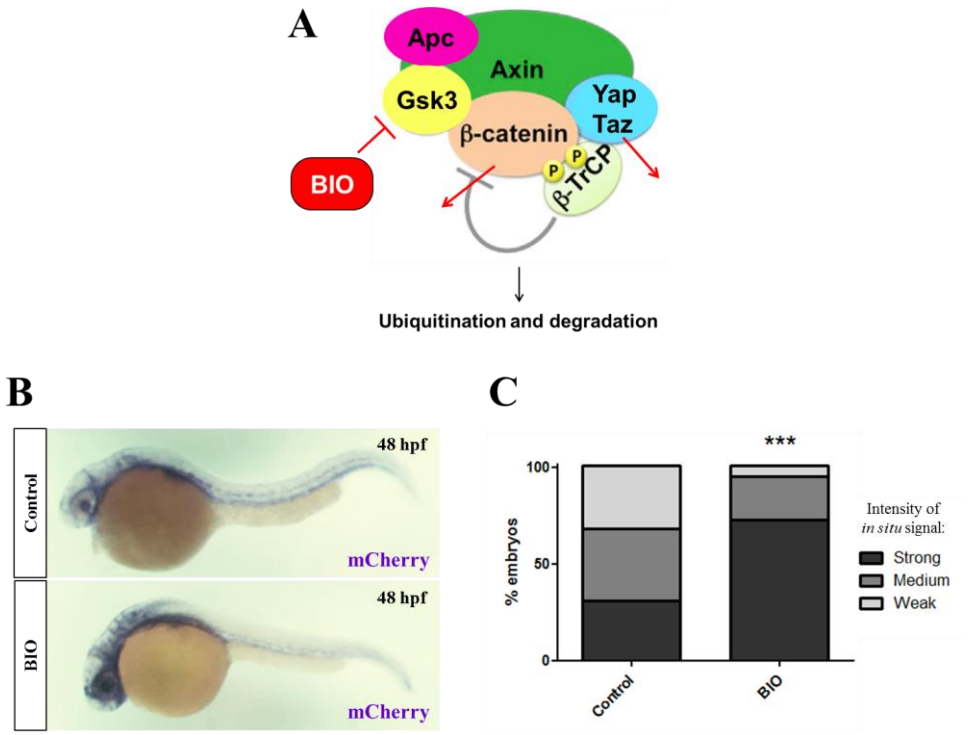

Figure 4.20. Wnt/β-catenin pathway activation via Gsk3 inhibition increases *Tg(Hsa.CTGF:nlsmCherry)* reporter signal. *Tg(Hsa.CTGF:nlsmCherry)* embryos were exposed to BIO from 24 to 48 hpf. (A) Schematic representation of Wnt/b-catenin pathway activation by BIO. BIO inhibit Gsk3-mediated β-catenin phosphorylation, promoting β-catenin and Taz escape from the destruction complex. (B) *In situ* hybridization for mCherry showing the increase of mCherry mRNA level after BIO treatment. The result is quantified in (C).

4.5.4 Genetic activation of Wnt/β-catenin pathway increases Yap/Taz reporter signal

A different approach to activate Wnt/β-catenin signaling pathway by blocking the destruction complex activity was also tried. We used the zebrafish mutant line *apc*^{hu745} (Hurlstone et al., 2003), which carries a mutation in *apc* gene resulting in a truncated protein similar to those observed in human tumors (Hurlstone et al., 2003). The loss of the Apc component causes the destabilization of the β-catenin destruction complex, with the subsequent release of its components, included β-catenin and, according to the model by Azzolin et al. (2014), also Yap/Taz (Figure 4.21 A).

Tg(Hsa.CTGF:nlsmCherry) Yap/Taz reporter fish were outcrossed to *apc*^{hu745} mutant line, in order to obtain, by incrossing the *apc*^{hu745} heterozygous mutants *Hsa.CTGF*:nlsmCherry positive of the following generation, Yap/Taz reporter embryos

harboring the *apc* mutation in homozygosis. Of note, *apc* mutants display an altered phenotype, lethal by 96 hpf. Specifically, as revealed by the alcian blue staining, they fail to develop the cartilage structures, that are predominant in the pharyngeal arches at 72 hpf. *Tg(Hsa.CTGF:nlsmCherry)* Yap/Taz reporter is strongly activated in this region in physiological conditions, while an evident reduction of the mCherry expression is observed in *apc* mutant background (Figure 4. 21 B). However, this effect is due to the complete lack of the cartilage structures and not to the regulation the β-catenin destruction complex exerts on Yap/Taz activity. To analyze this last aspect we are interested in, we moved our attention to the other anatomical districts in which the reporter is active. In particular, variations in the reporter signal are well evidenced by *in situ* hybridization for mCherry in the head dorsal region, the eye, the otic vesicle and the vascular system along the yolk extension. With the exception of the pharyngeal arches, a general upregulation of Yap/Taz reporter activity is clear in *apc* mutant background (Figure 4.21 C-D). This result confirms *in vivo* during development that Yap/Taz activity is positively regulated by the activation of Wnt/β-catenin signaling.

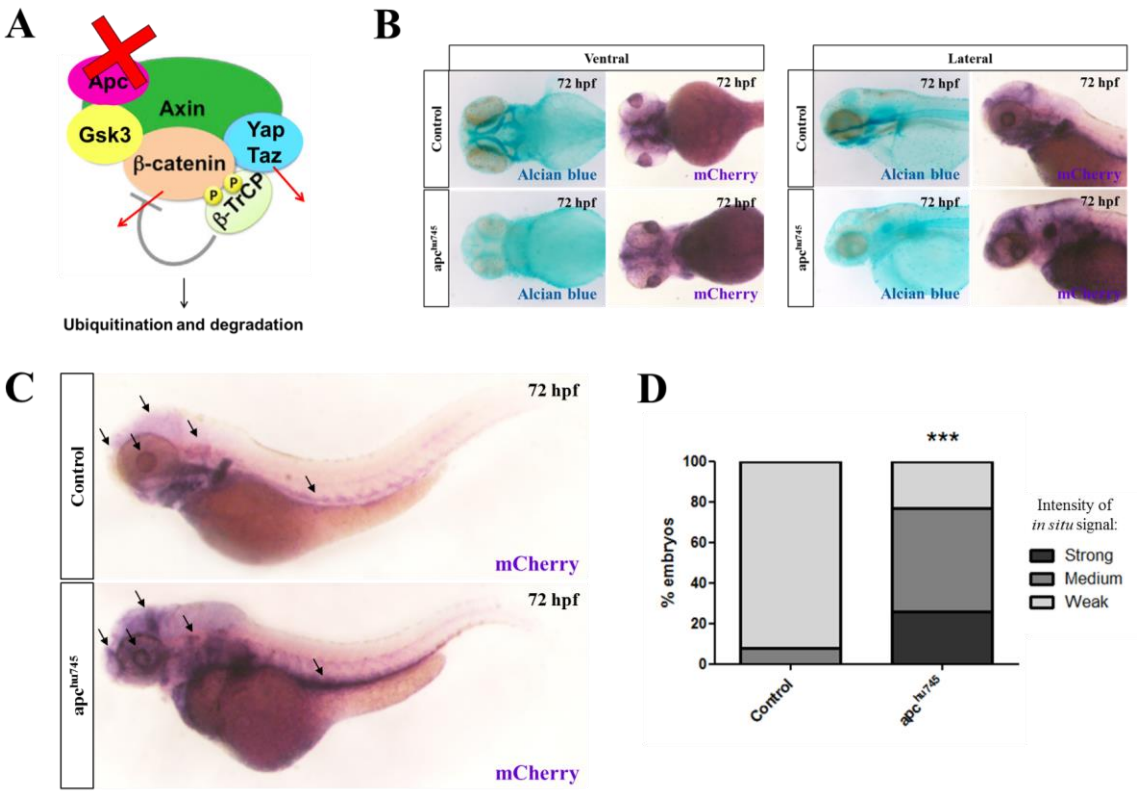

Figure 4.21. *Tg(Hsa.CTGF:nlsmCherry)* reporter activity is increased in *apc* mutant background. (A) Schematic representation of Wnt/b-catenin pathway activation in *apc* mutant background. The loss of Apc destabilizes the destruction complex, leading to the release of its components, included β-catenin and Yap/Taz. (B) *apc*^{hu745} mutants fail to develop any cartilage structure, as evidenced by the alcian blue

staining. In consequence of this, as shown by the *in situ* hybridization for mCherry, the reporter signal labelling the pharyngeal arches disappears in *apc* mutant background, due to the lack of these structures themselves. (C) *In situ* hybridization for mCherry displaying the general upregulation (with the exception of the pharyngeal arches) of Yap/Taz reporter activity in *apc* mutant background. The increase of mCherry mRNA level is evident in the head dorsal region, the eye, the otic vesicle and the vascular system along the yolk extension, as indicated by the arrows. (D) Quantification of the results obtained by *in situ* hybridization, showing the increase in Yap/Taz reporter activity in *apc* mutant background.

Different approaches with chemical compounds, transgenic and mutant lines were used both to inhibit and activate Wnt/β-catenin signaling at different levels of the pathway. By using the *Hsa.CTGF*-based Yap/Taz zebrafish reporter as a readout of Yap/Taz activation, we showed *in vivo* during development that Yap/Taz/Tead transcriptional activity is regulated both positively and negatively by Wnt signaling. Specifically, when acting on the β-catenin destruction complex – primarily, as we did with IWR-1, XAV939, BIO drugs and with *apc* mutants; or upstream in the signaling cascade, as in the case of Dkk1 overexpressing transgenics – any alteration of its activity resulted in a variation of Yap/Taz reporter signal. The direction of Yap/Taz activity variation followed that of β-catenin: the inhibition of Wnt/β-catenin signaling (via IWR-1, XAV939 and Dkk overexpression) determined an inhibition of Yap/Taz reporter expression, while, on the contrary, Wnt/β-catenin signaling activation (via BIO and Apc knockout) determined an increase of Yap/Taz reporter signal. All these results are in agreement with the last model depicted *in vitro*, that places Yap/Taz as integral components of the β-catenin destruction complex (Azzolin et al., 2014), and thus confirm in a living model such as zebrafish the Wnt/β-catenin-mediated regulation of Yap/Taz nuclear activity.

4.6 Yap/Taz nuclear activity promotes vessel sprouting cell-autonomously

As shown, one of the few tissues in which Yap/Taz reporter signal is strongly activated during early development and regeneration and persists throughout the development and in adulthood is the endothelium. The role of Yap/Taz in the endothelium is almost unknown, so we decided to explore the possible function of Yap/Taz in this tissue during development by taking advantage of the observations

derived from the *Hsa.CTGF*-based zebrafish reporter and of the genetic tools available for *in vivo* studies in the zebrafish model.

4.6.1 Hsa.CTGF*:nlsmCherry signal in the endothelium is* bona fide *reporting Yap/Taz activity*

Before to consider the functional reason why Yap/Taz are active in the endothelium, we wanted to confirm that the *Hsa.CTGF*:nlsmCherry signal is *bona fide* reporting Yap/Taz activity also specifically in this tissue. We have previously demonstrated that the *Hsa.CTGF*-based transgenic fish is a good readout of Yap/Taz activity, as the reporter protein expression can be up- and down-modulated as expected by perturbing Yap/Taz signaling. However, all these validation experiments were performed and analyzed at the organism level, without taking into account each different organ and tissue labelled by the reporter. Hence, we focused on the reporter signal in the endothelium, to confirm that it also varied in a Yap/Taz activity-dependent manner, as it happens for the total reporter signal in general.

Tg(Hsa.CTGF:nlsmCherry) fish were outcrossed to the *Tg(kdrl:GFP)* line labelling the endothelium and the offspring was injected at one-cell stage with either the splice morpholino targeting Yap pre-mRNA or the mRNA coding for the constitutively active version of Yap YAP-5SA, that work respectively by knocking down and upregulating Yap activity. *Hsa.CTGF*:nlsmCherry reporter fluorescence was acquired with the confocal microscope together with the *kdrl*:GFP fluorescence at 32 hpf in the midtrunk region. Fiji program was then asked to filter the mCherry signal using the GFP as a mask, to isolate the reporter expression in the endothelium. The intensity of fluorescence was quantified as usual and compared with the endothelium-specific mCherry signal of control injected *Tg(Hsa.CTGF:nlsmCherry)/Tg(kdrl:GFP)* embryos. A significant decrease of the reporter signal in the endothelium was detected after Yap morpholino injection compared to controls; on the contrary, a significant increase was observed after YAP-5SA mRNA injection (Figure 4.22). The system is quite sensitive, considering that the expected variations in the reporter expression were obtained by knocking down only Yap and conversely by injecting a dose of YAP-5SA mRNA causing only a mild phenotype.

These results confirm that *Tg(Hsa.CTGF:nlsmCherry)* reporter line represents a specific readout for Yap/Taz activity also in the single endothelial tissue. Consequently, we conclude that the constitutive mCherry expression we see in the endothelium is

reporting a sustained transcriptional activity of Yap/Taz, suggesting a possible role of Yap/Taz signaling during the development and the maintenance of the vascular system.

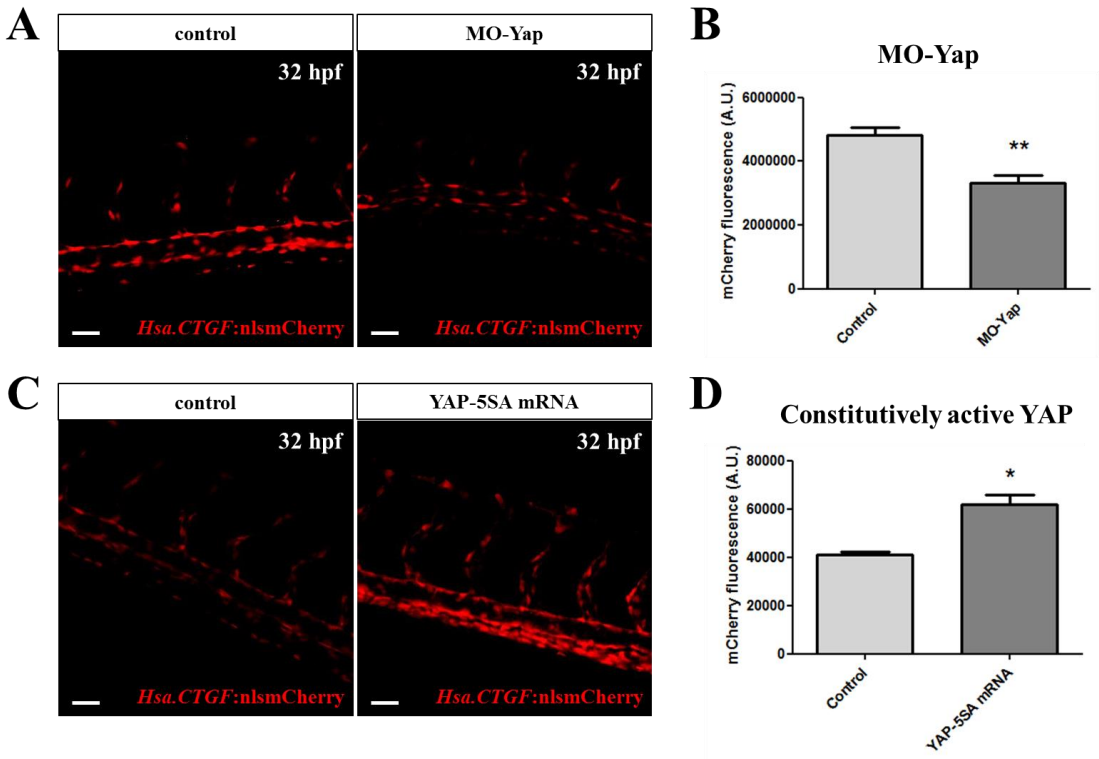

Figure 4.22. *Hsa.CTGF*:nlsmCherry signal in the endothelium is *bona fide* reporting Yap/Taz activity. (A, C) Confocal Z-stack projections of the midtrunk region of 32 hpf *Tg(Hsa.CTGF:nlsmCherry)/Tg(kdrl:GFP)* embryos. Lateral view, anterior to the left, dorsal to the top. Only mCherry reporter fluorescence in the endothelium is depicted. The images were obtained by isolating the reporter signal co-localizing with the *kdrl*:GFP from the reporter signal in the surrounding cells. (A) Yap knockdown by morpholino injection reduces *Hsa.CTGF*:nlsmCherry expression in the endothelium. The reduction of mCherry fluorescence intensity is quantified in (B). (C) Yap activity upregulation by YAP-5SA mRNA injection increases *Hsa.CTGF*:nlsmCherry expression in the endothelium. The increase of mCherry fluorescence intensity is quantified in (D). Scale bar: 100 µm.

4.6.2 Yap/Taz knockdown impairs intersegmental vessels (ISVs) growth

With the aim to see whether the perturbation of Yap/Taz activity has some effects on the vasculature during embryonic angiogenesis, we first knocked down Yap and Taz by morpholino injection in one-cell stage *Tg(kdrl:GFP)* embryos. Two considerations are needed: first, morpholino oligos are working everywhere in the embryo, without any specificity for the vascular system; second, the general downregulation of Yap/Taz has a very strong impact on zebrafish development (Hong et al., 2005; Khuchua et al., 2006; Jiang et al., 2009; Hu et al., 2013), given its fundamental role in regulating stemness,

proliferation, cell death and cell-fate decisions. Thus, to avoid a generalized developmental impairment, the experiment was performed by injecting an amount of morpholinos (1-3 ng each per embryo) that gives rise to a mild phenotype. The development of the vascular system was followed by looking in particular at the ISVs from 24 to 32 hpf. This time interval corresponds to the midtrunk ISVs growth, from their sprouting from the dorsal aorta (DA) to the formation of the DLAV.

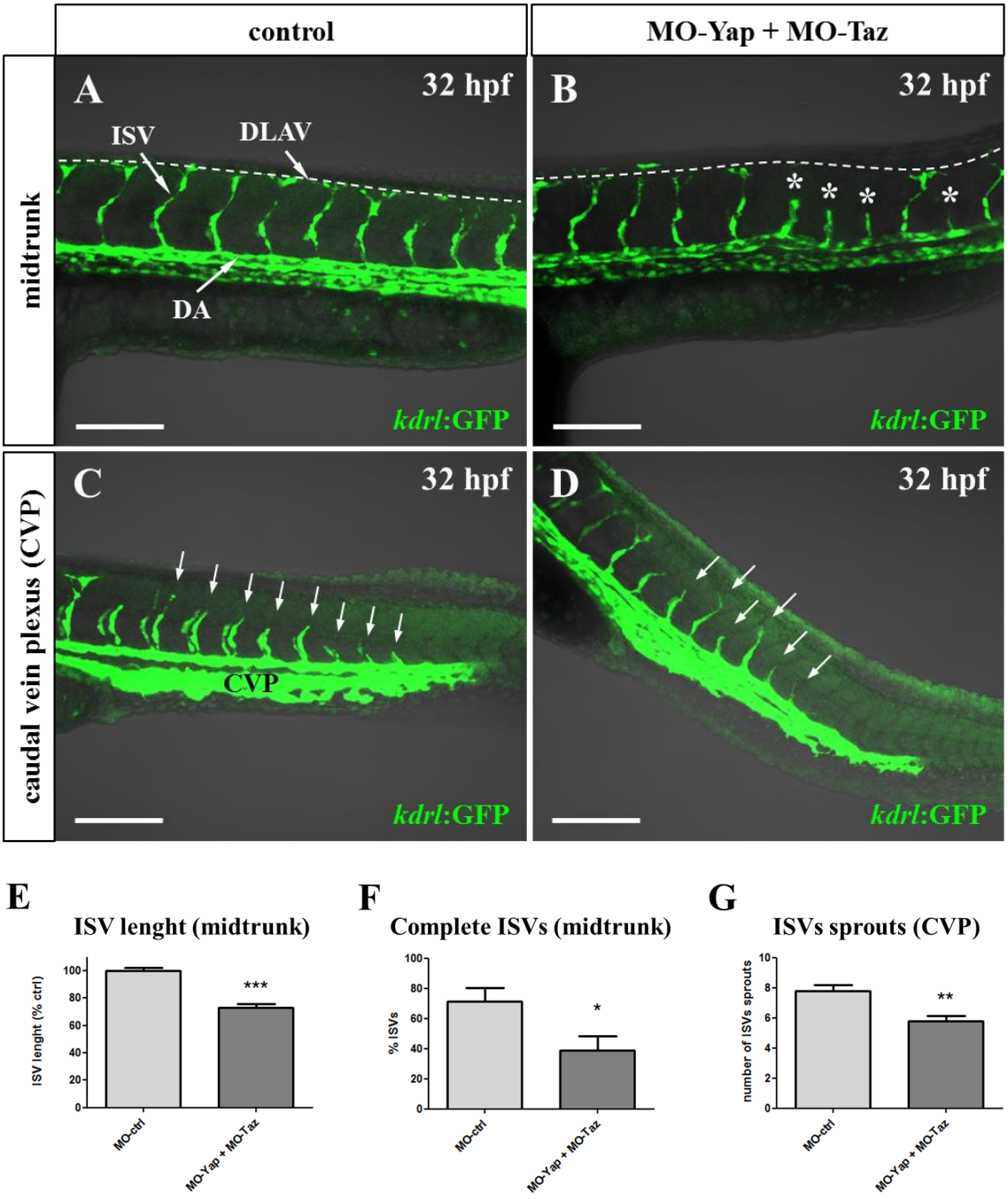

Figure 4.23. Yap/Taz knockdown by Yap and Taz morpholinos co-injection impairs ISVs growth. (A-D) Confocal Z-stack projections of *Tg(kdrl:GFP)* 32 hpf embryos. Lateral view, anterior to the left,

dorsal to the top. (A-B) Midtrunk region. Representative images of a control (A) and a Yap/Taz morphant embryo (B). The dorsal roof level, corresponding to the developing DLAV, is depicted (dashed line). The ISVs of the double morphant embryo display a delay in reaching this level. The asterisks indicate the ISVs stalled at mid-somite and shorter than the next ones (i.e. more caudal ones), showing a defect in the temporal order of the ISVs growth. (C-D) CVP region. Representative images of a control (C) and a Yap/Taz morphant embryo (D). The number of the ISVs sprouts (arrows) in this region is reduced in Yap/Taz morphants (only the lane of ISVs sprouts of one side of the embryo was considered). Again, their reciprocal length is less uniform in the morphants with respect to the controls. (E-G) Quantification of the ISVs angiogenesis impairment caused by Yap/Taz knockdown. Only embryos with a mild phenotype were considered. In the midtrunk region the length of the single ISVs (E) and the percentage of ISVs reaching the dorsal roof level (complete ISVs) (F) were evaluated. In the CVP region the number of ISVs sprouts was counted (G). All the three parameters are reduced by Yap/Taz knockdown. ISV, intersegmental vessel; DLAV, dorsal longitudinal anastomosing vessel; DA, dorsal aorta; CVP, caudal vein plexus. Scale bar: 100 μm.

A general delay in ISVs development was noticed in the double morphant embryos with respect to age-matched embryos injected with a control morpholino. At 32 hpf, when almost all the ISVs of the midtrunk region in the controls had already reached the dorsal roof level (corresponding to the developing DLAV), Yap/Taz morphants presented many ISVs that had not (Figure 4.23 A-B). Moreover, normal ISVs growth starts rostrally and continues toward the tail, so that the growth of each ISV is time-shifted with respect to the rostral adjacent one. Besides the simple developmental delay, Yap/Taz morphants showed some defects in the temporal order of the ISVs growth, as often we found ISVs shorter than the next ones (i.e. more caudal ones) (Figure 4.23 B, D). The defective ISVs sprouting angiogenesis was evaluated and quantified at 32 hpf by using three different parameters and by focusing on two different anatomical regions. At the midtrunk level (corresponding to the yolk extension region), we measured both ISVs length and the percentage of complete ISVs, meaning the ISVs that had already reached the dorsal roof level and had started to form the DLAV. We moved then caudally, at the level of the CVP, where at 32 hpf the last ISVs are sprouting, and we evaluated the number of ISVs sprouts per embryo. A significant decrease of all the three parameters considered was found in Yap/Taz morphant embryos when compared to controls, supporting the observation that Yap/Taz knockdown impairs ISVs growth along the trunk (Figure 4.23 E-G).

4.6.3 The upregulation of Yap/Taz/Tead-mediated transcription causes aberrant vessel sprouting

To further investigate the role of Yap/Taz in embryonic angiogenesis and specifically during ISVs growth, we upregulated Yap/Taz activity by injecting TAZ-4SA mRNA in one-cell stage *Tg(kdrl:GFP)* embryos. Again, due to the diffuse and strong action of this mRNA, as we did for the morpholinos, the experiment was performed by injecting a small amount of mRNA, such that only embryos with a mild general phenotype were analyzed. We then looked at the developing vascular system labeled by the *kdrl:GFP* expression, comparing its morphology in TAZ-4SA injected embryos with that in control injected embryos.

Interestingly, at 32 hpf we observed the appearance of anomalous sprouts emerging from some ISVs in TAZ-4SA injected embryos. The sprouts arose from around the middle of the ISVs and not from the tip cells of the developing ISVs, that at this stage had already anastomosed with the tip cells of the ISVs nearby to form the DLAV (Figure 4.24 A-B). The supernumerary sprouts pointed mostly toward the adjacent ISVs, and often gave raise to complete anastomosis between two adjacent ISVs of the same side of the trunk (Figure 4.24 B-C). Despite we reported this aberrant vessel sprouting in TAZ-4SA injected embryos with a low frequency with respect to the number of the ISVs, this phenomenon is almost absent during normal development. In particular, complete anastomosis between adjacent ISVs, like the ones described here, were never detected in more than 50 control embryos analyzed and represent a more clearly defined phenotype caused by TAZ-4SA injection (Figure 4.24 D-E). We therefore decided to focus on this peculiar phenotype; for the following experiments, we will show only evaluation and quantification of complete anastomosis between adjacent ISVs. The low frequency of the aberrant sprouting phenotype is likely due to the limited level of Yap/Taz upregulation we were forced to keep when using a non-tissue-specific upregulation system like the mRNA injection.

To confirm that the aberrant sprouting phenotype was specifically due to Yap/Taz overactivation, ruling out any possible side-effect of TAZ-4SA mRNA, the experiment was repeated using YAP-5SA mRNA instead of the TAZ-4SA one. Again, we obtained the same phenotype observed before (data not shown). Moreover, we asked whether the effect on ISVs angiogenesis we reported was due to the enhanced Yap/Taz/Tead-mediated transcription, as expected, or to other different actions of Yap and Taz proteins. To address this issue, *Tg(kdrl:GFP)* embryos were injected with TEAD-VP16

mRNA, which constitutively activates Tead target genes transcription independently of Yap/Taz. Aberrant vessel sprouting and anastomosis between ISVs were found also after the injection of TEAD-VP16 mRNA alone, thus confirming the vessel sprouting-promoting capacity of forced Yap/Taz/Tead-mediated transcription (Figure 4.25 C and data not shown).

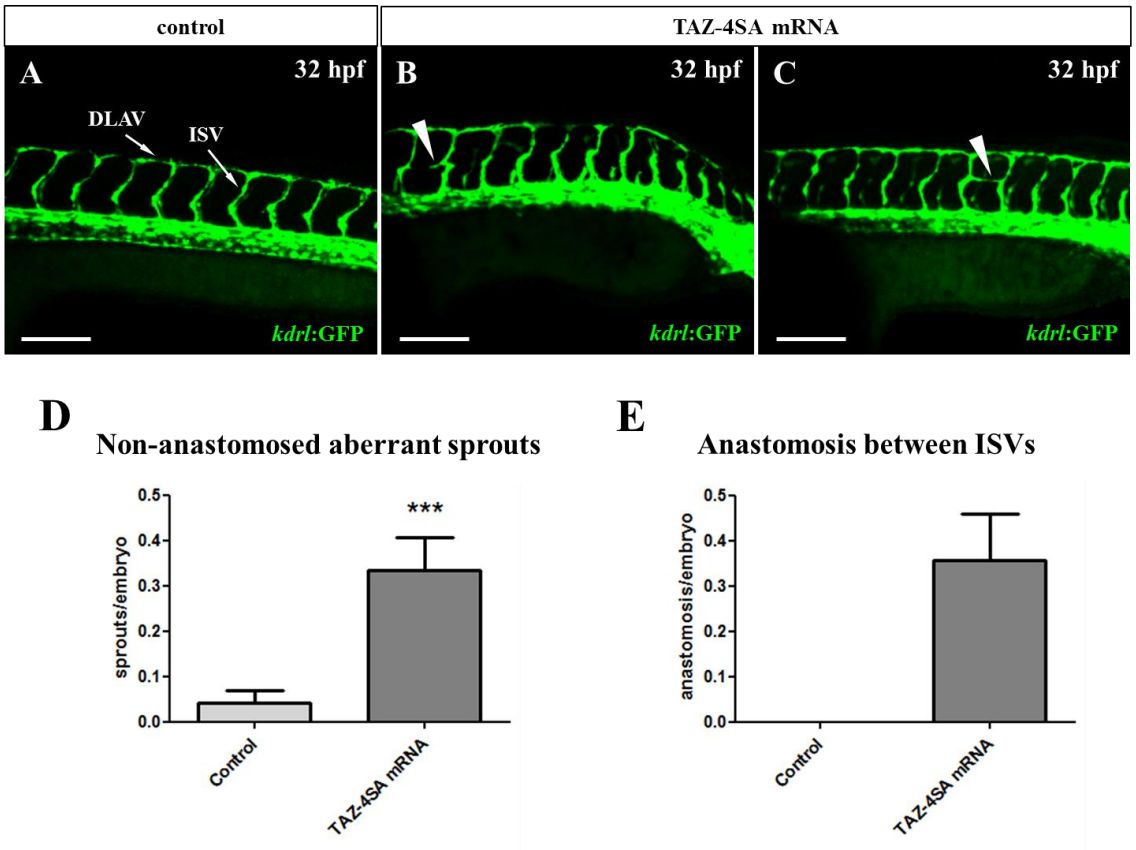

Figure 4.24. Yap/Taz activity upregulation by TAZ-4SA mRNA injection causes aberrant vessel sprouting. (A-C) Confocal Z-stack projections of the midtrunk region of *Tg(kdrl:GFP)* 32 hpf embryos. Lateral view, anterior to the left, dorsal to the top. Representative images of a control (A) and two TAZ-4SA mRNA injected embryos, showing an aberrant ISV sprout from one ISVs (arrowhead in (B)) and an anastomosis between adjacent ISVs (arrowhead in (C)). (D-E) Quantification of the aberrant sprouting caused by Yap/Taz activity upregulation. Only embryos with a mild phenotype and ISVs of the midtrunk region were considered. The number of non-anastomosed aberrant sprouts and the number of anastomosis between adjacent ISVs were evaluated. Both phenomena, observed in TAZ-4SA mRNA injected embryos, are extremely rare or absent at all in the controls. ISV, intersegmental vessel; DLAV, dorsal longitudinal anastomosing vessel. Scale bar: 100 µm.

4.6.4 The vessel sprouting-promoting capacity of Yap/Taz is cell-autonomous

As already said, TAZ-4SA mRNA, as well as the other mRNAs used to upregulate Yap/Taz transcriptional activity, is expected to diffuse everywhere in the embryo,

without any specificity for a cell type, an organ or a tissue. Thus, even though we analyzed the effects of Yap/Taz upregulation in the vascular system, the surrounding tissues were also undergoing the same upregulation. This raised the issue of what tissue is responsible for the aberrant vessel sprouting phenotype we observed in the vasculature. Is the overactivation of Yap/Taz in the endothelial cells enough to promote the supernumerary ISVs sprouts? Otherwise, is the phenotype a secondary effect due to the general Yap/Taz overactivation in the surrounding tissues or maybe to the paracrine action of a specific tissue nearby the endothelium? To answer these questions and prove if the vessel sprouting-promoting capacity of Yap/Taz is cell-autonomous or not, we designed a new transgenic construct, placing TAZ-4SA expression under the control of the *fli1a* promoter. TAZ-4SA-coding sequence was first cloned in a Gateway middle entry clone (pME-MCS). The pME-TAZ-4SA vector produced was recombined together with the 5' entry clone containing the *fli1a* promoter (p5E-fli1a) and the 3' entry clone p3E-polyA in the Tol2 destination vector pDestTol2CG2. The final product of the MultiSite Gateway LR recombination reaction was the destination vector *pDestTg(fli1a:TAZ-4SA),* which drives the expression of the constitutively active TAZ in all endothelial cells, but not in the surrounding tissues. The pDestTol2CG2 Tol2 transposon backbone used contains the *cmlc2*:eGFP transgenesis marker, which expresses the eGFP in the cardiomyocytes carrying the transgene. Considering that the *cmlc2*:eGFP transgene is placed in the same vector of the *fli1a*:TAZ-4SA transgene, the extension of eGFP expression in the cardiac muscle represents an estimation of the degree of the mosaicism in transiently injected mosaic fish.

The *pDestTg(fli1a:TAZ-4SA)* vector was injected in one-cell stage *Tg(kdrl:GFP)* embryos together with the Tol2 transposase mRNA and the effect of Yap/Taz transient overactivation in the endothelium was analyzed. Unfortunately, the degree of the mosaicism was estimated to be extremely low, because only a few eGFP positive cardiomyocytes were found in the heart of the injected embryos. We supposed a toxicity problem of the vector itself, since an higher dosage strongly increased the mortality rate. Nevertheless, we speculated that, although TAZ-4SA was expressed only in a few ISVs endothelial cells, if the vessel sprouting-promoting capacity of Yap/Taz is cell autonomous, we expected to observe anyway the aberrant sprouting phenotype, at least in some ISVs. We thus screened the injected embryos for mosaic *cmcl2*:eGFP expression and analyzed the vasculature morphology in the midtrunk region. About 60% of the *cmlc2*:eGFP mosaic expressing embryos do not display any alteration,

possibly due to complete or nearly complete absence of endothelial cells carrying the transgenic construct and expressing TAZ-4SA protein. However, the remaining embryos showed the peculiar anastomosis between ISVs observed after TAZ-4SA, YAP-5SA and TEAD-VP16 mRNAs injection, up to four anastomosis in a single embryo (Figure 4.25). Therefore, the upregulation of Yap/Taz nuclear activity only in the endothelium is sufficient to phenocopy the aberrant vessel sprouting effect caused by TAZ-4SA, YAP-5SA and TEAD-VP16 mRNAs injection, indicating that the vessel sprouting-promoting capacity of Yap/Taz/Tead-mediated transcription is cell-autonomous.

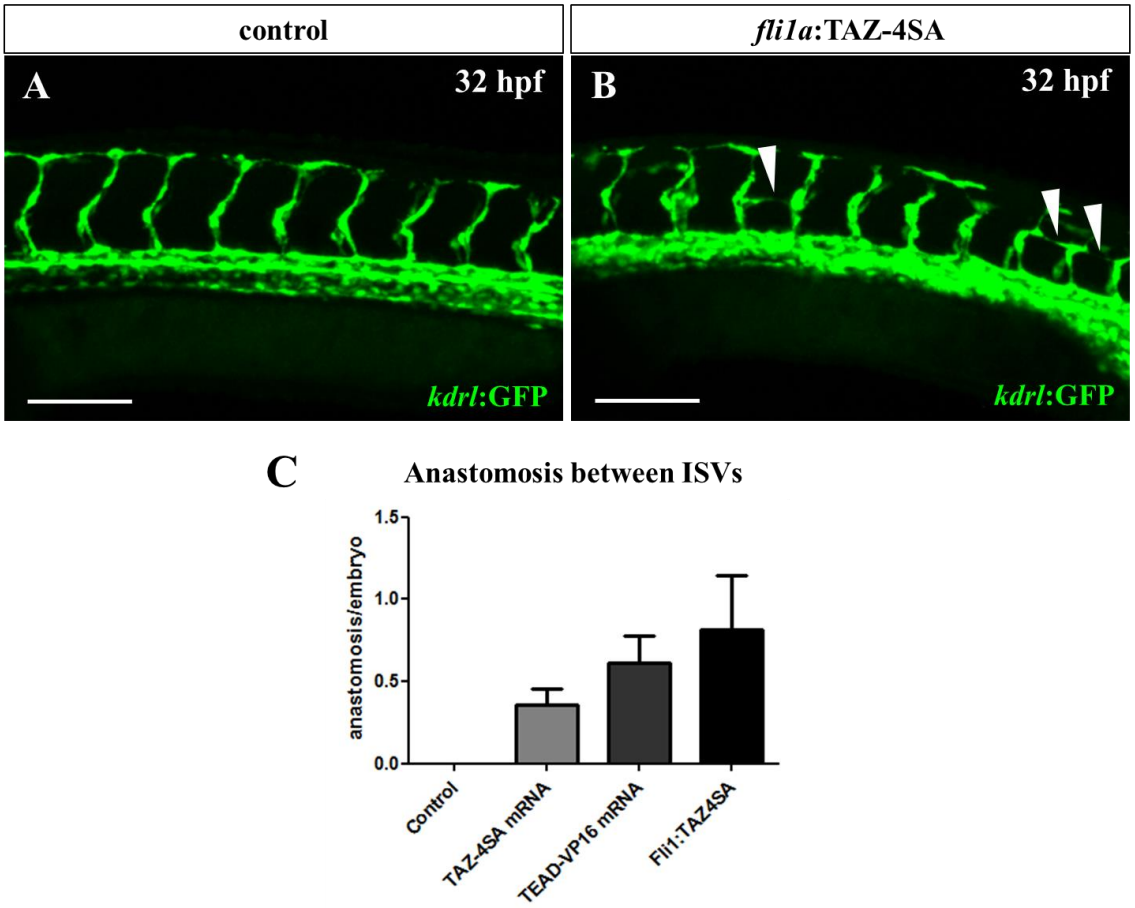

Figure 4.25. Yap/Taz activity upregulation only in the endothelium by *fli1a*:TAZ-4SA phenocopies the aberrant vessel sprouting caused by TAZ-4SA and TEAD-VP16 mRNAs injection. (A-B) Confocal Z-stack projections of the midtrunk region of *Tg(kdrl:GFP)* 32 hpf embryos. Lateral view, anterior to the left, dorsal to the top. Representative images of a control (A) and a *fli1a*:TAZ-4SA mosaic expressing embryo (B) showing three anomalous anastomosis between adjacent ISVs (arrowheads). (C) Quantification of the anastomosis between ISVs observed in TAZ-4SA mRNA (see also Figure 4.24), TEAD-VP16 mRNA and *pDestTg(fli1a:TAZ-4SA)* DNA injected embryos. Scale bar: 100 µm.

Overall, our observations *in vivo* in the *Hsa.CTGF*-based Yap/Taz zebrafish reporter and in *Tg(kdrl:GFP)* embryos after different perturbations of Yap/Taz activity suggest a new role of this signaling pathway in embryonic angiogenesis. Specifically, Yap/Taz are necessary for the proper growth of the ISVs, since Yap/Taz downregulation impairs their growth. Oppositely, forced Yap/Taz/Tead-driven transcription is able to cell-autonomously promote aberrant sprouting of endothelial cells from the ISVs and the formation of complete anastomosis between adjacent ISVs never detected during normal development.

5 DISCUSSION

5.1 A novel Yap/Taz zebrafish reporter

YAP/TAZ signaling has recently gained a central position in developmental, cancer and regeneration biology; the intense research of the last years is unravelling novel views on YAP/TAZ biology and exciting therapeutic perspectives are emerging. Nevertheless, our knowledge in the complexity of YAP/TAZ signaling is still limited, starting from the spatio-temporal pattern of YAP/TAZ activity during development and in adulthood. Here, we described the generation, validation and characterization of a Yap/Taz zebrafish reporter, which represents an initial but powerful answer to this issue, allowing to follow the signaling activity in the living organism.

Validation through knockdown and overexpression approaches showed that the *Hsa.CTGF*-based zebrafish transgenic lines we developed effectively reports *in vivo* Yap/Taz activity, being able to reflect variations in the signaling with a good sensitivity. Another Yap/Taz zebrafish reporter was published last year by Miesfeld and Link (2014), based on a different transgenic construct. To drive the expression of the reporter gene in the districts of Yap/Taz transcriptional activity, we used a fragment of the promotorial region of the Yap/Taz target gene *CTGF*, containing 3 Yap/Taz REs and the TATA box as minimal promoter. Miesfeld and Link, instead, used the established *4xGTIIC* promoter, containing 4 artificially multimerized Yap/Taz REs (Mahoney et al., 2005), followed by the minimal promoter of the chicken troponin T (cTNT) gene, a transcriptional target of TEAD. Besides the comparison among the progenies of different founder fish, the comparison among reporter lines of the same signaling pathway but based on a different design of the transgenic construct is an important step in the validation of a novel zebrafish reporter line. In this case, in fact, the consistency of the reporter expression pattern represents a strong indication of the responsiveness of the transgenic fish to the specific pathway, independently of other factors possibly linked to the particular transgenic construct used (Shimizu et al., 2012).

The expression of the *4xGTIIC* reporter was described in the epidermis, the heart (in cardiac progenitor cells as well as in differentiated cardiomyocytes), the otic and lens vesicles, the midbrain-hindbrain boundary (MHB) region and the striated muscle of the trunk (Miesfeld and Link, 2014). The *Hsa.CTGF*-based reporter presented in this thesis also displays a strong expression of the reporter protein in these districts. This confirms

the specificity of the reporter line for the pathway of interest and highlights relevant tissues of Yap/Taz activity, suggesting that this signaling may have there an important functional role during development.

Nevertheless, some differences in the reporter expression pattern are apparent between the two reporters. From 24 hpf, *Hsa.CTGF*:nlsmCherry expression is detected also in the rhombencephalon, the neural tube, the notochord, the floorplate and the vasculature and at 72 hpf the reporter signal is particularly strong in the pharyngeal arches and in the pectoral fin too. All these anatomical domains of expression are not described for the *4xGTIIC* transgenic. Overall, the *Hsa.CTGF*-based reporter displays a much wider expression pattern, whilst that of the *4xGTIIC* one is restricted to a few organs and tissues, with the reporter gene predominantly expressed in the skeletal and cardiac muscle. These differences might be ascribed to unknown signals derived from the cTNT minimal promoter contained in the *4xGTIIC* reporter construct, since the TEAD target gene cTNT is specifically expressed in both embryonic skeletal and cardiac muscle. It is important to note, however, that elements conferring muscle specificity to cTNT expression have been found in the sequences flanking the MCAT core motifs (Larkin et al., 1996), located upstream of the cTNT promoter fragment probably used as minimal promoter by the authors, and thus not present in the *4xGTIIC* reporter construct.

On the other hand, one could argue that the *Hsa.CTGF*-based lines might report the zebrafish *ctgf* gene expression pattern instead of Yap/Taz signaling activity, and this might explain the differences with the *4xGTIIC* Yap/Taz reporter. There are several reasons to exclude this hypothesis: i) the reporter expression is driven by a 200 bp fragment of the *CTGF* promoter, that represents only a minimal part with respect to the whole promoter regulating the expression of the *CTGF* gene; ii) we used the human *CTGF* promoter, in order to filter the reporter construct from other zebrafish-specific regulatory elements possibly present in the sequence among the desired MCAT sites; iii) the reporter expression pattern is not overlapping the *ctgf* one, neither represents a subset of it; iv) the *ctgf* and the reporter protein expression are not co-regulated. As expected by the fact that *ctgf* is a Yap/Taz target gene, there is a considerable overlap between the *ctgf* and the reporter expression during development, especially in the districts showing the strongest signals, such as the lens and otic vesicles, the heart, the pharyngeal arches, the pectoral fin, the floorplate. However, the *Hsa.CTGF*-based Yap/Taz reporter is also active in regions in which the *ctgf* expression is off: the

endothelium, the intestine, the epidermis, the MHB and the rhombencephalic regions. Moreover, we experimentally observed that in certain conditions the *ctgf* and the *Hsa.CTGF* reporter gene are not co-regulated, thus ruling out the hypothesis of dealing with a *ctgf* expression reporter instead of a Yap/Taz activity reporter. For example, treatment with either IWR-1 or XAV939 leads to a general downregulation of the reporter signal, but a concomitant upregulation of the *ctgf* expression, as assessed by *in situ* hybridization (data not shown), possibly due to other regulations impacting on the *ctgf* expression independently of Yap/Taz and acting on regulatory sequences other than the MCAT sites. Of note, *CTGF* is a well-known YAP/TAZ target gene and a good readout for YAP/TAZ activity in cultured cells and in mice, but studies validating the *CTGF* as a general readout of YAP/TAZ activity *in vivo* during development are still absent.

To further validate the *Hsa.CTGF* reporter, we evaluated the consistency of the reporter gene expression pattern with that of *yap* and *taz* described in the literature. It is important to underline here that the Yap/Taz reporter we developed is reporting the transcriptional activity of Yap/Taz, not their simple expression pattern as it could be analyzed by *in situ* hybridization. In fact, the presence of Yap/Taz proteins in the cell doesn't automatically mean a Yap/Taz activity inside the nucleus, given the prominent post-translational regulation that governs their nucleocytoplasmic shuttling. In zebrafish, *yap* is ubiquitously expressed at 6-somite stage; as development progresses, *yap* expression is associated to several tissues and organs, mainly the notochord, the brain (evident in particular at the level of the MHB and the rhombomeres), the eye, the branchial arches and the pectoral fins (Jiang et al., 2009). Similarly to *yap*, *taz* is ubiquitously expressed at early stages. At 24 hpf, an high expression is visible in the head, the eye and the tail, becoming restricted to the head, the eye, the heart and the endodermal tissue next to the yolk by 30 hpf. A general downregulation of both *yap* and *taz* expression is evident at 50-60 hpf. Overall, *yap*/*taz* expression pattern is consistent with the Yap/Taz activity pattern revealed by the *Hsa.CTGF* reporter. The strong downregulation and the restriction of the reporter activity pattern evidenced by the *Tg(Hsa.CTGF:VenusPEST)* line at 48 hpf probably reflect those of *yap*/*taz* expression. As already touched on, this is not appreciable with the mCherry and eGFP lines, likely because of the difference in the proteins half-life.

5.2 *In vivo* analysis of Yap/Taz activity

The first important application of the newly generated *Hsa.CTGF*-based Yap/Taz reporter is represented by the investigation of the spatio-temporal activation of Yap/Taz. A zebrafish biosensor like the one presented in this thesis enables to follow *in vivo* at single-cell resolution the activity of the signaling pathway during embryonic and larval stages of development, as well as in the superficial structures of the adult organism, but also in the internal organs after *post-mortem* dissection.

The reporter expression pattern described here highlights an almost ubiquitous activation of Yap/Taz signaling during early embryonic development, with a stronger signal in the proliferating and undifferentiated tail bud mesenchyme (Figure 4.12). This likely reflects the general role first discovered for YAP/TAZ as nuclear transducers of the Hippo pathway, regarding the promotion of cell proliferation and organ growth during development. The activation of the Hippo pathway and the consequent silencing of YAP/TAZ activity, on the contrary, is responsible for the growth arrest when the organs reach their appropriate size. In the adult organism, in fact, YAP/TAZ expression is strongly restricted, being enriched in the stem/progenitor cells niches (Ramos and Camargo, 2012). Consistently with this, the *Hsa.CTGF*-based zebrafish reporter is largely silenced in the adult fish with respect to the embryonic and larval development. However, further investigation is needed to see if these zebrafish lines report a specific activation in the anatomical compartments containing stem/progenitor cells.

A highly positive organ in the adult is represented by the ovary, where the reporter protein is present in the nuclei of the oocytes. The presence of Yap/Taz target genes in the eggs might contribute to the very first biological events occurring in the embryo. The other tissues and organs in which a reporter signal was observed in the adult are the lens, the heart and the endothelium. While in the lens the presence of the reporter protein could be simply due to the almost absent protein turnover of the lens cells (Stewart et al., 2013), the persistent activity of Yap/Taz in the cardiovascular system could suggest an important role of this signaling in the maintenance of cardiac and vascular functions. A fascinating possibility is that Yap/Taz activity there, and in the cardiac ventricle in particular, could be sustained by the continuous mechanical stress which the cells are subjected to.

By looking in more detail at the reporter activation throughout the development, some reporter-expressing anatomical districts come out over the others, mainly the lens,

the pharyngeal arches, the heart, the pectoral fin and the otic vesicle. Along the trunk, Yap/Taz activation was detected in all the structures recognizable of the region, with a strong reporter expression particularly in the floorplate. Interestingly, the developing nervous system appears as the least reporter-expressing domain. A very weak signal is detected in the neural tube, while in the brain the reporter is almost completely off. Intriguing, however, is the Yap/Taz activity revealed by the reporter expression in the MHB region (although the neural nature of the fluorescent cells remains to be determined) and in the rhombencephalic region, where six stripes of fluorescent cells seam to follow the metameric organization of the rhombomeres (Figure 4.13 C). As already mentioned, many of these districts are novel domains of Yap/Taz activity first revealed *in vivo* by the *Hsa.CTGF*-based zebrafish reporter lines here described. Among these, the endothelium in particular attracted our attention, since a general and sustained activity of the reporter is detected in this tissue from when it starts to develop till adulthood. Importantly, all these reporter-expressing domains might suggest a role of Yap/Taz signaling in the development of tissues and organs or in their maintenance in adulthood, which is worth investigating.

Despite the general silencing of YAP/TAZ activity reported in the adult organism, a number of studies are demonstrating how the reactivation of YAP/TAZ signaling plays an instrumental and apparently general role in the regeneration. This activation of Yap/Taz was reported by the *Tg(Hsa.CTGF:nlsmCherry)* line in the well-established regeneration models represented by the larval fin primordia and adult epimorphic fin regeneration after amputation. We showed that the Yap/Taz biosensor zebrafish is able to report the expected activation of the signaling pathway in these regenerative processes, opening wide possibilities to study the details of Yap/Taz involvement in regeneration. The availability of such a reporter, coupled to reporters for other signalings implicated in the regeneration (first of all the Wnt/β-catenin pathway) and to the advantages of the zebrafish model, will enable powerful *in vivo* imaging of different regenerative processes and analysis of the relationships among the different molecular players.

Furthermore, cancer could be seen as the corresponding pathological side of the coin, that as well in many cases requires the reactivation, although aberrant and out of control, of YAP/TAZ. An exciting application of the *Hsa.CTGF*-based zebrafish reporter lines is therefore in the study of Yap/Taz role in tumorigenesis, by the use of

zebrafish tumor models, which permit also to monitor in real time processes as tumor growth and metastasis.

5.3 Yap/Taz zebrafish reporter as a tool for drug screening

Different chemical compounds were used on the *Hsa.CTGF*-based reporter to demonstrate its regulation by the Wnt/β-catenin pathway. Treatments with IWR-1, XAV939 and BIO drugs showed how the fluorescent reporter signal can be modulated by the chemical activation or inhibition of molecular pathways. From here, it appears evident how such a living reporter line could be exploited as a versatile platform for the discovery, screening and validation of novel drugs acting as agonists or antagonists of YAP/TAZ signaling, as already proposed for other zebrafish reporter lines (Weger et al., 2012; Moro et al., 2012; Moro et al., 2013). The big advantages of the use of a zebrafish reporter line are represented by the possibility to perform large-scale chemical screens with automated systems (Walker et al., 2012), the easy delivery of orally active drugs directly added to the fish water and, above all, the analysis of the pharmacological effect on a molecular pathway *in vivo* in a vertebrate organism.

The need of safe and effective drugs able to modulate YAP/TAZ activity is particularly relevant for a scientifically young signaling pathway like this, which has a strong impact on development, cancer and regeneration. In fact, both agonists and antagonists of YAP/TAZ signaling could be therapeutically relevant: contrasting YAP/TAZ activity might help in combatting cancer; the possibility to tune them at will might improve tissue regeneration.

5.4 Wnt/β-catenin pathway regulates Yap/Taz activity during development

Very recently a model was depicted, that revolutionizes our vision on YAP/TAZ activity regulation and its relationship with other signaling pathways. YAP and TAZ were demonstrated to be integral component of the β-catenin destruction complex and to be regulated by this complex in response to a Wnt signal similarly to β-catenin. YAP/TAZ represent therefore a transcriptional branch of the Wnt pathway, mediating a

significant fraction of Wnt biological responses, as demonstrated in cultured cells and in particular conditions in mice, such as in the APC/YAP/TAZ triple knockout in the intestine (Azzolin et al., 2012; Azzolin et al., 2014).

The zebrafish *Hsa.CTGF*-based reporter permitted to show in a living organism during development the regulation that the Wnt/β-catenin pathway exerts on Yap/Taz activity. As expected from the model, the up- or downregulation of the β-catenin destruction complex activity should result respectively in an up- or downregulation of both β-catenin and Yap/Taz transcriptional outputs. Our results in terms of variations of the reporter signal, after either genetic or pharmacological modulation of the complex activity, are in perfect accordance with the model. This confirms that the regulation of Yap/Taz activity mediated by the β-catenin destruction complex may function also *in vivo* during development. Moreover, in the same Yap/Taz zebrafish reporter we tested also the importance of the phosphorylation of β-catenin to drive Taz to degradation. Consistently, inhibition of β-catenin phosphorylation by treatment with BIO resulted in an increased expression of the reporter, readout of Yap/Taz activity.

It is worth noting, however, that all the modulations of Yap/Taz activity observed with the reporter zebrafish by acting on the Wnt/β-catenin pathway, although significant, were never particularly robust. As a comparison, the same genetic and pharmacological tools are much stronger when used on a Wnt/β-catenin reporter fish (Moro et al., 2012). This cannot be due to the limited ability of the *Hsa.CTGF*-based reporter to sense Yap/Taz activity variations, since mild alterations of Yap/Taz signaling, as performed for example with Yap/Taz morpholinos or TAZ-4SA mRNA injections, were shown to strongly modulate the reporter signal. A possible explanation could be that, in the complexity of a vertebrate organism such as zebrafish, the control of Yap/Taz activity is very tight and finely tuned by the intersection of many regulatory networks. Within this view, the Wnt/β-catenin pathway is only one modality the cells have to regulate Yap/Taz activity, together with the Hippo pathway and the other known or still undiscovered molecular actors. On the other hand, the morpholino oligos and the mRNAs used act directly on Yap/Taz or downstream, at the level of the Yap/Taz/Tead-mediated transcription, and are so expected to have a more powerful impact on the reporter signal.

What shown here regarding the Wnt/β-catenin pathway can be extended to the study of other possible crosstalks among Yap/Taz and other signaling pathways involved in vertebrate development and in cancer. Firstly, for instance, we could see if also the

reverse regulation, i.e. the inhibitory action of cytoplasmic YAP/TAZ on β-catenin activity (Azzolin et al., 2014), can be reported *in vivo* in zebrafish, in this case by using a Wnt/β-catenin reporter line. Thanks to living biosensor fish like the Yap/Taz reporter described here, relationships in space and time of activation of different signalings can be investigated *in vivo* also in many pathological contexts, by using the variety of zebrafish models of human diseases becoming available.

5.5 A role for Yap/Taz in angiogenesis

The general and sustained reporter activity we observed in the endothelium during embryogenesis suggested a possible functional involvement of Yap/Taz signaling in developmental angiogenesis. In fact, we showed that Yap/Taz knockdown impaired ISVs angiogenesis, while Yap/Taz overactivation caused a cell-autonomous aberrant sprouting from the ISVs, proposing a pro-angiogenic role of Yap/Taz transcriptional activity.

Only incomplete and indirect associations between YAP/TAZ signaling and angiogenesis are present in the literature. Very recent works are unraveling now a link between YAP/TAZ and pathways involved in angiogenesis, such as the Wnt/β-catenin, the hypoxia response, the Angiomotins and the mevalonate/Rho GTPases signalings.

5.5.1 YAP/TAZ, Wnt/β-catenin pathway and angiogenesis

The tight link existing between the Wnt/β-catenin pathway and YAP/TAZ might have an interesting significance also in angiogenesis. The pro-angiogenic role of Wnt signaling, carried on by β-catenin-mediated transcription of genes such as *VEGF* (Choi et al., 2012), could be coherently supported by YAP/TAZ-mediated transcription, at least during development, where both Wnt/β-catenin and YAP/TAZ signalings are active in the vasculature. In fact, Wnt-mediated inhibition of the β-catenin destruction complex results in both β-catenin and YAP/TAZ nuclear translocation and transcriptional activation (Azzolin et al., 2014), as we also evidenced *in vivo* by using the *Hsa.CTGF*-based reporter. This means that Wnt signals could impact on angiogenesis by co-regulating two sets of target genes. Within this hypothesis, the Hippo pathway might also have a double effect on angiogenesis. Indeed, the activation of the Hippo pathway, by promoting YAP/TAZ cytoplasmic retention, thus inhibiting

their nuclear activity, inhibits also β-catenin, whose degradation requires the presence of YAP/TAZ in the complex (Azzolin et al., 2014).

5.5.2 YAP/TAZ, hypoxia and angiogenesis

Even if in different biological conditions, both YAP and TAZ were found to associate with HIF1α within the nucleus and to promote HIF1α transcriptional activity, included the expression of key pro-angiogenic genes such as *VEGF*. Functionally, YAP knockdown in a breast cancer xenograft mouse model led to a reduced microvessel density due to an impairment in HIF1α activity (Ma et al., 2014; Bendinelli et al., 2013). Thus, the delay in the ISVs growth we described after morpholino-mediated Yap/Taz knockdown could be explained by the YAP/TAZ action on HIF1α accumulation and its pro-angiogenic functions. Oppositely, the aberrant angiogenesis obtained by forced activation of Yap/Taz could be due to an increased HIF1α-driven transcription occurring in concomitance to the increased Yap/Taz/Tead-driven transcription highlighted by the *Hsa.CTGF*-based reporter.

However, the same phenotype seen after injection of the non-phosphorylatable versions of YAP and TAZ (YAP-5SA and TAZ-4SA) was reported also after injection of the constitutively active version of TEAD transcription factor (TEAD-VP16). But TEAD is not known to bind HIF1α transcriptional complex as done by YAP and TAZ, meaning that the vessel sprouting-promoting activity we observed in this case cannot be due to HIF1α-mediated transcription, but it should be triggered by the transcriptional activity of Yap/Taz/Tead. Hence, both the effects of Yap/Taz morpholinos and YAP-5SA or TAZ-4SA mRNAs cannot be ascribed totally to the regulation exerted by Yap/Taz on HIF1α activity in the nucleus. On the contrary, it is likely that the vascular phenotype is due mostly to Yap/Taz transcriptional activity, since TEAD-VP16 and YAP-5SA or TAZ-4SA were equally effective in causing the phenotype. This is also consistent with the strong Yap/Taz reporter signal in the endothelium, which suggested indeed a role for the transcriptional activity of Yap/Taz in embryonic angiogenesis.

Now, if we consider the picture delineated by Ma et al. (2014) and Bendinelli et al. (2013) in which, under hypoxia, increased levels of both YAP and TAZ were registered in the nucleus, an intriguing scenario opens. The hypoxia response pathway promotes HIF1α nuclear accumulation and activity, which lead to a pro-angiogenic response. In addition, at the same time, hypoxia promotes also YAP/TAZ nuclear accumulation and activity, which, on one hand, sustain HIF1α-mediated transcription, on the other hand

trigger YAP/TAZ/TEAD-mediated transcription, that might also lead to a pro-angiogenic response, in accordance with our observation in zebrafish. Therefore, the road bringing hypoxia to angiogenesis might be mediated by a double transcriptional activation and rely not only on HIF1α target genes but also on YAP/TAZ target genes.

Of course, further studies are needed first to clarify the impact of YAP/TAZ transcriptional activity on angiogenesis and then to explore the crosstalk between HIF1α and YAP/TAZ within the hypoxia response pathway, together with the mechanisms and the target genes effectively supporting angiogenesis.

5.5.3 YAP/TAZ, Angiomotins and angiogenesis

Other important players in angiogenesis are the Angiomotins, whose interaction with the Hippo/YAP/TAZ pathway is tight even if with controversial outcomes. Our findings about the requirement of Yap/Taz for the proper ISVs growth and their vessel sprouting-promoting capacity can fit well with the proposed models of AMOT crosstalk with the Hippo/YAP pathway (Dai et al., 2013; Yi et al., 2013). It was shown, in fact, that the Hippo pathway activation results in LATS-mediated AMOT phosphorylation, disruption of AMOT binding to F-actin and subsequent decrease of F-actin stress fibers and focal adhesion, that finally leads to inhibition of angiogenesis (Dai et al., 2013). Thus, given the positive role of YAP/TAZ toward angiogenesis that we proposed, the Hippo-mediated inhibition of angiogenesis via AMOT phosphorylation would be coherent with the Hippo-mediated inhibition of angiogenesis via YAP phosphorylation and inhibition of YAP/TAZ-driven transcription. This point of view presents LATS as a potent player in angiogenesis inhibition, realized both by detaching AMOT from F-actin and by excluding YAP from the nucleus. Moreover, AMOT phosphorylation promotes AMOT-YAP association, inhibiting YAP activity (Dai et al., 2013). This, again, would reinforce the negative regulation of angiogenesis exerted by the Hippo pathway.

AMOT-YAP association, however, was also shown to prevent YAP phosphorylation by LATS and promote YAP nuclear translocation and target genes transcription in liver-specific *Amot* knockout mice (Yi et al., 2013). This discrepancy could be ascribed to the phosphorylation status of AMOT, which, when phosphorylated by LATS, inhibits YAP, and when not behaves in the opposite way. In such a context, the reduction in YAP nuclear activity observed in *Amot* knockout background could couple the impairment in angiogenesis due to the lack of AMOT with that derived from the downregulation of YAP-mediated transcription.

Anyway, new studies with different experimental setups are necessary to clarify the interaction between AMOT and YAP and in particular its biological significance *in vivo*.

5.5.4 YAP/TAZ, mevalonate/Rho GTPases signaling and angiogenesis

Similarly to the Wnt/β-catenin and the hypoxia response pathways, the mevalonate/Rho GTPases signaling could represent another pathway regulating angiogenesis through two different mechanisms, in light of the pro-angiogenic role of YAP/TAZ suggested by our observations in the zebrafish model. The mevalonate pathway, in fact, sustains cell locomotion and angiogenesis through FPP-mediated Ras and GGPP-mediated Rho cytoplasm-to-membrane translocation and activation. In consequence of this, Rho was demonstrated to inhibit YAP/TAZ phosphorylation, thus leading to their nuclear accumulation and activity. Therefore, the mevalonate pathway might promote angiogenesis by activating Ras and Rho but also by stimulating YAP/TAZ transcriptional activity. Oppositely, the inhibition of the mevalonate pathway by treatment with drugs such as the statins, might block the angiogenesis by silencing both the responses.

From this analysis, it appears evident how a possible role of YAP/TAZ transcriptional activity in triggering angiogenesis could be interesting and place Hippo/YAP/TAZ signaling among a complex network of pathways converging on angiogenesis through different roads. Furthermore, if this novel role of YAP/TAZ will be confirmed and shown in different biological contexts, it will have a strong impact in YAP/TAZ biology, especially in the cancer field. In fact, despite the relevance of YAP/TAZ in cancerogenesis is undeniable, it is not known if the aberrant activation of YAP/TAZ in the progression toward malignancy is responsible also for the angiogenesis necessary to sustain tumor growth.

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