Transcriptional regulators in the Hippo signaling pathway control organ growth in *Xenopus* tadpole tail regeneration

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**A B S T R A C T**

The size and shape of tissues are tightly controlled by synchronized processes among cells and tissues to produce an integrated organ. The Hippo signaling pathway controls both cell proliferation and apoptosis by dual signal-transduction states regulated through a repressive kinase cascade. Yap1 and Tead, transcriptional regulators that act downstream of the Hippo signaling kinase cascade, have essential roles in regulating cell proliferation. In amphibian limb or tail regeneration, the local tissue outgrowth terminates when the correct size is reached, suggesting that organ size is strictly controlled during epimorphic organ-level regeneration. We recently demonstrated that Yap1 is required for the regeneration of *Xenopus* tadpole limb buds (Hayashi et al., 2014, Dev. Biol. 388, 57–67), but the molecular link between the Hippo pathway and organ size control in vertebrate epimorphic regeneration is not fully understood. To examine the requirement of Hippo pathway transcriptional regulators in epimorphic regeneration, including organ size control, we inhibited these regulators during *Xenopus* tadpole tail regeneration by overexpressing a dominant-negative form of Yap (dnYap) or Tead4 (dnTead4) under a heat-shock promoter in transgenic animal lines. Each inhibition resulted in regeneration defects accompanied by reduced cell mitosis and increased apoptosis. Single-cell gene manipulation experiments indicated that Tead4 cell-autonomously regulates the survival of neural progenitor cells in the regenerating tail. In amphibians, amputation at the proximal level of the tail (deep amputation) results in faster regeneration than that at the distal level (shallow amputation), to restore the original-sized tail with similar timing. However, dnTead4 overexpression abolished the position-dependent differential growth rate of tail regeneration. These results suggest that the transcriptional regulators in the Hippo pathway, Tead4 and Yap1, are required for general vertebrate epimorphic regeneration as well as for organ size control in appendage regeneration. In regenerative medicine, these findings should contribute to the development of three-dimensional organs with the correct size for a patient’s body.

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**Introduction**

In epimorphic regeneration (epimorphosis), such as amphibian limb or tail regeneration, the organ size is regulated so that the restored appendage is the same size as the original one. Organ size and shape are determined through the integration of cellular behaviors, including proliferation, apoptosis, cellular hypertrophy, oriented cell division, and regulation of the cell growth-supporting extracellular matrix (ECM) composition. Intracellular signaling triggered by secreted growth factors or by cell–cell contact directs these cellular behaviors, to generate an organ of the proper size and shape.

Among tetrapods, only amphibians along with fish can regenerate a lost organ or appendage such as a limb, tail, or fin ray (Brockes, 1997; Poss et al., 2003; Stoick-Cooper et al., 2007a; Straube and Tanaka, 2006; Tamura et al., 2010). In such animals, after an acute injury, organ reconstruction occurs by recapitulating developmental morphogenesis. While urodele amphibians (newts and salamanders)...
can regenerate a complete limb after limb amputation throughout their life cycles. *Xenopus*, an anuran amphibian, can regenerate a complete limb after limb bud amputation only at the early tadpole stages (Dent, 1962; Muneoka et al., 1986). Xenopus tadpoles also can regenerate their tail, which consists of multiple tissues, including muscle, notochord, and spinal cord (Beck et al., 2009; Love et al., 2014; Mochii et al., 2007; Slack et al., 2008; Tseng and Levin, 2008) (Fig. S1A). Tail regeneration of the *Xenopus* tadpole is a suitable model for examining molecular function in a comprehensive context since many molecular biological techniques including transgenesis are available for the *Xenopus* system. Furthermore, tail regeneration of amphibians is suitable for studying size regulation mechanisms in epimorphic regeneration because the total length of the regenerated tail is proportional to the length of the removed tail. Amputation at the proximal level of the tail (deep amputation) results in regeneration of a longer tail than does amputation at the distal level (shallow amputation) to restore the original size of the tail (Iten and Bryant, 1976). However, while recent studies have uncovered fascinating molecular details about the process of tail regeneration in amphibians, how the size of the regenerated tail is regulated at the molecular level remains to be elucidated.

The Hippo signaling pathway has attracted attention for its role in organ size control among bilaterians (Halder and Johnson, 2011; Zhao et al., 2010, 2011). This interesting signaling pathway mediates signal transduction from cell–cell or cell–ECM contact or from mechanical pressure on a cell to regulate target gene expressions (Mammoto et al., 2012). At high cell density, a kinase complex consisting of Mst and Lats phosphorylates the transcriptional regulator Yap1, and deposits it into the cytoplasm (Hao et al., 2008; Hayashi et al., 2014). At low cell density, Yap1 is dephosphorylated and translocates into the nucleus, where it acts as a transcriptional co-activator. Yap1 is widely expressed in almost all tissues in zebrafish, mouse, and *Xenopus* (Jiang et al., 2009; Morin-Kensicki et al., 2006; Nejigane et al., 2011). The gene product Yap1 is essential for organogenesis, tissue homeostasis, and stem cell regulation. *yap1* deficient mice and *yap1*-morphant embryos of *Xenopus* and zebrafish show severe defects during early embryogenesis (Gee et al., 2011; Jiang et al., 2009; Morin-Kensicki et al., 2006). Yap1 also plays a role in the size control of visceral organs, such as the liver and heart, during development. The transient overexpression of Yap1 causes liver expansion, which is reversible (Camargo et al., 2007; Dong et al., 2007), whereas the conditional knockout of Yap1 causes a reduced heart size (von Gise et al., 2012). Functional activation of Yap1 by its overexpression or by conditional knockout of the Yap1 inhibitor Salvador increases the heart size (Heallen et al., 2011; von Gise et al., 2012). Yap1 is a transcriptional co-activator that regulates cell behaviors and morphological growth by forming a transcriptional complex with DNA-binding transcription factors (Mauviel et al., 2012). Tea domain (Tead) transcription factors directly interact with Yap1 as a partner in the transcriptional complex (Vassilev et al., 2001; Zhao et al., 2008). This functional relationship is conserved in *Drosophila*, in which the Yap1 and Tead orthologs are called Yorkie and Scalloped, respectively (Halder and Johnson, 2011; Zhang et al., 2008). Several knockout experiments revealed that the Tead family transcription factors are essential for embryogenesis (Chen et al., 1994; Nishioka et al., 2008; Sawada et al., 2008; Yagi et al., 2007). Among the Tead family members, Tead4 promotes trophoderm development from the inner cell mass in the preimplantation mouse embryo (Nishioka et al., 2009).

The importance of Yap or Yap homologues in regenerative processes has been suggested in several species. In mouse, Yap1 is required for the proper regulation of intestinal stem cells and intestinal regeneration (Barry et al., 2013; Cai et al., 2010). The insect homologue Yorkie is involved in intestinal and imaginal disc regeneration in *Drosophila* (Grusche et al., 2011; Karpowicz et al., 2010; Shaw et al., 2010) and in leg regeneration in crickets (Bando et al., 2009). In our previous study, we provided the first evidence for the Yap1’s role in epimorphic regeneration of vertebrates. We transiently inhibited the function of Yap1 in limb bud regeneration of *Xenopus* tadpoles and revealed that Yap1 is required for the cell proliferation, gene regulation of pattern formation, and limb reconstruction in this process (Hayashi et al., 2014). Our findings suggested that Hippo signaling is required for the formation of multiple tissues of the precise size and pattern in *Xenopus* limb bud regeneration. Since Hippo signaling-mediated Yap1 activity is involved in a wide range of biological events, through its regulation of the progenitor cell populations of multiple tissues, we hypothesized that Yap1’s function in the mechanisms underlying organ size and shape determination is widely conserved in the organ or appendage regeneration (epimorphic) of vertebrates and invertebrates.

In this study, we examined the molecular mechanisms controlling size and shape in appendage regeneration. Focusing on the Hippo signaling pathway, we investigated the molecular functions of its downstream mediators, the transcriptional regulators Yap1 and Tead4, in *Xenopus* tadpole tail regeneration. Our results demonstrated that Yap1 and Tead4 are required for the precise regulation of appendage regeneration, suggesting that the Hippo pathway is an important part of the mechanism of morphological regeneration, which is spatiotemporally transduced through cell and tissue interactions.

**Materials and methods**

**Ethical treatment of animals and manipulation of Xenopus tadpoles**

All of the animal manipulations were performed under appropriate anesthesia, and all efforts were made to minimize the animals’ suffering. *Xenopus laevis* tadpoles were reared at 22–23 °C in dechlorinated tap water and manipulated as previously reported (Hayashi et al., 2014). The tadpoles were staged according to Nieuwkoop and Faber staging (Nieuwkoop and Faber, 1994). Heat shock treatment was performed at 34 °C for 30 min (Beck et al., 2003). For st41/42 tadpoles, the heat shock was performed once, on day 0 (3 h before tail amputation), and for st52 tadpoles, it was performed twice, on day 0 as described above, and 3 days after tail amputation. F1 or F2 transgenic animals that did not show tdTomato fluorescence in the lens nor induction of transgene expression by heat shock were used as matched sibling negative controls (wild-type) (Please see also “Plasmid construction and transgenesis” below). The tadpoles were moved to 18 °C water to cool down soon after each heat shock, and then returned to the rearing temperature (22–23 °C). The distal tip of the tadpole tail was amputated by a surgical knife after applying anesthesia [0.025% ethyl-3-aminobenzoate (Tokyo Chemical Industry, 886–86–2) dissolved in Holtfreter’s solution]. The tadpoles were then allowed to recover under the rearing conditions until analysis. For bleaching, tadpoles euthanized with ethyl-3-aminobenzoate were treated with a bleaching solution (30% H2O2:100% methanol, 2:1).

**Plasmid construction and transgenesis**

The establishment of a heat-shock-inducible dnYap transgenic (Tg) *Xenopus* line was reported previously (Hayashi et al., 2014). A heat-shock-inducible cassette under control of the *Xenopus hsp70* promoter (Wheeler et al., 2000) was used to generate a dominant-negative form of Tead4 (dnTead4) Tg *Xenopus*. The dnTead4 cDNA, encoding a chimeric protein of Tead4 and Engrailed repressor domain (kindly gifted by Drs Hiroshi Sasaki and Yoshikazu Hirate) (Nishioka et al., 2009) was bound to GFP with 2A peptide (Nojima et al., 2010). Plasmid construction and transgenesis

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generate founder dnTead4 Tg Xenopus embryos (F0), the sperm nuclear transplantation method was used, as previously reported (Kroll and Amaya, 1996; Ogino et al., 2008). In brief, oocytes were de-jellied by 2% cysteine/Marc’s modified Ringers (pH 8.9) and then the linearized plasmid construct mixed with sperm nuclei and oocyte extract was injected into the oocytes. We established stable F1 Tg lines, which were reproduced by crossing sexually mature F0 Tg male frogs with wild-type (WT) female frogs. F1 Tg tadpoles were used for the experiments. The hsp70-GFP F4 Tg Xenopus containing GFP under control of the hsp70 promoter (Mukaigasa et al., 2009) was gifted by Dr. Yumi Izutsu. The original plasmid used for the transgenesis of hsp70-GFP Xenopus was phS1/EGFP, described in (Michiue and Asashima, 2005). We obtained hsp70-GFP F5 Tg lines by crossing sexually mature F4 male frogs with WT female frogs, and used the F5 Tg Xenopus tadpoles for the IR-LEGO experiment. The heat-shock-inducible Dkk1 F0 Tg Xenopus was prepared as previously reported (Yokoyama et al., 2011; Yokoyama et al., 2007). Dkk1 was tagged with GFP to monitor the transgene expression, and tdTomato under control of the gamma-crystallin promoter was inserted downstream of hsp70-Dkk1GFP to select Tg individuals (Yokoyama et al., 2011). We obtained Dkk1 F2 Tg lines by crossing sexually mature F1 male frogs with WT female frogs, and F2 Tg Xenopus tadpoles were used for the Wnt/β-catenin-inhibition experiment.

SU4502 treatment

A 4 mM stock solution of SU5402 (Calbiochem, 572630) dissolved in DMSO was stored in the dark at −20°C. This stock solution was diluted to 100 μM with ultra pure water. Five microliters of 100 μM SU5402 solution were injected into the abdominal cavity of anesthetized st52 tadpoles with a glass needle (60–100-μm diameter at the tip). For control tadpoles, 5 μl of 2.5% DMSO diluted with ultra pure water was injected.

Immunofluorescent staining

Whole mount immunostaining was performed as described previously (Schreiber et al., 2001) with some modification. Euthanized tadpoles were fixed with 4% paraformaldehyde for 30 min at room temperature (RT) and treated with bleaching solution (30% H2O2:100% methanol, 2:1) at RT overnight and with 1% Triton X100/PBS at RT overnight. The following primary antibodies were applied at a 1/100 dilution: anti-Mycosin heavy chain (Developmental Studies Hybridoma Bank, MF20), anti-acetylated alpha Tubulin (Sigma, 6-11B-1), anti-phosphorylated Histone H3 (Millipore, 06-570), and active Caspase3 (BD Pharmingen, 559565). Secondary antibodies were applied at a 1/400 dilution (Molecular Probes, anti-mouse/rabbit IgG conjugated with Alexa 488/594). For anti-Yap1 (1/50, Cell Signaling Technology, #4912) and anti-phosphorylated Yap1 (1/100, Cell Signaling Technology, #4912), the fluorescent signal was amplified using a TSA kit (Cell Signaling Technology, #4912). Immunostaining of sectioned samples was performed as described previously (Hayashi et al., 2014).

Laser irradiation at the single-cell level using an infrared laser-evoked gene operator (IR-LEGO) microscope system

We used the IR-LEGO method to apply heat shock at the single-cell level as previously reported (Deguchi et al., 2009; Kamei et al., 2009) with slight modifications. We used an IX71 microscope (Olympus) and IR-LEGO-1000 system (Sigma-Koki). Tadpoles were anesthetized as described above and exposed to an infrared laser (32 mW, 1000 ms, LCPlan N based custom made 20 × objective) on a glass-bottom dish.

qRT-PCR

The total RNA was extracted by Trizol (Life Technologies, 15596-026), treated with RQ1 RNase-Free DNase (Promega, M6101), and used to synthesize cDNA with Superscript III reverse transcriptase (Invitrogen, 18080-044), as described in the manufacturer’s manual. Quantitative (q) RT-PCR was performed using the Power SYBR Green PCR master mix (Applied Biosystems, 4368702) and StepOnePlus (Applied Biosystems). The following primers were used: birc5.1 forward primer: GCT AAA GCT GGC TTT GGT CA, birc5.1 reverse primer: GGA ATG TTC AGT CCA AGG GT, odc1 forward primer: TCC ATT GAC AGC GTA GGA CT T, odc1 reverse primer: GAG CCT GCG CGG TGA AAT A.

Results

Transcriptional regulators in the Hippo signaling pathway regulate tail regeneration in the Xenopus tadpole

We first examined the Yap1 protein distribution in the whole body of the Xenopus tadpole by immunofluorescent staining. At st38 (tail bud stage) and st46 (early tadpole stage), Yap1 protein was detected broadly in multiple organs, including the brain and muscle (Fig. S1B–E). This systemic distribution of Yap1 suggests that multiple tissues were under the control of Hippo signaling and Yap1 protein. The phosphorylation of serine in a conserved motif of mammalian Yap1 promotes Yap1’s sequestration in the cytoplasm, and thus its transcriptional inactivation (Hao et al., 2008; Hayashi et al., 2014; Zhao et al., 2007). We cloned and sequenced Xenopus Yap1 and found that the motif containing the serine residue was identical to that of mammals. Phosphorylated Yap1 (pYap1) was also broadly distributed throughout the whole Xenopus tadpole body (Fig. S1F and G). This result suggests that signal transduction mediated by Hippo pathway components (e.g. Mst and Lats), which are repressive upstream regulators of Yap1, makes Yap1 protein to be in the inactive form (pYap1) in multiple organs.

Each tissue in the Xenopus tadpole tail, including spinal cord, notochord, muscles, and blood vessels, can be reconfigured to reconstruct a motile tail with essentially the same size and shape as the tail before amputation. To investigate Yap1’s contribution to tail regeneration, we amputated the tail of early-stage tadpoles (st41/42; before the tadpoles start feeding) and examined the Yap1 protein distribution in the intact and regenerating tail. In the intact tail, the Yap1 protein expression overlapped with muscles, indicated by sarcomeric Myosin Heavy Chain (MyHC) staining, and with the spinal cord, indicated by acetylated alpha Tubulin (αTub) staining (Fig. 1A–C and J–L). At 1 dp (day post amputation), the wound was already healed (Fig. 1D–F and M–O). At 3 dp, the muscles and spinal cord had begun to regenerate, and Yap1 was detected in the regenerating tail (Fig. 1G–I and P–R). In this system, neurogenesis and axogenesis in the central nervous system can also be examined (Tanaka and Ferretti, 2009). In the spinal cord of Xenopus tadpoles, both axogenesis (Fig. 1P) and neurogenesis (Fig. S2) occurred to regenerate a motile tail. Yap1’s transcriptional co-activator function was more likely to be involved in the neurogenesis of neural progenitors than in nerve axon elongation, because Yap1 often promotes the proliferation of progenitor cells and inhibits the terminal differentiation of cells (Cao et al., 2008; Gee et al., 2011; Jiang et al., 2009; Van Hateren et al., 2011). The pYap1 signal was strongly detected in the notochord in the intact and regenerating tail (Fig. 1S–U). Compared to the intact tail, the pYap1 signal was slightly higher in the spinal cord in the regenerating tail and stump at 3 dp (Fig. 1U), suggesting that the spatiotemporal, dynamic Yap1 regulation via phosphorylation played a role in the regenerating tail. The distribution of Yap1 in multiple tissues in the regenerating tail led us to investigate Yap1’s functional contribution to
the control of organ morphogenesis in tail regeneration as well as tail development.

To evaluate the functional contribution of the transcriptional regulators in Hippo signaling to tail regeneration, we used stable F1 Tg lines that carried a heat shock-inducible dominant-negative form of Yap (dnYap) (Hayashi et al., 2014) or of Tead4 (dnTead4) (Fig. S3), see also the Materials and methods section. Both of these dominant-negative molecules were first established in mice and were confirmed to have dominant-negative activity for Yap and Tead4, respectively (Nishida et al., 2009). St41/42 Tg tadpoles were heat shocked, and GFP fluorescence as the indicator for transgene expression was observed within 3 h (Fig. 2A and B). The tail tip of Tg tadpoles and WT (wild-type) siblings was amputated, and the regeneration phenotypes were analyzed at 7 dpa (Fig. 2C). The heat shock was applied to tadpoles 3 h prior to amputation to obtain sufficient transgene expression at the amputation site and to separate the amputation damage from side effects of the heat shock. In both dnYap1 and dnTead4 Tg tadpoles, the tail regeneration was significantly impaired (Fig. 2D–F). The average regenerated tail length was shorter in the dnYap1 and dnTead4 Tg than in WT tadpoles (Fig. 2G. 2.55 ± 0.15 mm for WT, n = 13; 1.32 ± 0.08 mm for dnYap, n = 10; and 1.25 ± 0.15 mm for dnTead4, n = 24). The proportion of tadpoles showing successful regeneration was also reduced in the dnYap1 and dnTead4 Tg groups (Fig. 2H, n = 287, 39 and 64 for WT, dnYap and dnTead4, respectively). Regeneration defects such as upper fin absence, lower fin absence, spinal cord bending, or a combination of these defects were categorized as “incomplete regeneration” (Table 1), and a worse defect, in which tail regeneration completely ceased, was categorized as “severe defect” (Fig. 2H). These “incomplete regeneration” and “severe defect” phenotypes could not be explained as delayed regeneration by dnYap1 or dnTead4, because they were morphologically distinct from the early phase of tail regeneration in WT tadpoles.

To further evaluate the regeneration defects caused by dnYap or dnTead4, we examined their effect on the tissue regeneration at 7 dpa, and found that the muscle and nerve axon regeneration were impaired (Fig. 3B–G). In the regenerated tail of WT tadpoles, clusters of muscle were observed, but in the dnYap and dnTead4 tadpoles, only dispersed fibrous muscle (non-clustered muscle) cells were observed (Fig. 3B–D. Non-clustered muscles, WT: n = 0/8, dnYap: n = 3/5, dnTead4: n = 4/4). The nerve axon elongation was also reduced in the dnYap and dnTead4 Tg, compared with the WT tails (Fig. 3E–G. Shortened spinal cord, WT: n = 0/8, dnYap: n = 6/6, dnTead4: n = 4/4). In addition, mitoses in the regenerating tail, indicated by phosphorylated Histone H3 (pH3) immunostaining, was reduced in the dnYap and dnTead4 tadpoles (Fig. 3H–J. Decreased mitosis, WT: n = 0/9, dnYap: n = 5/6, dnTead4: n = 3/6). Ectopic apoptosis indicated by an apoptosis marker, active Caspase3 (acCasp3) was observed in the dnYap1 and dnTead4 tadpoles, with stronger staining in the latter, at 7 dpa (Fig. 3K–M. Ectopic apoptosis, WT: n = 1/10, dnYap: n = 7/8, dnTead4: n = 4/4). These results suggest that Yap1 and Tead4 regulate cell proliferation and apoptosis in the regenerating tail to control the amount of regeneration of each type of tissue.

We next addressed the requirement of these transcriptional regulators in tadpole development rather than tail regeneration. We heat shocked dnYap and dnTead4 Tg tadpoles at st41/42, allowed them to grow without tail amputation, and then measured their body length 7 days after the heat shock. The body length was reduced by either dnYap or dnTead4 induction, but the tail length was less affected (Fig. S4, 23.5% reduction in body length in dnYap, 29.9% in dnTead4, n = 4/14, and 26 for WT, dnYap, and dnTead4, respectively, versus 6.5% reduction for the tail in dnYap and 5.9% reduction for the tail in dnTead4, n = 51, 14, and 26 for WT, dnYap, and dnTead4, respectively). These results indicate that tail elongation during development may be independent of the Hippo pathway, and that it occurs probably by other driving forces, such as notochord swelling and muscle elongation by its postmitotic maturation. To exclude the possibility that the body lengths of Tg tadpoles were shortened by leaky expression of dominant negative form of proteins prior to heat shock, we measured body length of Tg tadpoles without heat shock and that of WT siblings with heat shock (Fig. S4E, n = 39, 12 and 24 for WT, dnYap and dnTead4, respectively). There was no obvious difference in body length between Tg tadpoles (without heat shock) and WT ones (with heat shock). We also observed excessive apoptosis in the dnYap and dnTead4 tadpoles, that was more frequent in the latter, after the heat shock at st41/42 (Fig. S5A–D). Furthermore, we found that the amount of birc5.1 (survivin) transcripts in the tadpole whole body 2 days after heat shock at st41/42 was significantly reduced by the induced dnYap or dnTead4 expression (Fig. S5E. All samples were n = 4). birc5.1 (survivin) is a member of the inhibitor of apoptosis (IAP) gene family (Altieri, 2003). The expression of IAP family genes is positively regulated by transcriptional regulators in the Hippo pathway in both invertebrates and vertebrates (Dong et al., 2007; Landin Malt et al., 2013). Thus, Tead4 and Yap1 may regulate tadpole body growth by suppressing apoptosis through the positive regulation of IAP family gene(s) such as birc5.1 (survivin).

**Tead4 maintains the neural progenitor population during spinal cord regeneration, in a cell-autonomous manner.**

As shown in Fig. 2, the tail regeneration of tadpoles was inhibited by the induction of dnYap or dnTead4 expression at st41/42. In this
It was difficult to distinguish the cell-autonomous effects caused by inhibiting transcriptional regulators such as Yap1 or Tead4 from non-cell autonomous effects caused by surrounding cells in which Yap1 or Tead4 function is inhibited, because the Yap1 or Tead4 function was inhibited in all of the cells of the dnYap1 or dnTead4 Tg tadpole after heat shock. Therefore, to distinguish the cell-autonomous effects from the non-cell autonomous ones, we induced the transgene expression in a single cell. Single-cell gene induction using heat-shock promoters has been reported in vertebrates (Halloran et al., 2000; Kimura et al., 2013; Sato-Maeda et al., 2006). In particular, the IR-LEGO system uses an IR laser (1480-nm wavelength) to elicit highly efficient gene induction with minimal cellular damage (Kamei et al., 2009). In combination with Cre-loxP, this system enabled the long-term cell labeling in teleost fish (Shimada et al., 2013). We found that the IR-LEGO system could be applied to X. laevis (Kawasumi et al., unpublished results), and we optimized the experimental conditions for single-cell gene induction in Xenopus tadpoles (see the Materials and methods section for details).

To investigate the cell-autonomous function of Yap1 and Tead4 in regenerating tadpole tails, we examined the neural progenitors that contribute to spinal cord regeneration. We focused on the spinal cord region for two reasons. First, the spinal cord is required for proper tail regeneration (Taniguchi et al., 2008). Second, the spinal cord is structurally distinguishable at the cellular level, and thus is well suited for single-cell gene induction with the current IR-LEGO system. We amputated tadpole tails at st41/42, and then used an IR laser to apply heat shock to a single neural progenitor cell in the spinal cord at 2 dpa (Fig. 4A and B). At 2 dpa, the regenerating tail is transparent, and the

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**Table 1**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Upper fin absence</th>
<th>Lower fin absence</th>
<th>Spinal cord bending</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>69/287 (24.0%)</td>
<td>31/287 (10.8%)</td>
<td>37/287 (12.9%)</td>
</tr>
<tr>
<td>dnYap Tg</td>
<td>15/39 (38.5%)</td>
<td>13/39 (33.3%)</td>
<td>22/39 (56.4%)</td>
</tr>
<tr>
<td>dnTead4 Tg</td>
<td>22/64 (34.4%)</td>
<td>19/64 (29.7%)</td>
<td>15/64 (23.4%)</td>
</tr>
</tbody>
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*Note: The tadpole number is indicated for each phenotype. Since some tadpoles had multiple defects, the sum of all the phenotypes is greater than the total tadpole number.*
spinal cord region can easily be recognized and focused on under the inverted microscope in the IR-LEGO system (Fig. 4B).

As a control, GFP Tg (Mukaigasa et al., 2009) F5 line tadpoles containing GFP under control of the *hsp70* promoter were used. These tadpoles showed GFP fluorescence in a single cell in the spinal cord 1 day after laser irradiation (Fig. 4C). Fig. 4D shows a control tadpole, in which two GFP-positive cells were observed 1 day after laser irradiation. These cells had multiplied (6 cells) at 2 days (Fig. 4E). In this case, a laser-irradiated cell probably divided before the GFP protein deposition accumulated to a visible level, and then increased rapidly to six cells because neural progenitors are highly proliferative (Fig. S2) (Gaete et al., 2012). The GFP signal became obscure 3 days after irradiation, but it was still visible for 1 week.

In the *dnTead4* line (which was tagged with GFP via 2A peptides under the *hsp70* promoter, Fig. S3), the GFP-positive cells after laser irradiation had a crumpled shape, suggesting that they were dying cells (Fig. 4F). Compared with controls (the *hsp70*-GFP tadpoles), the GFP-positive cells in *dnTead4*-GFP tadpoles were more scarce, and the cell number on subsequent days was significantly lower. The *dnYap*-GFP tadpoles showed a similar effect, although it was less severe than in the *dnTead4*-GFP tadpoles (Fig. 4G. GFP: n = 19, dnYap-GFP: n = 19, dnT4-GFP: n = 17). These results suggest that the transcriptional regulators in Hippo signaling, especially Tead4, are cell-autonomously required for the cell survival of neural progenitors in the spinal cord. This result, however, does not exclude the possibility that Tead4 also affects the proliferation or apoptosis of neighboring cells in a non-cell autonomous manner. Notably, the *dnTead4* expression in st41/42 tadpoles caused the strong induction of ectopic apoptosis (Fig. S5), indicating that the phenotype in *dnTead4* tadpoles may be exerted through the loss of apoptosis inhibition.
Fig. 4. Yap and Tead4 are cell-autonomously required for the cell survival of neural progenitors in the spinal cord. (A) Experimental schedule for labeling neural progenitors at the single-cell level by IR-LEGO. (B) Cartoon of the regenerating tail. Green line represents the spinal cord, and light orange region represents the notochord. Red line indicates the amputation plane. Blue square marks the photographed region shown in C–F, and the red cross indicates the point targeted by IR-REGO. (C)–(F) A single neural progenitor cell in the spinal cord was induced to express GFP or dnTead4 tagged with GFP (dnT4-GFP) by infrared laser irradiation at 2 days post amputation. White arrow indicates the irradiated site. Insets indicate high-power views of the fluorescent images of the irradiated cell and its descendants. (C) A single cell was induced to express GFP by infrared laser irradiation. (D) and (E). Another example showing active cell division. Two GFP-positive cells appeared 1 day after irradiation, suggesting that division occurred before the GFP expression was detected (D) and the cell number was increased the next day (E). (F) A single cell was induced to express dnTead4 tagged with GFP by the same infrared laser irradiation as in (C)–(E). The dnTead4 expression cell-autonomously affected cell survival and mitosis. Note that the GFP-positive cell has a crumpled appearance, and looks like a dying cell. (G) Average number of GFP-expressing cells after each irradiation. The induced dominant-negative proteins significantly reduced the cell number in dnYap Tg 2 days and in dnTead4 Tg 1 and 2 days after irradiation. Error bar indicates s.e.m. Scale bar in C–F = 100 μm and in inset = 10 μm. Single and double asterisks indicate statistically significant differences between WT and Tg tadpoles at p < 0.05 and p < 0.01 (Welch’s t-test), respectively.

Fig. 5. Tead4 contributes to position-dependent growth control. Experimental schedule. The tail of st52 tadpoles was amputated 3 h after the first heat shock. Tadpoles were heat shocked again at 3 dpa. Note that the observation of resultant tail regeneration at 7 dpa is far earlier than the onset of metamorphic tail regression that normally starts at st62 (Nieuwkoop and Faber, 1994). (B) and (C). WT (heat shocked) regenerated tail 7 days after amputation at the proximal (B) and distal (C) level (50% and 25% of the distance from the tail tip to the cloaca, respectively). The growth rate in the tail amputated at the proximal level was faster than that in the tail amputated at the distal level during the same period. (D) and (E) dnYap Tg regenerated tail 7 days after amputation at the proximal (D) and distal (E) level (50% and 25% of the distance from the tail tip to the cloaca, respectively). The growth rate in the tail amputated at the proximal level was faster than that in the tail amputated at the distal level during the same period. (F) and (G) dnTead4 Tg regenerated tail 7 days after amputation at the proximal (F) and distal (G) level. Tail regeneration in the dnYap and dnTead4 Tg tadpoles was impaired and shortened after both proximal and distal amputation. Red line indicates the amputation plane. Scale bar (B–G) = 1 mm. (H) Scatter graph of the regenerated tail (the average length). The difference between the proximal and distal regenerated tail length was severely decreased in the dnYap and dnTead4 Tg tadpoles. Horizontal bars indicate average length of regenerated tail. Single and double asterisks indicate statistically significant differences at p < 0.05 and p < 0.01 (Welch’s t-test) between the distal and proximal growth, respectively. Minus mark indicates no significant difference (Welch’s t-test). Error bar indicates s.e.m.
Tead4 is involved in the position-dependent growth rate control in tail regeneration

The size of a regenerating organ must be strictly controlled and adjusted to ensure that the apparatus is properly functional and motile after regeneration. In the newt, the tail regenerates the correct number of tail segments after amputation anywhere along its length (Iten and Bryant, 1976). Similarly, in zebrafish, regardless of the depth of the amputation plane, a regenerating fin reaches the same size as the original one (Lee et al., 2005). Notably, the growth rate of the regenerating fin after proximal amputation is faster than that after distal amputation, so both situations take the same time to reach a final length that matches the original one (Lee et al., 2005). Transcriptional regulators in the Hippo signaling pathway such as Yap1 are known to control the size of visceral organs such as the liver and heart (Camargo et al., 2007; Dong et al., 2007; Heallen et al., 2011; von Gise et al., 2012). Furthermore, our single-cell gene manipulation by IR-LEGO (Fig. 4) showed that the transcriptional regulators control the regenerating organ size by directly regulating the cell proliferation and apoptosis within a tissue.

To investigate the role of the transcription regulators in controlling the size of the regenerating tadpole tail, we next examined whether Yap1 or Tead4 is required for the position-dependent size control. As mentioned above, the growth of the tadpole body was reduced by inducing dnYap or dnTead4 expression at st41/42 (Fig. S4). To alleviate secondary influences of body growth reduction, we used st52 tadpoles for this analysis, because the effects of dnYap and dnTead4 Tg tadpoles on body length are minimal at this stage (Hayashi et al., 2014 and data not shown). At 3 h after heat shock, the tail was amputated at 50% of the distance from the tail tip to cloaca (proximal amputation) or at 25% of this distance (distal amputation) (Fig. S4A). To enhance the transgene expression in the tail regeneration processes, we added another heat shock at 3 dpa (Fig. 5A), as we previously reported for limb bud regeneration in st52 tadpoles (Hayashi et al., 2014). In WT controls at 7 dpa, the proximally amputated tadpoles had regenerated more tail length than the distally amputated ones, reflecting the position-dependent differential growth rate (Fig. 5B and C). On the other hand, the regenerated tails were significantly shorter in the dnYap and dnTead4 tadpoles at both amputation levels (Fig. 5D–G). The difference in growth rate of the regenerating tail after proximal versus distal amputation was decreased (flattened) by the inhibition of the transcriptional regulators, especially Tead4 (Fig. 5H. WT: 5.59 ± 0.22 mm for proximal amputation and 3.77 ± 0.17 mm for distal, dnYap: 2.35 ± 0.12 mm for proximal and 1.53 ± 0.09 mm for distal, dnTead4: 1.37 ± 0.10 mm for proximal and 1.25 ± 0.08 mm for distal, n = 27, 30 for WT proximal and distal, n = 15, 13 for dnYap proximal and distal, n = 10, 11 for dnTead4 proximal and distal, respectively). These results suggest that the transcriptional regulators in Hippo signaling are involved in the position-dependent growth control in tail regeneration.

To clarify the unique function of the Hippo signaling transcriptional regulators, we performed the same tail amputations while inhibiting other major signals, Wnt/β-catenin or Fgf, at the same time points (Fig. S6A); these signals are both known to be required for Xenopus tadpole tail regeneration (Lin and Slack, 2008). To inhibit Wnt/β-catenin signaling, we used stable F2 Tg lines carrying heat shock-inducible Dkk1, an Wnt/β-catenin antagonist, tagged with GFP (Yokoyama et al., 2011; Yokoyama et al., 2007). We heat-shocked the Dkk1 Tg tadpoles at st52, amputated the tails at the proximal or distal level, and heat-shocked them again at 3 dpa. In the case of Wnt/β-catenin inhibition by Dkk1 induction, tail regeneration was almost completely blocked at both amputation levels (Fig. S6B–F). To inhibit Fgf signaling, we injected SU5402, a selective inhibitor of this signaling (Mohammadi et al., 1997), into tadpoles and amputated the tails at the proximal or distal level with the same experimental schedule as that for the Dkk1 Tg tadpoles (Fig. S6A). In the case of Fgf inhibition by SU5402, tail regeneration was reduced at both amputation levels, but a position-dependent differential growth rate was still observed (Fig. S6G–K). These results by inhibition of Wnt and Fgf signaling were qualitatively different from the results by inhibiting the transcriptional regulators in Hippo signaling, suggesting that the Hippo pathway has a unique role in tail regeneration. Hippo signaling might be involved in position-dependent growth control rather than initiation of tail regeneration.

Discussion

Transcriptional regulators in the Hippo signaling pathway control morphological growth in tail regeneration

Organ morphology is strictly regulated to achieve an organ-specific shape that is tightly correlated with its function. Signaling through the Hippo pathway regulates organ size and shape and tissue homeostasis through the control of cell proliferation, apoptosis, and differentiation (Halder and Johnson, 2011). Much evidence indicates that the Hippo pathway regulates visceral organ size in mammals. The size of the mouse liver is severely increased by the overexpression of constitutively active Yap1, and then it reverts to normal size, regulated by endogenous Hippo signaling, after the Yap1 overexpression is ceased (Camargo et al., 2007; Dong et al., 2007). Heart growth is also under Yap1 and Tead-mediated control through regulation of the cardiomyocyte cell cycle in mouse (Heallen et al., 2011; von Gise et al., 2012). These roles of Hippo function in organ size control are consistent with our results in appendage regeneration. In the case of appendage regeneration (epimorphosis), we previously reported that Yap1 is required for the limb bud regeneration of Xenopus tadpoles (Hayashi et al., 2014). The intercalary regeneration of amphibian and insect appendages involves rigorous size control, because a minimum sequence of positional values between the host and graft needs to be restored (French et al., 1976). We previously showed that dnYap induction significantly reduces the length of the intercalary regenerated limb region after limb bud grafting in Xenopus tadpoles (Hayashi et al., 2014). These results suggest that Yap1 is required for proper size control in epimorphosis. The results of our present study focusing on another appendage regeneration system, Xenopus tadpole tail, support the idea that the transcriptional regulators in Hippo signaling, Yap1 and Tead4, are required not only for appendage regeneration itself but also for proper size control during this process (Figs. 2–5). Together with the fact that Yorkie, an insect homolog of Yap, is required for intercalary regeneration in the cricket (Bando et al., 2009), the Hippo pathway appears to be generally associated with size control in appendage regeneration in bilaterians as well as in the growth of multiple organs in various species.

Morphogenesis is tightly regulated during embryonic development, so that organs are formed with the proper size and shape in a species-specific manner. This process is assured by a robustness against noise from the external environment or accidents. Since Hippo signaling regulates cellular processes in response to cell contact, positional information, and cell damage (Halder and Johnson, 2011), it is thought to be responsible for the robustness of morphogenesis. For example, the strength of Yap1 activity is regulated by the density of neighboring cells. By this mechanism, the mitosis rate is regulated to reflect the surrounding cell condition. Changes in the composition of the ECM, a scaffold substrate for cell adherence, also influence the regulation of cell growth mediated by Hippo signaling (Dupont et al., 2011; Wada et al., 2011; Zhao et al., 2012). During amphibian limb regeneration, matrix metalloproteinases are upregulated (Miyazaki et al., 1996; Satoh et al., 2011; Vinarsky et al., 2005; Yang and Bryant, 1994) and these enzymes may alter the ECM composition to affect the Hippo signaling activity. Thus, by sensing structural and chemical changes at the cell surface, Hippo signaling is thought...
to play an essential role in converting contact information into proliferation rate.

Previous studies indicated that Yap1 promotes the proliferation of progenitor cell populations not only in a cell-autonomous manner but also non-cell autonomously, through the transcriptional activation of secretory growth factors such as the matricellular protein Connective tissue growth factor (Zhao et al., 2008) and an epidermal growth factor receptor (EGFR) ligand, Amphiregulin (Dong et al., 2011; Zhang et al., 2009), both of which contribute to Yap-mediated cell proliferation. In this study, we found that Tead4 maintained the neural progenitor population in the regenerating tail in a cell-autonomous manner, at least in part (Fig. 4). Sox2-positive neural progenitor cells are reported to be proliferative and to contribute to spinal cord regeneration in the Xenopus tadpole tail (Gaete et al., 2012). The neural progenitor cell number is regulated by Yap1 via Tead in the neural tube of chick embryos (Cao et al., 2008). We found that the single-cell induction of dnTead4 expression markedly reduced the number of vital descendant neural progenitors in the spinal cord (Fig. 4). However, it is still possible that the Yap1/Tead4 complex promotes cell proliferation in both cell autonomous and non-cell autonomous manners in the appendage regeneration of Xenopus tadpoles, as in other vertebrates. Although inhibition of Yap1 and Tead4 showed similar effects on neural progenitor survival and position-dependent growth rate, the effect of dnTead4 was more severe than that of dnYap (Figs. 4 and 5). Ectopic apoptosis in tadpole growth was also strongly induced by dnTead4 Tg (Fig. 5S). These results suggest that Tead4 may sometimes regulate organ morphogenesis independently of Yap1, as seen in other organisms (Pobbaty and Hong, 2013).

With regard to muscle, satellite cells participate in the regeneration of muscle fibers in the Xenopus tadpole tail (Gargioli and Slack, 2004), while axolotl tail regeneration shows muscle fiber dedifferentiation (Echeverri et al., 2001). Several reports have indicated that Yap1 and Tead family genes regulate myogenic differentiation in addition to the proliferation of progenitor cells (Judson et al., 2012; Watt et al., 2010; Zhao et al., 2006). Our finding that muscle regeneration was impaired and/or delayed by the dnYap or dnTead4 induction (Fig. 3B–D) suggests that Yap1 and Tead4 also contribute to the myogenic cell differentiation in Xenopus tail regeneration.

Yap1 and Tead1 are distributed in the notochord in developing mouse embryos (Ota and Sasaki, 2008). Although Yap1 protein was also distributed in the notochord in the regenerating tail of Xenopus tadpoles, it appeared to be highly phosphorylated (Fig. 15–U). Yap1 functions are not only transcriptional regulation in the nuclei but also multiple functions in the cytoplasm (Mauviel et al., 2012). It is possible that Hippo signaling controls notochord morphology through the cytoplasmic functions in the tail regeneration of Xenopus tadpoles. Although evidence to date suggests that Yap1 and Tead are involved in muscle and notochord regeneration in the Xenopus tail, further studies are needed to unravel the complicated signaling events involving the Hippo pathway in appendage regeneration. In Xenopus tadpole tail regeneration, the Hippo pathway may regulate integrated processes involving multiple cell types and tissues, including neural progenitors, myogenic lineage cells, and notochord, by controlling undifferentiated and differentiated cell states.

Mechanism for converting positional information into morphological growth mediated by transcriptional regulators in the Hippo signaling pathway

Positional information along the proximo-distal axis is tightly related to reconstitute an appendage of the exact size and shape as the original one. In zebrafish fin regeneration, Fgf signaling conveys positional information to the injured tissue (Lee et al., 2005). Compared with distal amputation, proximal amputation results in stronger Fgf signaling, which induces faster growth of the regenerating fin. It is assumed that positional information is linked to organ size control in regeneration, since the size of the regenerating organ (appendage) is adjusted against various depths of the amputation plane, based on positional information. Our results indicated that Yap1 or Tead4 inhibition impaired the position-dependent differential growth rate (Fig. 5), suggesting that the Hippo pathway is tightly related to the positional information in tail regeneration. These regulations of tail regeneration by Hippo signaling were qualitatively different from other important signaling for tail regeneration such as Wnt and Fgf signaling (Fig. S6) (Lin and Slack, 2008; Stockick-Cooper et al., 2007b).

Several intriguing models have been proposed for the link between organ size control and positional information (Halder and Johnson, 2011; Mammoto et al., 2012; Nishioka et al., 2009). Findings in invertebrates may provide hints for unraveling the signaling crosstalk that integrates positional information and size control. For example, from the viewpoint of functional conservation, the Dachsous/Fat steepness model for the process upstream of the Hippo pathway in cricket (Bando et al., 2009) may be useful for elucidating the regulatory mechanism for positional information in amphibian tail regeneration.

The regeneration responses to tail amputation are not restricted to the amputation plane region, but are also found in distant tissues. Laser or surgical ablation of the spinal cord at a distant region from the amputation plane can disrupt tail regeneration in Xenopus tadpoles (Monda et al., 2011; Tamiguchi et al., 2008), although in axolotl tail regeneration, only cells close to the amputation plane participate in the regenerating spinal cord (McHedlishvili et al., 2007). The transcription factor sox2 is upregulated in tissues distant from the amputation plane, such as the anterior-most spinal cord, neuromasts of the lateral line, and olfactory epithelium (Gaete et al., 2012). These findings indicate that localized organ regeneration, in some cases at least, causes systemic responses. Since Yap1 and Tead family genes regulate Sox2-positive progenitor cells in the developing neural tube (Cao et al., 2008), the transcriptional regulators in the Hippo pathway may play a role in the systemic rearrangement of gene expression occurring during morphogenesis. Such systemic responses may help orchestrate the morphological growth of multiple tissues and organs, to produce integrated body parts in an individual animal, in both regeneration and development.

Conclusion

Regenerative organisms such as amphibians and hemimetabolous insects can adjust the size of their regenerating tissues in the context of the amputation site to form an appendage the same size and shape as the original, even if the amputation level and wound size vary. Accumulating evidence indicates that the Hippo signaling pathway is important for organ size control. We investigated the role of this pathway in Xenopus tail regeneration by inhibiting Yap1 and Tead4, the downstream transcriptional regulators in Hippo signaling. Our findings showed that Yap1 and Tead4 are required for proper regeneration of the tadpole tail. Furthermore, we found that these transcriptional regulators, especially Tead4 maintains the cell survival of neural progenitors in the regenerating spinal cord and direct the position-dependent growth rate of the regenerating tail. Thus, our data suggest that the Hippo pathway, mediated by the Yap1/Tead4 complex, has important roles in the size control of organogenesis during tail regeneration. Our study provides novel insight into the link between positional information and morphological growth in organ regeneration, in which Yap1 and Tead4 play an essential role.
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Appendix A

Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.09.018.

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