Regulation of mouse embryonic stem cell self-renewal by a Yes–YAP–TEAD2 signaling pathway downstream of LIF

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Summary

The cytoplasmic tyrosine kinase Yes has previously been shown to have an important role in maintaining mouse and human embryonic stem (ES) self-renewal through an unknown pathway downstream of leukemia inhibitory factor (LIF) and one or more factors in serum. Here, we show that TEAD2 and its transcriptional co-activator, the Yes-associated protein YAP, co-operate in a signaling pathway downstream of Yes. We show that YAP, TEAD2 and Yes are highly expressed in self-renewing ES cells, are activated by LIF and serum, and are downregulated when cells are induced to differentiate. We also demonstrate that kinase-active Yes binds and phosphorylates YAP, and activates YAP–TEAD2-dependent transcription. We found that TEAD2 associates directly with the *Oct-3/4* promoter. Moreover, activation of the Yes pathway induced activity of the *Oct-3/4* and *Nanog* promoters, whereas suppression of this pathway inhibited promoter activity. Nanog, in turn, suppressed TEAD2-dependent promoter activity, whereas siRNA-mediated knockdown of Nanog induced it, suggesting a negative regulatory feedback loop. Episomal supertransfection of cells with inhibitory TEAD2–EnR induced endodermal differentiation, which suggests that this pathway is necessary for ES cell maintenance.

Key words: Embryonic stem cells, Leukemia inhibitory factor, Self-renewal, Src family kinases, Yes-associated protein

Introduction

Pluripotent embryonic stem (ES) cells can be expanded in culture and induced to differentiate into all three germ layers. Self-renewal of mouse ES cells depends on the LIF-induced activation of the signal transducer gp130 (also known as interleukin 6 signal transducer, IL6ST), and withdrawal of LIF induces differentiation (Burdon et al., 2002). Gp130 activates at least four pathways: the signal transducer and activator of transcription 3 (STAT3) pathway, the phosphoinositide 3-kinase (PI3K) pathway, the mitogen-activated protein kinase (MAPK) pathway and the Src family kinase (SFK) pathway (Annerén, 2008; Burdon et al., 2002; Liu et al., 2007). The STAT3, PI3K and SFK pathways help to sustain self-renewal, whereas activation of the MAPK pathway induces differentiation (Burdon et al., 1999). It was recently shown that the STAT3 pathway activates mainly SOX2, whereas PI3K predominantly activates Nanog (Niwa et al., 2009). However, no downstream target of LIF-activated SFKs has to date been identified in ES cells.

The Src family consists of nine members and has been shown to regulate a wide variety of cellular functions, such as proliferation, differentiation and survival (Abram and Courtneidge, 2000; Thomas and Brugge, 1997). Because all these processes are involved in embryogenesis, it is not surprising that seven SFKs have so far been identified to be expressed in ES cells and at least three of these, Yes, Hck and Lck, undergo dynamic changes in transcriptional and post-transcriptional regulation during human and mouse ES cell differentiation and are activated by LIF (Annerén et al., 2004; Ernst et al., 1994; Meyn et al., 2005). Interestingly, it was recently proposed that individual SFKs control distinct and opposing pathways in ES cell with Yes, Hck and Lck supporting self-renewal, whereas Src, which remains highly expressed in ES cells during differentiation, promotes differentiation into primitive ectoderm (Meyn et al., 2005; Meyn and Smithgall, 2009).

We have focused our attention on Yes because several findings suggest that this SFK has a particularly important role in self-renewing ES cells: (1) two independent transcriptional profiling screens found that Yes was highly enriched in ES cells compared with differentiated cells (Ivanova et al., 2002; Ramalho-Santos et al., 2002); (2) a transcriptome study specifically identified Yes as a ‘Pluri’ gene (a LIF-dependent gene, highly expressed in pluripotent cells, whose expression levels decreases sharply upon LIF withdrawal) (Trouillas et al., 2009) and Yes kinase activity is downregulated in response to differentiation in mouse and human ES cells (Annerén et al., 2004); (3) LIF and serum activates Yes in ES cells (Annerén et al., 2004); (4) specific knockdown of Yes with small interfering RNAs leads to mouse ES cell differentiation and reduced expression of key ES cell pluripotency genes including *Nanog* and *Oct-3/4* (also known as POU domain, class 5, transcription factor 1 or *Pou5f1*) (Annerén et al., 2004); and finally, (5) Oct3/4 has been shown to associate with the *Yes* promoter and positively regulate Yes expression, suggesting that Yes is a downstream target gene of Oct3/4 (Zhang et al., 2007).

Whilst searching for potential signaling pathways downstream of Yes, we found that the Yes-associated protein YAP (YAP65/YAP1) and a member of the TEA DNA binding domain protein family TEAD2 (TEF4) co-operate in a signaling pathway downstream of Yes. YAP is a powerful transcriptional co-activator, which is heavily involved in many pathways that regulate proliferation and cell death, and there is currently an upsurge in studies examining the role of YAP in various cancers (Zhao et al.,

2008). As the name implies, YAP was first identified as a Yes binding partner (Sudol, 1994), but despite the fact that the proline-rich region of YAP was found to associate with the Src homology domain 3 (SH3) of Yes many years ago (Sudol, 1994), only one study has since demonstrated a specific role of SFKs in YAP-mediated signaling (Zaidi et al., 2004). YAP interacts with SFKs at the plasma membrane, with 14-3-3 in the cytoplasm and with transcription factors in the nucleus (Basu et al., 2003; Vassilev et al., 2001; Zaidi et al., 2004). Inhibition of the YAP-related pathway is mediated by serine phosphorylation of YAP by the hippo pathway or Akt, which induces binding of 14-3-3 and retention in the cytoplasm (Basu et al., 2003; Zhao et al., 2007). Levels of *Yap* mRNA are enriched in mouse ES cells (Ramalho-Santos et al., 2002), but YAP is also present in most mouse and human tissues and cell types ranging from two-cell embryos to adults (Sudol et al., 1995).

Mammals express four highly conserved TEAD proteins that regulate transcription of a variety of genes and serve different functions during development. TEAD proteins share a highly conserved 68 amino acid TEA/ATTS DNA-binding domain, which binds to reporter elements related to the consensus sequence G(A)GA(T/C)ATG in gene promoters or enhancers (Jiang et al., 2000). All TEAD proteins require a transcriptional co-activator to function, of which the predominant, if not exclusive one is YAP, and overexpression of TEAD proteins without YAP results in quenching of transcriptional activity (Jiang and Eberhardt, 1995; Vassilev et al., 2001). TEAD2 and TEAD4 are predominantly expressed during early embryogenesis (Kaneko et al., 1997; Yagi et al., 2007). However, although TEAD4 is primarily expressed in the trophectoderm, TEAD2 is expressed in both inner cell mass (ICM) and trophectoderm of the blastocysts at fairly equal levels (Kaneko et al., 1997; Kaneko et al., 2004; Yagi et al., 2007). Furthermore, TEAD2 is highly enriched in ES cells (Ramalho-Santos et al., 2002). However, TEAD2-knockout mice (*Tead2*$^{-/-}$) exhibit no obvious abnormalities and are fertile, which is believed to be due to functional redundancy with TEAD1 (Sawada et al., 2008). Additionally, although *Tead2*$^{-/-}$;*Tead1*$^{-/-}$ double mutants are morphologically identical to wild-type embryos up to day E6.5, they do, however, exhibit growth retardation and severe morphological abnormalities by E8.5 and have a phenotype that is similar to YAP mutant mice (Morin-Kensicki et al., 2006; Sawada et al., 2008). A role of YAP and TEAD4 for specification of trophectoderm in pre-implantation mouse embryos was recently established, and it was demonstrated that expression of constitutively active TEAD4 (TEAD4-vp16) is sufficient to promote trophoblast fate in ES cells (Nishioka et al., 2009; Nishioka et al., 2008; Yagi et al., 2007).

In the present study, we examine the role of Yes, YAP and TEAD2 in ES cells. We show that TEAD2 activity is necessary for ES cell self-renewal and that Yes, YAP and TEAD2 are parts of the same signaling pathway, downstream of LIF, that induces expression of Oct3/4.

Results

Dynamic changes in transcriptional and post-translational regulation of Yes, YAP and TEAD2 during mouse ES cell differentiation

Real-time quantitative PCR (qPCR) data confirmed that *Tead2* is the pre-dominantly expressed TEAD member in mouse ES cells, whereas small or near undetectable levels of *Tead1*, *Tead3* and *Tead4* mRNA were found (Fig. 1A). However, when ES cells were induced to differentiate for 3 days, a significant reduction in levels of *Tead2*, *Yap* and *Yes* mRNA were observed (Fig. 1B). The level of *Tead1* mRNA was slightly, although not significantly, increased in response to differentiation, albeit from a very low basal level, whereas transcription of *Tead3* and *Tead4*, with expression levels of less than 0.1% of *Tead2* expression was not increased in response to differentiation (Fig. 1C). Cells, induced to differentiate for 3 days, expressed lower total YAP protein levels but higher levels of serine-phosphorylated YAP compared with levels in undifferentiated ES cells (Fig. 1D). In addition, YAP was predominantly found in

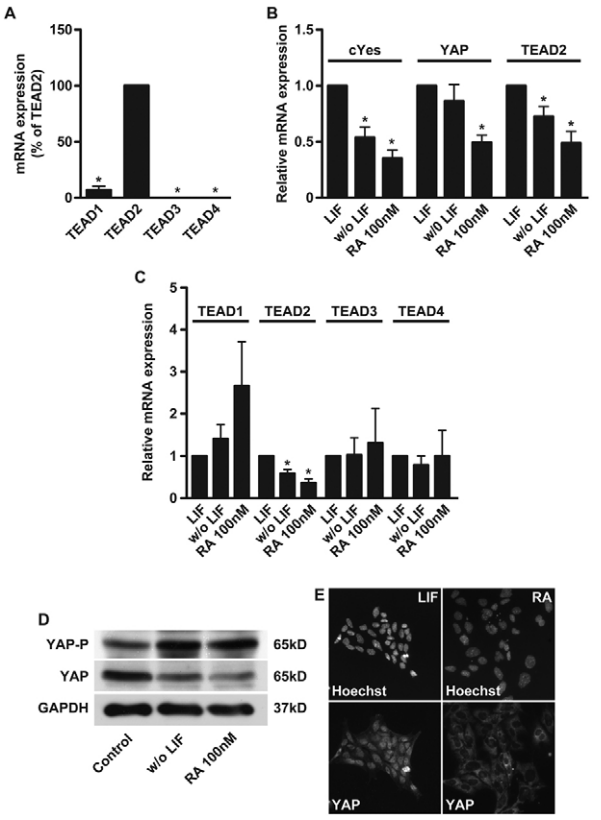

Fig. 1. Yes, YAP and TEAD2 are highly expressed in undifferentiated ES cells and are downregulated in response to differentiation. (**A**) QPCR analysis of *Tead1*, *Tead2*, *Tead3* and *Tead4* mRNA expression in E14/T cells. Transcript level comparison was based on primer efficiency estimated from five-point dilution curves and used for comparative C_t computation according to the PFAFFL method (Pfaffl, 2001). (**B**) QPCR analysis of *Yes*, *Yap* and *Tead2* mRNA expression in control E14/T cells cultured in the presence of LIF or induced to differentiate in the absence of LIF (w/o LIF) or with 100 nM retinoic acid (RA) for 3 days. Expression of β-actin is used for normalization, and the calibrator controls were cells grown in the presence of LIF. (**C**) qPCR analysis of the same cDNA samples as in B showing relative *Tead1*, *Tead2*, *Tead3* and *Tead4* mRNA expression in response to differentiation. Note that the mRNA expression levels are shown as percentage of internal control (i.e. expression of each gene in undifferentiated ES cells) and that the absolute mRNA levels of *Tead1*, *Tead3* and *Tead4* are negligible compared with the level of *Tead2* (as shown in A). (**D**) Western blot analysis of total YAP and serine-phosphorylated (Ser127) YAP (YAP-P) expression in cells shown in B. (**E**) Representative micrographs of undifferentiated E14/T cells (LIF, left panels) and differentiated ES cells exposed to 100 nM retinoic acid for three days (RA, right panels) stained with Hoechst 33342 (top panels) and anti-YAP (bottom panels) antibodies. All qPCR results are mean ± s.e.m. comparative C_t values (n=3); *P<0.05 (ANOVA; Tukey's MC Test).

the nuclei of undifferentiated ES cells, whereas retinoic-acid-induced (100 nM, 3 days) differentiated cells exhibited increased cytosolic YAP, as well as a clear decrease in nuclear YAP localization (Fig. 1E).

Reporter gene constructs expressing the firefly luciferase gene driven by the human chorionic somatomammotropin (CS) promoter with or without a TEAD enhancer region consisting of multiple (24) copies of the TEAD-binding GTIIC (GGAATG) site (pGTIIC–Luc and pCS–Luc, respectively) (Jiang and Eberhardt, 1995) were expressed in self-renewing or differentiating ES cells and the luciferase activities were measured. Barely detectable luciferase activity was found in cells transfected with pCS-Luc compared with pGTIIC–Luc, demonstrating that the CS promoter in the pGTIIC construct is mainly driven by the TEAD2 enhanson when expressed in ES cells (Fig. 2A). Moreover, TEAD-dependent transcription in ES cells decreased when cells were induced to differentiate for 3 days by withdrawal of LIF or by addition of 100 nM retinoic acid (RA) (Fig. 2B).

LIF induces TEAD2-dependent transcription in a Yes- and YAP-dependent manner

To determine whether LIF induces TEAD2-dependent transcription, ES cells transfected with pGTIIC–Luc with or without short hairpin RNA (shRNA) targeting TEAD2 were starved of serum and LIF overnight and then treated for 6 hours with LIF. The results show that LIF activates TEAD2, in agreement with previously described data showing LIF-induced Yes kinase activity (Annerén et al., 2004). shRNA targeting TEAD2 partly inhibited this effect (Fig. 2C). To exclude the possibility that our shRNA constructs have an effect upstream of the LIF receptor, cells were transfected with shRNA targeting YAP, TEAD2 or EGFP and then analyzed for tyrosine-phosphorylated Stat3 (Tyr705) and total Stat3 by western blotting. shRNA against YAP or TEAD2 showed no effect on phosphorylation of Stat3 (supplementary material Fig. S1). Next, the pGTIIC–Luc construct was co-expressed with YAP, Yes, a constitutively kinase active Y535F mutant of Yes (CA-Yes) or vectors expressing shRNA targeting Yes, YAP or TEAD2. Western blot analysis showed that ectopic expression of both Yes and CA-Yes dramatically increased the level of autophosphorylated (active) Yes in ES cells (supplementary material Fig. S2C). All shRNAs mediated a reduction of the respective mRNA and protein levels in transiently transfected cells as determined by qPCR and western blotting (supplementary material Fig. S2A,B). The endogenous TEAD-dependent transcriptional activity was increased by 50% in YAP-transfected cells, demonstrating that YAP is a rate-limiting factor for TEAD2-enhanced transcription in ES cells (Fig. 2D). In addition, overexpression of Yes or CA-Yes induced a two- to threefold increase in TEAD2-dependent transcription compared with that in cells transfected with pGTIIC alone, whereas expression of shTEAD2, shYAP or shYes decreased TEAD2 activity (Fig. 2D and supplementary material Fig. S3). Both shTEAD2 and shYAP significantly impaired CA-Yes-induced TEAD2-dependent transcription (Fig. 2E) and concurrently, pre-treatment of cells with the Src family tyrosine kinase inhibitor PP2 (10 μM) also markedly reduced the effect of LIF and CA-Yes (data not shown). Together, these results show that, similarly to YAP, Yes induces TEAD-dependent transcription and suggest that Yes is part of a signaling pathway upstream of YAP and TEAD2 in ES cells.

To determine whether LIF and/or SFKs also activate TEAD DNA binding activity, LIF- and serum-starved ES cells were treated with LIF and/or 5 μM of the selective SFK inhibitor SU6656 for

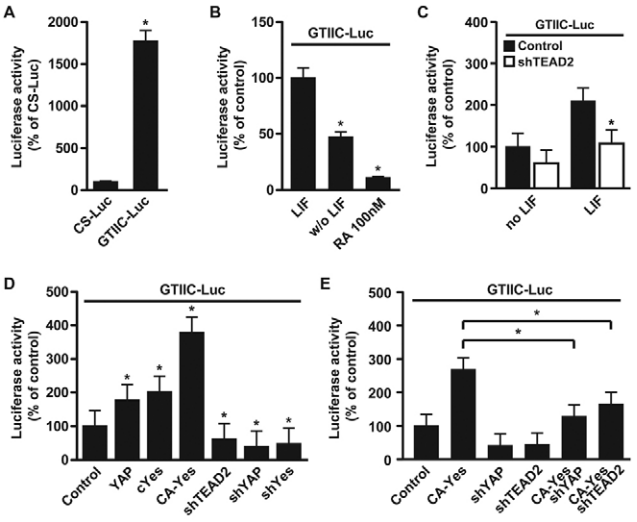

Fig. 2. TEAD2 activity is induced by LIF, YAP and Yes in ES cells. (**A**) Luciferase activity in E14/T cells transfected with constructs expressing the firefly luciferase gene driven by the human chorionic somatomammotropin (CS) promoter with (pGTIIC-Luc) or without (pCS-Luc) a TEAD2 enhancer region. (**B**) TEAD2-dependent promoter activity (pGTIIC) in E14/T control cells (LIF) and LIF-starved (w/o LIF) or RA-treated (RA 100 nM) for 3 days. (**C**) TEAD2 activity in E14/T cells starved of LIF and serum overnight and treated with LIF with or without the presence of shRNA against TEAD2. (**D**) TEAD2 activity in E14/T cells transfected with constructs expressing YAP, Yes, CA-Yes or shRNA against YAP, Yes and TEAD2 (analyzed 48 hours after transfection). (**E**) TEAD2 activity in E14/T cells transfected with CA-Yes and/or shRNA against TEAD2 and YAP. All luciferase data are presented as mean ± s.e.m. percentage of internal control (n=3); *P<0.05 (ANOVA; Tukey's MC Test).

30 minutes and subjected to electrophoretic mobility shift assays (EMSA) using [^{32}P]DNA probes containing the GT-IIC sequence (wt) or a probe containing the same sequence with a single base pair change that eliminates TEAD binding (mut), as previously described (Kaneko and DePamphilis, 1998). As expected, TEAD proteins from ES cell nuclear extracts bind the GT-IIC sequence, but not the mutant sequence (supplementary material Fig. S4A). Moreover, in agreement with the luciferase assays, TEAD DNA binding activity was increased in response to LIF, whereas SU6656 partially inhibited this effect. In addition, a lower TEAD-binding activity was found in 3-week-old embryoid bodies (EBs) compared with that in LIF-stimulated ES cells (supplementary material Fig. S4A,B). In conclusion, these results suggest that LIF activates TEAD2 via Yes and YAP and that this pathway is downregulated in response to differentiation.

Kinase-active Yes associates with YAP and induces its tyrosine phosphorylation

Interaction between the gp130 subunit of the LIF receptor and the Yes SH2 domain, but not the SH3 or unique domains, was induced by LIF in LIF- and serum-starved ES cells as confirmed by mixing cell lysates with GST-SH2–Yes, GST-SH3–Yes, and GST-SH3+unique–Yes fusion proteins (Fig. 3A). Additionally, co-immunoprecipitation of Yes and YAP was induced by LIF in LIF- and serum-starved cells (Fig. 3B). To elucidate whether YAP binds to and is phosphorylated by activated Yes, ES cells were transfected with CA-Yes, immunoprecipitated (IP) with anti-YAP antibody and subjected to western blot analysis for Yes, YAP,

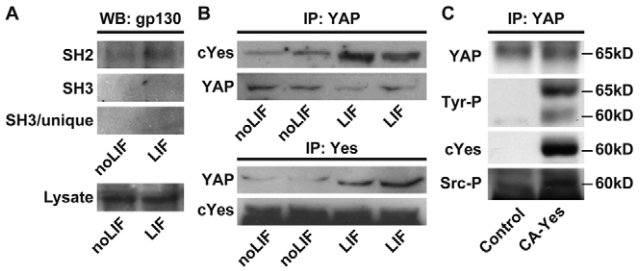

Fig. 3. The SH2 domain of Yes binds gp130 and activated Yes associates with YAP and induces its tyrosine phosphorylation. (**A**) Affinity absorption of gp130 by GST-tagged fusion proteins expressing the SH2, SH3 and SH3 + unique domains of Yes incubated with cell lysates from AV3 cells that were starved of LIF and serum overnight, and subsequently treated with LIF for 12 hours. Equal input of gp130 in lysates was confirmed by western blotting. (**B**) E14/T cells starved of LIF and serum overnight and subsequently treated with LIF for 2 hours, immunoprecipitated (IP) with anti-YAP and anti-Yes antibodies and subjected to western blot analysis. (**C**) E14/T cells transfected with control vector or CA-Yes-expressing vector, immunoprecipitated with anti-YAP antibody and subjected to western blot analysis 48 hours after transfection. Western blots were probed with anti-YAP, anti-PY (Tyr-P), anti-Yes, or anti-phosphorylated Y416 Src (Src-P, which recognizes all auto-phosphorylated kinase active SFKs) antibodies.

phosphotyrosine (PY) and PY416-Src (which recognizes all kinase-active, auto-phosphorylated SFKs) 48 hours after transfection. YAP did co-immunoprecipitate with Yes in cells transfected with CA-Yes, but did not in the control cells (Fig. 3C). Moreover, despite equal levels of YAP protein in control and CA-Yes-transfected cells, YAP was only tyrosine phosphorylated in cells expressing CA-Yes, suggesting that YAP is a substrate for Yes kinase activity (Fig. 3C).

Yes increases the activity of *Oct-3/4* and *Nanog* promoters in a YAP-dependent manner

We next addressed the question whether the Yes–YAP–TEAD2 pathway affects promoter activity of the key ES cell regulators Oct3/4 and Nanog. Reporter constructs expressing luciferase driven by the *Oct-3/4* (Okumura-Nakanishi et al., 2005) or *Nanog* promoters (Hattori et al., 2007) were co-expressed with activating or shRNA constructs for Yes, YAP and TEAD2. As expected, high *Oct-3/4* and *Nanog* promoter activities were confirmed in ES cells grown under normal ES cell culture conditions and these were decreased upon LIF withdrawal (data not shown). Moreover, activity of the *Oct-3/4* promoter increased as a response to LIF, similarly to what was observed for TEAD2-dependent promoter activities (data not shown). Interestingly, transient transfection of Yes induced a significant increase in *Oct-3/4* as well as *Nanog* promoter activities in ES cells (Fig. 4A,B). Concurrently, expression of shRNA targeting Yes, YAP and TEAD2 resulted in decreases in *Oct-3/4* and *Nanog* promoter activity (Fig. 4A,B) and additionally reduced the LIF-induced activity of the promoters (data not shown). Additionally, shYAP inhibited the Yes-induced activation of both the *Oct-3/4* and *Nanog* promoters, again suggesting that YAP is operating downstream of Yes (Fig. 4C,D).

Because *Nanog* transcription has previously been shown to be regulated through an interaction between Oct3/4 and SOX2 and their binding to Octamer and SOX elements in the *Nanog* promoter (Kuroda et al., 2005; Rodda et al., 2005), we next determined whether the effects on *Nanog* promoter activity was a direct effect

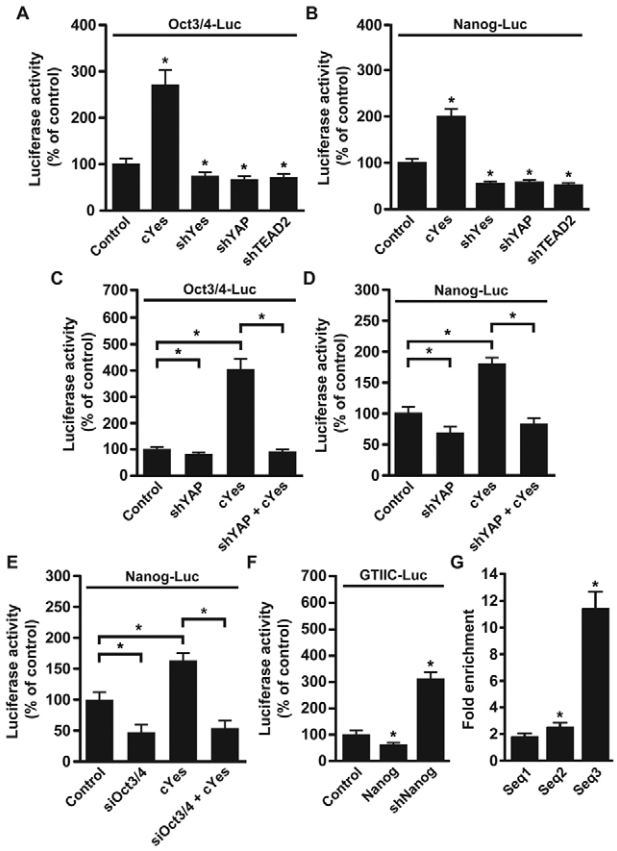

Fig. 4. Yes, YAP and TEAD2 increase *Oct-3/4* and *Nanog* promoter activity. (**A**) *Oct-3/4* and (**B**) *Nanog* promoter activity in E14/T cells transfected with Yes and shRNA constructs against Yes, YAP and TEAD2. *Oct-3/4* (**C**) and *Nanog* (**D**) promoter activity in E14/T cells transfected with Yes and/or shYAP. (**E**) *Nanog* promoter activity in E14/T cells transfected with Yes and/or siOct3/4. (**F**) TEAD2-dependent transcription (pGTIIC) in E14/T cells transfected with Nanog or shNanog. All Luciferase data are presented as mean ± s.e.m. percentage of internal control (n=3); *P<0.05 (ANOVA; Tukey's MC Test). (**G**) TEAD2 binds the *Oct-3/4* promoter. ChIP assays of ES cell chromatin immunoprecipitated with anti-TEAD2 or rabbit IgG (control) and subjected to qPCR using primers for three regions of the mouse *Oct-3/4* promoter. Note that Seq. 1 is a control sequence that does not contain any putative TEAD2 binding site. Results are comparative mean ± s.e.m. C_t values (n=3) and results are shown as fold difference in promoter DNA enrichment compared with unspecific binding by IgG. *P<0.05 (ANOVA; Tukey's MC Test).

of Yes signaling or an indirect effect caused by the increased expression of Oct3/4. Hence, a Nanog-Luc construct was co-transfected with Yes and small interfering RNA (siRNA) against *Oct-3/4* and luciferase activity was measured. *Oct-3/4* siRNA almost completely inhibited the Yes-induced activation of the *Nanog* promoter, implying that Yes signaling activates the *Nanog* promoter via the induction of Oct3/4 (Fig. 4E). Moreover, to our surprise, overexpression of Nanog significantly decreased the TEAD2-dependent transcriptional activity; whereas knockdown of Nanog with shRNA against Nanog dramatically increased this activity (Fig. 4F), suggesting the presence of a negative-feedback loop regulating YAP and TEAD2 activity in ES cells.

TEAD proteins activate or enhance gene expression by binding to various reporter elements related to the consensus sequence G(A)GA(T/C)ATG in gene promoters or enhancers (Jiang et al.,

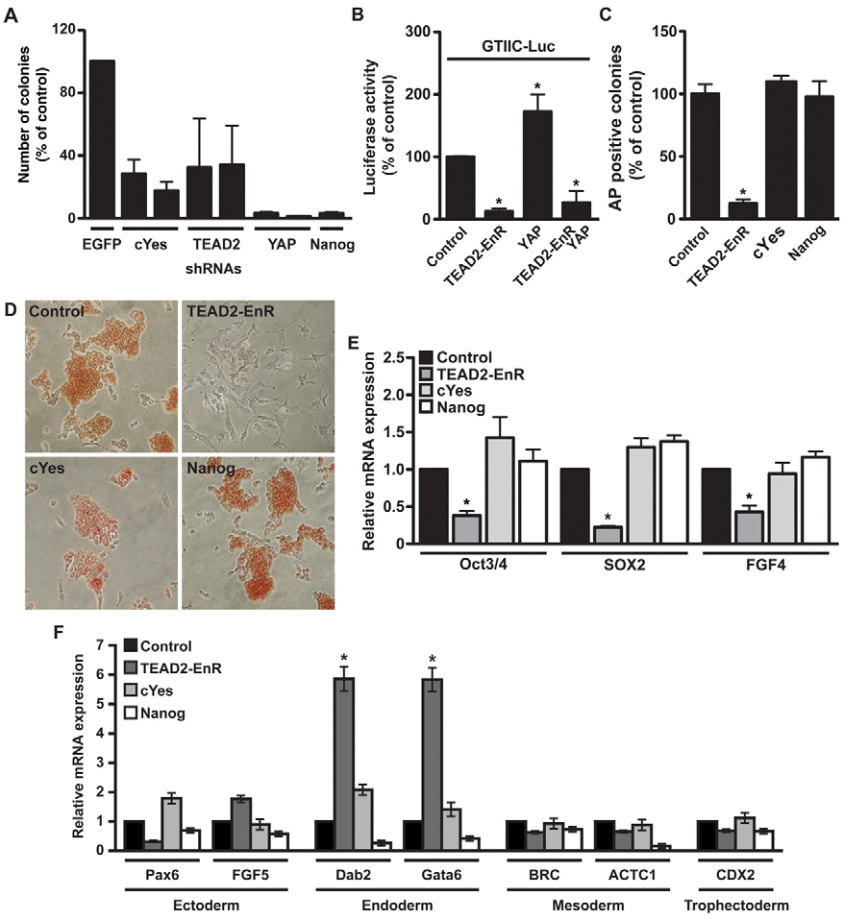

Fig. 5. The Yes–YAP–TEAD2 pathway is necessary for self-renewal of ES cells. (**A**) RNA interference of Yes, YAP, TEAD2 and Nanog reduces colony-forming ability in ES cells. AV3 cells were transfected with constructs expressing shRNAs against *Egfp* (control) *Yes*, *Tead2*, *Yap*, or *Nanog* and a plasmid containing a puromycin-resistance gene. After 2 weeks of puromycin selection (3 mg/ml), the number of colonies was counted and compared with control shEGFP-transfected cells. Results are mean ± s.e.m. (n=3). (**B**) Luciferase assay showing TEAD2-dependent activity (pGTIIC) in E14/T cells transfected with YAP and/or TEAD2-EnR. The results are mean ± s.e.m. (n=3); *P<0.05 (ANOVA; Tukey's MC Test). (**C**) Colony formation assays for alkaline phosphatase (AP)-positive E14/T cells. AP-positive colonies were quantified using ImageJ software. Results are mean ± s.e.m. (n=3); *P<0.05 (ANOVA; Tukey's MC Test). (**D**) Altered morphology and alkaline phosphatase activity staining of E14/T cells 5 days after transfection with TEAD2–EnR compared with cells transfected with empty, Yes and Nanog constructs. (**E**) qPCR analysis of pluripotency genes in E14/T cells 5 days after transfection with TEAD2–EnR, Yes and Nanog constructs. (**F**) qPCR analysis of lineage-specific differentiation markers in E14/T cells 5 days after transfection and puromycin selection. For all qPCR analyses, mRNA encoding β-actin was detected for normalization, controls transfected with empty plasmid were used as a calibrator and results are comparative mean ± s.e.m. C_t values (n=3); *P<0.05 (ANOVA; Tukey's MC Test).

2000). By searching the 5 kb upstream genomic region of the *Oct-3/4* promoter for these elements, we identified at least two putative TEAD binding sites, at –1.2 kb (GGAATG, nt. –1186 to –1181; seq. 2) and –4.7 kb (CATTCC, nt. –4712 to –4707; seq. 3; equivalent to GGAATG on the reverse strand) in the *Oct-3/4* promoter. Chromatin immunoprecipitation (ChIP) assays were carried out to assess whether TEAD2 associates with any of these regions of the *Oct-3/4* promoter. Sequence 1 is a control sequence, overlapping the codon start site and does not contain any TEAD2 binding site. Sequence 2 and sequence 3 overlap the putative –1.2 kb and –4.7 kb binding sites, respectively. In agreement with the luciferase data, the results demonstrated that TEAD2 did indeed bind to the identified response elements in the *Oct-3/4* promoter (Fig. 4G).

RNA interference of YAP and TEAD2 reduces formation of ES cell colonies

We have previously shown that inhibition or knockdown of Yes using chemical inhibitors or shRNAs induces differentiation of ES cells cultured in the presence of LIF and serum (Annerén et al., 2004). To establish whether YAP and TEAD2 are also necessary for ES cell self-renewal, two shRNAs were designed for each gene and expressed in ES cells together with a puromycin selection vector. *Nanog* shRNA was used as a positive control, whereas shEGFP (enhanced green fluorescent protein) was used as a negative control, as described previously (Annerén et al., 2004). Puromycin-selected colonies were stained for alkaline phosphatase (AP) activity and the number of colonies was assessed as a percentage of the shEGFP control. There were no significant changes in AP staining between cells transfected with shEGFP, and the other shRNA constructs, including shNanog (results not shown). However, cells transfected with shRNA against Yes, TEAD2, YAP and Nanog all generated significantly fewer colonies when compared with control shEGFP-transfected cells (Fig. 5A), suggesting that cells expressing reduced or abolished expression of these genes are unable to form colonies.

Decreased TEAD2 activity induces differentiation

To study sustained high-level blockade of TEAD2 activity in ES cells, a TEAD2 repressor fusion gene construct was generated (TEAD2–EnR) and supertransfected into E14/T cells using an episomal vector system, as previously described (Chambers et al., 2003). TEAD2–EnR completely repressed both endogenous and YAP-induced TEAD2 activity (Fig. 5B). Interestingly, prolonged expression of TEAD2–EnR in ES cells induced cells to acquire a fibroblast-like morphology, and there was a significant reduction in AP activity compared with that in cells transfected with empty plasmid, Yes or Nanog (Fig. 5C,D). TEAD2–EnR induced a clear decrease in the expression of all tested ES cell pluripotency genes, including Oct3/4, SOX2, FGF4, Nanog and GBX2 (Fig. 5E and results not shown). No phenotypical change could be seen in cells overexpressing TEAD2 without the EnR domain, and qPCR analysis failed to show any significant differences in the pluripotency genes 5 days after transfection (data not shown). We next examined whether cells with impaired TEAD2 activity differentiate in a spontaneous manner, similarly to the differentiation

pattern seen upon LIF withdrawal, or if they undergo a directed differentiation toward a specific germ layer. The same cDNAs used for the assessment of pluripotency gene expression were analyzed by qPCR for at least two genes specific for each germ layer as well as for trophectoderm differentiation. ES cells induced to differentiate in the absence of LIF for 5 days, showed increased expression of genes specific for all three germ layers (results not shown). Conversely, cells transfected with TEAD2–EnR exhibited a significant increase in expression of endoderm-specific genes (e.g. *Dab2* and *Gata6*), whereas increased expression of other germ-layer-related genes could not be detected (Fig. 5E).

Discussion

LIF activates four parallel pathways in mouse ES cells: the STAT3, MAPK, PI3K and SFK pathways (Fig. 6). It was recently shown that the STAT3 pathway mainly activates Krueppel-like factor 4 (KLF4), whereas the PI3K pathway stimulates T-box transcription factor (TBX3) (Niwa et al., 2009) and that these in turn activate SOX2 and Nanog, respectively. The MAPK pathway, however, was shown to antagonize the nuclear localization of TBX3 (Burdon et al., 1999; Niwa et al., 2009). In contrast to the other pathways, the SFK pathway has been less well studied and it is not clear how SFKs are linked to the core circuitry of pluripotency-associated transcription factors.

We have previously shown that LIF and serum activate Yes and that inhibition of this pathway disrupts self-renewal of ES cells (Annerén et al., 2004). In the present study, we show that the transcription factor complex YAP–TEAD2 is activated by Yes in ES cells and that TEAD2 in turn regulates expression of the crucial pluripotency genes *Oct-3/4* and *Nanog*. Yes, YAP and TEAD2 undergo dynamic changes in transcriptional and post-transcriptional regulation and activity during mouse ES cell differentiation. Moreover, downregulation of this pathway induces ES cells differentiation.

On the basis of these results, we propose the following model for how Yes, in concert with the above-described LIF-induced pathways, sustains self-renewal in ES cells (Fig. 6). (1) Yes is activated by LIF, through direct binding to activated gp130. (2) Active auto-phosphorylated Yes then transiently associates with and phosphorylates YAP on one or more tyrosine residues, which in turn binds to complexes of TEAD2 and DNA in the nucleus, inducing transcription of genes, including the key pluripotency factor *Oct-3/4*. (3) Finally, Oct3/4 activates transcription of *Nanog*, which in turn, negatively regulates TEAD2 through an unknown mechanism.

We have previously shown that LIF induces Yes kinase activity in mouse ES cells (Annerén et al., 2004). Here, we confirm that the SH2 domain of Yes associates with the LIF receptor subunit gp130. Moreover, we demonstrate that activated Yes associates with YAP and that both proteins in the complex are tyrosine-phosphorylated, suggesting that YAP is a direct substrate for Yes. We did not observe any significant changes in the subcellular localization of YAP in response to CA–Yes expression (data not shown), indicating that that Yes-mediated tyrosine phosphorylation of YAP does not affect its nuclear translocation. This contrasts with the well-described serine phosphorylation of YAP by Akt or Lats kinases, which induces binding of YAP to 14-3-3 proteins and cytoplasmic sequestering of YAP (Basu et al., 2003; Zhao et al., 2007). In fact, the role of YAP tyrosine phosphorylation is still unclear. Zaidi and co-workers showed that Src- and Yes-induced tyrosine phosphorylation of YAP is required for interaction of YAP

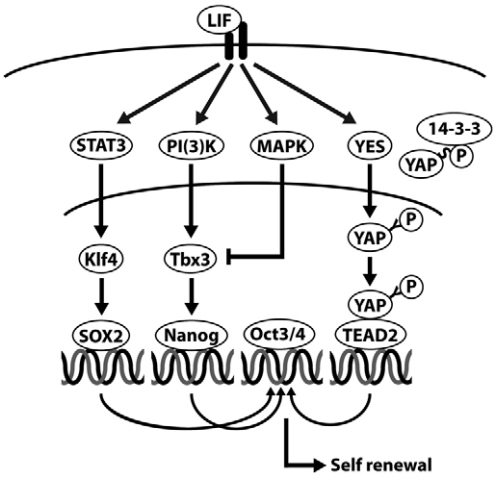

Fig. 6. Model of LIF-regulated self-renewal in mouse ES cells (modified from Niwa et al., 2009). The binding of LIF to the LIFR–gp130 receptor complex leads to recruitment and activation of at least four different downstream signaling pathways: the STAT3 pathway, the MAPK pathway, the PI3K pathway and the Yes pathway. See text for further details.

with the RUNX2 transcription factor in osteoblasts (Zaidi et al., 2004). In line with our observations, they did not observe any clear changes in the nucleocytoplasmic distribution of YAP. Levy and colleagues. demonstrated that Abl-mediated tyrosine phosphorylation of YAP in HEK293 cells stabilizes the YAP protein and increases the affinity of YAP to the p73 transcription factor (Levy et al., 2007). The tyrosine phosphorylation site in YAP was shown to be Y357, which is within the YAP transcriptional activation domain, suggesting that tyrosine phosphorylation directly regulates YAP activity. Interestingly, they also showed that the YAP phosphorylation state influences the p73 specificity of target gene activation, by switching between p73-mediated pro-apoptotic and growth arrest target genes. Thus, although the exact mechanism of Yes-induced activation of TEAD2 in ES cells remains to be shown, it can be speculated that Yes-induced tyrosine phosphorylation of YAP protects YAP from degradation, increases the affinity of YAP to TEAD2, and/or induces YAP-mediated transcriptional activity.

Additional evidence indicating that Yes directly activates the YAP–TEAD2 pathway includes: (1) transient expression of CA-Yes or Yes induces TEAD2-dependedent transcription whereas shYes inhibits it; (2) TEAD2 DNA-binding activity is increased in LIF-activated cells, a response that is partially impeded by the SFK inhibitor SU6656; and (3) shYAP inhibits Yes-induced activation of the *Oct-3/4* promoter. Our results suggest that the Yes, YAP and TEAD2 pathway is an integral component of the ES cell core regulatory circuitry, a label given to Oct3/4, SOX2 and Nanog because of their co-occupancy of a substantial portion of ES-cell-specific target genes and their involvement in various autoregulatory and feed-forward loops (Boyer et al., 2005; Loh et al., 2006). Interestingly, in a recent study published during revision of this paper, Lian and co-workers presented data strongly supporting our work. For instance, they showed that constitutively active YAP and overexpression of YAP prevented ES cell differentiation. In addition, YAP protein levels were expressed at significantly higher levels in induced pluripotent stem (iPS) cells than in the parental fibroblasts, and addition of YAP to the three

OSK factors (i.e. Oct3/4, Sox2, Klf4) increased the iPS reprogramming efficiency twofold (Lian et al., 2010). Together, our data mutually corroborate each other and further establish the crucial role of YAP in the self-renewal and differentiation of stem cells.

In the present study, we show that YAP–TEAD2 appears to directly induce Oct3/4 expression by binding to the *Oct-3/4* promoter. TEAD2 was found to exhibit strongest binding to the *Oct-3/4* promoter region approximately 4.7 kb upstream of the *Oct-3/4* transcriptional start, which, owing to the distant location, is probably enhancer region. This is not surprising because TEAD2 is known to be a strong transcriptional enhancer. Furthermore, transient overexpression and shRNA silencing of Nanog induced a distinct decrease and increase in TEAD2-dependent transcriptional activity, respectively. In addition, previous studies have shown that *Yap* is one of the top 20 upregulated genes in Oct3/4 tetracycline conditional knockout mouse ES cells (Matoba et al., 2006), and that post-translational modification of Oct3/4 regulates Yes expression by specific binding to the Yes gene promoter (Zhang et al., 2007). Hence, we speculate that the pathways presented in this study form an interconnected autoregulatory loop with Oct3/4 and Nanog. However, we should emphasize that this needs further investigation.

Interestingly, abnormally high expression levels of kinase-active Yes, YAP or constitutively active mutant TEAD2 (TEAD2-vp16), achieved by supertransfecting E14/T ES cells with episomal vectors, also induce ES cell differentiation (our unpublished observations). These results correlate with results obtained using a similar experimental setup for other key ES cell self-renewal transcription factors such as Tbx3 and Klf4 (Niwa et al., 2009), indicating the tight regulation and dosage effect of these genes.

We additionally show that TEAD2 activity is necessary for ES cell maintenance, because prolonged downregulation of TEAD2 activity induced differentiation. Moreover, this effect was forceful enough to override the LIF- and serum-regulated self-renewal and pluripotency machinery. In conclusion, we propose that Yes, YAP and TEAD2 are part of a tightly controlled signaling pathway downstream of LIF, which regulates the expression of key factors involved in the maintenance and self-renewal of ES cells.

Materials and Methods

Expression constructs

A dominant-negative TEAD2 construct was generated by fusing the repression domain of the *Drosophila melanogaster* homeodomain protein Engrailed to full-length TEAD2 (TEAD2–EnR) as follows: Full- length mouse *Tead2* cDNA was cloned using TOPO® (Invitrogen) immediately upstream of EnR in the SLAX–EnR vector and the TEAD2–EnR fusion gene was then blunt cloned into the *Xho*I-*Not*I site of the pPyCAGIP vector. The following expression constructs were also used: pPyCAGIP YAP, pPyCAGIP Nanog, pPyCAGIP TEAD2, pMIK Yes, pMIK Y535F (CA-Yes) (Espanel and Sudol, 2001), pGL3-basic vectors carrying the 2.1 kb upstream region of the mouse *Oct-3/4* gene (the upstream end at nt. –2136 relative to the translational start site) (Okumura-Nakanishi et al., 2005) or the 1 kb upstream region of mouse *Nanog* (the upstream end at nt. –983 relative to transcription start site) (Hattori et al., 2007), pCS-luciferase and pCS GT-IIC-luciferase (GTIIC) (Jiang and Eberhardt, 1995) and pCMV β-gal. The following shRNA sequences are shown in supplementary material Table S1: pSilencer 1.0-U6 shYes, pSuper shYAP, pSilencer 1.0-U6 shTEAD2, pSilencer 1.0-U6 shNanog and pSilencer 1.0-U6 EGFP. Oct3/4 was silenced using siRNA mOct3/4 (sense: GGAUGUGGUUCGAGUAUGGUU) with control ON-TARGET*plus* non-targeting siRNA to confirm silencing specificity (both from Dharmacon).

Embryonic stem cell culture

Feeder-dependent 129X1/SvJ-derived AV3 ES cells were cultured on mouse embryonic fibroblasts as previously described (Ramalho-Santos et al., 2002). The E14/T, a mouse embryonic stem cell line constitutively expressing polyoma large-T, was cultivated in 10% serum (fetal bovine serum and serum replacement in a 50/50 concentration) in the absence of feeder cells, as previously described (Smith, 1991). For simple differentiation assays, cells were grown in the absence of LIF or in the presence of retinoic acid (RA, 100 nM) for 72 hours before harvesting.

Electrophoretic mobility shift assay

Synthetic oligonucleotides (GT-IIC: 5′aagctt-TTCGGGACCCAGGCCTGG-AATGTTTCCACC-3′ with binding region underlined and GT-IIC Mut: 5′aagctt-TCGGGACCCAGGCCTAGAATGTTTCCACC-3′) were designed and generated as previously described (Kaneko and DePamphilis, 1998). Purified [γ-^{32}P]ATP-labeled GT-IIC and Mut probes were diluted to 20 nM in water and the radioactive signal was checked and normalized with cold oligonucleotide. Cytoplasmic and nuclear extracts were obtained using an NE-PER™ kit (Pierce) from 3-week-old embryoid bodies, serum starved AV3 ES cells and AV3 cells stimulated with 32 nM LIF for 30 minutes with or without the addition of 4 μM SU6656 (Calbiochem; added 30 min before LIF), according to the manufacturer's instruction. 5 μl of nuclear extracts were subjected to SDS-PAGE and stained with Coomassie Blue to ensure equal loading. Equal amounts (approx 5 μg) of nuclear extracts were mixed with reaction buffer (20 mM HEPES pH 7.6, 0.1 mM EGTA, 0.5 mM DTT, 10 mM NaCl, 0.1 mg/ml dGdC cold competitor, 5% glycerol and 2 μl [γ-^{32}P]ATP-labeled probe), incubated for 15 minutes at room temperature and loaded immediately on a 30% polyacrylamide (75:1; acrylamide:bis-acrylamide) gel. The gel was subsequently dried and exposed for autoradiography.

Transfection

Expression constructs (final concentration 3–5 μg DNA/well) were introduced into the mouse ES cells by transfection with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's recommendations. Cells were incubated at 37°C for 4 hours, after which ES cell medium was added (2:1). Cells transfected with episomal vectors (pPyCAGIP) containing a puromycin-resistance gene were selected with 2 μg/ml puromycin (Sigma) for 48 hours to eliminate untransfected cells and then cultured for an additional 72 hours. Colony-forming assays after expression of shRNA and subsequent puromycin selection were done as previously described (Annerén et al., 2004). In short, two different small interfering shRNA sequences for each gene were designed and inserted into pSilencer™ 1.0-U6 RNA, or pSuper siRNA expression vectors (Ambion). AV3 cells were co-transfected with siRNA constructs and a vector containing a puromycin-resistance gene, cultured for 14 days with puromycin, followed by the counting of colonies and staining for alkaline phosphatase. The shRNA efficiency and auto-phosphorylation of Yes upon ectopic expression of CA-Yes was assessed 48 hours after transfection without puromycin selection by qPCR and western blotting, respectively. To estimate basal levels of TEAD2 transcriptional activity in ES cells compared with that of differentiated cells, E14/T cells were grown in the absence of LIF with or without the presence of RA (100 nM) for 3 days before transfection with GTIIC-Luc.

Quantitative real-time polymerase chain reaction

Total RNA was extracted and purified with Qiagen RNeasy Mini kit (Qiagen) according to the manufacturer's instruction. First-strand cDNA was produced according to the manufacturer's protocol with SuperScript™II (Invitrogen) using 1 μg RNA and 100 ng random primers (Invitrogen). Quantitative real-time PCR was performed according to the manufacturer's instructions using the Miniopticon™ Real-Time PCR Detection System (Bio-Rad). The average C_t value for each gene was normalized against β-actin, calibrated against controls transfected with the empty plasmids, and the comparative C_t value (fold change) was calculated using $2^{-\Delta\Delta C(t)}$. Transcript level comparison of *Tead1*, *Tead2*, *Tead3* and *Tead4* was based on primer efficiency estimated from five-point dilution curves and used for comparative C_t computation according to the PFAFFL method (Pfaffl, 2001).

Alkaline-phosphatase assay

E14/T cells were transfected, puromycin selected and subsequently grown for 72 hours. Cells were fixed with 4% paraformaldehyde (PFA) for 1 minute at room temperature and then stained with Vector Red alkaline phosphatase substrate kit (Vector laboratories) according to the manufacturer's instructions. For the quantification of AP-positive colonies, plates were scanned and analyzed with ImageJ software. In parallel with AP analysis, cells were fixed with 4% PFA for 20 minutes at room temperature and stained with the nuclear stain Hoechst 33342 (Molecular Probes). Cells were mounted with Fluoromount (Sigma) and examined under fluorescent microscope for changes in morphology and nuclear size.

Western blot

Harvested cells were lysed in lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 150 mM NaCl, 0.05% NP40, 0.4 mM PMSF and 1 mM DTT) with the addition of Complete protease inhibitor cocktail (Roche) and 1 mM sodium orthovanadate (Sigma) and subsequently sonicated. Total protein concentration was measured using BCA™ Protein Assay Kit (Pierce) to ensure equal loading and subjected to western blot analysis. Membranes were probed with rabbit anti-phospho-Src family Y416 (1:1000, Cell Signaling), rabbit anti-phospho-YAP S127 (1:1000, Cell Signaling), rabbit anti-YAP (1:1000, Abcam), mouse anti-Yes (1:500, Santa Cruz), mouse anti-phosphotyrosine (1:1000, Millipore), rabbit anti-Yes (1:1000, Cell Signaling), Mouse anti-Stat3 (1:500, Santa Cruz), rabbit anti-phospho-Stat3 T705, rabbit anti-gp130 (1:500, Upstate Biotechnology) and mouse anti-GAPDH (1:2000,

Santa Cruz), followed by horseradish-peroxidase-conjugated secondary antibodies (1:10,000, Santa Cruz). Immunoreactivity was detected by enhanced chemiluminescence (GE Healthcare).

Immunoprecipitation

Protein concentration was measured and 1 mg of protein extract was incubated on ice with 2 μg rabbit anti-YAP or mouse anti-Yes (Cell Signaling) for 90 minutes, followed by capture with 40 μl 1:1 slurry lysis buffer and Immunosorb A (Medicago) for 1 hour at 4°C. Samples were washed four times with ice-cold lysis buffer, mixed with Laemmli loading buffer, boiled for 5 minutes, and analyzed by western blotting as described above.

Generation of GST fusion proteins

Gp130 binding experiments were carried out with ES cell lysates and either GST–SH2-Yes, GST–SH3-Yes, and GST–SH3+unique-Yes fusion proteins immobilized on glutathione–Sepharose beads (Summy et al., 2000). Preparation of bacterial lysates containing the encoded fusion proteins, and the absorption to glutathione–Sepharose beads were performed as follows: 1 ml sonicated and centrifuged bacterial lysates were mixed with 100 μl glutathione–Sepharose 4B (GE Healthcare) for 30 minutes on ice. The beads were washed three times with PBS and 1% Triton X-100. The beads conjugated to fusion proteins were mixed with ES cell lysates for 1.5 hours on ice, washed with PBS containing Triton, resuspended in Laemmli loading buffer and boiled for 5 minutes. The samples were then subjected to western blotting as described above using anti-gp130 (Millipore). Fusion proteins were quantified by staining with Coomassie Blue.

Luciferase reporter assay

24 hours after transfection with equal amounts of total plasmid DNA, including empty expression vectors and the pCMV β-gal reference plasmid containing a bacterial β-galactosidase gene, cells were harvested and lysed, and extracts were assayed for luciferase and β-galactosidase activities in a microplate luminometer and photometer reader (Wallac VICTOR 1420 Multilabel Counter: Perkin Elmer). Results are presented as the mean percentage of the control of at least three independent experiments made in triplicate.

Chromatin immunoprecipitation assay

E14/T DNA and proteins were crosslinked with formaldehyde (final concentration 0.37%) for 10 minutes at room temperature, and the reaction was stopped by the addition of 0.125 M glycine for 5 minutes. Chromatin was sheared in shearing buffer (Diagenode) to an average DNA fragment size of 0.5–1 kb using a Bioruptor sonicator (Diagenode). ChIP assays were performed using One Day ChIP assay kit (Diagenode) according to the manufacturer's protocol. Antibodies used for immunoprecipitation included anti-TEF4 1 polyclonal antibody sc-67115 (Santa Cruz) and IgG (Diagenode) as a negative control. DNA was analyzed by qPCR for various regions of the *Oct-3/4* promoter (primer sequences in supplementary material Table S1) and fold induction over input was calculated using $2^{-\Delta\Delta C(t)}$.

Statistics

At least three independent experiments were performed and data are presented as mean ± s.e.m. When applicable one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test was used to evaluate the statistical significance (*P<0.05) of the difference in values using the GraphPad Prism software.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/7/1136/DC1

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