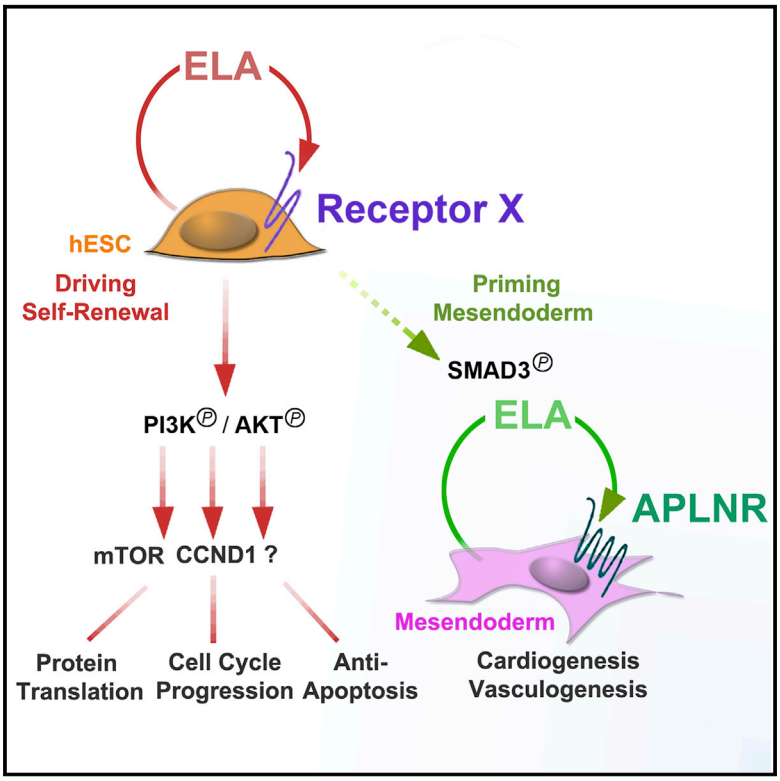
Article

Cell Stem Cell

ELABELA Is an Endogenous Growth Factor that Sustains hESC Self-Renewal via the PI3K/AKT Pathway

Graphical Abstract



Authors

Lena Ho, Shawn Y.X. Tan, Sheena Wee, ..., Jayantha Gunaratne, N. Ray Dunn, Bruno Reversade

Correspondence

lena.ho@reversade.com (L.H.), bruno@reversade.com (B.R.)

In Brief

Ho et al. show that ELA is an endogenous peptide hormone produced by human embryonic stem cells that supports self-renewal through auto/paracrine activation of PI3K/AKT signaling. ELA promotes cell-cycle progression and protein translation, prevents apoptosis upon cellular stress, and primes hESCs for endodermal differentiation via activation of TGFβ signaling.

Highlights

- ELA is a peptide hormone secreted by hESCs that activates the PI3K/AKT pathway
- ELA promotes self-renewal via cell-cycle progression and protein translation
- ELA potentiates the TGFβ pathway, priming hESCs toward the endoderm lineage
- hESCs do not express APLNR, so ELA may have an alternate unknown receptor

Accession Numbers

GSE71949

Ho et al., 2015, Cell Stem Cell *17*, 435–447
October 1, 2015
http://dx.doi.org/10.1016/j.stem.2015.08.010

Cell Stem Cell
Article

ELABELA Is an Endogenous Growth Factor that Sustains hESC Self-Renewal via the PI3K/AKT Pathway

Lena Ho,[1,*] Shawn Y.X. Tan,[1] Sheena Wee,[2] Yixuan Wu,[1] Sam J.C. Tan,[1] Navin B. Ramakrishna,[1] Serene C. Chng,[1] Srikanth Nama,[1] Iwona Szczerbinska,[3] Yun-Shen Chan,[3] Stuart Avery,[1] Norihiro Tsuneyoshi,[1] Huck Hui Ng,[3] Jayantha Gunaratne,[2] N. Ray Dunn,[1] and Bruno Reversade[1,2,*]
[1]Institute of Medical Biology, Human Genetics and Embryology Laboratory, A*STAR, Singapore 138648
[2]Institute of Molecular and Cell Biology, A*STAR, Singapore 138673
[3]Genome Institute of Singapore, A*STAR, Singapore 138672
*Correspondence: lena.ho@reversade.com (L.H.), bruno@reversade.com (B.R.)
http://dx.doi.org/10.1016/j.stem.2015.08.010

SUMMARY

ELABELA (ELA) is a peptide hormone required for heart development that signals via the Apelin Receptor (APLNR, APJ). ELA is also abundantly secreted by human embryonic stem cells (hESCs), which do not express *APLNR*. Here we show that ELA signals in a paracrine fashion in hESCs to maintain self-renewal. ELA inhibition by CRISPR/Cas9-mediated deletion, *shRNA*, or neutralizing antibodies causes reduced hESC growth, cell death, and loss of pluripotency. Global phosphoproteomic and transcriptomic analyses of ELA-pulsed hESCs show that it activates PI3K/AKT/mTORC1 signaling required for cell survival. ELA promotes hESC cell-cycle progression and protein translation and blocks stress-induced apoptosis. INSULIN and ELA have partially overlapping functions in hESC medium, but only ELA can potentiate the TGFβ pathway to prime hESCs toward the endoderm lineage. We propose that ELA, acting through an alternate cell-surface receptor, is an endogenous secreted growth factor in human embryos and hESCs that promotes growth and pluripotency.

INTRODUCTION

Human pluripotent embryonic stem cells (hESCs) represent a particular spatiotemporal state of human embryogenesis, i.e., the peri-implantation epiblast (Brons et al., 2007; Tesar et al., 2007). As such their study allows us to understand the cues and requirements that drive and regulate the earliest events of human development. Because of this biological significance and their clinical relevance for regenerative medicine, a great emphasis has been placed on elucidating the mechanisms underlying the "pluripotent circuitry network," which is composed mainly of transcription factors, chromatin regulatory proteins, and signaling pathways activated by extracellular growth factors (Warmflash et al., 2012). In particular, hESCs require a finely-tuned cocktail of exogenous basic FIBROBLAST GROWTH FACTOR (bFGF), NODAL/ACTIVIN, and INSULIN/INSULIN-LIKE GROWTH FACTORs (IGFs) for continued self-renewal (Vallier et al., 2005; Voskas et al., 2014; Xu et al., 2005). Aside from these, no other secreted factors have been isolated from hESCs or feeder-conditioned medium and proven to be necessary for maintaining self-renewal.

While examining the pluripotency gene signature of hESCs, we discovered a gene NM_001297550 that encodes an evolutionarily conserved 54-amino acid hormone named ELABELA (also known as APELA) (http://www.elabela.com). We and others have found that Ela signals through the Apelin Receptor (Aplnr, also known as Apj) to mediate endoderm differentiation during zebrafish embryogenesis (Chng et al., 2013; Pauli et al., 2014). In humans, *ELA* expression during development is highest in the blastocyst (Hs. 105196, LOC100506013) and is rapidly downregulated during hESC differentiation (Miura et al., 2004). To our knowledge, no hormonal peptide has ever been implicated in maintaining the self-renewal capacity of hESCs or their ability to differentiate into the three embryonic germ layers. Recently, murine *Ela* was reported to also function as a long non-coding RNA that promotes DNA-damage-induced apoptosis (DIA) mediated by p53 via sequestration of the p53 inhibitor, hnRNPL (Li et al., 2015). Intriguingly, this function was shown to be entirely independent of the translated Ela peptide or its cognate receptor Aplnr. Here, we demonstrate that in hESCs, ELA is a bona fide endogenous ligand necessary for maintaining growth and self-renewal. Through phospho-proteomic analysis, we find that ELA is necessary and sufficient to activate the Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT pathway to potentiate hESC growth and protect against cellular stress. While ELA can in part replace the need for exogenous INSULIN, the two are functionally distinct and non-redundant. Consistent with its role in promoting endoderm development in the zebrafish, ELA, unlike INSULIN, is required to poise hESCs toward the mesendoderm lineage, pointing to a dual function in maintaining the self-renewing state and facilitating early lineage commitment.

RESULTS

ELA Is a Conserved Hormone Associated with Human Embryonic Pluripotency

Within the human pluripotency circuitry network, which we delineated as the core intersection of the NANOG, POU5F1

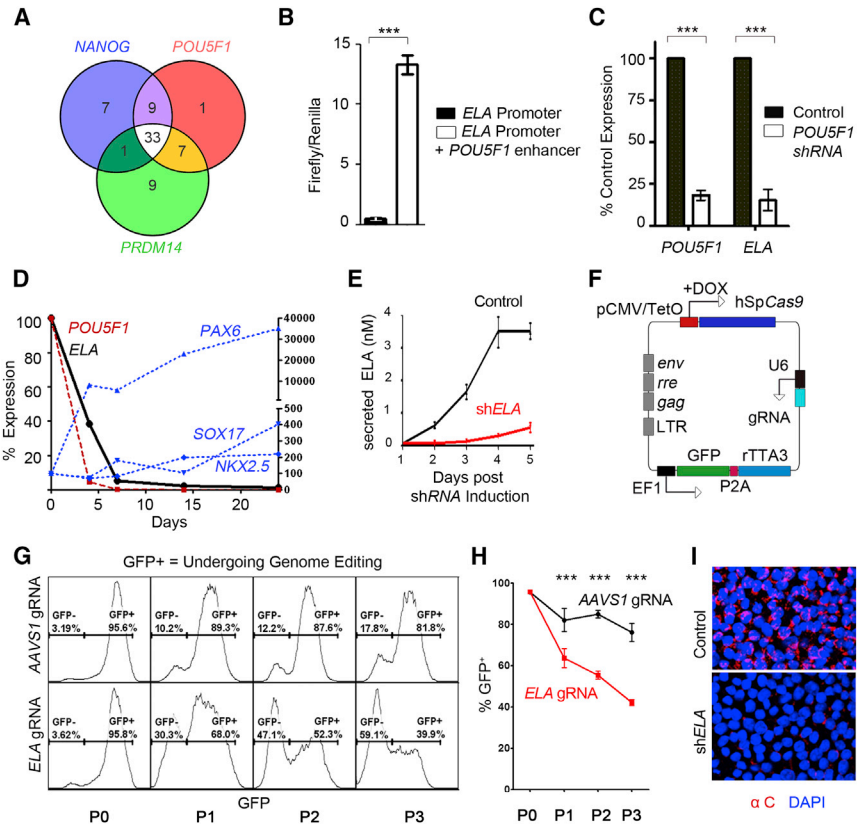


Figure 1. ELA Is Associated with Human Embryonic Stemness

(A) *NANOG*, *POU5F1*, and *PRDM14* syn-expression groups define a common list of 33 transcripts which are at the core of the human pluripotency circuitry. *ELA* is one of these genes.

(B) Luciferase reporter assay demonstrating that *ELA* is under direct transcriptional control by an upstream POU5F1 enhancer.

(C) *ELA* mRNA levels were measured by qPCR in control and *POU5F1*-knockdown hESCs.

(D) *ELA* mRNA expression in undifferentiated hESCs and during embroid body differentiation. Left axis: *ELA* and *POU5F1*; right axis: *PAX6*, *SOX17*, and *NKX2.5*, expressed as percentages relative to Day 0.

(E) Secreted ELA is detected in the supernatant of hESCs by ELISA. shRNA-mediated stable knockdown of *ELA* (sh*ELA*) reduces levels of secreted ELA by approximately 85%.

(F) Inducible CRISPR/Cas9 vector for derivation of *ELA*^{iCRISPR} and *AAVS1*^{iCRISPR} hESCs.

(G) FACS analysis of *ELA*^{iCRISPR} and *AAVS1*^{iCRISPR} hESCs serially passaged in the presence of DOX to track the persistence of GFP-positive genome-edited cells over four passages (P0 to P3).

(H) The percentage of GFP-positive *ELA*^{iCRISPR} hESCs rapidly declines over four passages compared to GFP-positive *AAVS1*^{iCRISPR} hESCs. Data are represented by the mean of six wells ± SEM.

(I) Immunofluorescence of ELA in control and sh*ELA* hESCs.

See also Figure S1.

(also known as OCT4), and PRDM14 syn-expression groups (Day et al., 2009; Niehrs and Pollet, 1999), lies a list of 33 transcripts (Figure 1A and Table S1), 6 of which are still unknown or uncharacterized. One, *ELABELA* (*ELA*), was previously reported to be specific to undifferentiated hESCs (Chng et al., 2013; Miura et al., 2004). According to UniGene, the Hs. 105196 transcript is highly and specifically expressed in human blastocysts before implantation (Figures S1A and S1B; UGID: 143461). Its expression in hESCs is dependent on an active *POU5F1* regulatory element lying 10 kb upstream of its promoter and is contingent on endogenous POU5F1 activity (Figures 1B and 1C). Consistent with this, *ELA* transcription is highest in undifferentiated hESCs and becomes rapidly silenced during embryoid body (Figure 1D), endodermal, and neuronal differentiation (Figures S1C and S1D). These data validate that *ELA* expression is correlated with the undifferentiated state of hESCs and is associated with pre-implantation human development.

ELA Is Secreted by hESCs and Is Required for Self-Renewal

ELA is translated into a bona fide protein that is readily detected in hESCs and in human embryonal carcinoma cells (ECs) by immunofluorescence using two custom ELA antibodies that recognize either the N or C terminus (Figures 1I and S1E) of the mature ELA peptide. ELA co-localizes with TGN46, a marker of the trans-Golgi network (Chng et al., 2013). We confirmed that endogenous ELA is indeed secreted because it is readily detected in the supernatant of cultured hESCs using a custom sandwich ELISA assay (Figure 1E). We estimated that over a period of 5 days, ELA reaches low nanomolar concentrations in the supernatant of hESCs (Figure 1E).

To assess the function of ELA in hESCs, we first attempted to generate hESC clones with homozygous genetic deletion of *ELA* using CRISPR/Cas9 technology (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013). To circumvent the appearance of culture-adapted hESC clones imposed by strong selective pressures (Avery et al., 2013), we devised an inducible CRISPR platform in which Cas9 expression is under doxycycline (DOX) control (Figure 1F). Coexpression of Cas9 and gRNAs targeting exon 1 of *ELA* (referred to as *ELA*^{iCRISPR}) or the *AAVS1* intronic locus resulted in similar mutation frequencies (primarily frameshifts in the *ELA* locus) as measured by droplet digital PCR (ddPCR) (Figure S1F). This method infers mutation frequency by measuring reduction in wild-type genomic DNA copy number as a result of Cas9 editting. Following dissociation and growth at clonal density, we were only able to recover 25.5% and 5.5% with mono- and bi-allelic mutations, respectively (Figures S1G and S1H). This represents a statistically significant reduction in the observed number of clonal outgrowths with mutations in *ELA* compared to the expected rate ($\chi^2 = 0.0008$), pointing to a severe growth disadvantage of *ELA*-het or *ELA*-null hESCs. Indeed, serial passaging of GFP-positive *ELA*^{iCRISPR} hESCs in the presence of DOX (such that the *ELA* locus is continuously edited) resulted in the rapid demise and concomitant differentiation of GFP-positive *ELA*^{iCRISPR} hESCs compared to

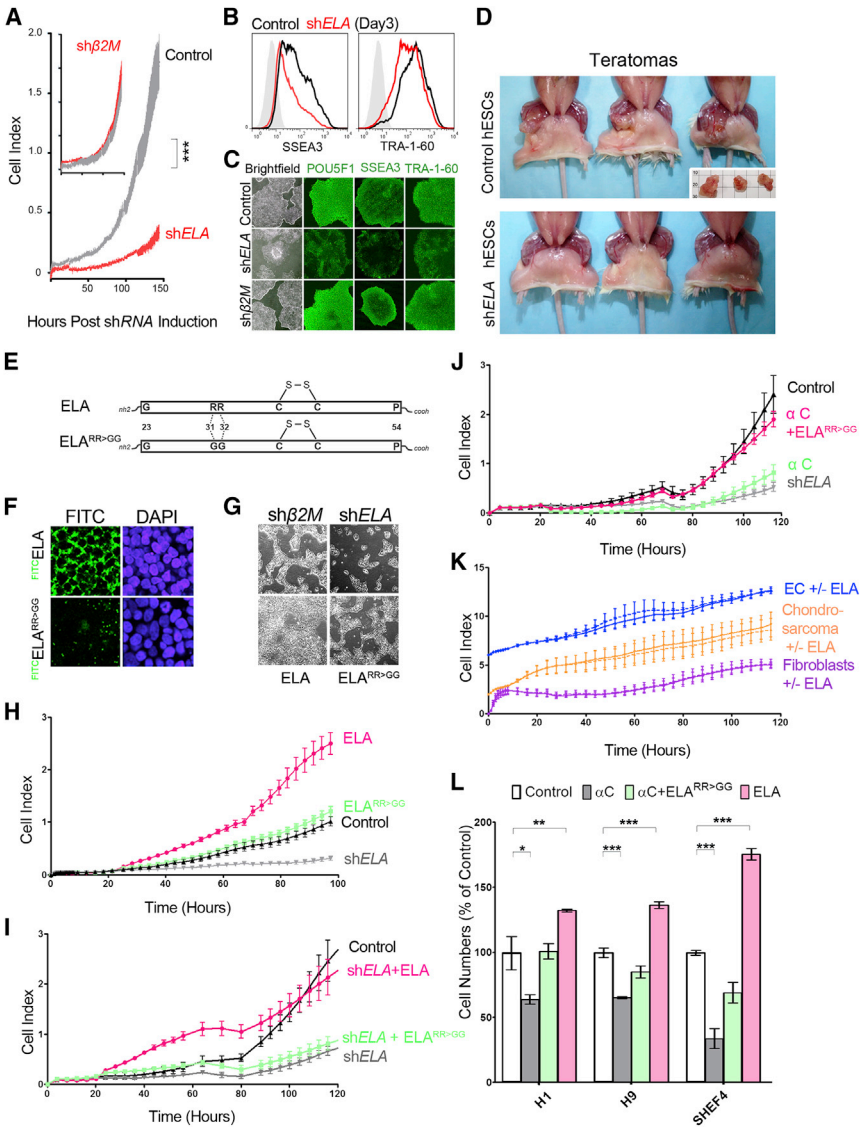


Figure 2. ELA Is Necessary and Sufficient for hESC Growth and Viability

(A) Cell index measurements (xCELLigence) of sh*ELA* and *shβ2M* (inset) hESCs seeded as single cells over 5 days. Cell Index is an approximation of cell numbers.

(B) FACS analysis of SSEA3 and TRA-1-60 3 days after induction of shRNA by DOX.

(C) Immunofluorescence for SSEA3, POU5F1, and TRA-1-60 in control and sh*ELA* hESC colonies after four passages of knockdown.

(D) Control and sh*ELA* hESCs were injected subcutaneously into NOD-SCID-GAMMA mice. Teratoma formation was visualized after 1 month.

(E) ELA and mutant ELA$^{RR > GG}$ (R31G, R32G), with an intramolecular cystine bond between conserved C39 and C44 residues, were synthetically produced to 98% purity.

(F) By immunofluorescence, recombinant ELA, but not ELA$^{RR > GG}$, labeled with N-terminal FITC is rapidly taken up by hESCs.

(G) Brightfield images of shRNA hESCs and wild-type hESCs cultured with exogenous ELA or ELA$^{RR > GG}$ after 4 days of shRNA or peptide treatment.

(H) Real-time cell index measurements of hESCs cultured with exogenous ELA or ELA$^{RR > GG}$ over 4 days.

(I) Real-time cell index measurements over 5 days of sh*ELA* hESCs rescued with exogenous ELA, but not ELA$^{RR > GG}$.

(J) hESCs were cultured with affinity purified α C antibody, which inhibited their growth. This neutralizing activity can be outcompeted by the mutant non-signaling ELA$^{RR > GG}$ peptide which competes for the α C antibody.

(K) Real time cell index measurements of multipotent human embryonal carcinoma cells (ECs) or unipotent human chondrosarcoma and primary fibroblast cells cultured with exogenous ELA over 5 days, with no apparent effect.

(L) H1, H9, and SHEF4 hESC lines were grown with exogenous ELA or α C antibody. Cell numbers were measured after 4 days. Data are represented by the mean of six wells ± SEM.

See also Figure S2.

contaminating CRISPR/Cas9/GFP-negative cells, indicating a selective disadvantage of *ELA*-null hESCs in culture over wild-type non-edited hESCs (Figures 1G and S1J). In contrast, this effect was not observed in GFP-positive *AAVS1*^{iCRISPR} or GFP-positive hESCs lacking a gRNA (Figures 1G and 1H and S1)I–S1J.

In order to circumvent the difficulty of obtaining *ELA*-null hESCs without the artificial selective pressure of culture adaptation, we chose instead to knock down *ELA* and a control non-essential gene, *β2-MICROGLOBULIN* (*β2M*) (Figure S2A), using stable DOX-inducible shRNA (Zafarana et al., 2009). sh*ELA* knockdown achieved approximately 85% depletion of *ELA* mRNA and extracellular ELA relative to control levels (Figures 1E and S2A). The intensity of ELA staining was also markedly reduced upon siRNA- and shRNA-mediated *ELA* knockdown (Figures 1I and S2B). sh*ELA* hESCs displayed significantly reduced growth rates compared to control or *shβ2M* hESCs when seeded as single cells as shown by real-time cell index analysis on the xCELLigence platform (Figure 2A). Slower growth rates were also documented in sh*ELA* hESC colonies that were on average less than half the size of *shβ2M* hESCs (Figure S2C). After 3 days of knockdown, sh*ELA* hESCs showed reduced surface levels of pluripotency markers SSEA3 and TRA-1-60 (Figure 2B). Over the course of four passages in both single-cell and colony format, depletion of *ELA*, but not *β2M*, resulted in a loss of hESC colony morphology and pluripotency markers POU5F1, NANOG, SSEA3, and TRA-1-60 (Figures 2C, S2D, and S2E). In line with these results and in contrast to control hESCs, sh*ELA* hESCs injected into NOD-SCID-GAMMA mice did not form teratomas (Figure 2D). All together, these findings argue that the endogenous peptide ELA is key to hESC self-renewal.

Exogenous ELA Is Sufficient to Promote Growth of hESCs

We next assessed the bioactivity of mature ELA. To this end we synthetically produced ELA at 98% purity as a 32-amino

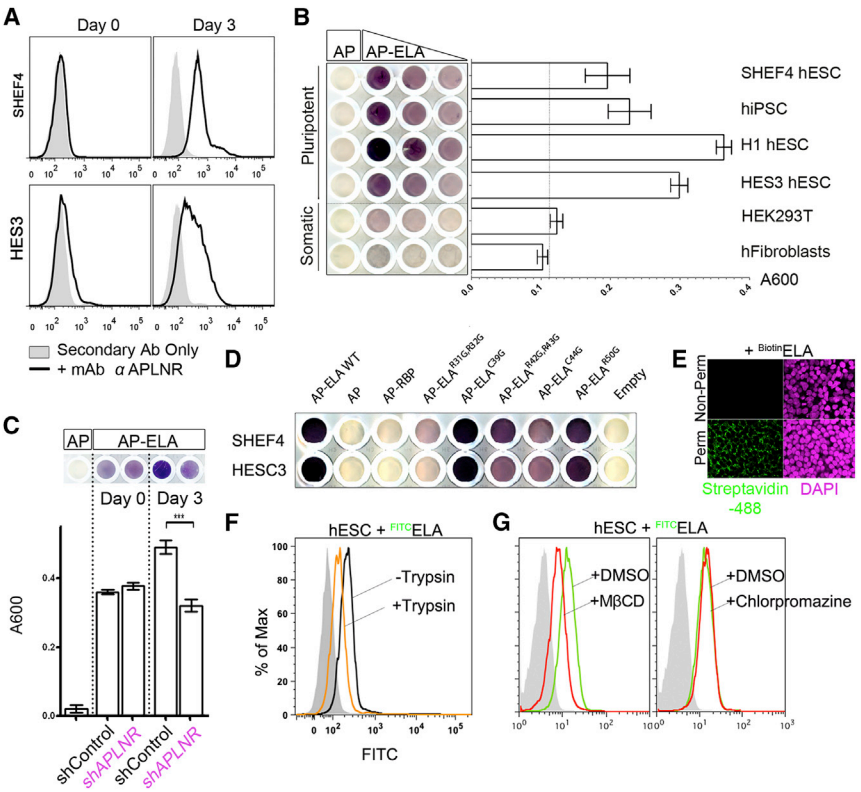


Figure 3. APLNR Is Not the ELA Receptor in hESCs

(A) FACS analysis of surface-expressed APLNR in undifferentiated (Day 0) versus Day 3 hESC-derived mesoendoderm (Day 3).

(B) AP-ELA binding assay on a variety of hESCs and also differentiated human cell lines. Data represent intensity of colorimetric readout and are means of three wells ± SEM.

(C) AP-ELA binding assay on shControl and sh*APLNR* undifferentiated hESCs (Day 0) versus Day 3 hESC-derived mesoendoderm (Day 3).

(D) Mutation of the indicated residues to glycine affects binding of AP-ELA to SHEF4 and HES3 hESCs. Input supernatants were normalized by their AP activity to ensure that equal amounts of each AP-ELA mutant were used for binding.

(E) Biotinylated ELA peptide applied to hESCs was detected using streptavidin with or without prior permeabilization of the cell surface membrane by digitonin.

(F) FITC-labeled ELA was bound to hESCs with or without a 5 min pretreatment of cells with a low concentration of trypsin, followed by FACs analysis.

(G) FITC-labeled ELA was bound to hESCs in the presence or absence of methyl-β-cyclodextrin (MβCD) or Chlopromazine, followed by FACs analysis.

See also Figure S3.

acid peptide bearing an intramolecular cysteine bond between cysteine residues 39 and 44 (Figure 2E). Synthetic FITC-labeled ELA was readily taken up by hESCs (Figure 2F). We discovered that mutation of two invariant arginines into glycines (R31G and R32G) completely abolished the uptake of ELA (Figures 2E and 2F). While sh*ELA* hESCs showed reduced growth, hESCs pulsed with wild-type ELA peptide showed dose-dependent enhanced growth relative to untreated hESCs. This was independently documented by cell counts (Figure S2F), colony size (Figure 2G), and real-time measurement of cell indices (Figures 2H and S2G). The doubly mutated ELA mutant peptide (referred to as ELA$^{RR > GG}$) had no effect in these assays (Figures 2E and 2H). Notably, the growth of sh*ELA* hESCs was entirely rescued by the addition of recombinant ELA, but not ELA$^{RR > GG}$, showing that ELA can be provided exogenously (Figure 2I). We therefore hypothesized that direct inhibition of ELA in the extracellular space should yield results similar to its depletion at the transcript or genetic level. Indeed, we found that addition of antigen affinity-purified α C and α N antibodies to hESC medium recapitulated the effects of sh*ELA* (Figures 2J and S2H), indicating that these antibodies have potent ELA-neutralizing activity. ELA$^{RR > GG}$ peptide was used as a competitive inhibitor to the α C antibody to prove the specificity of this assay (Figure 2J). Moreover, the combined use of sh*ELA* and α C neutralizing antibodies resulted in an exacerbated loss of growth compared to each inhibition alone (Figure S2F).

It is noteworthy that recombinant ELA and α C antibody promoted and inhibited, respectively, only the growth of hESCs and no other differentiated cell types such as ECs, a human chondrosarcoma cell line, or primary human fibroblasts (Figure 2K). With respect to cell growth, H1, H9, and SHEF4 hESCs all responded similarly to the loss or gain of function of ELA (Figure 2L), indicating that ELA is generally required for the human "primed" pluripotent state. Primed hESCs can be reprogrammed by a variety of methods to a more "naive" state believed to capture an earlier stage of human embryonic development (Hanna et al., 2010). We employed the "3iL" method to derive naive ESCs that resemble preimplantation epiblast (Chan et al., 2013) and found that *ELA* expression was unchanged in the 3iL state compared to the primed state (Figure S2I). ELA depletion using α C antibodies or shRNA had a negligible effect on 3iL ESCs, while exogenous ELA had only a modest, albeit statistically significant, effect on cell growth (Figures S2J–S2L). All together, these data suggest that ELA is a specific growth factor of the human primed ESCs (i.e., hESCs) and not naive hESCs.

APLNR Is Not the ELA Receptor in hESCs

We and others have shown that ELA serves as a cognate ligand to the cell surface APLNR to mediate endoderm development and subsequent heart morphogenesis. However, in agreement with previous reports (Vodyanik et al., 2010; Yu et al., 2012), *APLNR* is absent in undifferentiated hESCs. Unlike *ELA*, which is marked by H3K4me3 and actively transcribed, the *APLNR* locus is methylated and not transcribed in hESCs (Figure S3A). We confirmed the absence of *APLNR* transcripts in both SHEF4 and HES3 hESC lines by qPCR and flow cytometry (Figure 3A). In contrast, *APLNR* transcripts were upregulated nearly 2,500-fold upon mesendoderm differentiation, when

cell surface APLNR became robustly detectable (Figures 3A and S3B). Nonetheless, we performed shRNA-mediated depletion of *APLNR* in undifferentiated hESCs to ensure that trace levels of APLNR could not mediate the effects of ELA on hESCs (Figures S3B and S3C). We adapted our ELA cell surface binding assay (Chng et al., 2013) to several hESC lines including induced pluripotent ESCs (hiPSCs) and found that they readily bound to the Alkaline Phosphatase-ELA (AP-ELA) fusion protein (Figure 3B), indicating the presence of an endogenous cell-surface receptor. The level of AP-ELA binding to undifferentiated hESCs was not affected by sh*APLNR*, whereas sh*APLNR* hESC-derived mesendoderm cells had significantly less binding (Figure 3C). These data suggest that while APLNR is necessary to confer cell-surface binding to ELA in mesendoderm cells, it is not the receptor for ELA in undifferentiated hESCs. Consistent with this conclusion, the growth of sh*APLNR* hESCs was not compromised (Figure S3D). From these experiments, we predict that an alternate ELA receptor exists in hESCs and is responsible for maintaining self-renewal. We also documented AP-ELA binding to a variety differentiated cell types such as HEK293T and primary skin fibroblasts and found that ELA binds only to undifferentiated hESCs (Figure 3B). This receptor binding activity is impaired or abrogated by mutations in several conserved residues in ELA, namely C44 and R31R32, demonstrating specificity for this assay. The binding of ELA to this receptor results in its internalization, since biotin-labeled ELA can only be detected using streptavidin if ELA-pulsed hESCs are permeabilized (Figure 3E). Internalization of ELA, quantified using flow cytometry, is abrogated by pre-treatment of hESCs with trypsin (Figure 3F), indicating that the receptor activity is dependent on a cell-surface protein. ELA uptake is also impaired by treatment of hESCs with methyl-β-cyclodextrin (MβCD) but not chlorpromazine (Figure 3G), two widely used inhibitors of clathrin-independent and clathrin-dependent endocytosis, respectively (Conner and Schmid, 2003). Alt together, these results demonstrate that ELA binds to an alternate (non-APLNR) protein receptor present on the surface of human pluripotent cell types and is internalized upon binding.

ELA Activates the PI3K/AKT Pathway in hESCs

To identify the components of the ELA signal transduction pathway in hESCs, we used the proteomic approach Stable Isotope Labeling by Amino acids in Cell culture (SILAC) (Ong et al., 2002). We analyzed the phosphoproteome of hESCs pulsed with ELA for 10 min in order to identify immediate targets, using the inactive $ELA^{RR>GG}$ peptide as a baseline control (Figure 4A). The experiment was performed twice in both forward and reverse configurations, and only hits that were replicated were further investigated. We focused our attention on LNpTSDFQK, which was among the top phosphopeptides activated by ELA, but not $ELA^{RR>GG}$, and derived from PRAS40 (proline-rich Akt substrate of 40 kDa, a.k.a AKT1S1) (Figure 4B). PRAS40 is an immediate downstream substrate of AKT, which is in turn activated by PI3K (Vander Haar et al., 2007). This suggests that ELA activates the PI3K/AKT pathway. Indeed, addition of recombinant ELA to hESCs was sufficient to trigger the immediate phosphorylation of AKT at serine 473, leading to phosphorylation of PRAS40 at threonine 246 (Figure 4C). This was not observed in cells treated with mutant $ELA^{RR>GG}$ or vehicle control (Figure S3E) and was abrogated by pre-treating the cells with pan-PI3K inhibitor LY249004 (LY), but not by shRNA-mediated *APLNR* depletion (Figures 4D and 4E). This demonstrates that ELA activation of AKT is APLNR independent and requires PI3K. Once phosphorylated, PRAS40 is inactivated, relieving its repressive effect on the mammalian target of Rapamycin (mTORC1) complex (Wang et al., 2012). The subsequent activation of the mTORC1 by ELA is evidenced by phosphorylation of its prototypical substrate p70S6K (Figure 4C) (Peterson et al., 1999). Hence, ELA activates the PI3K/AKT pathway and subsequently the mTORC1 pathway. PI3K/AKT and mTORC1 are both potent regulators of growth and viability and contribute integrally to the self-renewal capacity of hESCs (Armstrong et al., 2006; Zhou et al., 2009). Consistent with the loss of self-renewal and viability of sh*ELA* hESCs, we find that sh*ELA* hESCs indeed have lower levels of endogenous PI3K/AKT and mTORC1 activity, which can be revealed by gradually lowering levels of exogenous INSULIN in the growth media (Figure 4F). These results suggest that the growth deficiency seen in sh*ELA* hESCs is attributable to the loss of paracrine and autocrine PI3K/AKT signaling mediated by endogenous ELA.

Murine and human ESCs are known to differ in their requirements for exogenous growth factors (Rao, 2004). We next tested if the requirement for ELA extends to murine pluripotent stem cells. mESCs express low levels of *Ela*, and relative to hESCs, they do not secrete Ela peptide at detectable levels (Figures S4A and S4B). Although *Ela* mRNA levels are upregulated during the transition from naive mESCs to epiblast-like stem cells (Epi-like SCs) (Figure S4A), we found that neither naive mESCs nor Epi-like SCs are affected by α C-mediated Ela inhibition or the addition of exogenous ELA (Figures S4C and S4D). Consistently, Ela does not activate PI3K/AKT and does not bind to the surface of mESCs, suggesting that Ela is not functional in these cells (Figures S4E–S4G). These results are in line with recent findings that in mESCs, *Ela* functions as a non-coding RNA independently of the translated Ela peptide to promote p53-mediated apoptosis (Li et al., 2015).

ELA and INSULIN/IGF Have Overlapping and Distinct Roles in hESCs

Unlike mESCs, hESCs are dependent on exogenous INSULIN and endogenous IGF2 (Bendall et al., 2007), both of which activate the PI3K/AKT pathway to mediate self-renewal and prevent differentiation (Singh et al., 2012). For this reason, mTSER1 (used in this study) and most formulations of hESC medium contain high levels of INSULIN. To investigate the functional interplay between INSULIN and ELA, we cultured hESCs in a defined TSERe8 medium (Chen et al., 2011), which only contains four growth factors, INSULIN, bFGF, TGFβ1, and TRANSFERRIN, to allow precise control over its composition. INSULIN is the sole activator of AKT in this formulation (Figure S4H). In SHEF4 and HES3 hESCs, we observed a high rate of cell death after 24 hr of growth in the absence of INSULIN. Addition of ELA rescued cell viability by 80%–90%, while $ELA^{RR>GG}$ showed no effects (Figures S4I and S4J). On the contrary, sh*ELA* hESCs were exquisitely sensitive to INSULIN withdrawal compared to

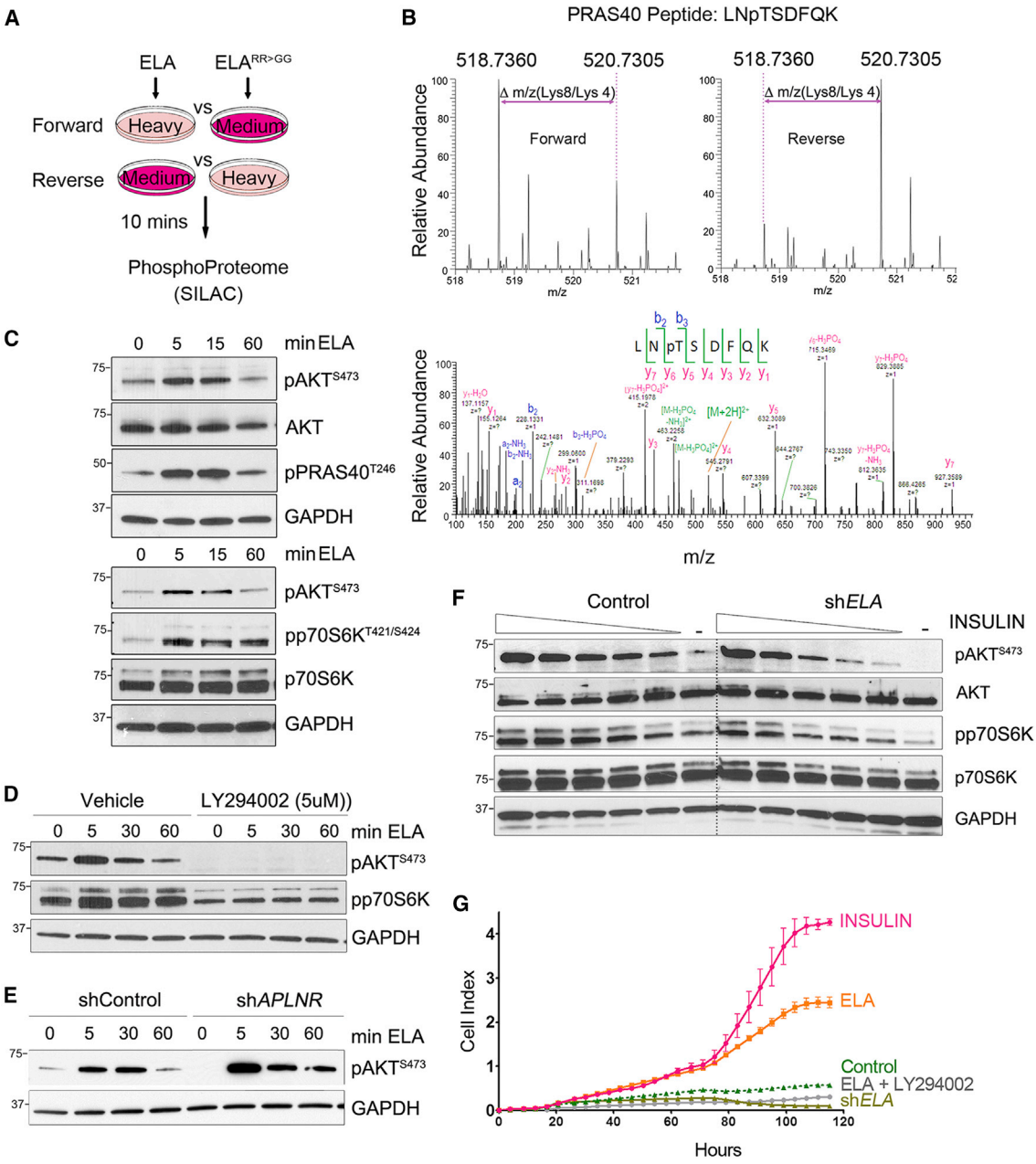


Figure 4. ELA Is the Endogenous Signal for Activation of PI3K/AKT in hESCs

(A) Schematic of SILAC-based phospho-proteomic analysis to elucidate immediate signal transduction of ELA in hESCs.

(B) Mass spectra of a PRAS40-derived peptide showing phosphorylation on T246 by ELA, but not ELA$^{RR > GG}$, suggesting activation of the AKT pathway.

(C) hESCs were pulsed with ELA and lysed at the indicated time points. Western blots show immediate activation of the PI3K/AKT and mTORC1 pathways. Lysates for the top and bottom panels were derived from separate technical replicates.

(D) Activation of AKT by ELA is dependent on PI3K and is abrogated by pan-PI3K inhibitor LY294002 (LY).

(E) Activation of AKT by ELA in sh*APLNR* hESCs is not impaired.

(F)Western blots of pAKT in Control and sh*ELA* hESCs grown in decreasing INSULIN concentrations for 24 hr reveal the requirement for ELA-mediated AKT activation.

(G) By real-time cell index analysis over 5 days, ELA, but not ELA$^{RR > GG}$, can partially rescue the requirement for INSULIN in hESCs growth medium.

See also Figure S4.

control hESCs, and addition of ELA provided an intermediate growth rescue (Figure S4K). ELA partially replaced INSULIN over 5 days of growth in a PI3K-dependent manner, since addition of LY abrogated the rescuing effect of ELA (Figure 4G). However, ELA cannot entirely replace INSULIN, since hESC cultures lacking INSULIN eventually display slower growth and decline,

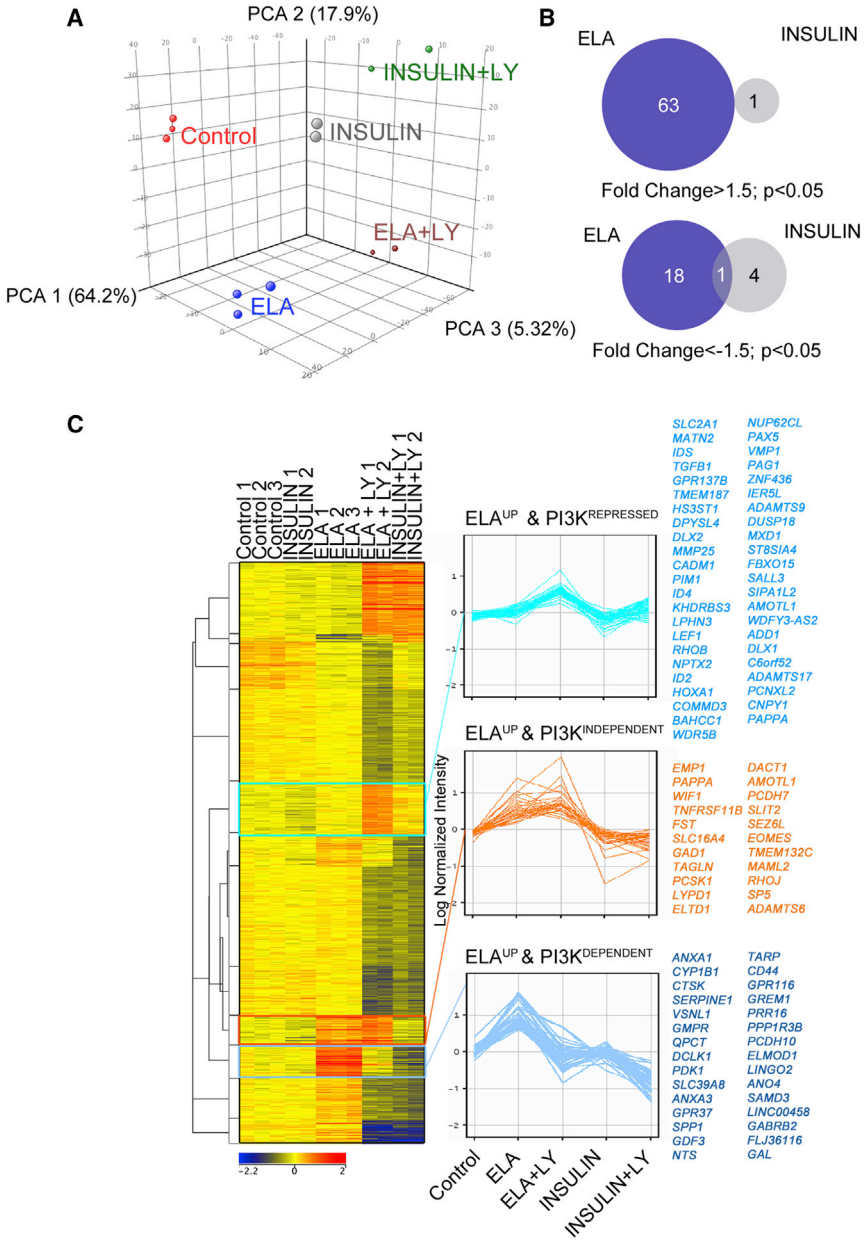


Figure 5. ELA and INSULIN Are Functionally Distinct

(A) PCA analysis of microarray data using probesets that showed at least greater than 1.5-fold change (one-way ANOVA, $p < 0.05$) between at least one pair of conditions.

(B) Venn diagrams depicting the overlap of probesets changed by more than 1.5-fold in ELA-treated (representing 42 upregulated and 16 downregulated genes) and INSULIN-treated (1 upregulated and 5 downregulated genes) hESCs.

(C) Self-organizing map of all probesets in the dataset with greater than 1.5-fold change between at least one pair of conditions (one-way ANOVA, $p < 0.05$). Selected clusters of ELA-dependent genes are highlighted to depict their variable dependence on PI3K/AKT activity.

See also Figure S5.

even if exogenous ELA is supplied (Figure 4G and data not shown). The opposite is also true, since ELA depletion cannot be fully rescued by exogenous INSULIN. This prompted us to carefully examine the differential downstream effects of ELA and INSULIN.

To this end, we profiled the global transcriptional response of hESCs toward ELA or INSULIN 12 hr following stimulation in the presence and absence of LY in order to delineate PI3K-dependent gene targets. ELA and INSULIN activated AKT to an equal extent (Figure S5A). Microarray data were validated and verified by qPCR using several representative genes (Figure S5B). Two major themes arise from analysis of these data. First, principle component analysis (PCA) and hierarchical clustering of the data indicate that ELA and INSULIN-treated hESCs are distinct and are equally distant from control-treated hESCs, while LY was very potent and had major and overriding effects on gene expression (Figures 5A and S5C). Although ELA and INSULIN activated AKT equally, ELA elicited a greater transcriptional response compared to that of INSULIN, with little overlap in transcriptional targets (Figure 5B). This result points to the distinct potency and activity of the two growth factors on hESC gene expression via the PI3K/AKT pathway. Second, self-organizing map (SOM) analysis of ELA-activated genes revealed variable dependence of ELA activity on PI3K/AKT activity. ELA-activated genes can either be sensitive to PI3K inhibition (PI3K dependent), insensitive to PI3K inhibition (PI3K independent), or enhanced by PI3K inhibition (PI3K repressed) (Figure 5C). These results demonstrate that while ELA can activate the PI3K pathway, its downstream effects are not exclusively mediated through PI3K. In fact, a part of its activity appears to be antagonized and held in check by PI3K. These observations indicate a clear distinction in the nature and activity of ELA relative to INSULIN, consistent with our observation that the two growth factors can only partially rescue one another.

ELA Impacts Cell Cycle and Protein Translation Downstream of PI3K/AKT

To explore how ELA functions specifically during self-renewal, we made use of the gene signature discovery tool Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005). Consistent with activation of the PI3K/AKT pathway, genes in the mTORC1 signaling pathway were positively enriched or upregulated by ELA gain-of-function (Figure S5D). Conversely, microarray analysis of sh*ELA* hESCs showed that ELA loss-of-function led to a statistically significant downregulation of

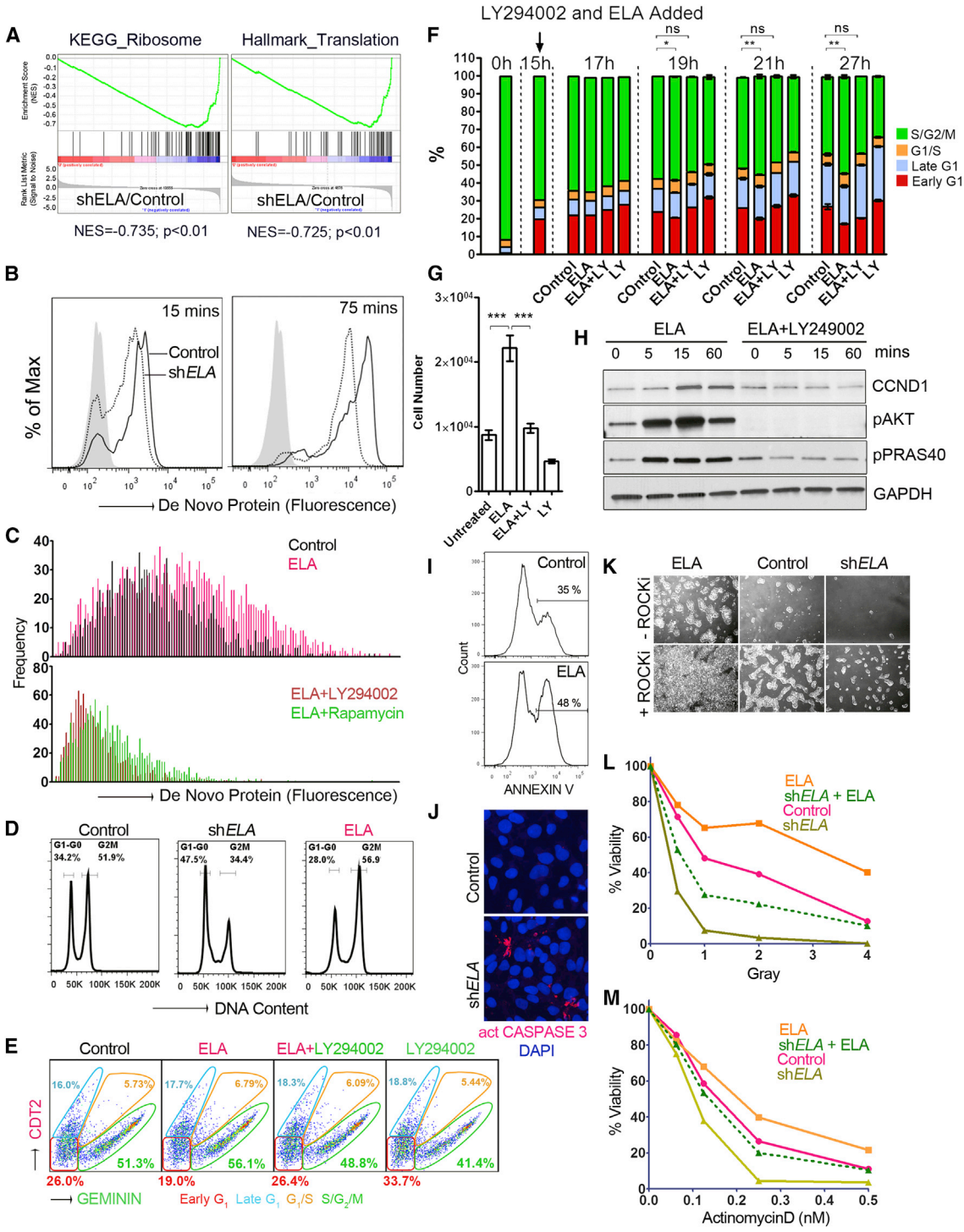


Figure 6. ELA Promotes Translation and Proliferation and Protects against Stress-Induced Apoptosis

(A) GSEA profile plots depicting negative enrichment of ribosomal genes and genes involved in translation in sh*ELA* compared to control hESCs. NES, normalized enrichment score.

(B) Pulse-chase analysis by metabolic labeling to measure the rate of newly synthesized proteins in shControl versus sh*ELA* hESCs. Cells were harvested for FACs analysis of incorporated fluorescent amino acid 15 and 75 min after pulsing.

(C) Metabolic labeling to measure the rate of newly synthesized proteins in Control versus ELA-treated hESCs in the absence (top) or presence (bottom) of Rapamycin and LY. Cells were harvested for microscopic measurement of label incorporation 15 min after the addition of the amino acid label.

(D) 23 hours following release from a double thymidine block, sh*ELA* hESCs show an accumulation of cells in the G1 phase as measured by DNA content.

(E) Cell-cycle analysis using FUCCI-H9 hESCs synchronized by a double thymidine block following treatment with ELA, LY, or both 19 hr post-release.

(legend continued on next page)

the same mTORC1 gene set (Figure S5D). A key function of the mTORC1 pathway is to regulate protein translation in response to growth factors, through the activation of p70S6K and transcription of ribosomal genes (Workman et al., 2014). In agreement, GSEA analysis showed a statistically significant downregulation of genes involved in protein translation and ribosome biogenesis in sh*ELA* hESCs (Figure 6A). Indeed, sh*ELA* hESCs exhibited reduced protein synthesis as assayed by incorporation of fluorescently labeled methionine (Figure 6B). Conversely, ELA treatment increased the proportion of cells with a higher fluorescence intensity following pulsing with fluorescently labeled methionine (Figure 6C). This effect was completely reversed by the mTOR inhibitor Rapamycin or the PI3K inhibitor LY. Hence, ELA promotes the growth of hESCs through PI3K/mTOR-dependent activation of protein translation.

A second function of the PI3K/AKT pathway is control of the cell-cycle progression through the G1/S phase, in part through post-translational stabilization of CYCLIN D (Muise-Helmericks et al., 1998). Unlike mESCs, in which Cyclin D is mostly inactive (Stead et al., 2002), the pRB-CYCLIN D/CDK4/6 cascade in hESCs is operational and is indispensable for G1/S transition and pluripotency (Pauklin and Vallier, 2013). We hypothesized that the loss of PI3K/AKT signaling in sh*ELA* cells results in an accumulation of cells in the G1 phase. Careful analysis of the cell cycle of sh*ELA* hESCs following release from a double thymidine block (which synchronizes cells at the beginning of the S phase) revealed a noticeable increase of cells in G1 (Figure 6D). Exogenous ELA had the opposite effect (Figure 6D). We confirmed this observation using FUCCI-H9 hESCs, which afford real-time and live observation of cell-cycle progression (Pauklin and Vallier, 2013). Synchronized ELA-treated hESCs had a statistically significant decrease in the proportion of cells in the early G1 phase and an increase in the proportion of cells in the S/G2/M phase (Figures 6E and 6F), suggesting a promotion of G1/S transition. Conversely, treatment of hESCs with LY lengthened the residence time in the early G1 phase and reversed the effects of ELA (Figures 6E and 6F). ELA treatment for 48 hr clearly decreased cell doubling time, resulting in a 2-fold increase in cell numbers relative to control and LY-treated hESCs (Figure 6G). Consistent with these results, ELA pulsing led to an increase in CYCLIN D1 (CCND1) protein levels in a PI3K-dependent manner (Figure 6H). All together, these data indicate that ELA acts through the PI3K/AKT/mTORC1 pathway in hESCs to potentiate growth by promoting cell-cycle progression and optimal protein translation.

ELA Protects Against Apoptosis and Prevents Stress-Induced Cell Death

PI3K/AKT is equally recognized for its anti-apoptotic properties in both normal and cancer cells (Fresno Vara et al., 2004). For this reason, we hypothesized that ELA may prevent apoptosis and thereby potentiate hESC growth. In support of this hypothesis, a significantly higher proportion of sh*ELA* hESCs underwent apoptosis, as marked by surface expression of ANNEXIN V (Figure 6I) and intracellular activated CASPASE 3 (Figure 6J and Figure S6A). This suggests that endogenous ELA is required to protect against apoptosis induced by routine in vitro culture conditions, such as dissociation-induced cell death, also known as anoikis (Watanabe et al., 2007). Indeed, sh*ELA* hESCs do not survive single-cell dissociation in the absence of Y-27632, which inhibits anoikis by inhibition of Rho-associated kinase (ROCK) (Watanabe et al., 2007) (Figure 6K). Conversely, exogenous ELA was able to increase survival following dissociation compared to untreated hESCs, partially replacing Y-27632 (Figure 6K). Furthermore, sh*ELA* hESCs were twice as sensitive to γ-irradiation, which inflicts DNA damage, relative to control, while ELA-treated hESCs were five times more resistant (Figure 6L) despite comparable numbers of DNA double-stranded breaks marked by γH2AX (Figure S6B). These findings in hESCs stand in contrast to the reported function of *Ela* in mESCs, where instead it appears to promote apoptosis in response to DNA damage (Li et al., 2015). Similar results were obtained when hESCs were treated with nanomolar concentrations of Actinomycin D to inflict translational stress (Perry, 1962) (Figures 6M and S6C). In both cases, sh*ELA* hESCs had increased levels of activated CASPASE 3/7 (Figures S6B and S6D) and CASPASE 9 (Figure S6E), indicating increased activation of the intrinsic apoptotic cascade (Gillies and Kuwana, 2014). This was due to higher levels of BAX in the mitochondria and CYTOCHROME C in the cytoplasm (Figure S6F), which was suppressed by addition of exogenous ELA (Figure S6F). All together, our results indicate that exogenous ELA acting through PI3K/AKT protects against the intrinsic apoptosis pathway activated by a variety of cellular stress, while hESCs depleted of ELA are highly sensitized to stress.

ELA Primes Cells toward the Mesendoderm Lineage

We next sought to delineate the functions of ELA that are distinct from INSULIN. *Ela* is known to be required for mesendoderm differentiation during zebrafish embryogenesis (Chng et al., 2013), a process that is highly dependent on Nodal/Tgfβ. In ELA-pulsed hESCs, we observed upregulation of several genes related to the NODAL/TGFβ pathway such as *EOMES*, *GDF3*,

(F) Quantitation of data in (E) at the indicated time points following thymidine block release.
(G) Cell numbers 48 hr following thymidine block release.
(H) Western blot analysis of CYCLIN D1 levels following an ELA pulse.
(I) By FACS analysis, a larger proportion of sh*ELA* hESCs are positive for ANNEXIN V, which marks apoptotic cells.
(J) Activated CASPASE 3 can be detected in sh*ELA* hESCs by immunofluorescence, but not in control hESCs.
(K) ELA can partially replace the ROCK inhibitor to prevent anoikis following single-cell dissociation.
(L) ELA-treated hESCs are more resistant to γ-irradiation compared to control hESCs. sh*ELA* hESCs are sensitized to γ-irradiation, which can be rescued by the addition of ELA. Data are representative of three independent experiments.
(M) ELA-treated hESCs are more resistant to low levels of Actinomycin D treatment, which induces transcriptional stress and p53-dependent cell death. sh*ELA* hESCs are more sensitive but can be rescued by addition of ELA. Data are representative of six independent experiments.
See also Figure S6.

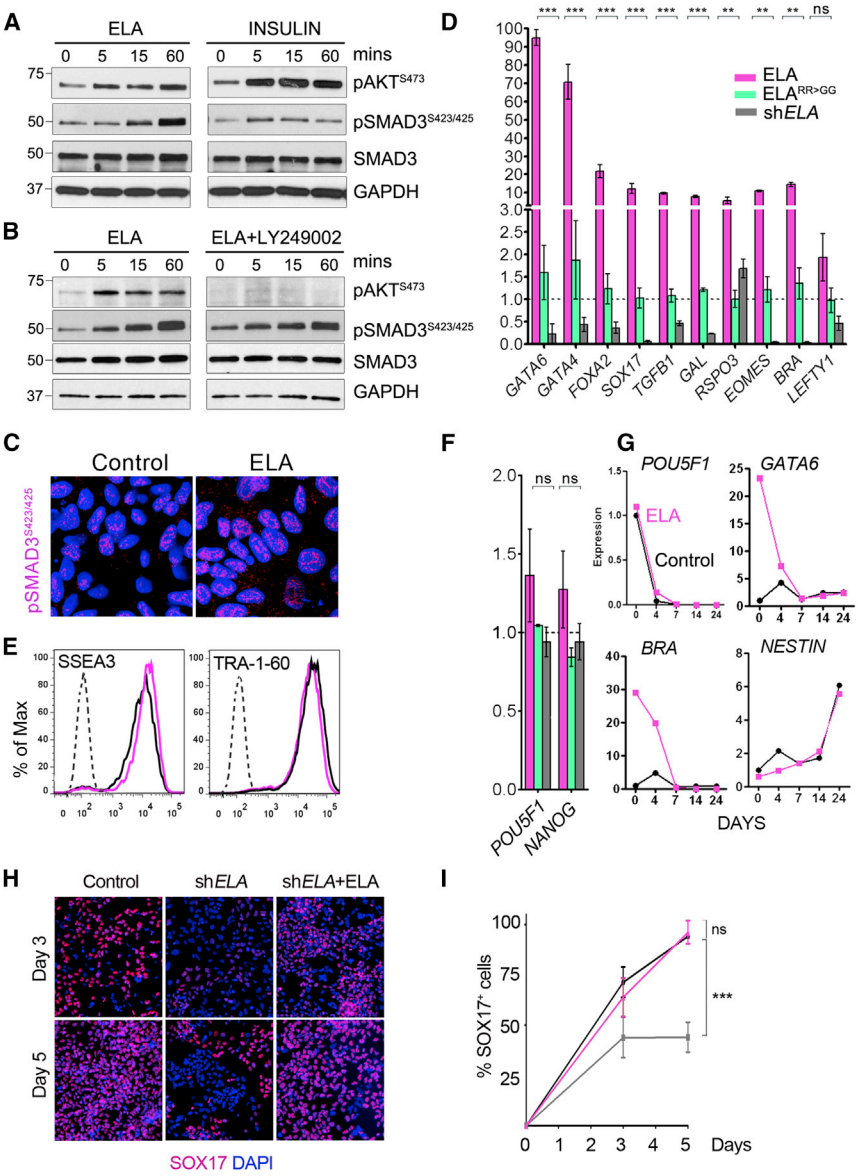


Figure 7. ELA Poises hESCs toward the Mesendodermal Lineage

(A) ELA, but not INSULIN, activates pSMAD3, as shown by western blot.

(B) ELA-mediated activation of pSMAD3 is insensitive to PI3K inhibition by LY.

(C) Immunofluorescence of pSMAD3 in hESCs pulsed with ELA.

(D) qPCR analysis of mesendoderm lineage genes in hESCs grown in the presence of ELA or depleted of *ELA* for 72 hr.

(E) FACs analysis of SSEA3 and TRA-1-60 levels on hESCs grown in the presence of ELA for 72 hr.

(F) qPCR analysis of *POU5F1* and *NANOG* in hESCs grown in the presence of ELA for 72 hr.

(G) qPCR analysis of germ layer markers during embroid body formation from hESCs grown in the presence (pink) or absence (black) of exogenous ELA prior to differentiation.

(H) SOX17 immunofluorescence of hESC-derived definitive endoderm at Day 3 and Day 5 of differentiation.

(I) Quantitation of SOX17-positive cells in (H).

See also Figure S7.

FST, and *TGFβ1* (Figure 5B). Using GSEA analysis, we found that transcripts that were upregulated by ELA were enriched for genes upregulated during in vitro differentiation of hESCs to definitive endoderm (DE) (Figure S7A; GEO: GSE25557). Conversely, genes that were downregulated in sh*ELA* hESCs are in fact upregulated during DE differentiation. Together, these observations implicate ELA in DE lineage commitment, possibly through modulation of NODAL/TGFβ. Consistent with this possibility, ELA-pulsed, but not INSULIN-pulsed, hESCs showed increased levels and nuclear localization of carboxy phospho-SMAD3 (Figures 7A and 7C), indicating activation of the NODAL/TGFβ pathway in a PI3K-independent manner (Figure 7B). After 72 hr, ELA-treated hESCs upregulated many markers of the mesendoderm lineage, including *BRA*, *EOMES*, and *GATA4/6* (Figure 7D). The same set of differentiation markers was downregulated in sh*ELA* hESCs (Figure 7D). Despite these changes, ELA was not sufficient to commit hESCs grown in mTSER1 toward the DE lineage, as cells maintained an hESC morphology (Figure 2G) and expressed normal, if not higher, levels of the pluripotency markers (Figures 7E and 7F). In addition, hESCs treated with ELA were still able to give rise to all three germ lineages during embroid body differentiation (Figure 7G), indicating that they remained pluripotent. Hence, we propose that ELA, while not sufficient to induce differentiation in the absence of other cues such as BMP4 and ACTIVIN A, is required to maintain a transcriptional profile that is permissive for endoderm development. On the contrary, sh*ELA* showed compromised in vitro differentiation of SOX17+ definitive endoderm, which could be fully rescued by the addition of exogenous ELA in the culture medium (Figures 7H and 7I). We conclude that ELA, working through the NODAL/TGFβ pathway, is required for endoderm differentiation by keeping hESCs primed toward the mesendoderm lineage.

DISCUSSION

ELA-Mediated Regulation of Embryonic Cell Growth and Viability

Besides the well-studied NODAL/BMP, IGF/FGF, and WNT, very few additional secreted factors with proven roles in pluripotency have been discovered in the last decade. Here we present evidence that the extracellular peptide ELA plays an important and specific role in self-renewing hESCs. ELA, like INSULIN and IGFs, can activate the PI3K/AKT pathway and is either the endogenous or an alternate activator of this crucial pathway during early embryogenesis. Unlike FGF, which needs

to be added exogenously or secreted by feeder cells, ELA is endogenously synthesized and secreted by hESCs in sufficient quantities. Inhibition of ELA caused loss of cell proliferation, apoptosis, and subsequent differentiation, suggesting that it serves as an essential factor to counteract the high levels of spontaneous apoptosis and differentiation inherent to hESC cultures. In line with this, we note that the depletion of ELA in hESCs grown as single cells is more detrimental than in hESCs grown in colonies due to the higher rate of anoikis following dissociation (Watanabe et al., 2007). Alternatively, this might point to the extracellular activity of the peptide, which may be more readily captured in a paracrine manner by hESC colonies than it is by single cells. Also, we note that while endogenous ELA is present in hESC-conditioned medium in the nM range, our recombinant peptide, although specific and bioactive, works in the μM range. This finding suggests existence of possible post-translational modifications on endogenous ELA that are required for its full potency, as is the case for other peptide hormones such as GHRELIN (Kojima et al., 1999).

ELA and INSULIN Are Functionally Distinct

INSULIN and IGFs are well known to stimulate pre-implantation embryonic growth in vitro, increasing the number of cells in the inner cell mass of mouse embryos and protecting them against apoptosis induced by oxidative stress (Kurzawa et al., 2002; Markham and Kaye, 2003; Rappolee et al., 1992). While ELA and INSULIN both activate PI3K/AKT in hESCs, their functions are only partially complementary. ELA does not bind to IGF or INSULIN receptors expressed abundantly in hESCs (unpublished data), arguing against its role as an alternate ligand of the INSULIN/IGF pathway. ELA and INSULIN elicit different transcriptional responses in hESCs, pointing to a different mode of action and set of downstream effectors. Lastly, ELA's ability to potentiate the NODAL/TGFβ pathway is unique and does not appear to be PI3K dependent. In fact, canonical PI3K/AKT signaling activated by IGFs maintains self-renewal by restraining the prodifferentiation functions of SMAD2/3 (Singh et al., 2012). In our hands, inhibition of PI3K/AKT as a result of ELA depletion does not lead to increased SMAD2/3-mediated differentiation (unpublished data). Rather, ELA depletion impairs subsequent endoderm differentiation, a process that is SMAD2/3 dependent. We therefore propose a model whereby ELA, acting through an alternate unknown receptor, promotes growth and survival through PI3K/AKT while potentiating mesendoderm differentiation through direct or indirect regulation of the NODAL/TGFβ pathway.

An Alternate Receptor for ELA Mediates Its Function in hESCs

Our observation that recombinant ELA is rapidly taken up by hESCs and that endogenous ELA can be found in the cytoplasm suggests that ELA signals via a dedicated receptor in these cells. We do not favor the possibility that ELA behaves as a self-penetrating peptide (Green and Loewenstein, 1988) despite its very basic amino-acid makeup (Chng et al., 2013) because its rapid cellular uptake is only observed in hESCs and not in other tested cell types. As previously reported and confirmed by us, *APLNR* is silent in hESCs. Furthermore, APELIN13, the other endogenous ligand for APLNR, which can activate PI3K/AKT in certain cell types (Tang et al., 2007), has no effect on hESCs (data not shown). We therefore believe that another cell-surface receptor mediates ELA's activity in hESCs.

Is Mouse Ela Lost In Translation?

A recent study reported a non-coding role of *Elabela* in promoting p53-mediated DIA in embryonic stem cells of mouse origin (mESCs). Intriguingly, this function was entirely dependent on the 3′ UTR of the murine *Ela* mRNA, which was predicted to form a secondary structure that interacts with the p53-inhibitor hnRNPL protein (Li et al., 2015). This function was shown to be entirely independent of the *Ela* ORF or its peptide product. This finding draws the question of whether human *ELA* also bears such a non-coding RNA function. Several key differences exist between primate and other vertebrates in regards to the structure of *ELA*'s 3′ UTRs, which is entirely encoded by its ultimate third exon (Figure S4L). The 3′ UTRs are of very different lengths: 2,113 bp in human versus 573 bp in mouse. Moreover, the 3′ UTR of human *ELA* has acquired during primate evolution the insertion of two anti-parallel 300 bp *Alu* repeats. Even without these *Alu* repeats, the sequence conservation is poor between the two species with an overall homology of less than 10%. Hence, we propose that the secondary structures and protein-binding properties of *ELA* and *Ela* mRNAs are likely to be species specific. Indeed, our results show that in hESCs, *ELA* mRNA prevents rather than promotes DIA as reported by Huang and colleagues in mESCs. Consistent with the authors' conclusions, we found that Ela had no effects on mESC self-renewal and growth under normal conditions. In fact, *Ela* knockout mice have no overt pre-implantation defects (B.R., unpublished data). These data point to a possible different role of *Ela* in mouse compared to *ELA* in human with respect to pre-implantation development and ESC maintenance. Nevertheless, we do not rule out the idea that the human ELA mRNA may have non-coding functions and we look forward to addressing this question more in the future.

Taken together, our data suggest that mature ELA functions as an endogenous hormonal peptide secreted by hESCs. It is then taken up by hESCs in a paracrine manner and signals through an unknown receptor to the PI3K/AKT pathway to sustain survival and self-renewal of ESCs. As ELA is highly expressed in the human blastocyst, the stage from which hESCs are derived, we speculate that it may play a similar protective role in primate pre-implantation embryos. What endogenous stress signal in the mammalian embryo ELA might protect against is not entirely clear. Normoxia, replicative stress, nutrient deprivation, and temperature are all potential stimuli that could equally solicit the need for ELA during embryogenesis. Lastly, given the potent ability of ELA to activate PI3K/AKT, promote growth, and prevent apoptosis, it is tempting to speculate that cancer stem cells may also display a similar dependence on ELA for tumor initiation and progression. Thus, defining and studying the role of ELA in human neoplasms seems a high priority given its attractiveness as a potential therapeutic target.

EXPERIMENTAL PROCEDURES

Teratoma studies were undertaken with prior approval from Biological Resource Centre under IACUC#110703.

Cell Culture and Assays

The SHEF4 cell line was used throughout and exhibits standard morphological and surface marker characteristics of hESCs and a normal 46XY karyotype (Inniss and Moore, 2006). Recombinant ELA or ELA$^{RR > GG}$ was added at 2.5 μM (or 10 μg/ml) where indicated. Refer to the Supplemental Information for details on assay protocols and reagents.

shRNA-Mediated *ELA* Knockdown

To generate stable inducible knockdown of *ELA* in hESCs, the sequence GTGATTCTCGTGCCTCAAC targeting the 3′ UTR of *ELA* was cloned into pSUPERIOR (Oliogoengine) and nucleofected (LonzA) into SHEF4$_{TetR5}$ hESCs (Zafarana et al., 2009). Refer to the Supplemental Information for details.

ELISA

A custom sandwich ELISA assay was developed for detecting secreted ELA. An in-house goat α C antibody (4 μg/ml) was used as the capture antibody and a rabbit α C (0.8 μg/ml) was used as the detection antibody. Refer to the Supplemental Information for details.

SILAC Cell Culture and Cell Lysis

SHEF4 hESCs were cultured in custom-made mTSER1 (Stem Cell Technologies) containing either stable medium isotopes of L-lysine-(2H4) (K4) and L-arginine-(13C6) (R6) or heavy isotope L-lysine-(13C615N2) (K8) and L-arginine-(13C615N4) (R10) for three passages to allow complete exchange of isotopes. Cells were then starved in DMEM/F12 for 2 hr, which was followed by a 10 min pulse with 5 μM of ELA or ELA$^{RR > GG}$. Lysates were subjected to phospho-proteomic analysis. Refer to the Supplemental Information for details.

Microarray Analysis

100 ng of purified RNA was used for cRNA generation and hybridization to the Affymetrix HG-U133_Plus_2.0 platform according to the manufacturer's protocol. Raw data were analyzed using GeneSpring GX (Agilent) to generate normalized intensity readings. Refer to the Supplemental Information for details.

Statistical Analysis

Unless otherwise indicated, all values were expressed as mean ± SEM. Comparison of means was performed using two-tailed Student's unpaired t test in Prism GraphPad, with significance levels indicated as follows: n.s. $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

ACCESSION NUMBERS

The accession number for the microarray data reported in this paper is GEO: GSE71949.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes three tables, seven figures, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2015.08.010.

AUTHOR CONTRIBUTIONS

L.H. and B.R. designed experiments and wrote the manuscript. S.Y.X.T, Y.W., S.A., N.T., S.J.C.T., N.B.R., S.C.C., and N.R.D. assisted with hESCs experiments. S.W. and J.G. performed SILAC experiments. S.A. made sh*ELA* hESCs lines. I.S., W.C.Y.S., and H.H.N. derived 3iL cells. S.N. contributed intellectually. All authors are funded by the Agency of Science Technology and Research, Singapore. B.R. is a fellow of the Branco Weiss Foundation, an A*STAR Investigator, and a Young EMBO Investigator. This work was partly funded by a Strategic Positioning Fund on Genetic Orphan Diseases from A*STAR, Singapore.

ACKNOWLEDGMENTS

We thank Barbara Knowles and Davor Solter for initiating this project and for their intellectual input. We thank Ludovic Vallier (Cambridge) for his kind gift of FUCCI-H9 hESCs and Theresa K.Y. Teng and Kimberle Shen for technical assistance. We thank all members of the Reversade laboratory for comments.

Received: November 25, 2014
Revised: May 12, 2015
Accepted: August 12, 2015
Published: September 17, 2015

REFERENCES

Armstrong, L., Hughes, O., Yung, S., Hyslop, L., Stewart, R., Wappler, I., Peters, H., Walter, T., Stojkovic, P., Evans, J., et al. (2006). The role of PI3K/AKT, MAPK/ERK and NFkappabeta signalling in the maintenance of human embryonic stem cell pluripotency and viability highlighted by transcriptional profiling and functional analysis. Hum. Mol. Genet. *15*, 1894–1913.

Avery, S., Hirst, A.J., Baker, D., Lim, C.Y., Alagaratnam, S., Skotheim, R.I., Lothe, R.A., Pera, M.F., Colman, A., Robson, P., et al. (2013). BCL-XL mediates the strong selective advantage of a 20q11.21 amplification commonly found in human embryonic stem cell cultures. Stem Cell Reports *1*, 379–386.

Bendall, S.C., Stewart, M.H., Menendez, P., George, D., Vijayaragavan, K., Werbowetski-Ogilvie, T., Ramos-Mejia, V., Rouleau, A., Yang, J., Bossé, M., et al. (2007). IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. Nature *448*, 1015–1021.

Brons, I.G., Smithers, L.E., Trotter, M.W., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S.M., Howlett, S.K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R.A., and Vallier, L. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature *448*, 191–195.

Chan, Y.S., Göke, J., Ng, J.H., Lu, X., Gonzales, K.A., Tan, C.P., Tng, W.Q., Hong, Z.Z., Lim, Y.S., and Ng, H.H. (2013). Induction of a human pluripotent state with distinct regulatory circuitry that resembles preimplantation epiblast. Cell Stem Cell *13*, 663–675.

Chen, G., Gulbranson, D.R., Hou, Z., Bolin, J.M., Ruotti, V., Probasco, M.D., Smuga-Otto, K., Howden, S.E., Diol, N.R., Propson, N.E., et al. (2011). Chemically defined conditions for human iPSC derivation and culture. Nat. Methods *8*, 424–429.

Chng, S.C., Ho, L., Tian, J., and Reversade, B. (2013). ELABELA: a hormone essential for heart development signals via the apelin receptor. Dev. Cell *27*, 672–680.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., and Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science *339*, 819–823.

Conner, S.D., and Schmid, S.L. (2003). Regulated portals of entry into the cell. Nature *422*, 37–44.

Day, A., Dong, J., Funari, V.A., Harry, B., Strom, S.P., Cohn, D.H., and Nelson, S.F. (2009). Disease gene characterization through large-scale co-expression analysis. PLoS ONE *4*, e8491.

Fresno Vara, J.A., Casado, E., de Castro, J., Cejas, P., Belda-Iniesta, C., and González-Barón, M. (2004). PI3K/Akt signalling pathway and cancer. Cancer Treat. Rev. *30*, 193–204.

Gillies, L.A., and Kuwana, T. (2014). Apoptosis regulation at the mitochondrial outer membrane. J. Cell. Biochem. *115*, 632–640.

Green, M., and Loewenstein, P.M. (1988). Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. Cell *55*, 1179–1188.

Hanna, J., Cheng, A.W., Saha, K., Kim, J., Lengner, C.J., Soldner, F., Cassady, J.P., Muffat, J., Carey, B.W., and Jaenisch, R. (2010). Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. Proc. Natl. Acad. Sci. USA *107*, 9222–9227.

Inniss, K., and Moore, H. (2006). Mediation of apoptosis and proliferation of human embryonic stem cells by sphingosine-1-phosphate. Stem Cells Dev. *15*, 789–796.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science *337*, 816–821.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature *402*, 656–660.

Kurzawa, R., Głabowski, W., Baczkowski, T., and Brelik, P. (2002). Evaluation of mouse preimplantation embryos exposed to oxidative stress cultured with insulin-like growth factor I and II, epidermal growth factor, insulin, transferrin and selenium. Reprod. Biol. *2*, 143–162.

Li, M., Gou, H., Tripathi, B.K., Huang, J., Jiang, S., Dubois, W., Waybright, T., Lei, M., Shi, J., and Zhou, M. (2015). An Apela RNA-Containing Negative Feedback Loop Regulates p53-Mediated Apoptosis in Embryonic Stem Cells. Cell Stem Cell *16*, 669–683.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M. (2013). RNA-guided human genome engineering via Cas9. Science *339*, 823–826.

Markham, K.E., and Kaye, P.L. (2003). Growth hormone, insulin-like growth factor I and cell proliferation in the mouse blastocyst. Reproduction *125*, 327–336.

Miura, T., Luo, Y., Khrebtukova, I., Brandenberger, R., Zhou, D., Thies, R.S., Vasicek, T., Young, H., Lebkowski, J., Carpenter, M.K., and Rao, M.S. (2004). Monitoring early differentiation events in human embryonic stem cells by massively parallel signature sequencing and expressed sequence tag scan. Stem Cells Dev. *13*, 694–715.

Muise-Helmericks, R.C., Grimes, H.L., Bellacosa, A., Malstrom, S.E., Tsichlis, P.N., and Rosen, N. (1998). Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway. J. Biol. Chem. *273*, 29864–29872.

Niehrs, C., and Pollet, N. (1999). Synexpression groups in eukaryotes. Nature *402*, 483–487.

Ong, S.E., Blagoev, B., Kratchmarova, I., Kristensen, D.B., Steen, H., Pandey, A., and Mann, M. (2002). Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol. Cell. Proteomics *1*, 376–386.

Pauklin, S., and Vallier, L. (2013). The cell-cycle state of stem cells determines cell fate propensity. Cell *155*, 135–147.

Pauli, A., Norris, M.L., Valen, E., Chew, G.L., Gagnon, J.A., Zimmerman, S., Mitchell, A., Ma, J., Dubrulle, J., Reyon, D., et al. (2014). Toddler: an embryonic signal that promotes cell movement via Apelin receptors. Science *343*, 1248636.

Perry, R.P. (1962). The Cellular Sites of Synthesis of Ribosomal and 4s Rna. Proc. Natl. Acad. Sci. USA *48*, 2179–2186.

Peterson, R.T., Desai, B.N., Hardwick, J.S., and Schreiber, S.L. (1999). Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycinassociated protein. Proc. Natl. Acad. Sci. USA *96*, 4438–4442.

Rao, M. (2004). Conserved and divergent paths that regulate self-renewal in mouse and human embryonic stem cells. Dev. Biol. *275*, 269–286.

Rappolee, D.A., Sturm, K.S., Behrendtsen, O., Schultz, G.A., Pedersen, R.A., and Werb, Z. (1992). Insulin-like growth factor II acts through an endogenous growth pathway regulated by imprinting in early mouse embryos. Genes Dev. *6*, 939–952.

Singh, A.M., Reynolds, D., Cliff, T., Ohtsuka, S., Mattheyses, A.L., Sun, Y., Menendez, L., Kulik, M., and Dalton, S. (2012). Signaling network crosstalk in human pluripotent cells: a Smad2/3-regulated switch that controls the balance between self-renewal and differentiation. Cell Stem Cell *10*, 312–326.

Stead, E., White, J., Faast, R., Conn, S., Goldstone, S., Rathjen, J., Dhingra, U., Rathjen, P., Walker, D., and Dalton, S. (2002). Pluripotent cell division cycles are driven by ectopic Cdk2, cyclin A/E and E2F activities. Oncogene *21*, 8320–8333.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., and Mesirov, J.P. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. USA *102*, 15545–15550.

Tang, S.Y., Xie, H., Yuan, L.Q., Luo, X.H., Huang, J., Cui, R.R., Zhou, H.D., Wu, X.P., and Liao, E.Y. (2007). Apelin stimulates proliferation and suppresses apoptosis of mouse osteoblastic cell line MC3T3-E1 via JNK and PI3-K/Akt signaling pathways. Peptides *28*, 708–718.

Tesar, P.J., Chenoweth, J.G., Brook, F.A., Davies, T.J., Evans, E.P., Mack, D.L., Gardner, R.L., and McKay, R.D. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature *448*, 196–199.

Vallier, L., Alexander, M., and Pedersen, R.A. (2005). Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J. Cell Sci. *118*, 4495–4509.

Vander Haar, E., Lee, S.I., Bandhakavi, S., Griffin, T.J., and Kim, D.H. (2007). Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat. Cell Biol. *9*, 316–323.

Vodyanik, M.A., Yu, J., Zhang, X., Tian, S., Stewart, R., Thomson, J.A., and Slukvin, I.I. (2010). A mesoderm-derived precursor for mesenchymal stem and endothelial cells. Cell Stem Cell *7*, 718–729.

Voskas, D., Ling, L.S., and Woodgett, J.R. (2014). Signals controlling un-differentiated states in embryonic stem and cancer cells: role of the phosphatidylinositol 3′ kinase pathway. J. Cell. Physiol. *229*, 1312–1322.

Wang, H., Zhang, Q., Wen, Q., Zheng, Y., Lazarovici, P., Jiang, H., Lin, J., and Zheng, W. (2012). Proline-rich Akt substrate of 40kDa (PRAS40): a novel downstream target of PI3k/Akt signaling pathway. Cell. Signal. *24*, 17–24.

Warmflash, A., Arduini, B.L., and Brivanlou, A.H. (2012). The molecular circuitry underlying pluripotency in embryonic stem cells. Wiley Interdiscip. Rev. Syst. Biol. Med. *4*, 443–456.

Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., Takahashi, J.B., Nishikawa, S., Nishikawa, S., Muguruma, K., and Sasai, Y. (2007). A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat. Biotechnol. *25*, 681–686.

Workman, J.J., Chen, H., and Laribee, R.N. (2014). Environmental signaling through the mechanistic target of rapamycin complex 1: mTORC1 goes nuclear. Cell Cycle *13*, 714–725.

Xu, C., Rosler, E., Jiang, J., Lebkowski, J.S., Gold, J.D., O'Sullivan, C., Delavan-Boorsma, K., Mok, M., Bronstein, A., and Carpenter, M.K. (2005). Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. Stem Cells *23*, 315–323.

Yu, Q.C., Hirst, C.E., Costa, M., Ng, E.S., Schiesser, J.V., Gertow, K., Stanley, E.G., and Elefanty, A.G. (2012). APELIN promotes hematopoiesis from human embryonic stem cells. Blood *119*, 6243–6254.

Zafarana, G., Avery, S.R., Avery, K., Moore, H.D., and Andrews, P.W. (2009). Specific knockdown of OCT4 in human embryonic stem cells by inducible short hairpin RNA interference. Stem Cells *27*, 776–782.

Zhou, J., Su, P., Wang, L., Chen, J., Zimmermann, M., Genbacev, O., Afonja, O., Horne, M.C., Tanaka, T., Duan, E., et al. (2009). mTOR supports long-term self-renewal and suppresses mesoderm and endoderm activities of human embryonic stem cells. Proc. Natl. Acad. Sci. USA *106*, 7840–7845.