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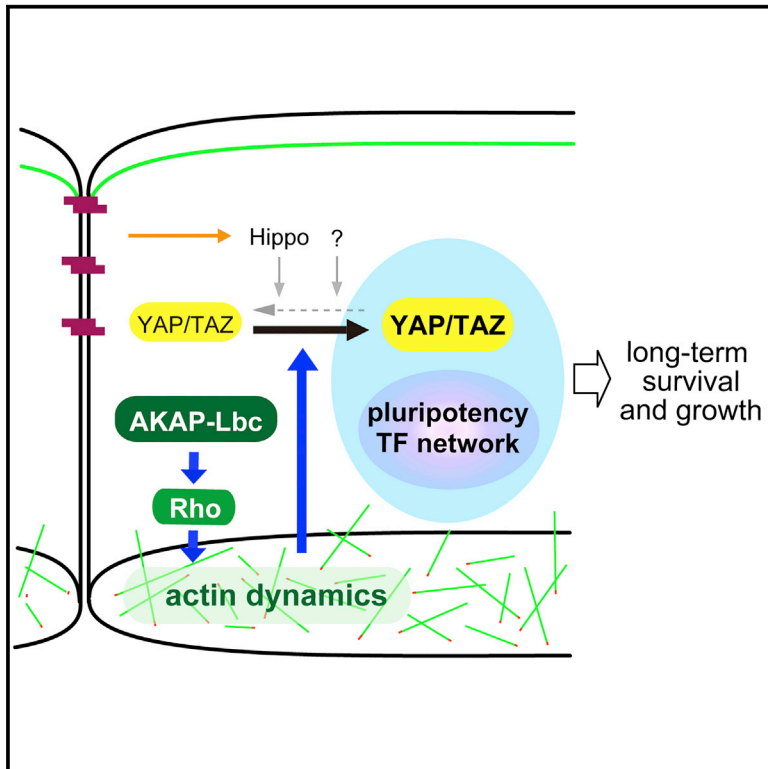
Rho-Signaling-Directed YAP/TAZ Activity Underlies the Long-Term Survival and Expansion of Human Embryonic Stem Cells

Graphical Abstract

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In Brief

Ohgushi et al. show that a regulatory pathway involving Rho, YAP/TAZ, and the actin cytoskeleton that is specific for the pluripotent state enables human embryonic stem cells to undergo long-term survival and expansion in culture.

Highlights

- Rho activation by AKAP-Lbc is required for hESC survival

- Depletion of AKAP-Lbc attenuates YAP/TAZ activity

- Cofilin suppression ameliorates depletion of AKAP-Lbc

- Dependence on AKAP-Lbc is associated with the pluripotent state

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Rho-Signaling-Directed YAP/TAZ Activity Underlies the Long-Term Survival and Expansion of Human Embryonic Stem Cells

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SUMMARY

Human embryonic stem cells (hESCs) can survive and proliferate for an extended period of time in culture, but unlike that of tumor-derived cells, this form of cellular immortality does not depend on genomic aberrations. In this study, we sought to elucidate the molecular basis of this long-term growth property of hESCs. We found that the survival of hESCs depends on the small GTPase Rho and its activator AKAP-Lbc. We show that AKAP-Lbc/Rho signaling sustains the nuclear function of the transcriptional cofactors YAP and TAZ by modulating actin microfilament organization. By inducing reprogramming and differentiation, we found that dependency on this Rho signaling pathway is associated with the pluripotent state. Thus, our findings show that the capacity of hESCs to undergo long-term expansion in vitro is intrinsically coupled to their cellular identity through interconnected molecular circuits that link cell survival to pluripotency.

INTRODUCTION

Embryonic stem cells (ESCs) have the potential to give rise to cells of all three germ layers, a characteristic known as pluripotency (Nichols and Smith, 2012). They provide an excellent in vitro system to understand early mammalian developmental processes including cell differentiation or fate determination. In addition, human pluripotent stem cells, including human ESCs (hESCs) and induced pluripotent stem cells (hiPSCs), hold great potential as replenishable resources for the development of cell therapies and for use in drug discovery (Murry and Keller, 2008). Extensive studies of the molecular basis for the establishment and maintenance of pluripotency have revealed that intrinsic and environmental factors, such as growth factors and cell-cell/cell-matrix signals, coordinate to regulate the pluripotency network by fine-tuning the activity of a defined set of transcriptional factors and epigenetic modifiers (Nichols and Smith, 2012).

ESCs are able to undergo long-term expansion in culture, a phenomenon that is orchestrated by the sustainable proliferation and survival of individual cells. Such "immortality" is observed only in ESCs and cancer cells, and sharply contrasts with primary cultured normal cells, which can be expanded for only limited periods in vitro before undergoing senescence or death (Schmitt, 2003). The in vitro expansion of tumor-derived cells is known to be strictly dependent on multiple genetic mutations, which irreversibly cause misactivation in oncogenes or dysfunction in tumor suppressors. ESCs, on the other hand, exhibit long-term expansion while retaining a normal karyotype. Importantly, ESCs lose this property during differentiation, demonstrating the plasticity of their immortal phenotypes. Although some of the mechanisms that overcome barriers to unlimited growth in mouse ESCs have been reported (Takahashi et al., 2003; Zalzman et al., 2010), the mechanism by which hESCs adapt to ex vivo environments and undergo long-term expansion remains unknown.

We previously reported that the small GTPase Rho promotes cell death of dissociated hESCs via ROCK-dependent myosin hyperactivation (Watanabe et al., 2007; Ohgushi et al., 2010). In the present study, we introduce an unexpected aspect of Rho function, namely that Rho signaling is required for the survival and expansion of hESCs cultured *en bloc*. This raises the possibility that the Rho pathway plays culture context-dependent roles in hESCs. To understand the molecular mechanisms underlying this finding, we sought to answer the following questions: Which aspect of Rho signaling is relevant for survival? Which downstream pathways are involved in the Rho-mediated survival promotion? How is the dependency of hESC survival on Rho signaling related to the pluripotent state?

To characterize the pro-survival activity of the Rho signaling pathway, we performed a candidate functional screen and identified AKAP-Lbc, a Rho activator, as essential for hESC survival. We further identified YAP and TAZ as major downstream factors in the pro-survival Rho signal pathway (YAP and its paralog TAZ are transcriptional cofactors; for simplicity, we refer to them as a YAP/TAZ). We then found that actin dynamics transmit the AKAP-Lbc/Rho signal to regulate YAP/TAZ function. Finally, we found that the dependency of cultured hESCs on Rho signaling is specifically connected to pluripotent cellular identity.

RESULTS

Rho Activity Is Required for hESC Survival

Small GTPases in the Rho subfamily (RhoA, RhoB, and RhoC in mammal) function as molecular switches cycling between guanosine diphosphate (GDP)-bound inactive and guanosine triphosphate (GTP)-bound active forms (Burridge and Wennerberg, 2004). We previously showed that inhibition of ROCK or myosin, downstream effectors of Rho, promotes the survival of dissociated hESCs (Watanabe et al., 2007; Ohgushi et al., 2010). We therefore reasoned that Rho inhibition might have a similar pro-survival effect. Contrary to this expectation, however, we found that treatment of KhES-1 hESCs with a Rho-specific inhibitor C3 (Morii et al., 1990) resulted in massive cell death within 24 hr, even under non-dissociation conditions (Figure 1A and Movie S1, part1). Prior to cell death, cell morphology was strongly altered within several hours (Figures 1A and 1B). Quantitative analyses indicated that after 24 hr treatment with C3, most of the cells became positive for a cell death marker (Figure 1C; representative flow cytometric histogram was shown in Figure S1A). This cell death also occurred in the presence of a ROCK inhibitor (Y-27632; Figure 1C) and in another hESC line (KhES-3; Figure S1B). Notably, overexpression of anti-apoptotic Bcl-X$_L$ effectively inhibited cell death (Figures S1C and S1D), indicating that the response was driven through an active signaling process rather than an uncontrolled cellular collapse.

We next examined the role of Rho in hESC survival more stringently using drug-inducible RNAi-knockdown (see Figures S1F and S1G for the strategy). Following this strategy, we obtained a polyclonal population in which each Rho isoform could be effectively and selectively depleted in an optional timing (Figure 1D; note that RhoB protein is effectively undetectable in our hESC line; Figure S1E). Hereafter, we denote these engineered hESCs as "tet-sh[Target]" (for instance, tet-shRhoA). Doxycycline (dox)-induced depletion of RhoA and RhoC reduced the expansion of alkaline phosphatase (AP)-positive colonies (Figures 1E, 1F, and S1H show the entire view of the stained plate). In particular, RhoC depletion almost totally eliminated growing colonies from the culture. These phenomena were also seen in a feeder-free culture condition (on Matrigel substrate in mTeSR1 media; Figure S1I) and confirmed by time-course quantitative analyses (Figure 1G). Using live imaging, we observed cell death upon RhoC depletion (Figures 1H and S1J for control; quantitative data were shown in Figure 1I; Movie S1, part 2), suggesting that defective colony expansion is caused mainly by cell death rather than by proliferation defects or differentiation.

Thus, Rho activity—that of RhoC in particular—is required for hESC survival. Our previous study showed that E-Cadherin-mediated cell-cell contact prevents cellular Rho activity from being aberrantly activated (Ohgushi et al., 2010). Our new observations suggested the interesting possibility that Rho acts as a survival factor in these conditions.

AKAP-Lbc Is a Rho-GEF Responsible for the Rho-Mediated Survival Signal

The Rho pathway is controlled by the cooperation of a large number of distinct types of modulators, GEFs (activators), GAPs (inactivators), and GDIs (inhibitors) (Burridge and Wennerberg, 2004; Bos et al., 2007). The large diversity of regulators and

effectors may enable context-specific distinct Rho functions. Given the dual role of Rho in cell survival, we speculate that multiple Rho signaling pathways are active in hESCs. We previously found that a specific GEF ABR activates the pro-apoptotic Rho/ROCK/Myosin pathway following cellular dissociation (Ohgushi et al., 2010). With this in mind, we hypothesized the presence of a unique GEF factor that specifically acts in the pro-survival Rho pathway.

We first evaluated the mRNA expression of known Rho isoform-activating GEFs (Rossman et al., 2005) (Figure S2A) and focused on 22 candidates whose expression was detected in both hESC and hiPSC lines. To identify the factor(s) involved in hESC survival, we performed a small-scale RNAi screen (Figure 2A) and found that the expression of a short hairpin (sh) RNA against *AKAP13* strongly suppressed AP$^+$ colony expansion (Figures 2B and S2B), a phenotype that was reversed by co-expression of an active RhoC mutant (Figure S2C). Although it has been reported that *AKAP13* produces several Lbc protein isoforms (Olson et al., 1997; Diviani et al., 2001; Figure S2D), western blotting analyses revealed that hESCs mainly expressed one AKAP-Lbc isoform (Figure S2E). The reliability of this screening result was confirmed by the drug-inducible RNAi strategy described above (Figures 2C, 2D, 2F, and S2G; in figures, the engineered hESCs are labeled as a "tet-shLbc", instead of the full protein name AKAP-Lbc). Similar phenotypes were observed when another shRNA was used and were seen in another hESC line and in feeder-free culture (Figures S2F–S2H). The expansion defect was also verified by time-course growth analyses (Figure 2F). Similar to the case of RhoC, loss of AKAP-Lbc caused cell death that could be reversed by Bcl-X$_L$ overexpression (Figures 2G and 2H and Movie S2), indicating that this GEF factor is required for hESC survival.

AKAP-Lbc is a very large protein (over 2,800 amino acids) containing a Rho-GEF domain (DH/PH) and a PKA-anchor domain (Figure S2C) (Diviani et al., 2006). To examine the relationship between AKAP-Lbc and Rho, we performed rescue experiments. Overexpression of an RNAi-resistant AKAP-Lbc mutant (AKAP-Lbc*) substantially suppressed cell death in AKAP-Lbc-depleted hESCs, but a GEF-dead mutant form (AKAP-Lbc*(YF); Diviani et al., 2001) had little effect (Figures S2I and S2J), indicating that AKAP-Lbc controls hESC survival by activating the Rho-mediated signal pathway.

Immunostaining analyses showed that AKAP-Lbc is distributed in the cytosol in a punctate pattern (Figures 2I, 2J, S2L, and S2K show antibody specificity). Structured illumination microscope (SIM) images showed that some of AKAP-Lbc proteins partially colocalize with F-actin (Figure 2K), as demonstrated in previous studies (Olson et al., 1997; Diviani et al., 2001). z axis scanning along the apical-basal axis using confocal imaging indicated that AKAP-Lbc distribution is biased to the basal side, with little signal at the apical (Figures 2L and S2M and Movie S3).

Collectively, these data suggest that AKAP-Lbc, a basally localized Rho-GEF, is responsible for the survival-supporting axis of Rho signaling.

Transcriptional Cofactor YAP/TAZ Is Attenuated in AKAP-Lbc-Depleted hESCs

To search for the specific intracellular pathways that are affected by AKAP-Lbc-depletion, we compared gene expression in
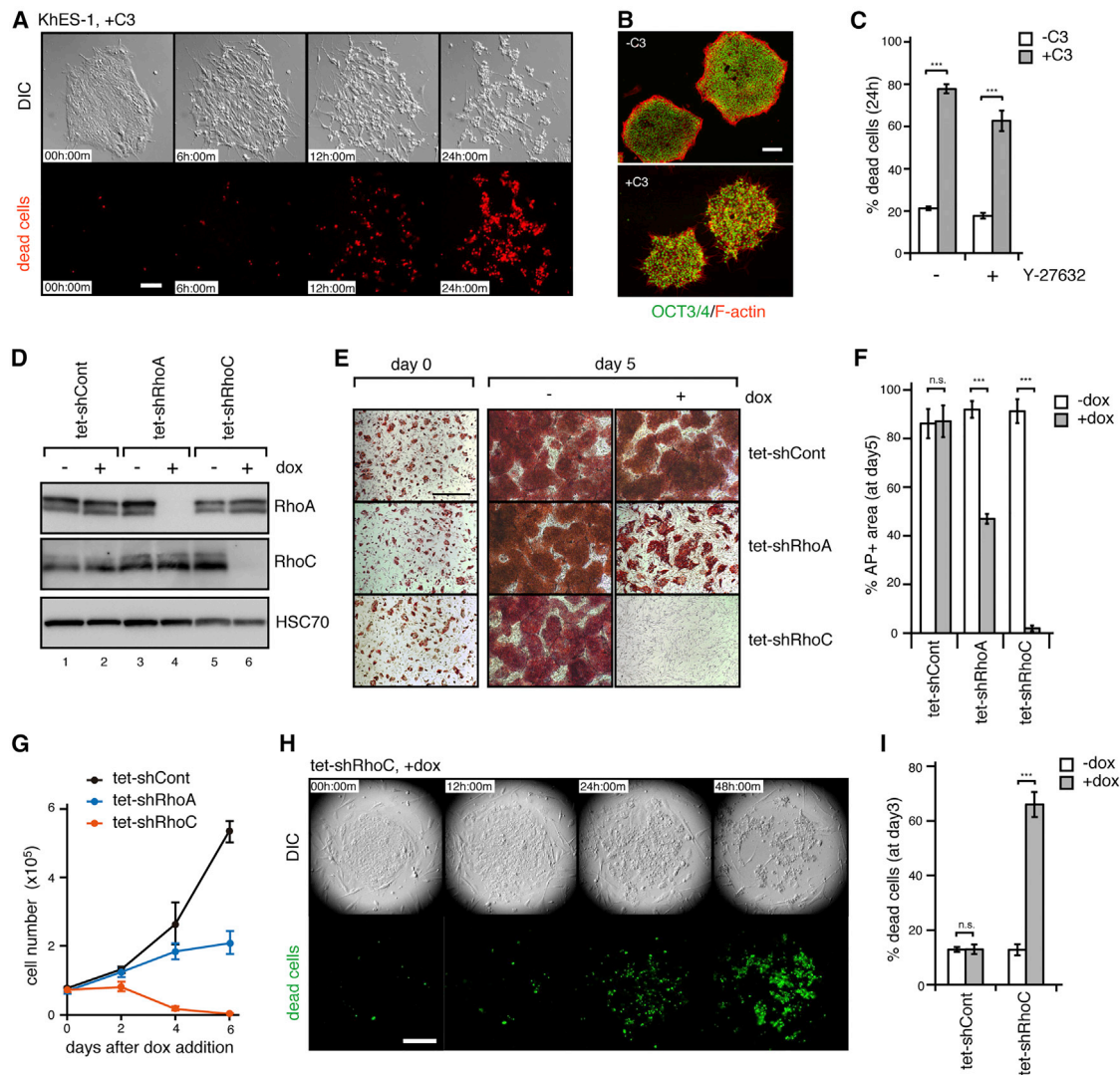
Figure 1. Rho Activity Is Required for hESC Survival

(A) Live imaging analyses of KhES-1 cells treated with C3 (2 μg/ml) for 24 hr. The dead cells were visualized with Propidium iodide uptakes (red, bottom). The recording was started immediately after the dox addition. The scale bar represents 100 μm.

(B) Immunostaining of KhES-1 cells treated with or without C3 for 8 hr. The OCT3/4 (green) and F-actin (red) were shown. The scale bar represents 200 μm.

(C) Cell death assay. The KhES-1 cells were cultured with or without C3 for 24 hr and the percentage of DRAQ7-positive dead cells was determined by flow cytometric analyses.

(D) Western blotting analyses. The engineered KhES-1 cells, indicated as tet-shCont (for control), tet-shRhoA (for inducible depletion of RhoA), and tet-shRhoC (for inducible depletion of RhoC) were cultured with or without dox for 3 days. The HSC70 was examined as a loading control.

(E) Colony expansion assay. The left images show colony morphology before the dox addition (day 0). The right images were obtained after 5 days culture in the presence or absence of dox (day 5). The colonies were visualized by AP staining. The scale bar represents 500 μm.

(F) Area occupancy of AP positive colonies within each stained well at day 5.

(G) Time course quantitation of living cells after dox addition. In this case, cells were maintained on Matrigel with MEF-conditioned medium.

(H) Live imaging of dox-treated tet-shRhoC KhES-1 cells. The dead cells were visualized with YOYO-1 uptakes (green, bottom). The recording was started from 24 hr after dox addition. The scale bar represents 200 μm.

(I) Cell death assay. The indicated cells were cultured with or without dox for 3 days.

The bars in the graphs represent SD (Student's t test, n = 3, n.s., not significant, and ***p < 0.001 in C, F, and I). See also Figure S1 and Movie S1.

control and AKAP-Lbc-depleted hESCs by RNA-sequencing analysis (Figure 3A). Taking into account the concern that several pathways could be perturbed by death-associated secondary effects, masking primary responses, we overexpressed Bcl-X$_L$ to preserve the transcriptional profile that exists before catastrophic activation of a cytolethal program in the AKAP-Lbc-depleted population. From this analysis, we identified 166 genes that were significantly up (Log2 fold > 2) or downregulated (Log2 fold < −2) after AKAP-Lbc-depletion (Figure 3B; Table S1). Looking at the downregulated genes, we found that two of the top ten genes with the highest fold-change, *CTGF* and *AMOTL2*, were known target genes of the transcriptional cofactor YAP/TAZ
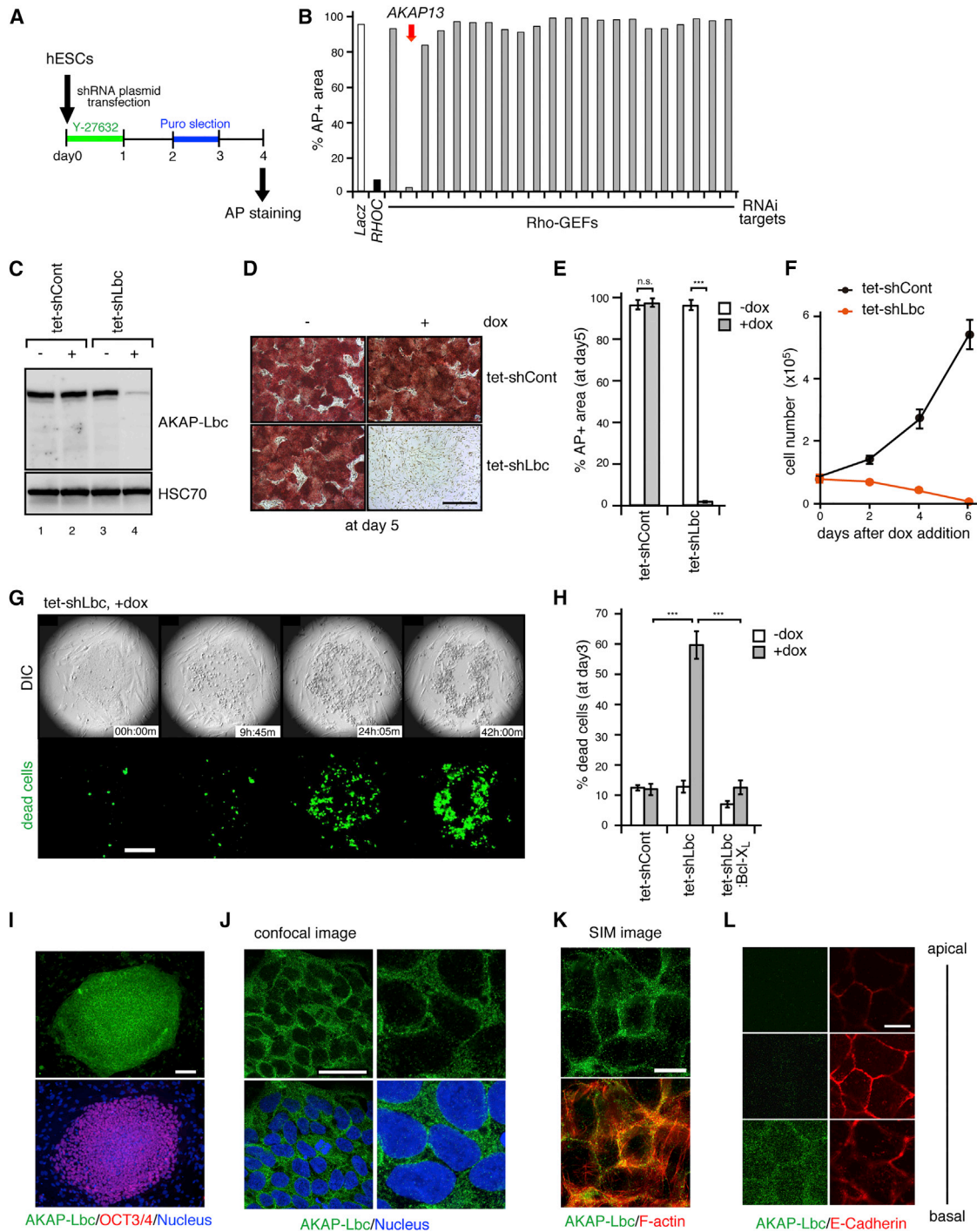
Figure 2. AKAP-Lbc Is a Rho-GEF Essential for hESC Survival

(A) Schematic of the RNAi screening experiments. This procedure was based on the prediction that depletion of responsible GEF may phenocopy the Rho inhibition. The cells were transiently transfected with each shRNA plasmid. After eliminating non-transfected cells by puromycin treatment, surviving cells were visualized by AP staining.

(B) Screening result is summarized by area occupancy of the AP-positive cells. The LacZ or RhoC knockdown was a negative and positive control, respectively.

(C) Western blotting analyses. The tet-shCont and tet-shLbc KhES-1 cells (for inducible depletion of AKAP-Lbc) were cultured with or without dox for 3 days. The HSC70 was examined as a loading control.

(D and E) Colony expansion assay (D) and area occupancy of AP-positive colonies (E). The scale bar represents 500 μm.

(F) Time course quantitation of living cells after dox addition.

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(Zhao et al., 2008; Mohseni et al., 2014). Quantitative (q)PCR analyses validated the decreased expression of *CTGF* and *AMOTL2*, as well as *CYR61*, another representative target gene (Lai et al., 2011) (Figure 3C). These data suggest that there may be YAP/TAZ dysfunction in AKAP-Lbc-depleted cells.

YAP/TAZ has been shown to promote cell proliferation and survival in several experimental systems, so we regarded these factors as good candidates for downstream effectors in the putative survival-promoting signaling pathway. In hESCs, YAP/TAZ is predominantly distributed in the nucleus (Figures S3A–S3C) and associated with TEAD1, a nuclear partner of YAP/TAZ (Figure S3D), consistent with active function as transcriptional cofactors. Next, we examined whether AKAP-Lbc-depletion affects YAP/TAZ behavior. Western blot analyses showed that protein levels of both YAP and TAZ were reduced in AKAP-Lbc-depleted cells (Figure 3D). In addition to transcriptional downregulation, there may have been enhanced protein degradation, as TAZ protein levels were restored in the presence of a proteasome inhibitor (Figures S3E and S3F). In addition, the residual YAP/TAZ was delocalized and showed reduced nuclear localization (Figures 3E, 3F, S3G, and S3H). Identical results were obtained in another hESC line (Figures S3I and S3J). These data indicate that this GEF is required for the function of YAP/TAZ as transcriptional cofactors.

To investigate whether YAP/TAZ has any impact on hESC survival and expansion, we performed RNAi-mediated loss-of-function experiments for YAP/TAZ (Figures 3G, S3K, and S3L show the functional control experiments for our double knockdown strategy). Depletion of either YAP or TAZ alone partially reduced colony expansion. In particular, YAP depletion had only a marginal effect. However, simultaneous depletion of both proteins caused a dramatic decrease in colony expansion (Figures 3H, 3I, and S3H) that was reproduced in other experimental setups (Figures S3N–S3P). YAP/TAZ double knockdown caused cell death that was rescued by Bcl-X$_L$ (Figures 3J and 3K and Movie S4), as well as an RNAi-resistant TAZ (Figure S3Q).

These results indicate that YAP/TAZ is functionally compromised in AKAP-Lbc-depleted hESCs, which may explain the defective survival and expansion of these cells.

YAP/TAZ Attenuation Is a Major Cause for the Impaired Survival of AKAP-Lbc-Depleted hESCs

To ascertain whether YAP/TAZ attenuation was a direct cause of the cell death, rather than merely an unrelated epiphenomenon or an artifact of the death-suppressive conditions, we performed rescue experiments by promoting YAP/TAZ activity.

YAP/TAZ attenuation is often correlated with activation of the Hippo signaling pathway (Yu and Guan, 2013). This evolutionarily conserved pathway activates the kinases LATS1 and LATS2

(hereafter, they are referred together as LATS). Activated LATS phosphorylates YAP/TAZ at several residues, priming YAP/TAZ for inhibition by at least two mechanisms: cytoplasmic sequestration by anchoring proteins such as 14-3-3, which is triggered by N terminus phosphorylation, and proteolytic degradation, which is primed by phosphorylation at the C termini (Figure 4A; Yu and Guan, 2013). We examined the magnitude of Hippo pathway activation in AKAP-Lbc-depleted cells and found that substantial elevation of YAP, MST1/2, or LATS1/2 phosphorylation was not observed (Figure 4B). Consistent with this, an in vitro kinase assay revealed that LATS activity is not substantially altered (Figure 4C). These results indicate that AKAP-Lbc-depletion does not elevate LATS activity or, alternatively, that LATS is already active before AKAP-Lbc-depletion.

To address whether this observed LATS activity affected the attenuation of YAP/TAZ, we simultaneously depleted AKAP-Lbc and LATS (Figure 4D; triple knockdown strategy was shown in Figure S4A). As expected, the YAP phosphorylation level was reduced by LATS1 and LATS2 double knockdown (Hippo-OFF condition, see below). In a colony expansion assay, LATS inhibition caused no significant increase relative to AKAP-Lbc-depleted cells (Figures 4E, 4F, and S4B). Consistently, YAP/TAZ delocalization and dysfunction were not substantially reversed by reducing LATS expression (Figures 4G and S4D). Thus, YAP/TAZ activity was still depressed even without priming by LATS-mediated phosphorylation, indicating that YAP/TAZ attenuation in AKAP-Lbc-depleted hESCs occurs in a LATS-independent manner.

Because YAP/TAZ function is not restored by derepression strategies, including LATS knockdown (Figures 4E and 4F) or overexpression of LATS-insensitive TAZ (Figure S4C), we next performed a direct gain-of-function study of TAZ (TAZ has more prominent effects on hESC survival than YAP, Figure 3I). To achieve enforced TAZ activation, we utilized ER^{T2}-TAZ, a fusion molecule that can be localized to the nucleus by 4-hydroxytamoxifen (4OHT) exposure (Figures S4E and S4F; Indra et al., 1999). We confirmed that 4OHT promotes nuclear accumulation of ER^{T2}-TAZ and transcriptional activation of *CTGF* and *CYR61* (TAZ-ON condition, Figures S5G and S5H). However, when 4OHT was added, only a few small AP+ colonies formed in the AKAP-Lbc-depleted cells (Figures 4H, 4I, and S4I).

Considering the possible inhibitory function of LATS in TAZ activation, we next tested TAZ gain of function combined with LATS loss of function by expressing TAZ or ER^{T2}-TAZ in the tet-shLbc/Lats hESCs (TAZ-ON/Hippo-OFF). Interestingly, a simple overexpression of TAZ under the Hippo-OFF condition resulted in a subtle, but significant, rescue on AP+ colony expansion. More importantly, forced TAZ activation using ER^{T2}-TAZ + 4OHT showed a more dramatic increase in colony expansion (Figures

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(G) Live imaging analyses of dox-treated tet-shLbc KhES-1 cells. The dead cells were visualized with YOYO-1 uptakes (green, bottom). The recording was started from 24 hr after dox addition. The scale bar represents 200 μm.

(H) Cell death assay. The indicated cells were treated with dox or not for 3 days. The Bcl-X$_L$-expressing tet-shLbc KhES-1 cells (referred as a tet-shLbc:Bcl-X$_L$) were also examined.

(I–L) Immunostaining for AKAP-Lbc. (J) Confocal images. (K) Super-resolution SIM images. Both images show medial sections. (L) Z-scanning images along apical-to-basal axis. The top, middle, and bottom represent most-apical, sub-apical, and most-basal sections, respectively. The AKAP-Lbc (green in all images), OCT3/4 (red in I), F-actin (red in K), and E-cadherin (red in L) were shown. The scale bars show 100 μm (I), 50 μm (J), and 10 μm (K and L), respectively.

The bars in the graphs represent SD. The Student's t test (n = 3) (E); Dunnett's test (n = 3) versus lane 2 (H); not significant (n. s.); and ***p < 0.001. See also Figure S2 and Movies S2 and S3.
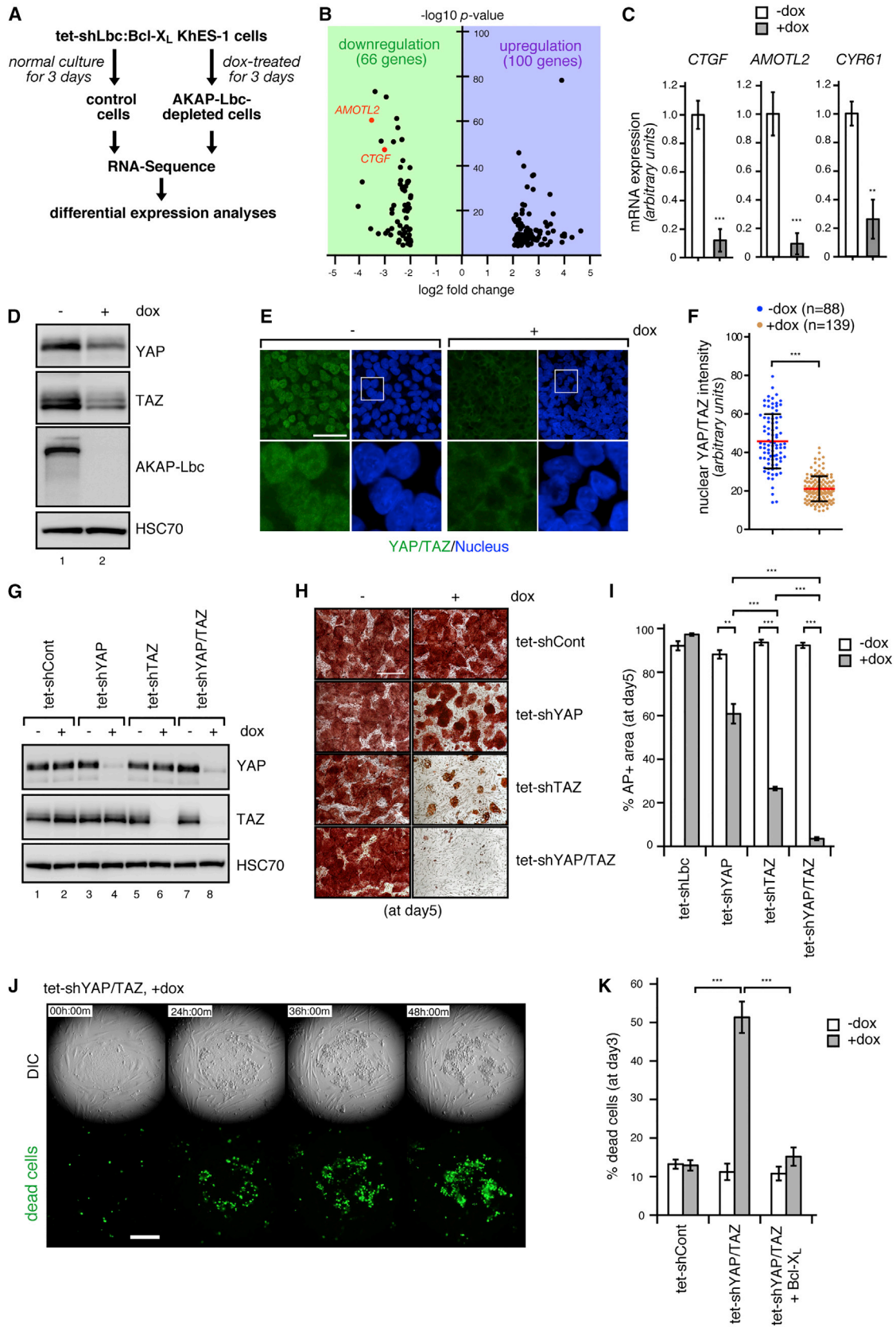
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4J, 4K, and S5J). Consistent with these results, total and nuclear abundance of ER^{T2}-TAZ recovered nearly to the control level under the TAZ-ON/Hippo-OFF condition (data not shown). Thus, forced TAZ activation rescues AKAP-Lbc-depletion-induced phenotypes when supported by Hippo pathway inactivation.

In conclusion, our data provide strong evidence that YAP/TAZ attenuation plays a major role in the impaired survival of AKAP-Lbc-depleted hESCs.

Role of the Hippo Pathway in YAP/TAZ Control

Some of the results regarding interaction between LATS and YAP/TAZ activity are somewhat difficult to reconcile. LATS inhibition failed to restore YAP/TAZ function in AKAP-Lbc-depleted cells (Figures 4E and 4G), clearly indicating that YAP/TAZ is inhibited by a LATS-independent mechanism in this experimental condition. However, LATS knockdown is required for TAZ-mediated rescue of AKAP-Lbc-depleted cells (Figures 4J and 4K), suggesting that LATS does nevertheless counteract ER^{T2}-TAZ nuclear accumulation in this context. These results may reflect the existence of multiple redundant regulatory mechanisms for YAP/TAZ regulation in hESCs.

Notably, contact-dependent LATS activation (Figure S4L) and LATS-mediated YAP phosphorylation (Figure S4K) were clearly detected, suggesting that the Hippo kinase cascade is activated in growing hESC colonies to a level sufficient for this to occur. In this regard, we noted an intriguing behavior of YAP in hESCs; despite the expectation that phosphorylated YAP is excluded from nuclei, immunostaining using a phospho-YAP antibody exhibited nuclear signals as well as faint cytosolic ones (Figures S4M and S4N show biochemical analyses). Thus, LATS phosphorylates YAP/TAZ, but does not impede its nuclear localization unless the AKAP-Lbc/Rho pathway is inhibited, suggesting that LATS is a modulator, but not a main determinant, of YAP/TAZ function in this context.

Taken together, our data indicate that the AKAP-Lbc/Rho pathway regulates YAP/TAZ behavior via a LATS-independent mechanism.

The Contribution of F-Actin Dynamics in AKAP-Lbc/Rho-Mediated Survival Signal Transduction

Next, we sought to identify the mechanistic connection between AKAP-Lbc/Rho and YAP/TAZ. Recent studies suggested micro-filaments as a strong candidate (Dupont et al., 2011; Wada et al., 2011; Zhao et al., 2012; Aragona et al., 2013). Of interest, impressive morphological alterations occurred in death-suppressed hESCs after AKAP-Lbc was depleted (Figures 5A and 5C). Although hESC colonies show a typical epithelial-like morphology polarized along the apical-to-basal axis (Figure S5A), upon AKAP-Lbc-depletion, irregular gaps spontaneously arose inside the hESC epithelial sheet, leading the colony to split into several fragments (Figure 5A and Movie S5, part 1). In these fragmented colonies, epithelium-characteristic microfilament structures (e.g., apical actin bundle) disappeared and they piled up into a disorganized cell mass (Figures 5B and 5C and Movie S5, part 2). To study this process in more detail, we focused on F-actin organization in the basal area, as AKAP-Lbc localization has a basal bias and it may be associated with actin filaments (Figures 2K and 2L). Immunostaining showed numerous thick stress fibers with tips that were anchored to focal adhesions (FAs, indicated by p-Paxillin$^+$) in dox-untreated cells (Figure 5D, left). After AKAP-Lbc-depletion, the actin fibers were thinner and more disorganized and showed much less FA anchoring (Figure 5D, right). Using live imaging, we found that the well-developed actin meshwork was gradually shifted to the less contractile fibers (Figure 5E and Movie S5, part 3).

To examine the relationship between actin dynamics, YAP/TAZ, and hESC expansion, we tested simultaneous depletion of AKAP-Lbc and cofilin, a major F-actin severing factor (Figure 5F) (Aragona et al., 2013). Immunostaining showed that cofilin codepletion rescues stress fiber formation and FA-dependent signal initiation (Figures 5G and S5B). Interestingly, the impaired colony expansion in AKAP-Lbc-depleted cells was partially reversed by cofilin suppression (Figures 5H, 5I, and S5C). Consistent with this result, the reduced transcription of YAP/TAZ target genes and the decreased nuclear YAP/TAZ is also restored significantly (Figures 5J and 5K). These data suggested a causal relationship between altered actin dynamics, YAP/TAZ attenuation and impaired hESC expansion. Actin dynamics thus provides a mechanistic link connecting AKAP-Lbc/Rho signaling to YAP/TAZ nuclear function.

Taken altogether, these data indicate that a Rho-mediated survival pathway that consists of an AKAP-Lbc/Rho/F-actin molecular cascade supports hESC survival and expansion by promoting nuclear activity of YAP/TAZ (Figure 5L).

Figure 3. Transcriptional Cofactor YAP/TAZ Is Attenuated in AKAP-Lbc-Depleted hESCs

(A) Schematic of RNA-seq analyses. The Bcl-X$_L$-expressing cells (tet-shLbc:Bcl-X$_L$) were used to preserve the AKAP-Lbc-depleted population.

(B) Genes identified by the RNA-seq experiment. The two red dots show *AMOTL2* and *CTGF*.

(C) qPCR assay for YAP/TAZ target genes. The tet-shLbc:Bcl-X$_L$ KhES-1 cells were treated with dox or not for 3 days.

(D) Western blotting analyses for YAP/TAZ in control and AKAP-Lbc-depleted KhES-1 cells. The HSC70 was examined as a loading control.

(E) The tet-shLbc:Bcl-X$_L$ KhES-1 cells were treated or not with dox for 3 days and stained with an antibody that recognizes both YAP and TAZ (green). The images were obtained with a confocal microscopy. The bottom images show the enlarged views squared in the upper images. The scale bar represents 50 μm.

(F) Quantification of nuclear YAP/TAZ abundance by confocal image data processing.

(G) Western blotting analyses. The engineered KhES-1 cells, indicated as tet-shCont, tet-shYAP (for inducible depletion of YAP), tet-shTAZ (for inducible depletion of TAZ), and tet-shYAP/TAZ (for simultaneous inducible depletion of YAP and TAZ), were treated or not with dox for 3 days. The HSC70 was examined as a loading control.

(H and I) Colony expansion assay (H) and area occupancy of AP-positive colonies (I). The scale bar represents 500 μm.

(J) Live imaging analyses of tet-shYAP/TAZ KhES-1 cells during 24–72 hr period of dox-treatment. The dead cells were visualized with YOYO-1 uptakes (green, bottom). The scale bar represents 200 μm.

(K) Cell death assay. The indicated cells were cultured with or without dox for 3 days. The Bcl-X$_L$-expressing cells (referred as a tet-shYAP/TAZ:Bcl-X$_L$) were also examined.

The bars in the graphs represent SD. Student's t test (n = 3) (C and F); Durnett' test (n = 3) versus lane 2 (I); Tukey's test (n = 3) among all groups (K); **p < 0.01; and ***p < 0.001. See also Figure S3 and Movie S4; Table S1.
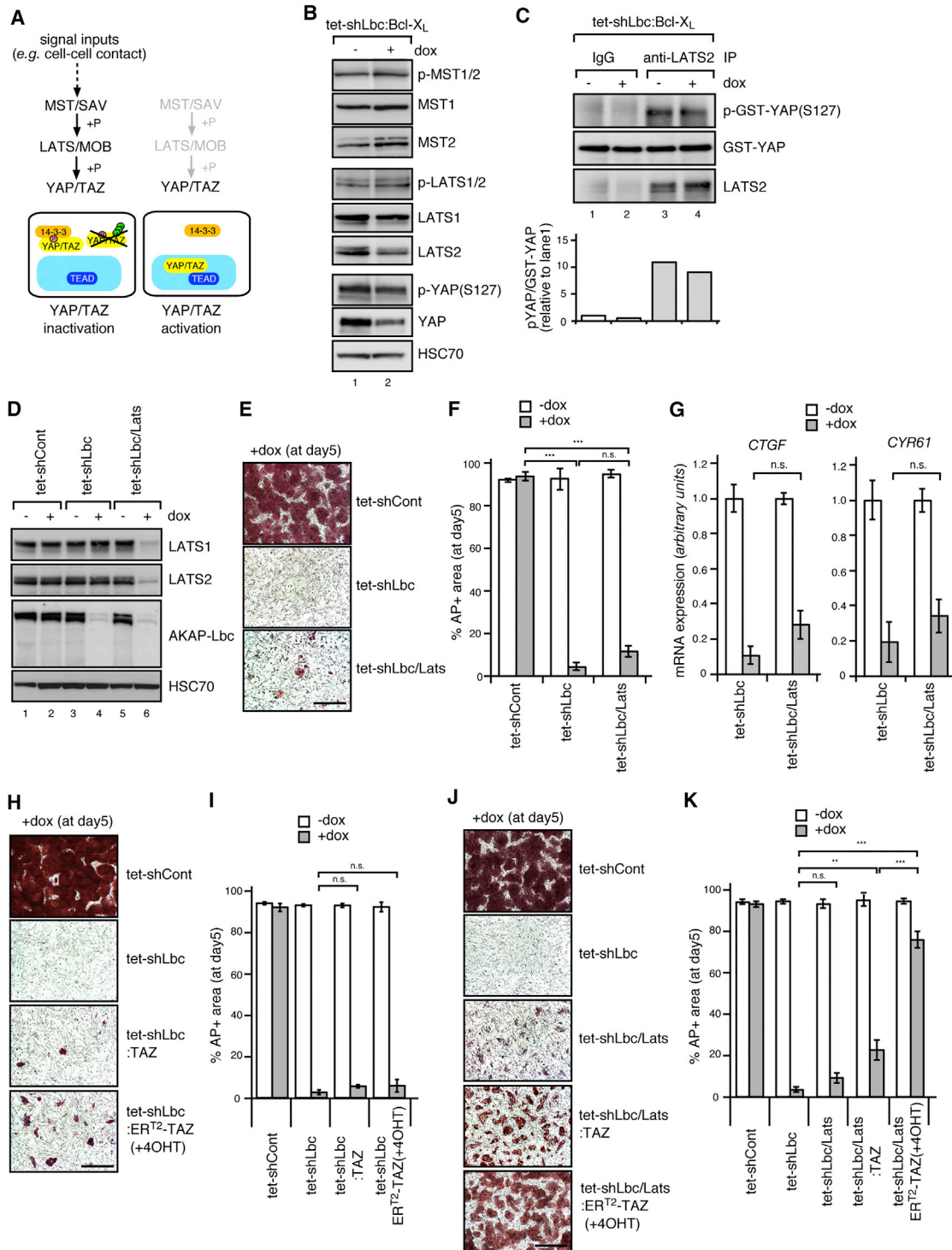
Figure 4. Hippo-OFF and TAZ-ON Experiments on AKAP-Lbc-Depleted hESCs

(A) Overview of the Hippo-mediated inhibition of YAP/TAZ. The Hippo-pathway activation results in LATS-mediated YAP/TAZ phosphorylation. The phosphorylated form of YAP/TAZ is sequestered in cytosol or degraded by proteasome.

(B) Western blotting analyses of components of the Hippo pathway. The tet-shLbc:Bcl-X$_L$ KhES-1 cells were cultured with or without dox for 3 days. The HSC70 was shown as a loading control.

(C) In vitro kinase assay for endogenous LATS2. As a negative control, immunoprecipitation was performed using an IgG antibody. The quantitative changes in YAP phosphorylation were determined by each signal intensity and were shown as relatives to lane 1.

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Coordination of the Rho-Mediated Pathway and Molecular Circuit of hESCs

Having shown that hESCs depend on the AKAP-Lbc/Rho pathway for survival, we sought to investigate the potential relevance of this phenotype for the pluripotent stem cell state.

By profiling the expression of key factors of the Rho-survival signaling pathway, AKAP-Lbc, RhoC, and YAP/TAZ, in several human cell lines, we found that they are present in all cell lines examined (Figure S6A). We also confirmed that their expression exhibited minor fluctuation during differentiation of pluripotent cells into either endoderm or neuroectoderm lineages (Figure S6B). Thus, expression of these factors does not appear to be restricted to pluripotent cell types. However, the cellular responses to AKAP-Lbc-depletion were dramatically different: cell death occurred in human iPSC (253G1) and mouse ESC (EB5) lines, but not in human carcinoma HeLa cells or human dermal fibroblasts (HDFs) (Figures 6A–6D). This suggests that dependence on AKAP-Lbc/Rho signaling is a specific characteristic of ESCs/iPSCs, but is not determined by cell-type specific transcriptional control of pathway components.

How then is this cell-type specificity accomplished? We hypothesized that cell identity is relevant and tested this possibility by shuffling cell identity via gain- or loss-of-pluripotency experiments. In the gain-of-pluripotency studies, HDFs were converted to a hESC-like state by cellular reprogramming methods (Figures 6E and S6D), while in the loss-of-pluripotency assay, hESCs were differentiated into retinal pigmented epithelial-like cells (RPELCs), as a representative example of hESC-derived differentiated somatic cells (Figures 6F, S6G, and S6H). Interestingly, HDFs became vulnerable to AKAP-Lbc-depletion-induced cell death after they were reprogrammed (Figures 6G, S6E, and S6F), while the hESC-derived somatic cells could survive when AKAP-Lbc was depleted (Figure 6I). To further characterize the apparent correlation between cellular identity and survival dependency on this Rho pathway, AKAP-Lbc was depleted during a specific period of reprogramming process (Figure S6I), showing that AKAP-Lbc-depletion during the later phase (day 8 onward) substantially impaired the emergence of AP+ colonies (Figures 6H and 6I). These findings provide evidence that cellular differentiation state, controls the phenotypic response to AKAP-Lbc-depletion.

Together, these data suggest that human pluripotent stem cell survival is intrinsically directed by the coordinated action of an AKAP-Lbc/Rho pathway and the pluripotent cell state (Figure 6J). The hESC-specific transcription factor circuit, which is already regarded as critical for pluripotency maintenance, may also play an important role in conferring immortality on hESCs.

DISCUSSION

Rho-Mediated Survival Signaling Pathway for hESC Expansion

Sustained growth in vitro is a characteristic of hESCs. In this study, we showed that Rho activity is essential for this capacity of hESCs when they are grown as colonies. This finding may seem somewhat paradoxical considering the known pro-apoptotic function of Rho/ROCK signaling in dissociated hESCs (Ohgushi et al., 2010). A striking difference, however, between these two pathways is the GEF factors involved: ABR for pro-apoptotic signaling in dissociated cells and AKAP-Lbc for pro-survival signaling in colonies, which function non-redundantly in the two different situations. In addition, ROCK has no effect on hESC viability in colonies, and forced activation of TAZ (LATS knock down + nuclear TAZ overexpression) does not prevent dissociation-induced cell death (data not shown). Thus, two functionally distinct Rho pathways seem to be independently activated in hESCs depending on the intercellular adherence status (clumping versus dissociation) and the presence or absence of connecting neighbor cells.

When looking to understand the downstream components of the Rho-mediated survival pathway, we identified a role of sustained nuclear activity of YAP/TAZ, transcriptional cofactors known as effector of the Hippo pathway. Consistent with the cell contact-induced activation model, the Hippo pathway was activated within hESC colonies and phosphorylated YAP/TAZ in a contact-dependent fashion. Nevertheless, YAP/TAZ was shown to be functionally active in this context, indicating an unexpected decoupling of LATS-mediated phosphorylation and YAP/TAZ function in which, although it is always primed for inactivation, YAP/TAZ is able to escape from cytosolic sequestration and degradation. A series of loss- and gain-of function studies suggested that AKAP-Lbc/Rho signals render YAP/TAZ refractory to these regulatory surveillances. We showed that the AKAP-Lbc/Rho/F-actin molecular cascade functions as a deterministic upstream controller of YAP/TAZ and is dominant over other counteracting upstream signaling, including cell-contact/Hippo signals (Figure 5L). Recent related works revealed that YAP/TAZ influenced cytoskeleton architecture (Morikawa et al., 2015; Porazinski et al., 2015). In addition, it has been reported that in some situations LATS regulates polarized accumulation of F-actin (Fernández et al., 2011; Lucas et al., 2013). Thus, the Hippo pathway, the cytoskeleton, and YAP/TAZ may form a complex molecular network via multilayered interactions with feedback mechanisms. How these are integrated to allow YAP/TAZ nuclear accumulation and sustained hESC expansion within high-density colonies, an unfavorable condition for cell survival and proliferation, remains an interesting question for future work.
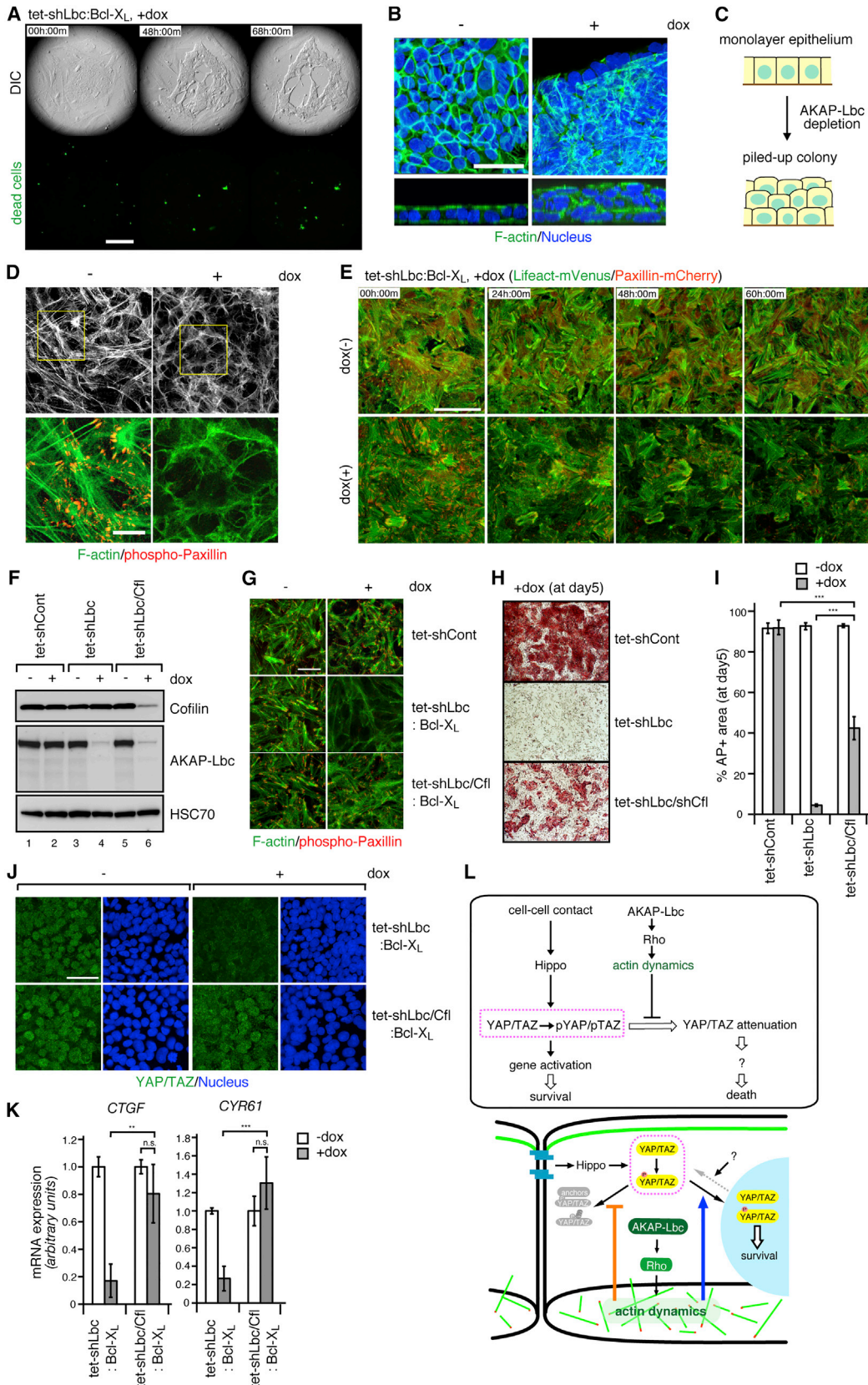
(D) Western blotting analyses. The engineered KhES-1 cells, referred to as tet-shCont, tet-shLbc, and tet-shLbc/Lats (for triple inducible depletion of AKAP-Lbc, LATS1, and LATS2), were cultured with or without dox for 3 days. The HSC70 was shown as a loading control.

(E and F) Colony expansion assay (E) and area occupancy of AP-positive colonies (F). The scale bar represents 500 μm.

(G) qPCR assay for the YAP/TAZ target genes. The indicated cells were cultured with or without dox for 3 days.

(H–K) Colony expansion assays. The tet-shLbc (H and I) or tet-shLbc/Lats KhES-1 cells (J and K) were engineered to express a wild-type or an ER^{T2}-fused TAZ (indicated as tet-shLbc:TAZ, tet-shLbc:ER^{T2}-TAZ, tet-shLbc/Lats:TAZ, and tet-shLbc/Lats:ER^{T2}-TAZ, respectively). For TAZ activation, 4-OHT (1 μM) was added from day 2 to 5. The scale bar represents 500 μm. (I and K) Area occupancy of AP-positive colonies.

The bars in the graphs represent SD. Tukey's test (n = 3) among all groups (F, G, I, and K); not significant (n. s.); **p < 0.01; and ***p < 0.001. See also Figure S4.

A tet-shLbc:Bcl-X$_L$, +dox

DIC

dead cells

B — + dox

F-actin/Nucleus

C monolayer epithelium

AKAP-Lbc depletion

piled-up colony

D — + dox

F-actin/phospho-Paxillin

E tet-shLbc:Bcl-X$_L$, +dox (Lifeact-mVenus/Paxillin-mCherry)

dox(-)

dox(+)

F

tet-shCont / tet-shLbc / tet-shLbc/Cfl

- + / - + / - + dox

Cofilin

AKAP-Lbc

HSC70

1 2 3 4 5 6

G — + dox

tet-shCont

tet-shLbc : Bcl-X$_L$

tet-shLbc/Cfl : Bcl-X$_L$

F-actin/phospho-Paxillin

H +dox (at day5)

tet-shCont

tet-shLbc

tet-shLbc/shCfl

I

□ -dox ■ +dox

% AP+ area (at day5)

tet-shCont / tet-shLbc / tet-shLbc/Cfl

J — + dox

tet-shLbc :Bcl-X$_L$

tet-shLbc/Cfl :Bcl-X$_L$

YAP/TAZ/Nucleus

K *CTGF*   *CYR61*

mRNA expression (arbitrary units)

□ -dox ■ +dox

tet-shLbc : Bcl-X$_L$ tet-shLbc/Cfl : Bcl-X$_L$

L

cell-cell contact   AKAP-Lbc

↓   Rho

Hippo   actin dynamics

YAP/TAZ → pYAP/pTAZ ⊣ YAP/TAZ attenuation

↓   ⇓

gene activation   ?

⇓   ⇓

survival   death

Hippo → YAP/TAZ

anchors YAP/TAZ

AKAP-Lbc

Rho

actin dynamics

survival

(legend on next page)

Plastic Immortality in Culture

In this study, we showed that a specific Rho signaling pathway governs hESC survival. Dependency on this signaling pathway for survival seems to be restricted to pluripotent cells. In addition, mouse genetic studies revealed that neither *Akap13* nor *Rhoc* is required for survival of pluripotent cells in vivo (Hakem et al., 2005; Mayers et al., 2010). This finding supports the hypothesis that the molecular pathways presented here are specifically required for PSC survival in vitro.

In general, cells derived from animals are difficult to grow and maintain in vitro, as they readily undergo senescence or death in culture. However, ES and tumor cells are special exceptions to this rule. In contrast to tumor-derived cells, ESCs show characteristic reversibility of their immortality during differentiation. As YAP/TAZ is a potent driver of proliferation and survival, we reasoned that sustained nuclear activity of YAP/TAZ, which can otherwise drop in response to various stimuli (e.g., cytoskeletal reorganization), represents one critical mechanism ensuring the reversible immortalized phenotype of hESCs. On the other hand, YAP/TAZ are considered as "stemness factors" in several types of stem cells (Tremblay and Camargo, 2012) and in ESCs they have been incorporated into the transcriptional circuit for pluripotency (Varelas et al., 2008; Lian et al., 2010; Beyer et al., 2013, and our unpublished data). Notably, when cell death is circumvented, prolonged AKAP-Lbc-depletion causes correlated changes in differentiation-associated marker expression (data not shown), suggesting the interesting possibility that the AKAP-Lbc/Rho signaling modulates the integrity of self-renewal and survival competency by regulating the dual action of YAP/TAZ as stemness and survival factor. Within this molecular framework, YAP/TAZ stands at the crossroads of two essential modules underlying hESC identity: a Rho-mediated survival signal and a transcriptional network supporting the maintenance of pluripotency (Figure 6J). In this system, the survival signal input branches to stabilize the network, while the stemness-associated circuit underpins YAP/TAZ-driven expansion of these untransformed cells. This coordinated feedback may serve to safeguard the robustness of these striking properties of hESCs.

Whether the ESC immortality is acquired property during their derivation remains an open question. A report that genomic lesion-free immortality was induced during the conversion of somatic cells, including peripheral blood cells, to a pluripotent state may provide a fruitful hint for this question (Okita et al., 2013). In the present study, we provide data suggesting that AKAP-Lbc was required for the efficient propagation of reprogrammed cells (Figures 6H and 6I), which is in line with the reported positive roles of YAP and TAZ in reprogramming (Lian et al., 2010; Qin et al., 2012). Another hallmark shared by AKAP-Lbc and YAP/TAZ is that they are known oncoproteins whose hyperactivation can immortalize primary cells (Onco-Lbc, a constitutively active mutant of AKAP-Lbc, was originally identified in human leukemia; Figure S2D; Zheng et al., 1995). Together, our findings suggest the possibility that the AKAP-Lbc/Rho-mediated promotion of YAP/TAZ activation is conducive to the generation of ESCs/iPSCs by reversibly activating the intrinsic program for the establishment of in vitro immortality.

Although successful ex vivo cultivation and scalable expansion is currently restricted to a few types of stem/progenitor cells (Wang et al., 2015; Huch et al., 2015), it will be important to test the potential for application of this technology to a broader range of cells. A deeper understanding of the molecular basis of the intrinsic immortality of ESCs/iPSCs could help to provide fundamental insights useful for addressing the practical challenge of maintaining viability across a variety of different cell types.

EXPERIMENTAL PROCEDURES

Cell Culture

All experiments using hESC lines were approved by an institutional ethics committee and done following the hES cell guidelines of the Japanese government. The hESC (KhES-1 and KhES-3) and hiPSC lines (253G1 and in-house lines) were maintained as described previously (Watanabe et al., 2007). They were cultured on feeder layers of mouse embryonic fibroblasts (MEF) in DMEM: nutrient mixture F-12 (DMEM/F12) (Sigma) supplemented with 20% knockout serum replacement (KSR), 2 mM glutamine, 0.1 mM non-essential amino acids (Invitrogen), 5 ng/ml recombinant human bFGF (Wako), and 0.1 μM 2-mercaptoethanol (2-ME). For feeder-free condition, cells were

Figure 5. Actin Dynamics Links AKAP-Lbc/Rho Signal to YAP/TAZ Function

(A) Live imaging analyses of the dox-treated tet-shLbc:Bcl-X_L KhES-1 cells. The dead cells were validated with YOYO-1 uptakes (green, bottom). The recording was started from 24 hr after dox addition. The scale bar represents 200 μm.

(B) F-actin staining (green) in the tet-shLbc:Bcl-X_L KhES-1 cells treated with dox or not for 3 days. The snapshots of 3D reconstitution displays from z series of confocal images are shown. The upper and bottom images are the x-y and x-z views, respectively. The scale bar represents 50 μm.

(C) Schematic diagram of morphological transformation upon AKAP-Lbc-depletion. The illustrations show side-views of the colony.

(D) Immunostaining of basal actin meshwork. The tet-shLbc:Bcl-X_L KhES-1 cells were treated with dox or not for 3 days. The basal surface was imaged with confocal microscopy. The F-actin (green) and phospho-Paxillin (red) are shown. The bottom images show enlarged views squared in the upper images. The scale bar represents 10 μm.

(E) Live imaging analyses of the dox-treated or untreated tet-shLbc:Bcl-X_L KhES-1 cells. The F-actin and FA were visualized by Lifeact-mVenus and Paxillin-mCherry, respectively. The recording was started from 24 hr after dox addition.

(F and G) The engineered KhES-1 cells, indicated as tet-shCont, tet-shLbc, and tet-shLbc/Cfl (for double inducible depletion of AKAP-Lbc and cofilin), were cultured with or without dox for 3 days.

(F) Western blotting analyses. The HSC70 was a loading control.

(G) Immunostaining for basal actin meshwork. The F-actin (green) and phospho-Paxilin (red) were shown. The images were obtained with a confocal microscopy. The scale bar represents 50 μm.

(H and I) Colony expansion assay (H) and area occupancy of AP-positive colonies (I). The scale bar represents 500 μm.

(J and K) YAP/TAZ localization and function. The indicated cells were cultured with or without dox for 3 days (J). The immunostaining for YAP/TAZ is shown (green). The images were obtained using a confocal microscopy. The scale bar represents 50 μm. (K) qPCR assay for the YAZ/TAZ target genes.

(L) Signaling network of the Rho-mediated survival pathway.

The bars in the graphs represent SD. Tukey's test (n = 3) among all groups (I and K); not significant (n. s.); **p < 0.01; and ***p < 0.001. See also Figure S5 and Movie S5.
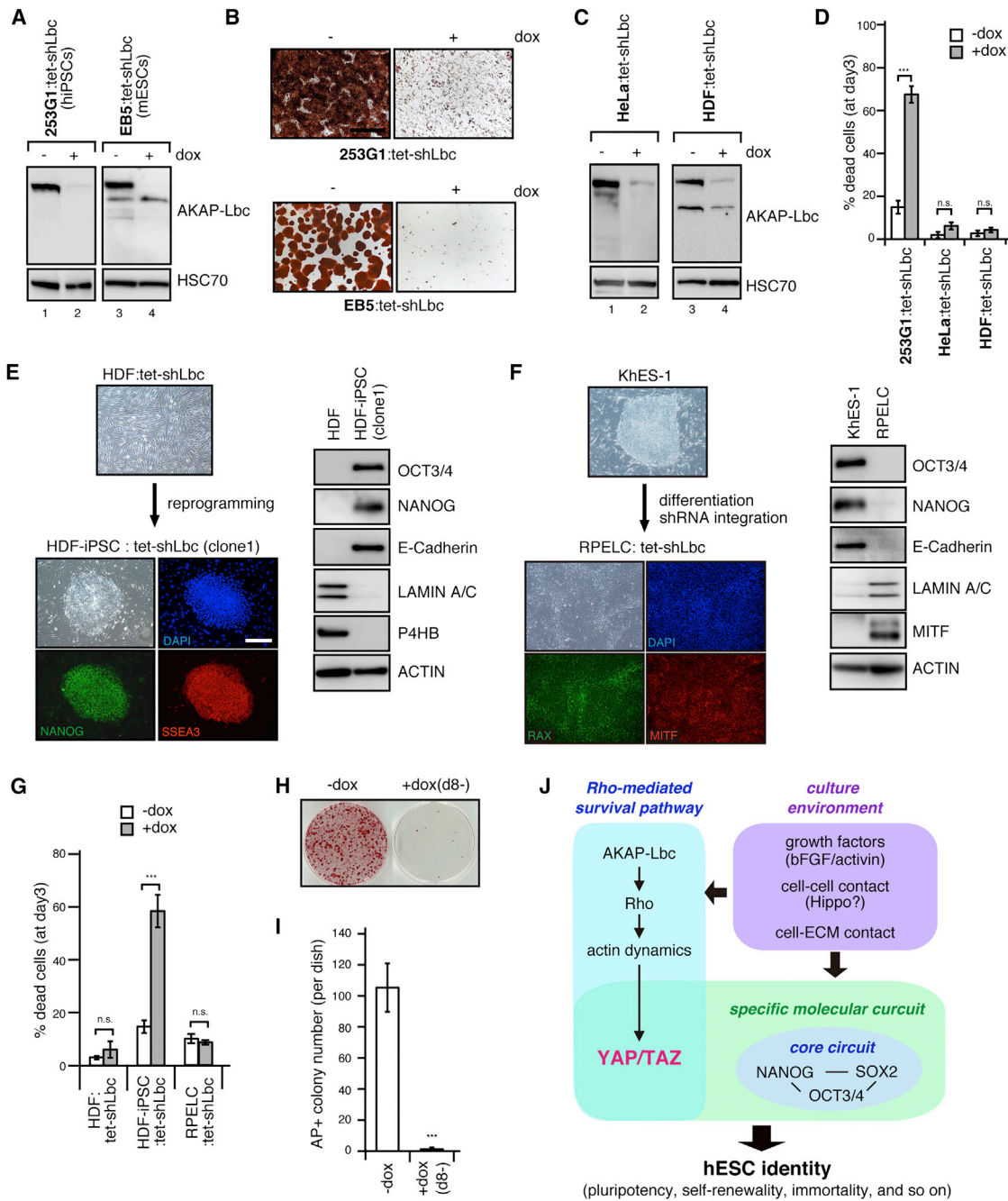
Figure 6. Cellular Identity Dictates Survival Dependency on the Rho Signaling

(A–D) Human iPSCs (253G1), mouse ESCs (EB5), HeLa, and HDFs were engineered for conditional knock down of AKAP-Lbc (indicated as 253G1:tet-shLbc, EB5:tet-shLbc, HeLa:tet-shLbc, and HDF:tet-shLbc, respectively). These cells were cultured with or without dox for 3 days (for western blotting analyses and cell death assay) or 5 days (for colony expansion assay). (A and C) Western blotting analyses for AKAP-Lbc. The HSC70 was a loading control. (B) Colony expansion assay. (D) Cell death assay. For a positive control, 253G1:tet-shLbc hiPSCs were also examined.

(E–G) Gain- and loss-of-pluripotency experiments. (E) The tet-shLbc HDFs (top) were reprogrammed by iPS technology (indicated as a HDF-iPSC:tet-shLbc, clone 1 was shown). The differential interference contrast (DIC) and immunostaining images for pluripotent markers, NANOG (green), and SSEA-3 (red) are shown (bottom). (F) KhES-1 cells (top) were differentiated into somatic-like cells positive for RPE markers, RAX (green), and MITF (red) (bottom) and then they were engineered for the conditional knock down of AKAP-Lbc (indicated as RPELC:tet-shLbc). Western blotting analyses are shown for the further confirmation of cell state conversion. The actin was examined as a loading control. (G) Cell death assay. The indicated cells were cultured with or without dox for 3 days. The scale bars represent 200 μm.

(H and I) Effects of AKAP-Lbc-depletion on reprogramming. The transfectants were divined into two dishes on day 7 and from day 8 the cells were cultured in the presence or absence of dox (0.1 μg/ml).

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cultured on Matrigel substrates (Invitrogen) in MEF-conditioned medium or mTeSR1 medium (STEMCELL Technologies).

Gene Manipulation
The plasmid transfection was performed as described previously (Ohgushi et al., 2010). To obtain stable transfectants, the cDNA expression cassettes were integrated into genomes using a PiggyBac transposon system. A few days after transfection, stable transfectants were selected by drug treatment. To avoid clone biases, a drug-resistant pool was used for experiments without single cell-derived colony pick-up. For an inducible gene silencing, we used dox-inducible shRNA expression lentivirus vectors. For a more detailed outline of the strategy for double or triple knockdown and target mRNA sequences, see Supplemental Information.

Colony Expansion Assay, Immunostaining, and Biochemical Analyses
For colony expansion assay, cells were dissociated and seeded onto MEF-coated 12 well plates in the presence of 10 μM Y-27632. After complete Y-27632 removal on the next day, cells were cultured for 24 hr to form small colonies. Using this strategy, we succeeded in reproducibly making small colonies with uniform size and equal cell numbers in each well. At 2 days after seeding, 1 μg/ml of dox was added and the colonies were further cultured for 5 days with daily media changes. Undifferentiated colonies were visualized by AP staining. Immunostaining was performed as previously described (Watanabe et al., 2007). For western blot analyses, cells were once transferred onto the Matrigel-coated dishes before being lysed. For a complete list of antibodies used in this work, see Supplemental Information.

ACCESSION NUMBERS

The accession number for the RNA-sequence data reported in this paper is GEO: GSE67128.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, one table, and five movies and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2015.07.009.

AUTHOR CONTRIBUTIONS

M.O. designed the project, performed experiments, and wrote the manuscript; M.M. supported M.O. and performed experiments; and Y.S. supervised the project.

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(H) AP-staining was done to visualize reprogrammed colonies. The experiments were performed in triplicate.
(I) Graph shows the average number of AP-positive colony in each dish.
(J) The AKAP-Lbc/Rho pathway supports hESC survival and expansion in collaboration with the hESC-unique molecular circuit.
The bars in the graphs represent SD. Tukey's test (n = 3) among all groups (D and G); Student's t test (n = 3) (I); not significant (n. s.); and ***p < 0.001. See also Figure S6.

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