

# Mammalian Tead proteins regulate cell proliferation and contact inhibition as transcriptional mediators of Hippo signaling

Mitsunori Ota and Hiroshi Sasaki\*

Regulation of organ size is important for development and tissue homeostasis. In *Drosophila*, Hippo signaling controls organ size by regulating the activity of a TEAD transcription factor, Scalloped, through modulation of its co-activator protein Yki. Here, we show that mouse Tead proteins regulate cell proliferation by mediating Hippo signaling. In NIH3T3 cells, cell density and Hippo signaling regulated the activity of endogenous Tead proteins by modulating nuclear localization of a Yki homolog, Yap1, and the resulting change in Tead activity altered cell proliferation. Tead2-VP16 mimicked Yap1 overexpression, including increased cell proliferation, reduced cell death, promotion of EMT, lack of cell contact inhibition and promotion of tumor formation. Growth-promoting activities of various Yap1 mutants correlated with their Tead-co-activator activities. Tead2-VP16 and Yap1 regulated largely overlapping sets of genes. However, only a few of the Tead/Yap1-regulated genes in NIH3T3 cells were affected in *Tead1*<sup>−/−</sup>; *Tead2*<sup>−/−</sup> or *Yap1*<sup>−/−</sup> embryos. Most of the previously identified Yap1-regulated genes were not affected in NIH3T3 cells or mutant mice. In embryos, levels of nuclear Yap1 and Tead1 varied depending on cell type. Strong nuclear accumulation of Yap1 and Tead1 were seen in myocardium, correlating with requirements of *Tead1* for proliferation. However, their distribution did not always correlate with proliferation. Taken together, mammalian Tead proteins regulate cell proliferation and contact inhibition as a transcriptional mediator of Hippo signaling, but the mechanisms by which Tead/Yap1 regulate cell proliferation differ depending on the cell type, and Tead, Yap1 and Hippo signaling may play multiple roles in mouse embryos.

**KEY WORDS:** Hippo signaling, TEF, Tead, Yap1, Cell proliferation, Contact inhibition

## INTRODUCTION

In multicellular organisms, organ sizes are strictly controlled during development to ensure proportional growth of organs. Regulation of organ size is also important for tissue homeostasis of adults, and its disruption may lead to cancer. Genetic screening in *Drosophila* has identified the Hippo signaling pathway as a key mechanism of organ size control (for reviews, see Harvey and Tapon, 2007; Pan, 2007; Reddy and Irvine, 2008; Saucedo and Edgar, 2007). The core components of this pathway are an Ste20-like kinase Hippo (Hpo) (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003) and its regulatory protein Salvador (Tapon et al., 2002), NDR family kinase Warts (Wts) (Justice et al., 1995; Xu et al., 1995) and a Mob1-related regulatory protein Mats (Lai et al., 2005). Wts phosphorylates a transcriptional co-activator Yorkie (Yki) and suppresses its nuclear accumulation (Dong et al., 2007; Zhao et al., 2007). Yki promotes organ growth by stimulating cell proliferation and inhibiting apoptosis; this is achieved by activating Cyclin E, the apoptosis inhibitor Diap1 and the *bantam* microRNA (Huang et al., 2005; Nolo et al., 2006; Thompson and Cohen, 2006). Recent studies have revealed that the TEAD/TEF family transcription factor Scalloped (Sd) interacts with Yki and mediates Hippo signaling (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008). Upstream of the Hpo kinase cascade, the FERM domain-containing family of proteins, Merlin (Mer) and Expanded, and atypical cadherin Fat are involved (Bennett and Harvey, 2006; Cho et al., 2006; Hamaratoglu et al., 2006; Silva et al., 2006; Willecke et

al., 2006), suggesting that Hippo signaling is linked to extracellular spaces through these proteins, although the underlying mechanisms are not known.

The Hippo signaling pathway appears to be conserved in mammals. Mammals have multiple Hippo pathway-component counterparts, and some of them rescued fly mutants and/or showed similar activities in flies (Lai et al., 2005; Tao et al., 1999; Wu et al., 2003). The human ortholog of Mer is encoded by a tumor suppressor gene, neurofibromatosis 2 (*NF2*), mutations of which lead to neurofibromatosis (McClatchey and Giovannini, 2005). Mice that are mutant for a *Wts* homolog, *Lats1*, develop soft-tissue sarcomas and ovarian tumors (St John et al., 1999). The Yki homolog yes-associated protein 1 (*Yap1*) is involved in cancer. A genomic region containing *Yap1* and *clAP2/Birc3* is amplified in mouse models of liver cancer and human cancers, and these genes contribute to tumorigenesis (Overholtzer et al., 2006; Zender et al., 2006). Yap1 overexpression in liver reversibly increases liver size and prolonged overexpression causes liver tumor (Camargo et al., 2007; Dong et al., 2007). In cultured cells, Yap1 alters subcellular localization depending on cell density and Hippo signaling, and mediates the cell contact inhibition of proliferation (Zhao et al., 2007). A Yap1-related protein Wwtr1/TAZ also has similar functions (Lei et al., 2008). Therefore, the framework of the Hippo signaling cascade appears to be conserved in mammals to regulate cell proliferation and to control organ size.

In *Drosophila*, the Hippo signal converges with the activity of TEAD/TEF family transcription factor Sd through interaction with Yki (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008). In mammals, Tead proteins also interact with Yap1 (Vassilev et al., 2001). Mice have four Tead genes (*Tead1–Tead4*), and they are expressed widely during development. Tead proteins regulate development of various tissues, including heart, skeletal muscles, neural crest, notochord and trophoblast (Chen et al., 1994; Maeda

Laboratory for Embryonic Induction, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-Minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan.

\*Author for correspondence (e-mail: sasaki@cdb.riken.jp)

et al., 2002; Milewski et al., 2004; Nishioka et al., 2008; Sawada et al., 2008; Sawada et al., 2005; Yagi et al., 2007). Our recent study on *Tead1*/*Tead2* double-mutant embryos revealed the genetic interactions between *Tead1/2* and *Yap1* during embryogenesis, and their necessity in cell proliferation and apoptosis (Sawada et al., 2008). This observation supports the hypothesis of *Tead1/2* involvement in the regulation of cell proliferation and Hippo signaling.

In this study, we first examined the role of *Tead* proteins in Hippo signaling using cell culture systems. Although Zhao et al. (Zhao et al., 2008) recently reported involvement of *Tead* in *Yap1*-dependent gene expression in cultured cells, we took complementary approaches and further extended our analyses to mouse embryos. Cell density and Hippo signaling regulates nuclear *Yap1* and endogenous *Tead* activity. Modulation of *Tead* activity altered cell proliferation and cell death. The diverse effects of *Yap1* overexpression were mimicked by *Tead2*-VP16, and *Tead* and *Yap1* regulated common sets of genes in NIH3T3 cells. Thus, *Tead* is a key mediator of Hippo signaling in mouse. However, the *Tead*/*Yap1*-regulated genes varied between experimental systems, suggesting the complexity of Hippo signaling. Protein distribution in embryos suggests that *Tead*, *Yap1* and Hippo signaling may regulate both proliferation and differentiation of cells.

## MATERIALS AND METHODS

### Cell lines

NIH3T3, MTD1A (Hirano et al., 1987), pam212 (Yuspa et al., 1980) and HEK293T cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal calf serum (DMEM + 10% FCS). PLAT-E cells were maintained as described previously (Morita et al., 2000).

### Mouse mutants

*Tead1* and *Tead2* mutant mice have been described previously (Sawada et al., 2008). *Yap1<sup>tm1Smil</sup>* mice (Morin-Kensicki et al., 2006) were crossed with *Actb:Cre* transgenic mice to remove the neomycin cassette flanked by *loxP* sites. The resulting mice (*Yap1<sup>Δmi</sup>*) are referred to as *Yap1* mutant mice in this paper. Mice were housed in environmentally controlled rooms in the Laboratory Animal Housing Facility of the RIKEN Center for Developmental Biology (CDB), under the institutional guidelines for animal and recombinant DNA experiments.

### Antibody staining

Rabbit anti-*Yap1* antibody (No. 1) was raised by T. K. Craft (Gunma, Japan) and affinity purified by Qiagen using the following peptide as the antigen: CKLDKESFLTWL. Cells were cultured in LAB-TEK II chamber slides (Nunc) coated with 0.1% gelatin. Cryosections (15 μm) of embryos were prepared. Immunofluorescent staining was performed according to standard procedures. Briefly, slides were incubated with rabbit anti-*Yap1* antibody (1:300) or rabbit anti-*Tead1* antibody (1:430) (Nishioka et al., 2008) at 4°C for 16 hours, followed by detection with anti-rabbit IgG-Alexa 594 (1:2000) (Invitrogen). Nuclei were counterstained with 1 μg/ml of 4',6'-diamidino-2-phenylindole (DAPI).

### Quantification of fluorescent signals

Average intensities of the *Yap1*, *Tead1* and DAPI signals in the nucleus area were measured with MetaMorph software (Molecular Devices). The *Yap1*/*Tead1* signals were normalized to the DAPI signal.

### Luciferase assay

8×GT-IIC-Luc was constructed by cloning eight copies of the following oligonucleotides straddling the GT-IIC motif of SV40 enhancer (Davidson et al., 1988) into p851-LucII (Kamachi and Kondoh, 1993): 5'-CCAGCTGTGGAATGTGTGTC-3' and 3'-ggGGTCGACACCTTACACACA-5' (underlines indicate GT-IIC, the additional c or g was added to facilitate directional cloning). NIH3T3 cells were plated into 12-well plates at a density of  $0.25 \times 10^5$  cells/well 24 hours before transfection. A DNA mixture consisting of effector (0.1 μg), reporter (0.1 μg), reference (pCS2-β-gal, 0.1 μg) and pBluescript (Stratagene) (0.5 μg) were transfected for

24 hours using 2 μl of lipofectamine2000 (Invitrogen). Preparation of lysates, luciferase and β-galactosidase assays were as described (Sasaki et al., 1999). Luciferase activities were normalized to β-galactosidase activities. Results were shown by averaging two samples with standard errors.

### Transfection assay

pcDNA-HA-Lats2 was constructed by cloning the coding sequence of mouse Lats2 cDNA (GenBank: BC053028) into pcDNA3-HA (a gift from Dr A. Shimono). Cells were seeded into LAB-TEK II chamber slide (Nunc 154461) coated with 0.1% gelatin at a density of  $0.5 \times 10^5$  cells/well 24 hours before transfection. DNA mixture consisting of pCMV-EGFP (0.2 μg), pcDNA3-HA-Lats2 or pcDNA3.1-MST2 (a gift from Dr Georg Halder) (0.2 μg) and pBluescript (0.4 μg) were transfected for 24 hours with 2 μl of lipofectamine2000, and were stained with antibodies as described above.

### Bromodeoxyuridine (BrdU)-labeling

NIH3T3 cells were plated at the density of  $0.5 \times 10^5$  (low density) or  $2 \times 10^5$  (high density) cells/well in gelatin-coated LAB-TEK II chamber slides and were cultured for 48 hours. Cells were incubated with 10 μg/ml BrdU for 30 minutes, followed by fixation, and incubation with 2 M HCl for 30 minutes and 0.1 M sodium borate (pH 8.9) for 10 minutes. The pretreated cells were immunostained as described above using mouse monoclonal anti-BrdU antibody (1:500 dilution, Sigma) and anti-mouse IgG-Alexa488 as primary and secondary antibodies, respectively. For embryos, pregnant mice were injected intra-peritoneally with BrdU equivalent to 200 μg/g of body weight, 2 hours prior to dissection. Immunostaining was performed on paraffin-embedded sections as described previously (Liu et al., 2000; Megason and McMahon, 2002).

### Preparation of *Tead*/*Yap1* virus infected cells

Retroviral vectors, pMYs-*Tead*/*Yap1*-IRES-EGFPs, were generated by cloning the coding sequences of full-length or modified *Tead1*, *Tead2*, *Tead4* or *Yap1* cDNAs into pMYs-IRES-EGFP (Kitamura et al., 2003). Identities of the PCR-amplified cDNAs were verified with DNA sequencing. *Tead*/*Yap1*-viruses were produced by transfecting pMYs-*Tead*/*Yap1*-IRES-EGFP plasmids into PLAT-E packaging cells as described (Morita et al., 2000). Forty-eight hours after infection of *Tead*/*Yap1*-viruses into NIH3T3 cells, EGFP-positive cells were selected with FACSaria cell sorter (BD Biosciences). For growth curve analysis, EGFP-positive cells ( $0.5 \times 10^5$ ) were seeded into 35 mm dishes coated with gelatin, and the total cell numbers in each dish were counted. Results are shown as the average of two samples with standard errors.

### Leishman stain

Virus-infected NIH3T3 cells were plated in 35 mm dishes as growth curve analysis. On the 18th day, plates were stained with 0.2% Leishman's stain (Sigma).

### Three-dimensional (3D) culture

pMYs-IRES-puro was constructed by cloning the IRES-puro fragment into pMYs vector (Kitamura et al., 2003). pMYs-*Tead*/*Yap1*-IRES-puros were generated by cloning full-length or modified *Tead2*/*Yap1* cDNA. *Tead*/*Yap1*-viruses were prepared and infected into MTD1A cells as above, and were selected with 2 μg/ml puromycin for 7 days from 48 hours post-infection. The 3D culture was performed as described (Debnath et al., 2003), with a change to the culture medium to DMEM supplemented with 5% FCS and 10 ng/ml EGF (Peprotech).

### Immunoprecipitation assay

The immunoprecipitation assay was performed as previously described (Yamamoto et al., 2008). To prepare the lysates, the DNA mixture consisting of 0.45 μg of pcDNA-HA-*Yap1*-polyA83 or pcDNA-HA-*Yap1*-Δ*TeadBD*-polyA83 and 0.45 μg of pCMV-Flag-*Tead1* or pCMV-Flag-*Tead2* were transfected into HEK293T cells with FuGENE HD (Roche).

### Tumorigenesis assay

BALB/cAJc1 nude mice (8-week-old male) were obtained from CLEA Japan (Japan). Virus-infected NIH3T3 cells ( $100 \mu\text{l}$ ,  $1 \times 10^6$ ) were injected subcutaneously through 23-gauge needles into the dorsal flank area.

### Microarray analysis

Proliferating Yap1/Tead2-VP16-expressing cells were harvested at a density that slightly exceeds the confluency of normal cells. For low- and high-density cultures, control virus-infected cells were harvested at 30% or complete confluency. Tead2-EnR-expressing cells were harvested at 30% confluency of these cells. RNA was extracted with RNeasy kit (Qiagen) followed by further purification. Biotinylated cRNAs were prepared according to the Affymetrix standard labeling protocol, followed by fragmentation and hybridization to the Affymetrix GeneChip Mouse Genome 430 2.0 Array. Chips were washed and stained with Streptavidin R-phycoerythrin (Invitrogen). After scanning the chips, expression values of probe sets were summarized with the RMA algorithm (Irizarry et al., 2003). Differently expressing probe sets were identified with the eBayes method ( $FDR < 0.1$ ) (Smyth, 2004). The results of two independent experiments were used for analysis. The microarray analysis was carried out at the Functional Genomics Unit of RIKEN CDB. Genes that show significant differences ( $P < 0.0001$ ) were used for analysis. Yap1/Tead2-VP16 cells were compared with high-density cells. Tead2-EnR cells were compared with low-density cells. All of the microarray data have been submitted to the Gene Expression Omnibus (Accession Number GSE12498).

### Quantitative RT-PCR (qRT-PCR)

Total RNAs (1  $\mu$ g, cells; 0.5  $\mu$ g, embryos) isolated from virus-infected NIH3T3 cells, E8.0 *Yap1 $\Delta$ mi1/ $\Delta$ mi1* and *Tead1 $^{-/-}$* ; *Tead2 $^{-/-}$*  embryos were used for cDNA synthesis using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare) and random hexamer (Invitrogen) following the manufacturer's instructions. The resultant cDNA was diluted at 1:100 (for cells) or 1:10 (for embryos) for quantitative PCR. Primers used were as follows: *Acta2*, 5'-AGGGCTGTTTCCCATCCATCG-3' and 5'-TCTCTTGCTCTGGGCTTCATCC-3'; *Ctgf*, 5'-CAAGGACGCCACAGCAGTT-3' and 5'-AGAACAGGCGCTCCACTCTG-3'; *Etv5*, 5'-TAGCGGAGACTTTGGAAGCACC-3' and 5'-AATCAAAGGTCGCCCTCGACAG-3'; *Gapdh*, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'; *Hmga2*, 5'-CAGCAAAAACAAGAGCCCC-3' and 5'-AGCAGGCTTCTCTGAACG-3'; *Il1r1l*, 5'-TGGGCTTTGGCAATTCTGACAC-3' and 5'-TAAGTCGAGCGTCTCTTTGGG-3'; *Klhd8a*, 5'-TCCAAGATCTATGTGCTGGGGG-3' and 5'-GGAATGTTGGGGAACCTGGTCC-3'; *Serpine1*, 5'-GCGGCAGATCCAAGATGCTATG-3' and 5'-TCTCATTCTTGTTCACGGCCC-3'; *Tagln*, 5'-TGGATTGTAGTGACAGTGGC-3' and 5'-TTCGATCCCTCAGGATACAGGC-3'; *Tnfrsf1b*, 5'-CGCCTGCACTAAACAGCAGAAC-3' and 5'-TTGCTCAGCCTCATGCACTGTC-3'; *Vcl*, 5'-TCCTATCCACAGTGAAGGCCAC-3' and 5'-CACAGACTGCATGAGGTTCTGG-3'. For qPCR, 1.6  $\mu$ l of diluted cDNAs were amplified using the SYBR Premix Ex Taq (Takara Bio, Japan) in a total volume of 20  $\mu$ l on a ABI PRISM 7900HT (Applied Biosystems). The PCR conditions were 95°C for 10 seconds, and 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. qPCR was carried out in duplicate, using *Gapdh* as a housekeeping control.

### Cell death assay

Virus-infected NIH3T3 cells ( $5 \times 10^5$ ) were cultured in six-well plates for 24 hours, followed by treatment with 500 nM Taxol (Sigma) for 24 hours. Both floating cells and attached cells were collected and combined, stained with propidium iodide following the procedure of Flow Cytometry Core Facility of University of Michigan (<http://www.med.umich.edu/flowcytometry/PDF%20files/HYPOpi.pdf>), and were analyzed on a FACSCantoII (BD Bioscience) to determine percentage of cells with sub-G1 DNA content.

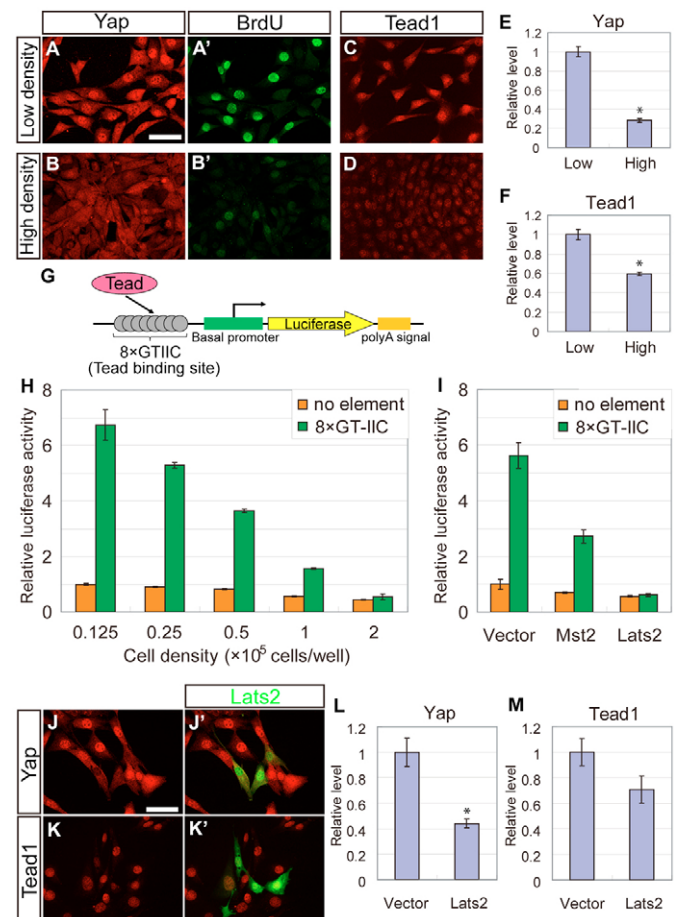
### Statistics

Statistical analyses, with the exception of microarray analysis, were performed with Prism5 statistical software (GraphPad) using an unpaired, two-tailed *t*-test or a one-way ANOVA followed by Tukey's multiple comparison test.

## RESULTS

### Cell density and Hippo signaling regulate Tead activity

To examine the role of Tead genes in Hippo signaling, we used a mouse embryonic fibroblast cell line, NIH3T3, which expressed all four Tead genes and *Yap1* (data not shown). Yap1 proteins showed dynamic changes in subcellular localization depending on cell densities, as previously reported (Zhao et al., 2007). At a low cell density, the cells proliferated actively and Yap1 showed strong nuclear localization (Fig. 1A,A'). At a high cell density, cell proliferation was suppressed, and nuclear levels of Yap1 were reduced (Fig. 1B,B',E). Similar behavior of Yap1 was also observed in two mouse epithelial cell lines, pam212 and MTD1A,

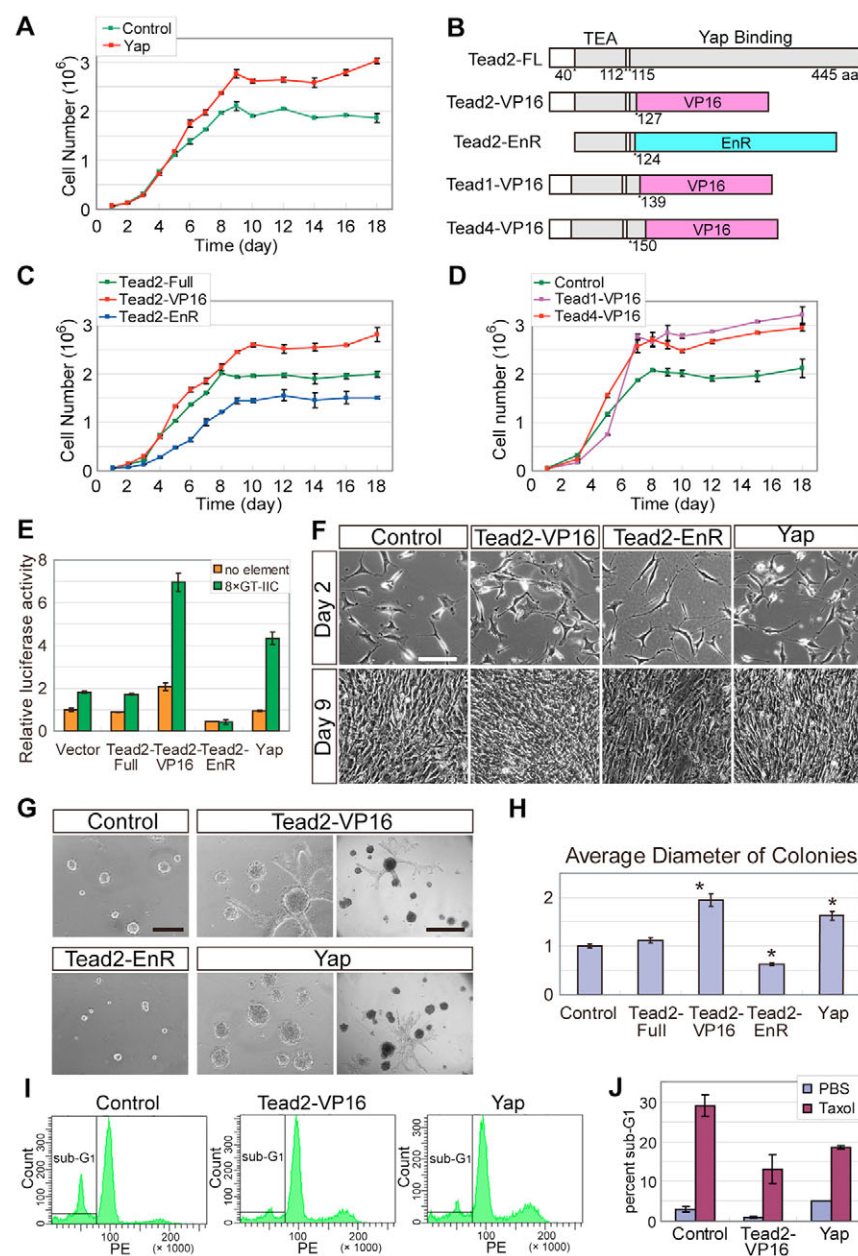


**Fig. 1. Regulation of Tead activity by cell density and Hippo signaling.** (A-F) Subcellular distribution of Yap1 and Tead1 proteins in NIH3T3 cells. Distribution of Yap1 (Yap; A,B) and Tead1 (C,D) proteins, and incorporation of BrdU (A',B') at low (A,C) or high (B,D) cell density. (E,F) Signal intensities of Yap1 (E,  $n=27$ ) and Tead1 (F,  $n=24$ ) proteins in the nuclei. (G) Schematic representation of the reporter plasmid monitoring the transcriptional activity of endogenous Tead proteins. (H,I) Regulation of Tead activity by cell density (H) or Hippo signaling components (I). (J-M) Reduction of nuclear Yap1 and Tead1 by Lats2. Distribution of Yap1 (J) and Tead1 (K) proteins. Lats2-overexpressing cells were labeled with EGFP expression (green) in J' and K'. Signal intensities of Yap1 (L,  $n=14$ ) and Tead1 (M,  $n=16$ ) proteins in the nuclei of Lats2-expressing cells. Empty vector-expressing cells were used as controls. The graphs show averages with standard errors. An asterisk indicates that the difference is statistically significant ( $P < 0.05$ ). Scale bars: in A, 50  $\mu$ m for A-D; in J, 50  $\mu$ m for J,K.



indicating the generality of this observation (see Fig. S1 in the supplementary material). Although Tead1 proteins constantly localized to the nuclei, the level of nuclear Tead1 was reduced at a high cell density (Fig. 1C,D,F). To monitor the transcriptional activity of endogenous Tead proteins, we exploited a Tead reporter (8×GT-IIC-Luc) containing an 8-mer of a Tead-binding motif, GT-IIC (Davidson et al., 1988) (Fig. 1G). As a similar GT-IIC-containing reporter (pGT<sub>4</sub>Luc) is activated by all four Tead proteins (Vassilev et al., 2001), the reporter should monitor the total activity of four Tead proteins. The Tead reporter showed high activity at low cell density, whereas the activity levels decreased with an increase in cell density (Fig. 1H). Activity of the reporter without the GT-IIC motif remained relatively unaffected. There is, therefore, a good correlation between nuclear localization of Yap1, cell proliferation, transcriptional activity of Tead proteins and cell density.

To correlate these observations with Hippo signaling, we examined the effects of known upstream components of Hippo signaling. Mst2 (Stk3 – Mouse Genome Informatics), an Hpo homolog, and Lats2, a Wts homolog, suppressed Tead activity, moderately and strongly, respectively (Fig. 1I). Lats2 also reduced nuclear levels of Yap1 and Tead1 (Fig. 1J,M). Lats2 phosphorylates Ser127 of human YAP1, promoting its interaction to a cytoplasmic scaffold protein 14-3-3, thereby reducing nuclear YAP1; changing this Ser into Ala (YAP1-S127A) increases nuclear Yap1 (Zhao et al., 2007). Based on the identity of surrounding sequences, we introduced a similar point mutation into mouse Yap1 (Yap1-S112A). Overexpression of Yap1-S112A increased nuclear Tead1 (see Fig. S2 in the supplementary material), suggesting that the amount of nuclear Yap1 has an effect on levels of nuclear Tead1. These results suggest that the Hippo signal negatively regulates Tead activity by inhibiting the nuclear localization of Yap1 and accumulation of Tead proteins.



**Fig. 2. Regulation of proliferation, EMT and cell death by Tead and Yap1.**

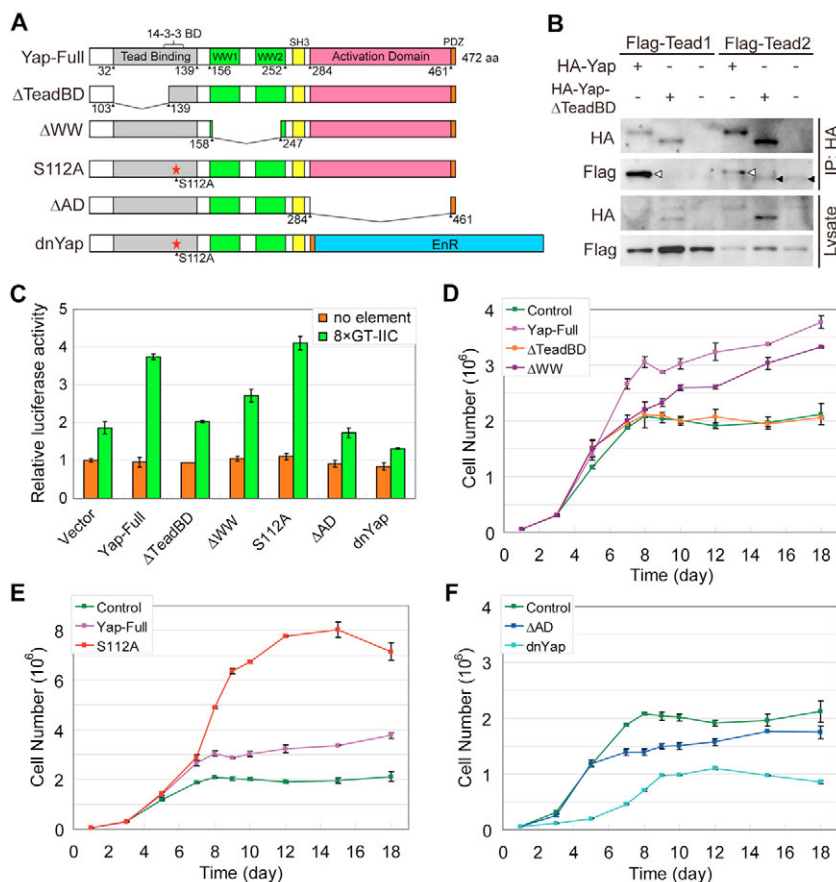
(A) Growth curve of Yap1-overexpressing NIH3T3 cells. Control in A,D,F-J indicates cells infected with the empty virus. (B) Schematic representation of structures of modified Tead proteins. (C) Growth curve of modified Tead2-expressing cells. (D) Growth curve of Tead1-VP16- and Tead4-VP16-expressing cells. (E) Effects of modified Tead2 and Yap1 on transcriptional activity of Tead. (F) Effects of altered Tead activities on cell morphology. (G,H) Effects of modified Tead2 and Yap1 on growth of MTD1A cells in matrigel. Morphology (G) and size (H) of colonies. The bar graph shows an average of 50 colonies with standard errors. Asterisks indicate that the differences from control were statistically significant ( $P < 0.05$ ). (I,J) Effects of Tead2-VP16 and Yap1 on Taxol-induced apoptosis. Distribution of DNA amounts (I) and ratio of sub-G1 cells (J). Scale bars: 200 μm in F,G (left); 500 μm in G (right).

## Increased Tead activity mimics the effects of Yap1 overexpression

If Tead is a downstream effector of Hippo signaling, altering its transcriptional activity would modulate cell proliferation. As *Tead1/2* double-mutants display growth defects (Sawada et al., 2008), we expressed modified forms of Tead2 in NIH3T3 cells and compared their effects with those of Yap1. We used a bi-cistronic retrovirus vector, which also expresses EGFP, and selected the *Tead*- or *Yap1*-overexpressing cells with a cell sorter by EGFP expression. To avoid clonal-selection effects, all experiments were performed with short-term culture of EGFP-selected and uncloned pools of cells. Overexpression of Yap1 increased Tead activity (Fig. 2E), and such cells continued to proliferate even after reaching the density of normal confluency and resulted in a higher saturation density than control virus-infected cells, as previously reported (Zhao et al., 2007) (Fig. 2A, day 9-14; Fig. 2F, day 9). Similarly, increasing Tead activity by expressing the activator-modified Tead2, a fusion protein of the N-terminal region of Tead2 containing the TEA domain and the activation domain of herpes simplex virus VP16 (Tead2-VP16) (Fig. 2B,E), promoted cell proliferation beyond normal confluency and resulted in a higher saturation density (Fig. 2C, day 9-14; Fig. 2F, day 9). As other Tead proteins (Tead1-VP16 and Tead4-VP16) also promoted cell proliferation (Fig. 2B,D), the growth-promoting activity of Tead2-VP16 may represent the general activity of Tead family proteins as a whole. By contrast, suppression of Tead activity by the repressor-modified Tead2, a fusion protein of the TEA domain of Tead2 and the repression domain of *Drosophila* Engrailed (Tead2-EnR), resulted in slower cell proliferation and reached a lower saturation density than that of control cells (Fig. 2B,C,E). Morphologically, Tead2-EnR-expressing cells tend to have longer

processes than control cells at day 2, and stopped proliferation at day 9, leaving open spaces between cells (Fig. 2F). Expression of full-length Tead2 had no effect on Tead activity, cell proliferation and saturation density (Fig. 2C,E). In summary, increased Tead activity mimicked effects of Yap1 overexpression on cell proliferation and saturation density, and reduced Tead activity displayed opposite effects. As Tead-modulated cells stopped proliferation at different cell densities, the cell contact inhibition system is likely to be operating in these cells with a change in sensitivity to the inhibition signal.

To examine whether Tead proteins also control cell proliferation rate, we used a three-dimensional culture system, in which cell contact inhibition is not involved. An epithelial cell line, MTD1A, forms acinar colonies when cultured in a reconstituted basement membrane (matrigel). Yap1- or Tead2-VP16-overexpressing MTD1A cells formed larger colonies (approximately double the diameter length, i.e. approximately eight times larger in volume), while Tead2-EnR-expressing cells formed smaller colonies (~60% of diameter, i.e. ~20% in volume) than control cells (Fig. 2G,H). The colony size of full-length Tead2-expressing cells was comparable with that of control cells (Fig. 2H). As colony sizes reflect proliferation rates, the results suggest that Tead and Yap1 also regulate cell proliferation rate. Yap1-overexpressing mammary epithelial cells, MCF10A, often form invasive colonies in matrigel culture, an indication of epithelial-to-mesenchymal transition (EMT) (Overholtzer et al., 2006). Some of the Yap1-overexpressing MTD1A cells and Tead2-VP16-overexpressing cells also formed similar invasive colonies, whereas such colonies were not observed with vector control or full-length Tead2-overexpressing cells (Fig. 2G; data not shown). Therefore, EMT-inducing activity of Yap1 was also mimicked by Tead2-VP16.



**Fig. 3. Correlation of Tead-co-activator activity and growth-promoting activity of mutant Yap1 proteins. (A)** Schematic representation of structures of modified Yap1 proteins. **(B)** Absence of interactions between Yap1-ΔTeadBD and Tead1/2.

White arrowheads indicate co-precipitated Tead1 and Tead2. Black arrowheads indicate non-specific bands. **(C)** Effects of modified Yap1 proteins on transcriptional activity of Tead proteins.

**(D-F)** Growth curves of modified Yap1-expressing NIH3T3 cells. Effects of ΔWW and ΔTeadBD (D), S112A (E), and ΔAD and dnYap1 (F). Control indicates cells infected with the empty virus.



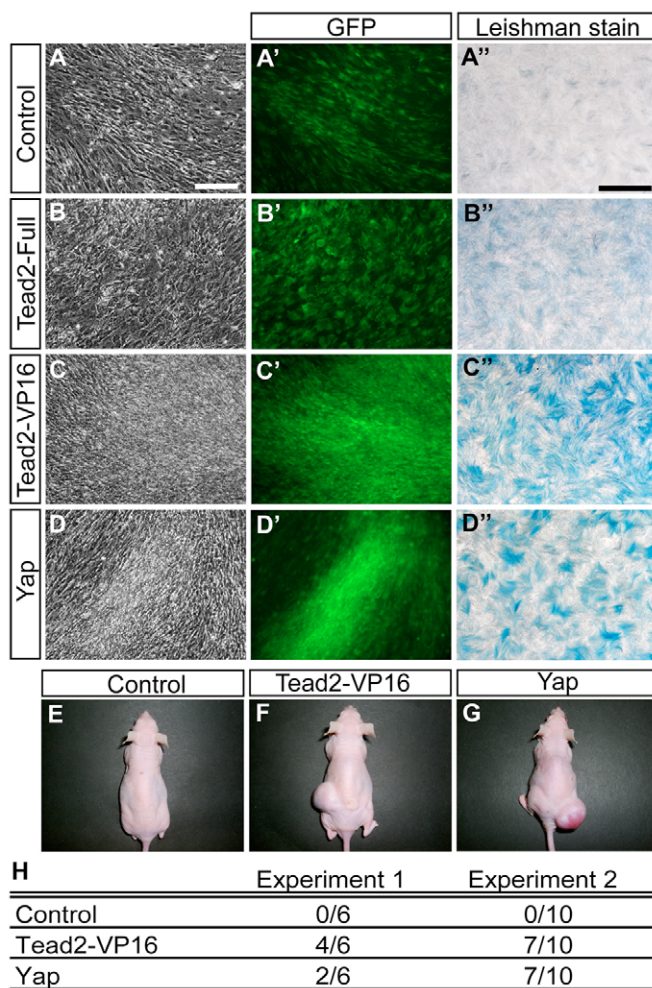
Although Yap1 promotes cell proliferation, it also suppresses apoptosis. Yap1-overexpressing NIH3T3 cells showed reduced apoptosis after treatment with a pro-apoptotic reagent, Taxol, as previously reported (Overholtzer et al., 2006); a similar anti-apoptotic effect was also observed with Tead2-VP16-expressing cells (Fig. 2I,J). In summary, Yap1 controls cell proliferation rate, contact inhibition, EMT and apoptosis, and all of these activities were mimicked when Tead activity was increased.

### Growth regulatory activity of Yap1 depends on its Tead co-activator activity

To further corroborate the relationship between Tead and Yap1, we next examined the effects of various mutant forms of Yap1 in NIH3T3 cells. A deletion in the Tead-binding domain of Yap1 (Yap1- $\Delta$ TeadBD) abolished interaction with Tead1/2 (Fig. 3A,B). Yap1- $\Delta$ TeadBD had no effect on Tead activity or cell proliferation (Fig. 3C,D). Yap1 interacts with other transcription factors through two WW domains. A Yap1 mutant lacking WW domains (Yap1- $\Delta$ WW) showed weaker co-activator activity for Tead proteins with unknown reasons, and also displayed weaker enhancement of cell proliferation (Fig. 3A,C,D). The Yap1-S112A mutant is supposedly insensitive to inhibition by Hippo signaling. At low cell density or under conditions where Hippo signaling is assumed to be weak, Yap1-S112A was not significantly different from Yap1 in its Tead co-activator activity and cell proliferation (Fig. 3A,C,E). However, once the cells reach 100% confluency or under conditions where Hippo signaling is assumed to be strong, Yap1-S112A-expressing cells continued to proliferate far beyond the confluence reached by Yap1-expressing cells (Fig. 3E), demonstrating the importance of S112 for regulation of Yap1 by Hippo signaling or cell-cell contact. Deletion of the activation domain of Yap1 (Yap1- $\Delta$ AD) or replacement of activation domain of Yap1-S112A with EnR (dnYap1) converted Yap1 into weak or strong co-repressors of Tead, respectively (Fig. 3A,C). Yap1- $\Delta$ AD slightly reduced saturation density, whereas dnYap1 strongly reduced cell proliferation rate and saturation density (Fig. 3F). Correlation of Tead co-activator activity of various mutant forms of Yap1 and their effects on cell proliferation further supports the hypothesis that Yap1 regulates cell proliferation by modulating the transcriptional activity of Tead proteins.

### Increased Tead activity transforms cells

Yap1 has oncogenic activities and its persistent overexpression in mouse liver results in tumorigenesis (Dong et al., 2007). Therefore, we next asked whether increased Tead activity is sufficient for cell transformation. When Yap1-overexpressing cells were cultured for an extended period, they stopped proliferating at one point when they reached saturation density, and then re-initiated cell proliferation (Fig. 2A, day 14-18). At this time, some cells form nodules, in which cells become piled up (compare Fig. 4A-A'' with Fig. 4D-D''), indicating anchorage-independent growth of these cells; this is an indication of transformation. Similar late-onset proliferation and nodule formation was also observed with Tead2-VP16-expressing cells, but not with control virus-infected or Tead2-expressing cells (Fig. 2A,C; Fig. 4B-B'',C-C''). Therefore, the nodule-forming activity of Yap1 was mimicked by increasing Tead activity. To further corroborate the oncogenic activities of Tead, we examined tumorigenic activities of Tead2-VP16 expressing NIH3T3 cells by subcutaneously transplanting them into nude mice. Control virus-infected cells formed no tumor, whereas Yap1-overexpressing and Tead2-VP16-expressing cells formed tumors (Fig. 4E-H and data not shown). Taken together, increased Tead activity also mimicked transforming activity of Yap1.

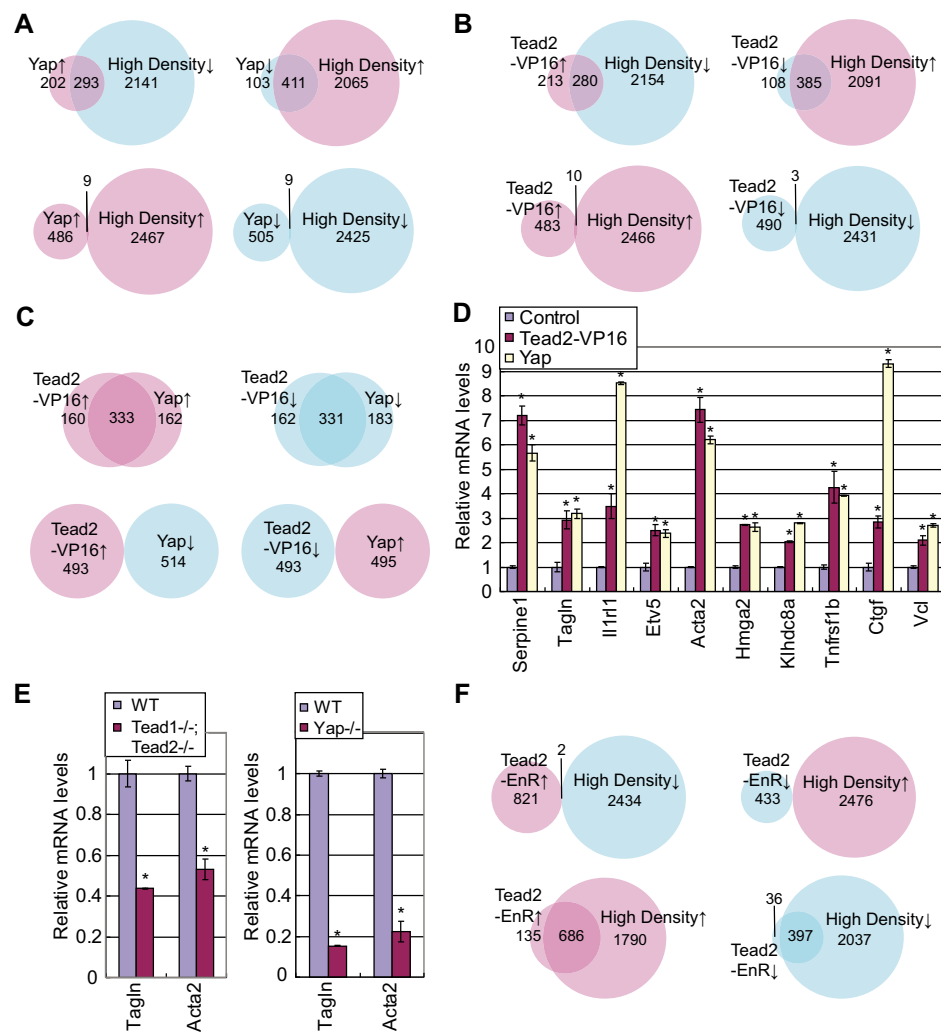


**Fig. 4. Transformation of NIH3T3 cells by Tead2-VP16 and Yap1.**

(A-D'') Effects on cell morphology in culture dish. NIH3T3 cells were infected with empty virus (A) or viruses expressing full-length Tead2 (B), Tead2-VP16 (C) or Yap1 (D). Left (A-D) and middle (A'-D') panels are the phase-contrast and the fluorescent images of the same area. Scale bar in A: 200  $\mu$ m for A-D and A'-D'. Right panels (A''-D'') are low magnification images of the cells stained with Leishman stain. Scale bar in A'': 5 mm for A''-D''. (E-H) Tumor formation assay. Representative mice transplanted with NIH3T3 cells infected with empty virus (E), Tead2-VP16 virus (F) or Yap1 virus (G). (H) Summary of two independent experiments. The tumor was counted on 100th day after subcutaneous injection of cells.  $n$ =(number of tumor formation)/(number of transplantation).

### Tead, Yap1 and cell density regulate common sets of target genes

To compare the genes that are regulated by Yap1, Tead2-VP16 and cell density, we examined gene expression profiles with microarrays. Consistent with previous observations (Zhao et al., 2007), the set of genes induced by Yap1 overlapped with the set of genes repressed by high cell density (Fig. 5A). Similarly, the majority of the genes repressed by Yap1 overlapped with the genes induced by high cell density (Fig. 5A). By contrast, the set of genes induced or repressed by Yap1 did not significantly overlap with the set of genes induced or repressed by high cell density, respectively (Fig. 5A). Similar results were also obtained with the genes regulated by Tead2-VP16 and cell density. Namely, the majority of



**Fig. 5. Comparison of genes regulated by Yap1, Tead2-VP16, Tead2-EnR and cell density.** (A) Yap1 and high cell density affect gene expression in an opposing manner. (B) Tead2-VP16 and high cell density affect gene expression in an opposing manner. (C) Yap1 and Tead2-VP16 affect gene expression in the same manner. (D) Quantitative RT-PCR confirmation of Yap1- and Tead2-VP16-regulated genes. Control indicates cells infected with the empty virus. (E) Quantitative RT-PCR showing the altered expression of the two Yap1- and Tead2-VP16-regulated genes in *Tead1* $^{-/-}$ ; *Tead2* $^{-/-}$  and *Yap1* $^{-/-}$  embryos. Asterisks in D and E indicate that the differences from the control are statistically significant ( $P < 0.05$ ). (F) Tead2-EnR and high cell density affect gene expression in the same manner.

the genes induced or repressed by Tead2-VP16 overlapped with the genes repressed or induced by high cell density, respectively (Fig. 5B). No significant overlap was observed between the set of genes induced or repressed by Tead2-VP16 and the set of genes induced or repressed by high cell density, respectively (Fig. 5B). Furthermore, the majority of the set of genes induced or repressed by Tead2-VP16 was also induced or repressed by Yap1, respectively (Fig. 5C). There is no overlap between the set of genes induced or repressed by Tead2-VP16 and repressed or induced by Yap1, respectively (Fig. 5C). These results suggest that Tead2-VP16 mimics the effects of Yap1 overexpression at the transcriptional level.

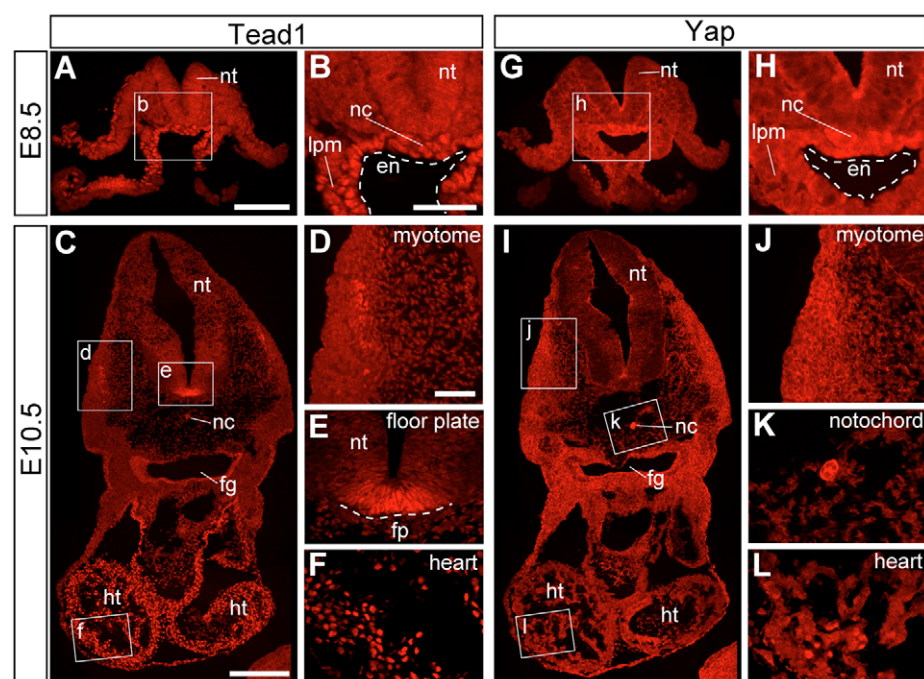
### Tead and Yap1 regulate different target genes depending on the cell types

To examine to what extent the results of overexpression of Tead2-VP16 or Yap1 in NIH3T3 cells represent the roles of Tead and Yap1 in normal development, we next examined the expression of Tead2-VP16/Yap1-regulated genes in *Tead1* $^{-/-}$ ; *Tead2* $^{-/-}$  (Sawada et al., 2008) and *Yap1* $^{-/-}$  (Morin-Kensicki et al., 2006) embryos at E8.0. qRT-PCR analysis of the ten representative genes commonly induced by Tead2-VP16 and Yap1 in NIH3T3 cells confirmed their induction in NIH3T3 cells (Fig. 5D). By contrast, among the seven genes expressed in E8.0 embryos, only two (*Tagln* and *Acta2*) showed a significant decrease in both mutants (Fig. 5E; see Fig.

S3A,B in the supplementary material). Although the levels of *Ctgf* were decreased in *Yap1* mutants, they were increased in *Tead1/2* mutants (see Fig. S3A,B in the supplementary material). Therefore, the genes regulated by Tead/Yap1 in NIH3T3 cells may be divergent from those in vivo. Consistent with this hypothesis, none of the eight genes previously identified as Yap1-regulated genes in mouse liver (Zender et al., 2006) were induced in NIH3T3 cells by Yap1 or Tead2-VP16 (see Fig. S3C in the supplementary material, and data not shown). Therefore, it is likely that expression of the Tead/Yap1-regulated genes varies depending on the cell type, and the results obtained from overexpression of Tead2-VP16 or Yap1 in NIH3T3 cells has revealed only some of the Tead/Yap1-regulated genes. In further support of this hypothesis, the four growth-related genes induced by Yap1 in mouse liver (*Myc*, *Birc2*, *Birc5* and *Mcl1*) (Dong et al., 2007) were not downregulated in *Tead1* $^{-/-}$ ; *Tead2* $^{-/-}$  or *Yap1* $^{-/-}$  embryos (see Fig. S3D,E in the supplementary material).

As the analysis with increased Tead activity represented only part of the roles of Tead/Yap1, we next asked whether decreasing Tead activity by expression of Tead2-EnR better represents these roles. Similar to the results of Tead2-VP16 and Yap1, the majority of the Tead2-EnR-regulated genes overlapped with the cell density-regulated genes; namely, the majority of the genes repressed or induced by Tead2-EnR overlapped with the genes repressed or induced by high cell density, respectively (Fig. 5F). Interestingly, however, only one-quarter of the Tead2-EnR-regulated genes were





**Fig. 6. Distribution of Tead1 and Yap1 proteins in developing mouse embryos.** (A-L) Distribution of Tead1 (A-F) and Yap1 (G-L) proteins in E8.5 (A,B,G,H) and E10.5 (C-F,I-L) embryos. (B,D-F,H,J,L) Enlarged images of boxed areas in A,C,G,I, respectively. Abbreviations: fg, foregut; ht, heart; lpm, lateral plate mesoderm; nc, notochord; nt, neural tube. Scale bars in A: 100  $\mu$ m for A,G; in C, 250  $\mu$ m for C,I; in B, 50  $\mu$ m for B,H; in D, 50  $\mu$ m for D-F,J,L.

also regulated by Tead2-VP16 or Yap1 (see Fig. S3F,G in the supplementary material), suggesting that, even in a single cell type, growth regulations imposed by increasing or decreasing Tead activities are achieved through distinct target genes. Taken together, the results are consistent with the model that Tead and Yap1 regulate cell proliferation through diverse mechanisms that are dependent on cell types and/or conditions of cells.

### Distribution of Tead1 and Yap1 proteins in developing mouse embryos

In order to understand how Hippo signaling is regulated during mouse development, and whether Tead and Yap1 also play additional roles besides regulating cell proliferation, we examined distribution of Tead1 and Yap1 proteins in developing mouse embryos. At E8.5, both *Tead1* and *Yap1* RNAs were expressed widely throughout embryos (data not shown). Tead1 proteins were also observed widely, essentially in all nuclei (Fig. 6A). However, strong signals were observed in the nuclei of restricted tissues, including the heart, posterior notochord, posterior endoderm and adjacent lateral plate mesoderm (Fig. 6A,B; data not shown). Yap1 protein was also detected throughout the embryos, but the signals were mostly restricted to the cell membrane or cytoplasm, and were weak in the nuclei (Fig. 6G,H). The notochord, heart and posterior endoderm showed stronger signals for Yap1 proteins, and, in these cells, nuclear Yap1 signals were stronger than those in the surrounding cells (Fig. 6G,H; data not shown).

The cell type-dependent accumulation of Tead1 and Yap1 was more evident at E10.5. Although Tead1 signal was widely detected in most nuclei, an especially strong signal was observed in the nuclei of the myocardium, notochord, floor plate of the neural tube and myotomes (Fig. 6C-F). Relatively strong signals were also observed in the endoderm and epidermis. Yap1 was also expressed widely and was excluded from the nuclei of the majority of cells (Fig. 6I). A particularly strong signal was observed in the notochord, and relatively strong signals were observed in the mesenchymal cells, including myotomes (Fig. 6I-K). In the myocardium, Yap1 was not clearly excluded from the nuclei, and some cells showed clear nuclear

accumulation of Yap1 (Fig. 6L). Similar results were also obtained with a commercially available anti-Yap1 antibody (data not shown). Therefore, dynamic regulation of subcellular localization of Yap1 proteins also takes place in mouse embryos, and simultaneous increase of nuclear Yap1 and Tead1 levels in the myocardium and the notochord suggests that the Hippo signal is weak in these cells. Strong nuclear Tead1 levels in the floor plate and myotomes were not accompanied by strong nuclear Yap1 levels, suggesting that Tead1 is also regulated by mechanisms other than Hippo signaling.

As myocardium showed strong nuclear Yap1 and Tead1, an indication of low Hippo signaling, we next examined whether Tead1 promotes cell proliferation in these cells. *Tead1*<sup>-/-</sup> embryos die at E11.5 with severe heart defects (Chen et al., 1994; Sawada et al., 2008). At E9.5, the *Tead1* mutants are slightly smaller than control littermates, and BrdU incorporation was slightly reduced throughout embryos (Fig. 7A,B). The myocardium showed strong reduction of BrdU labeling (Fig. 7B,D), whereas BrdU labeling of the endocardium, in which Tead1 was absent (Fig. 7E), was not significantly affected (Fig. 7B,D). These results are consistent with the hypothesis that Tead1-Yap1 complex regulates cell proliferation as a mediator of Hippo signaling in these cells.

### DISCUSSION

#### Tead family proteins as transcriptional mediators of mammalian Hippo signaling

In cultured cells, cell density and Hippo signaling alter subcellular localization of Yap1 and the transcriptional activity of the Gal4-TEAD4 fusion protein (Zhao et al., 2007). Expanding on these observations, we first showed that cell density and Hippo signaling also affect the level of nuclear Tead1 without altering its subcellular distribution, and demonstrated that cell density and Hippo signaling actually modulate transcriptional activity of endogenous Tead proteins. Recently, others have also reported involvement of mammalian Tead proteins in Hippo signaling (Zhao et al., 2008). Whereas Zhao et al. (Zhao et al., 2008) used Tead1/3/4 knockdown and ChIP-on-chip in cultured cells to show requirement of Tead proteins for Yap1 activities, we used gain-of-function and dominant-



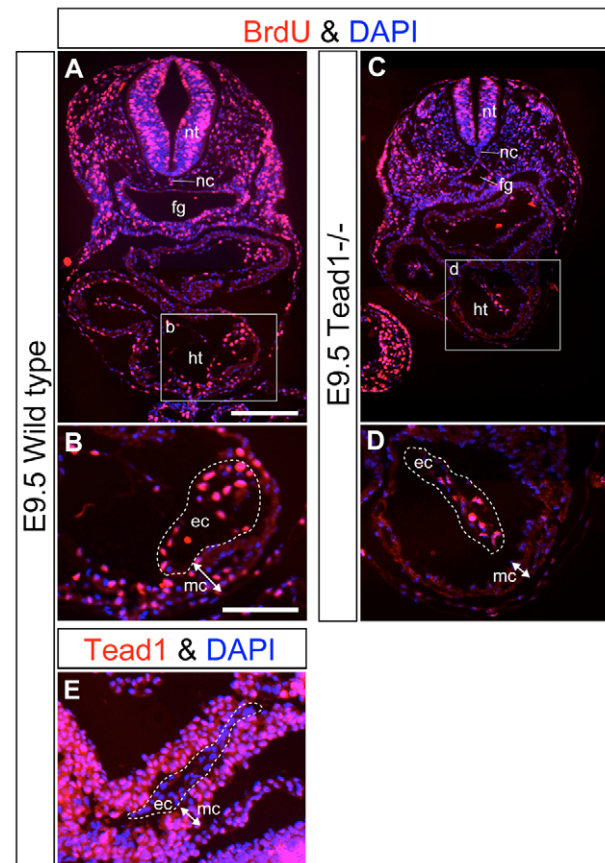
negative approaches, and microarray analysis in NIH3T3 cells to examine the effects of modulating Tead activity. These complementary studies reached the same conclusion that mammalian Tead proteins mediate Yap1 activity. Both studies showed the involvement of Tead in growth promoting and EMT-inducing activities of Yap1, and additionally, we showed the involvement of Tead in two other Yap1 activities: suppression of apoptosis and cell transformation/tumor formation. Therefore, information about cell density, which likely originates from cell-cell contacts, ultimately converges toward Tead activity by modulating nuclear accumulation of Tead and Yap1 through Hippo signaling. Then, the Tead activity regulates cell proliferation, apoptosis and EMT. Constitutive activation of Tead proteins was found to overcome cell contact inhibition and to promote tumor formation. Although the analysis by Zhao et al. (Zhao et al., 2008) was restricted to cultured cells, we have extended our analysis to mouse embryos, including *Tead1/2* and Yap1 mutants, and have revealed diversity of growth regulating mechanisms dependent on cell types, and also showed that Tead, Yap1 and Hippo signaling may play multiple roles in mouse embryos.

### Tead and Yap1 may regulate cell proliferation through multiple mechanisms

Our microarray analysis showed that Tead2-VP16 and Yap1 induced mostly overlapping set of genes in NIH3T3 cells, which supports the hypothesis of Tead being a transcriptional mediator of Hippo signaling. Unexpectedly, however, these genes did not contain the genes previously identified as Yap1-induced genes in mouse liver or NIH3T3 cells, except for *Ctgf* (Dong et al., 2007; Zhao et al., 2007). Only two genes (*Tagln* and *Acta2*) out of the 10 genes induced by Tead2-VP16/Yap1, and none of the previously identified Yap1-induced genes were downregulated in both *Tead1*<sup>-/-</sup>; *Tead2*<sup>-/-</sup> and *Yap1* mutant embryos. Furthermore, only one-fifth of the Tead2-VP16/Yap1-induced genes in NIH3T3 cells were suppressed by Tead2-EnR in the same cell line. As *Tagln* and *Acta2* were not suppressed by Tead2-EnR, the commonly regulated genes from all of the assays have yet to be determined. One interpretation of these complex observations is that Tead/Yap1 has an ability to control a wide range of growth regulators, and regulates different subsets of the targets, depending on the types and/or conditions of cells. An alternative interpretation is that the small number of genes commonly regulated in various types of cells and under various conditions constitute core batteries of the growth regulators of Hippo signaling, and variable genes are secondarily regulated by these core regulators. Although we do not rule out the latter possibility, we prefer the former model, because similar differential regulation of multiple cell-cycle regulators by a single signaling pathway was observed when cell proliferation was modulated by increasing or decreasing Sonic hedgehog signaling in chicken limb buds (Towers et al., 2008). In further support of this model, although *Ctgf* is a direct target gene of Tead-Yap1, which is essential for proliferation of cultured cells (Zhao et al., 2008), it is not downregulated in *Tead1*<sup>-/-</sup>; *Tead2*<sup>-/-</sup> embryos, and *Ctgf*<sup>-/-</sup> embryos can develop to term (Ivkovic et al., 2003), suggesting that regulation of *Ctgf* expression is not a general mechanism for growth regulation by Hippo/Tead-Yap1.

### Potential roles of Tead, Yap1 and Hippo signaling in developing mouse embryos

In NIH3T3 cells, Tead and Yap1 mediate Hippo signaling and regulate cell contact inhibition of proliferation. In mouse embryos, the majority of the cells showed weak Yap1 signal in the nuclei, suggesting that Hippo signaling is active and negatively modulates cell proliferation. Although the Yap1-related protein Wwtr1 also



**Fig. 7. Reduced proliferation of myocardium in *Tead1*<sup>-/-</sup> embryos.** (A-D) Distribution of BrdU-labeled cells in wild-type (A,B) and *Tead1*<sup>-/-</sup> (C,D) embryos at E9.5. (B,D) Enlarged images of the boxed areas in A and C. (E) Expression of Tead1 proteins in E9.5 heart. Endocardium lacks Tead1 expression. Abbreviations: ec, endocardium; fg, foregut; ht, heart; mc, myocardium; nc, notochord; nt, neural tube. Scale bars: in A, 100 μm for A,C; in B, 50 μm for B,D,E.

regulates cell proliferation and is inhibited by Hippo signaling in cultured cells (Lei et al., 2008), Wwtr1 proteins are always localized in the nuclei in mouse embryos (M.O. and H.S., unpublished). Therefore, the role of Wwtr1 in Hippo signaling in embryos may differ from that of Yap1. The cell proliferative role of Tead1 and Yap1 is most evident in the myocardium. Strong levels of nuclear Yap1 and Tead1 indicate weak Hippo signaling in the myocardium, and *Tead1* is required for the proliferation of myocardium. The role of Tead/Yap1, however, may not be restricted to growth regulation. For example, strong Tead1 signal was also observed in the notochord, the floor plate of the neural tube and the myotomes. In the notochord, Tead1/2 activates enhancer of *Foxa2*, a key regulator of notochord differentiation (Sawada et al., 2008; Sawada et al., 2005). As accumulation of Yap1 and Tead1 proteins indicates suppression of Hippo signaling in the notochord, Hippo signaling may suppress differentiation of the notochord. A similar cell fate specification role for Hippo signaling is also present in *Drosophila* photoreceptor cells (Mikeladze-Dvali et al., 2005). In the myotomes, Tead1 accumulation was not accompanied by clear increase in Yap1. Instead, another Tead co-factor protein, Vgl12, is specifically expressed in the myotomes and promotes skeletal muscle differentiation through Tead proteins (Chen et al., 2004; Maeda et al., 2002). Therefore, in the myotome, Tead regulates skeletal

muscle differentiation independently of Hippo signaling. In the floor plate, strong Tead1 signal is not accompanied by increased Yap1 or Vgll. Although the role of Tead proteins in the floor plate is currently unknown, considering that the floor plate is a mitotically inactive tissue, it is tempting to speculate that Tead1 suppresses its targets in order to suppress cell proliferation in this region. In fact, overexpression of Tead proteins inhibits their activator functions by 'squelching' co-activator proteins in cultured cells (Xiao et al., 1991).

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#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/24/4059/DC1>

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