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Establishment of transgenic lines to monitor and manipulate Yap/Taz-Tead activity in zebrafish reveals both evolutionarily conserved and divergent functions of the Hippo pathway

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ABSTRACT

To investigate the role of Hippo pathway signaling during vertebrate development transgenic zebrafish lines were generated and validated to dynamically monitor and manipulate Yap/Taz-Tead activity. Spatial and temporal analysis of Yap/Taz-Tead activity suggested the importance of Hippo signaling during cardiac precursor migration and other developmental processes. When the transcriptional co-activators, Yap and Taz were restricted from interacting with DNA-binding Tead transcription factors through expression of a dominant negative transgene, cardiac precursors failed to migrate completely to the midline resulting in strong cardia bifida. Yap/Taz-Tead activity reporters also allowed us to investigate upstream and downstream factors known to regulate Hippo signaling output in *Drosophila*. While Crumbs mutations in *Drosophila* eye disc epithelia increase nuclear translocation and activity of Yorkie (the fly homolog of Yap/Taz), zebrafish *crb2a* mutants lacked nuclear Yap positive cells and down-regulated Yap/Taz-Tead activity reporters in the eye epithelia, despite the loss of apical-basal cell polarity in those cells. However, as an example of evolutionary conservation, the Tondu-domain containing protein Vestigial-like 4b (Vgll4b) was found to down-regulate endogenous Yap/Taz-Tead activity in the retinal pigment epithelium, similar to *Drosophila* Tgi in imaginal discs. In conclusion, the Yap/Taz-Tead activity reporters revealed the dynamics of Yap/Taz-Tead signaling and novel insights into Hippo pathway regulation for vertebrates. These studies highlight the utility of this transgenic tool-suite for ongoing analysis into the mechanisms of Hippo pathway regulation and the consequences of signaling output.

1. Introduction

The Hippo signaling pathway is tightly controlled and critical during development and is often deregulated in disease. This evolutionary conserved signaling network influences tissue growth by regulating cell proliferation, apoptosis, and cell fate decisions. The core components of the pathway include the Serine Threonine Kinases 3 and 4 (Stk3/4; Hippo in

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Drosophila); two other serine threonine kinases called Large Tumor Suppressor 1 and 2 (Lats1/2; Warts in *Drosophila*); two scaffolding proteins Sav1 (Salvador in *Drosophila*) and Mps One Binder Kinase Activator-like 1A and 1B (Mobkl1a/1b; Mob as Tumor Suppressor in *Drosophila*). These proteins form a complex whose activity regulates the phosphorylation state, stability, and localization of the downstream transcriptional co-activators Yes-associated protein and WW Domain-containing transcriptional regulator 1 (Yap and Wwtr1 or more commonly, Taz; Yorkie in *Drosophila*). When the core kinases are inactive, Yap/Taz are free to translocate to the nucleus to activate transcription via interaction with members of the Tea Domain Family of transcription factors (Tead1–4; Scalloped in *Drosophila*) (Halder and Johnson, 2011; Yu and Guan, 2013). Regulatory factors both upstream and downstream of the core kinase complex are not well understood. However, some recently discovered components have been investigated in invertebrates and cell culture, but their role and significance has not been fully evaluated within vertebrates. Examples include Crumbs as an upstream component for controlling Hippo signaling activity, and the role of Vestigial-like 4 (Vgll4) as a co-repressor of Tead transcription factors.

Crumbs homologs (Crb1-3) are transmembrane proteins that contribute to a protein complex associated with apical cell–cell junctions of epithelial tissues and regulate various aspects of polarity. The Crumbs polarity complex was recently linked to the Hippo pathway in *Drosophila* through the effectors Kibra, Expanded, and Merlin. Crumbs manipulation in *Dropsophila* revealed tissue and developmental timing specificity on Hippo signaling output. Specifically, it was reported that either Crumbs overexpression or deletion in eye and wing imaginal discs resulted in mis-localized Expanded, increased nuclear Yorkie, and tissue overgrowth (Chen et al., 2010; Ling et al., 2010). In mammalian cell culture, several protein-binding assays showed that Yap and Taz interacted with the Crumbs polarity complex. When *crb3* was knocked down, phosphorylation of the cytoplasmic retention domain for Yap was reduced and there was a concomitant increase in nuclear Yap (Varelas et al., 2010). Together, the authors concluded that the Crumbs complex can sequester Yap/Taz at apical junctions in cultures of high-density, therefore preventing Yap/Taz-mediated proliferation. These observations in flies and cell culture provide strong rationale for investigation into the role of Crumbs and other upstream components on Hippo signaling in vertebrate animals.

The Tondu-domain containing protein Tgi was recently characterized as a downstream regulator of Hippo signaling in *Drosophila* (Koontz et al., 2013). Tgi interacts with Yorkie and competes for Scalloped binding, suggesting a model where Tgi acts as a co-factor to enhance Scalloped-mediated default repression. Interestingly, the mammalian ortholog of Tgi, Vestigial-like 4 (Vgll4) did not interact with Yap, but was found to bind Tead2 and block transcription within in vitro assays. Consistent with its role as a co-repressor of Scalloped/Tead-type transcription factors, overexpression of Vgll4 in mouse transgenic livers that also over-expressed Yap, reduced the Yap-mediated overgrowth phenotype. It will be important to confirm the role of Vgll4 as a co-repressor in other contexts in vivo and investigate whether this occurs through endogenous Yap/Taz-Tead signaling.

We are interested in how the Hippo network and other polarized signaling pathways function during development. To augment our understanding of Hippo signaling, particularly across different tissues in vivo, we have generated a tool-suite for monitoring and manipulating the Hippo-Yap/Taz-Tead signaling network in zebrafish. In particular, we generated fluorescent Hippo-Yap/Taz-Tead responsive transgenic lines based on the previously characterized *4xGTIIC* enhancer (Mahoney et al., 2005). This synthetic transcriptional enhancer contains four copies of the GTIIC sequence of the SV40 proximal promoter. The GTIIC element, as well as the multimerized variant, was found to bind Tead proteins, which subsequently interact with Yap or Taz to strongly activate transcription (Davidson et al., 1988; Mahoney et al., 2005; Sawada et al., 2005). We have verified the *4xGTIIC* transgenic lines as Hippo-Yap/Taz-Tead reporters by using gain and loss of function experiments, along with the analysis of endogenous Yap localization. We next used these lines to investigate the significance of Hippo signaling during early heart morphogenesis and to test the role of Crb2a and Vgll4b as potential endogenous, upstream and downstream regulators of vertebrate Hippo signaling.

2. Results

2.1. *Establishment of Hippo-Yap/Taz-Tead responsive transgenic reporter lines*

The *4xGTIIC* promoter contains 4 multimerized SV40 proximal promoter GTIIC sequences, which are consensus Tead binding sites (Fig. 1A) and was previously reported to be responsive to Yap/Taz-Tead activity (Davidson et al., 1988; Mahoney et al., 2005). The *4xGTIIC* and other Tead and Scalloped multimerized binding site promoters have been shown to be responsive to Hippo pathway manipulation in other models and contexts (Dupont et al., 2011; Ota and Sasaki, 2008; Zhang et al., 2008). Therefore, we generated stably transgenic zebrafish that contain the *4xGTIIC* promoter driving expression of d2GFP, eGFP, or mCherry. Multiple founders were generated, isolated, and characterized to ensure consistency in the pattern of expression. For each transgenic construct, offspring from one founder was used to establish stable expressing lines. High expression in the developing larvae was noted in the epidermis, cardiac progenitor cells, presumptive sinus venosus, undifferentiated endoderm, otic and lens vesicles, retinal pigmented epithelium (RPE), cranial mesenchymal cells, multiple cell types in the heart, and within striated muscle of the trunk (Fig. 1). Several of the *4xGTIIC* positive cell types have been shown to express Yap/Taz-Tead target genes *ctgfa* anc *cyr61* in zebrafish (Fernando et al., 2010). At 1 day post fertilization (dpf) the *4xGTIIC* reporter is highly active in migratory cells located at the midbrain/hindbrain boundary and in the craniofacial region (Fig. 1C′, Fig. 2, Fig. 3A′ and B′). Co-expression analysis of these migratory cells revealed active transgene expression in *foxc1b*-positive mesenchymal cells, but not *sox10*-positive neural crest cells (Fig. 2). Expression profile comparisons between the d2GFP, eGFP, and mCherry transgenes revealed significant overlap in the areas of high expression (Supplementary Fig 1). In order

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