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VGLL4 is a Novel Regulator of Survival in Human Embryonic Stem Cells

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Abstract

Human embryonic stem cells (hESCs) are maintained in a self-renewing state by an interconnected network of mechanisms that sustain pluripotency, promote proliferation and survival, and prevent differentiation. We sought to find novel genes that could contribute to one or more of these processes by using a gain-of-function screen of a large collection of human open reading frames. We identified Vestigial-like 4 (VGLL4), a co-transcriptional regulator with no previously described function in hESCs, as a positive regulator of survival in hESCs. Specifically, VGLL4 overexpression in hESCs significantly decreases cell death in response to dissociation stress. Additionally, VGLL4 overexpression enhances hESC colony formation from single cells. These effects may be attributable, in part, to a decreased activity of initiator and effector caspases observed in the context of VGLL4 overexpression. Additionally, we show an interaction between VGLL4 and the Rho/Rock pathway, previously implicated in hESC survival. This study introduces a novel gain-of-function approach for studying hESC maintenance and presents VGLL4 as a previously undescribed regulator of this process.

Keywords

VGLL4 protein; Pluripotent Stem Cells; Apoptosis; Rho-Associated Kinases

Introduction

Human pluripotent stem cells lie at the center of the model for regenerative medicine due to their ability to self-renew indefinitely and differentiate into multiple lineages. However, survival upon dissociation has been a roadblock in the application of hESCs to methods where clonal cells are needed, including gene manipulation, the study of clonal populations, survival after thaw, and even routine passaging. A chemical inhibitor of Rho-associated kinase (Rock) was recently shown to increase survival of dissociated hESCs [1] and is now commonly used in the maintenance and derivation of hESCs as well as induced pluripotent stem cells (iPSCs). The biological underpinnings of its mechanism of action have begun to be elucidated [2–4]. However, hESC survival remains a challenge and uncovering other mechanisms that contribute to this process is necessary.

Results

Identification of VGLL4 as a regulator of hESC maintenance through a gain-of-function screen

To uncover novel mechanisms regulating hESC maintenance, we conducted a biological gain-of-function screen to identify genes that, when overexpressed, could enhance hESC maintenance following the inhibition of known self-renewal pathways. We sought to screen a broad sample of the open reading frames (ORFs) in the human genome, and thus began with version 1.1 of the human ORFeome library –containing 8,076 ORFs, representing 7,263 genes– from the Center for Cancer Systems Biology Human ORFeome Collection

(horfdb.dfci.harvard.edu). ORFs were subcloned into a modified lentiviral pHAGE vector [5, 6] that had been adapted for Gateway cloning and to permit efficient expression in hESCs (Figure 1A and Supporting Information Fig. S1).

We next established screening conditions that would permit the identification of novel regulators of hESC maintenance. Given the importance of the TGFβ signaling pathway for the maintenance of pluripotency in hES cells [7–10], we predicted that inhibition of TGFβ signaling would impair self-renewal in these cells, thereby providing a robust platform to detect genes whose overexpression could enhance self-renewal. Treatment of HUES6 hESCs [8, 9, 11] for three passages with 10 μM SB-431542, a specific inhibitor of the TGFβ Type I receptors ALK4, 5, and 7 [12], caused a loss of hESC morphology and pluripotency marker expression as assessed by microscopy and FACS analysis (Fig. 1B, 1C). Nanog – unique among the core transcription factors in hESCs in that its overexpression maintains cells in a pluripotent state even in the presence of differentiation conditions [13, 14] – was chosen as a positive control for maintenance of self-renewal. GFP and Tubulin were used as negative controls.

To screen for novel regulators of hESC maintenance, hESCs were transduced with viral pools containing parts of the ORFeome library, or with positive and negative controls. Transduced cells were selected using puromycin, and then maintained in medium with SB-431542 for three passages. Colonies that retained hESC morphology following this treatment were isolated and subjected to PCR amplification and sequencing to identify virally-encoded ORFs. This analysis resulted in a list of 75 preliminary candidate ORFs with potential roles in self-renewal (Fig. 1D, Supplementary Information Table 1).

As a more stringent secondary assay, each candidate was tested for its ability to maintain self-renewal during treatment with inhibitors of both TGFβ and FGF signaling (TGFβi + FGFi). TGFβ and FGF signaling have both been shown to be necessary for hESC pluripotency [7, 8, 15–17], and simultaneous inhibition of both pathways proved to rapidly and efficiently induce differentiation (Fig. 2a and Supplementary Information Fig. S2, S3). Of the 75 candidates identified in our primary screen, only one gene involved in transcriptional control was able to sustain self-renewal under these more stringent conditions: Vestigial-like 4 (VGLL4; Fig. 2B, 2C, 2D). Maintenance of self-renewal as assessed by pluripotency marker expression and colony morphology was only temporary (cells transduced with Vgll4 differentiated when treatment with TGFβi + FGFi was extended to 7 days). However, we identified an important role for Vgll4 in promoting the survival of hESCs. These observations could suggest that self-renewal and survival in hESCs are more intricately related than previously thought.

VGLL4 is a putative transcriptional co-regulator that regulates cell survival pathways

VGLL4 is a member of the mammalian Vestigial-like protein family, which contains four genes (VGLL1–4). These genes are orthologs of the Drosophila gene Vestigial (Vg), whose loss impairs wing formation by interfering with cell proliferation in the wing imaginal disc [18, 19]. Vestigial-like proteins are transcriptional co-regulators that mediate the activity of Transcriptional Enhancer Factors (TEFs), also known as TEA domain-containing factors (TEADs) [20–22]. VGLL4 has not been previously implicated in hESC maintenance and its

function remains largely unstudied apart from a described role in regulating transcription in developing cardiomyocytes [20].

To gain insight into the potential role of VGLL4 in hESCs, we compared the global transcriptional profiles of hESCs overexpressing VGLL4 with wildtype hESCs. Multiple pathways involved in cell adhesion and apoptosis were significantly differentially regulated in the presence of VGLL4 (Table 1, Supplementary Information Fig. S4, Fig. S11). Interestingly, one of those pathways was signaling through Myosin Light Chain Phosphatase, which has been previously implicated in regulating hESC survival in response to dissociation [2–4]. Interestingly, our cells do not display changes suggesting adaptation to cell culture conditions. For instance, we did not observe changes in genes such as BclX-L and BIRC5. Additionally, our cell lines retained the ability to give rise to derivatives from the three germ layers as assessed by teratoma formation analysis (Supplementary Information Fig. S9).

Over-expression of VGLL4 promotes hESC survival following dissociation to single cells

To directly test the ability of VGLL4 to promote survival in the face of dissociation stress, we treated either VGLL4-overexpressing hESCs or control hESCs with the calcium-chelating agent ethylenediaminetetraacetic acid (EDTA), which disrupts E-cadherin mediated cell-cell junctions. VGLL4 overexpression resulted in a significantly higher proportion of live cells in both our EDTA-treated cultures as well as untreated controls as determined by AnnexinV and propidium iodide (PI) staining (Fig. 3A–3D). In addition to maintaining a higher proportion of live cells, VGLL4 overexpression decreased the proportion of apoptotic cells in both conditions (Fig. 3A–3D). Moreover, dissociated VGLL4-hES cells had significantly higher colony formation efficiency when plated at low densities both in the presence and absence of Rock inhibitor (Y-27632) (Supplementary Information Fig. S5).

Consistent with the idea that VGLL4 promotes survival of hESCs, we observed that cells overexpressing VGLL4 displayed an increased doubling rate as compared to controls that was consistent across multiple human pluripotent stem cell lines (Supplementary Information Fig. S6). This doubling rate was independent from a change in the distribution of cells in the cell cycle or an increase in the proportion of mitotic cells (Supplementary Information Fig. S7).

We next assessed whether the decrease in the proportion of apoptotic cells in populations overexpressing VGLL4 was correlated with a decrease in the activity of effector or initiator caspases, Caspase-3/7 or Caspase-9.VGLL4-hESCs showed a significant decrease in activated Caspase-3/7 and 9 activity in both the untreated and EDTA-treated populations (Fig. 3E, and Supplementary Information Fig. S8A). Together, these experiments suggest that VGLL4 reduces caspase activation resulting in increased survival.

Since VGLL4 is sufficient to promote hESC survival, we wondered whether VGLL4 was also necessary to regulate this process. To this end, we identified a short-hairpin RNA (shRNA) that achieved a VGLL4 knockdown of 60% (V78) (Supplementary Information Fig. S8B). Consistent with a potential interaction with the pluripotency network, decreased

levels of Vgll4 caused a modest decrease in pluripotency gene expression (Supplementary Information Fig. 10), although the effect of these changes was not studied further. Knockdown of Vgll4 by V78 caused a significant increase in Caspase 3/7 and 9 activities both under maintenance and dissociation conditions (Fig. 3F, and Supplementary Information Fig. S8C), suggesting that VGLL4 is necessary for normal regulation of caspases in hESCs.

Finally we addressed whether the activity of VGLL4 was required for the beneficial effects of Rock inhibition on hESC survival. No significant differences were detected between control and shRNA-treated cells in a colony-forming assay either in the absence or at high concentrations of Rock inhibitor. However, at intermediate concentrations of the inhibitor (1 μM–7.5 μM), we observed that cells deficient for VGLL4 have a decreased colony-forming efficiency compared to wildtype cells (Fig. 3G). This result demonstrates that VGLL4 is necessary to fully benefit from the inhibition of Rock activity, and suggests the existence of a genetic interaction between VGLL4 and the Rock-signaling pathway in maintaining hESC survival.

Discussion and Conclusions

Through a gain-of-function screen in hESCs, we identified VGLL4, a co-transcriptional regulator with no previously described role in hESCs, as a novel regulator of hESC maintenance. We demonstrated that VGLL4 over-expression promotes survival of hESCs in the context of dissociation stress by decreasing Caspase activation. Conversely, reduction in VGLL4 by shRNA knockdown results in an increase in Caspase activation, and impairs the ability of hESCs to respond to the pro-survival effects of Rock inhibition.

A fascinating open question is the interaction between mechanisms controlling cell survival, pluripotency, and developmental state. Interestingly, some of the candidates from our initial screen have been reported to have a role in regulating apoptosis and the stress response (Supplementary Information Table S2). High levels of cell death are consistently observed in the first few days of hES cell differentiation. It is therefore possible that by inhibiting self-renewal signals, our screening assay not only created permissive conditions for certain pluripotency or self-renewal genes, but also for genes involved in survival. Future work may elucidate the extent to which VGLL4 bridges these processes in hESCs. Previous studies have shown an interaction between VGLL4 and TEAD family members [20], which are also transcriptional effectors of Hippo signaling together with YAP and TAZ. Whether the VGLL4-TEAD interaction is independent of Hippo pathway signaling remains to be determined. It will be of significant interest to explore the possibility that VGLL4 regulates this pathway since Hippo signaling has been previously implicated in apoptosis, replication (reviewed in [23, 24]), early embryonic development [25], pluripotency [26], and reprogramming [27], and there is increasing evidence for its regulation via cytoskeleton proteins [28, 29].

The discovery of Rock inhibitor and its ability to increase the survival of dissociated hESCs provided an important tool to begin to address the problem of low viability of hESCs upon dissociation [1]. However, survival remains a challenge for many applications of pluripotent

cells. We found that VGLL4 can improve hES cell survival after dissociation, a finding with important implications for improving the efficiency of hES cultures, especially at lower densities. Given that VGLL4 is a co-transcriptional regulator, we hypothesize that VGLL4 may act in the nucleus to mediate transcriptional changes that prevent apoptosis. Alternatively, VGLL4 could be exported out of the nucleus to carry out a transcription-independent role for modulating cell survival, perhaps by directly interacting with members of the Rock pathway in the cytoplasm (Figure 3H). This study contributes to a deeper understanding of the mechanisms controlling cell survival in hESCs and represents a novel avenue to improve survival of hESCs. Developing more robust and efficient culture conditions for hESCs will undoubtedly aid the future therapeutic application of these cells.

Materials and Methods

Gain-of-function screen

Titrated and concentrated lentivirus carrying version 1.1 of the human ORFeome was used to transduce HUES6 hES cells. 2×10^6 HUES6 hES cells were transduced with 7×10^6 viral particles by incubating cells and virus in a low-attachment dish for 2 hours and subsequently plating onto three 15-cm dishes of MEFs as a feeder layer. This methodology ensured a low transduction rate of MEFs to maximize the proportion of transduced hES cells and resulted in an MOI of 3 or 4. We estimate that our library was covered close to 1000 times, ensuring adequate coverage.

Following transduction, cells were incubated in hES medium for 48 hours before selecting with 2μg/ml of puromycin. This incubation period ensured enough time for cell attachment, viral integration, and viral gene expression to begin. The GFP control allowed us to assess EF1α promoter in transduced cells. Additionally, by following the proportion of GFP-positive cells throughout selection, we were also able to assess PGK promoter activity (Supporting Information Fig. S1). Following puromycin selection cells were treated with SB-431542 for three weeks. After this treatment, the colonies that retained hES cell morphology were isolated manually and lysed to obtain genomic DNA. Primers surrounding the ORF region were used for PCR. Gel-purified PCR products were then sequenced and the ORF was identified using BLAST.

We identified 75 open reading frames (Supplementary Information Table S1) that were able to maintain hES cell colony morphology under the differentiation conditions that we used for our screen. None of these genes had been previously implicated in pluripotency and they belong to diverse gene ontology groups. Interestingly, Vestigial-like 4 (Vgll4) was identified 3 independent times, an event with a probability of 1×10^{-6}. All other candidates were identified only once.

Culture of hES cells

HUES6 were plated on irradiated murine embryonic fibroblasts (MEFs) and grown in media containing KO-DMEM (Invitrogen), 10% Knockout Serum Replacement (KOSR, GIBCO), 10% human plasma fraction (Talecris), 2 mM L-glutamine (Invitrogen), 0.1 mM non-essential amino acids (GIBCO), 0.055 mM β-Mercaptoethanol (GIBCO), and 10 ng/ml

bFGF (Invitrogen). Cultures were passaged using 0.05% Trypsin-EDTA (GIBCO) at a ratio of 1:6–1:10 every 5–7 days. Where indicated, SB-431542 (Sigma Aldrich) was used at a concentration of 10 μM, SU-5402 (Tocris) at 20 μM. Both of these were resuspended in DMSO. DMSO vehicle controls were made with the equivalent volume of DMSO of the two chemicals combined. Retinoic Acid was used at a final concentration of 10 μM. MEFs were plated on plates coated with 0.1% gelatin. MEF media contained 1X DMEM (Mediatech), 10% FBS, 2mM L-glutamine (Invitrogen), and 0.1mM non-essential amino acids (GIBCO).

Lentivirus production

293T/17 cells were grown in DMEM (Mediatech), 10%FBS, 2 mM L-glutamine (Invitrogen), and 0.1 mM non-essential amino acids (GIBCO). A day before transfection, cells were plated at a concentration of ~85000 cells/cm2 (or 8.0 × 105 per well of a 6-well dish). 20 hours later a media change was done on the cells. 2 hours after adding fresh media, the cells were transfected with a total of 2 μg DNA per well of a 6-well dish. A third-generation packaging system consisting of tat, rev, gag/pol and VSVG was mixed in a ratio of 5:1:1:1:2 (DNA:tat:rev:gag/pol:VSVG). As per manufacturer's instructions, 5 μl of TRANS-IT 293 (MirusBio) and 167 μl Optimem (GIBCO) were mixed thoroughly and incubated at room temperature for 5–10 minutes. Following this incubation, 172 μl of Mirus/Optimem mix was added to the DNA/packaging plasmid mix with very gentle mixing and incubated for 20 minutes at room temperature. After this incubation, the mix was added dropwise onto the cells and mixed gently by moving the plate back and forth. A media change was performed 24 hours later. Virus was harvested 24 hours later, filtered through a low-protein binding 0.45 μm filter, and added to cells or flash frozen in liquid nitrogen and stored at −80°C until use.

hES cell transduction

Cells were MEF-depleted for 30–45 min on gelatin-coated plates. Cells in suspension were collected, counted, and mixed with harvested lentivirus at an MOI of 2. Cells were incubated with the virus in a low-attachment dish for 2–3 hours at 37°C and 5% CO2 with occasional rocking. After this time, the cells were pelleted by centrifugation at 1000 rpm for 5 minutes at room temperature. The cells were then plated onto Puromycin-resistant MEFs (BioPioneer, GlobalStem). 48 hours later, transduced cells were selected with 2 μg/ml of puromicyn for 2 days, generating a population of >98% transduced cells.

Flow cytometry analysis

Cells treated with DMSO or 10 μM SB-431542 were harvested using 0.5% trypsin. Cells were washed once in PBS and then incubated in 100 μl of a 1:100 dilution of mouse IgM anti-TRA 1–60 (Millipore) or rat IgM anti-SSEA-3 (Santa Cruz Biotechnology)in FACS buffer (2% Hyclone Fetal Calf Serum in PBS). Cells were incubated in primary antibody for 15–30 minutes on ice. After this incubation, cells were washed with PBS and stained with secondary antibodies conjugated to APC (Jackson Immunoresearch) at a dilution of 1:300 for 15 minutes on ice. Cells were washed with PBS and resuspended in FACS buffer. Cells were filtered immediately before analysis through a 35 μm filter. An LSRII was used for analysis.

Immunofluorescent staining

Cells were washed once with PBS and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. Two washes were performed after fixation. Cells were blocked for 1 hour at room temperature or overnight at 4°C in 5% donkey serum in 0.1% PBST (0.1% TritonX-100 in PBS). Cells were incubated with primary antibodies in block for 2 hours at room temperature or overnight at 4°C. Primary antibodies used are: goat anti-Oct-4, 1:200 (Santa Cruz biotechnology); rabbit anti-Nanog, 1:50 (R&D); mouse anti-Sox2 1:200 (Cell Signaling Technologies), mouse IgM anti-TRA 1–60 (Millipore), rabbit anti-HA, 1:50 (Cell Signaling Technologies). Primary antibody was washed 2–3 times with 0.1% PBST. Next, cells were incubated for 1 hour at room temperature with secondary antibodies raised in donkey and conjugated to Alexa fluorescent probes. All secondary antibodies were used at a 1:300 dilution. Cell nuclei were stained using DAPI at 1 μg/ml.

Real-time PCR analysis

RNA was harvested using a Qiagen RNeasy kit. DNAse I treatment was used as indicated by the manufacturer to eliminate genomic DNA. Purified RNA was used as input for the reverse transcription reaction using the Superscript III First Strand kit (Invitrogen). TaqMan assays (Applied Biosystems) were used for human VGLL4 and samples were prepared following manufacturer instructions. GAPDH was used as a control. All samples were analyzed using a 7900HT machine (Applied Biosystems) using the Fast protocol as specified by the manufacturer (Applied Biosystems). Cycling parameters were the following: UNG incubation, 2 minutes at 50°C; Polymerase activation, 20 seconds at 95°C; denaturing, 1 second at 95°C; anneal/extend, 20 seconds at 60°C with 40 cycles of denaturing/extending/annealing. Resulting Ct values were then processed using the ΔΔCt method to obtain relative changes in expression.

Microarray analysis

RNA was purified from total cultures in quadruplicates (biological replicates) using a Qiagen RNeasy kit and 200 ng of starting material were used as input for the Illumina TotalPrep Amplification Kit. Samples were then hybridized to an Illumina microarray. A selection of genes with a p-value < 0.05 on at least 5 arrays was analyzed using SAM (http://www-stat.stanford.edu/~tibs/SAM) with an FDR=7%. Network analysis was performed using GeneGO (http://www.genego.com).Data has been deposited in the NCBI Gene Expression Omnibus under accession number GSE44590.

Growth curve analysis

Cells were passaged as described above. An aliquot of cell suspension was counted using a Vi-Cell XR counter (Beckman Coulter). The exponential growth formula ($N = N_o * e^{k\Delta t}$) was used to determine the growth rate (k).

Apoptosis analysis by Annexin V and PI

Cells were treated with 500 μM EDTA or 10 μM etoposide for 12–18 hours at 37°C and 5% CO_2. All floating cells were collected. Remaining adherent cells were then trypsinized and washed with ice cold PBS. Cells were stained following manufacturer instructions

(Invitrogen) as follows. First, cells were resuspended in Annexin V binding buffer and aliquoted where necessary to have 1–2 × 10^6 cells per sample of 100 μl. Cells were then stained with 1 μg/ml PI and 5 μl of APC Annexin V. Samples were incubated at room temperature for 15 minutes protected from light. After the incubation period, cells were resuspended in 400 μl of binding buffer and analyzed by FACS in a BD LSR II machine. Unstained and single-color controls treated with etoposide were used to perform compensation and set gates.

shRNA knockdown

ssDNA oligos were obtained from Invitrogen for VGLL4 (V78: Hmi423278) and processed using the instructions for the BLOCK-iT Pol II miR RNAi Expression Vector Kits. shRNAs were cloned into the EF1α-pDEST using Gateway cloning to use the same vector used in our screening strategy. Sequences were verified using Geneious (http://geneious.com/). Lentiviral particles were made as outlined in Chapter 2. Cells were transduced with the lentiviral shRNA constructs as indicated in the transduction section and selected with puromycin. Cells were maintained in hESC medium for 7 days after selection. After this timepoint cells were collected for RNA analysis or maintained as described above for subsequent analyses.

Clonality assay

Cells were dissociated with 0.05% Trypsin-EDTA as described above for passaging. Cells were washed once in PBS and then incubated in 100 μl of a 1:100 dilution of mouse IgM anti-TRA 1–60 (Millipore) in FACS buffer (2% Hyclone Fetal Calf Serum in PBS). Cells were incubated in primary antibody for 15–30 minutes on ice. After this incubation, cells were washed with PBS and stained with secondary antibodies conjugated to APC (Jackson Immunoresearch) at a dilution of 1:300 for 15 minutes on ice. Cells were washed with PBS and resuspended in FACS buffer. Cells were filtered immediately before sorting through a 35 μm filter. A BD Aria was used for sorting TRA 1–60^+ singlets. Cells were sorted onto 96-well plates previously coated with BD Matrigel for hESCs and containing 100 μl of MEF-conditioned media supplemented with 16 ng/ml of bFGF. Rock inhibitor was used in the indicated wells at a final concentration of 10 μM or at the indicated concentration. Media was changed every third day. After 10 days, cells were fixed with 4% PFA and stained with DAPI at 1μg/ml. Each well was inspected for the presence of colonies. A 4-cell minimum was considered for calling a colony.

Immunofluorescence was performed on positive wells using a goat anti-OCT4 or a rabbitanti-HA as described above.

Caspase Activity

Cells were treated as described for the AnnexinV/PI analysis. Fifty thousand cells were collected for Caspase activity analysis. Caspase-Glo Assays (Promega) were used following manufacturer instructions. Caspase activity was measured using a FLUO Star Optima luminometer (BMG Labtech).

Cell cycle dynamics by flow cytometry

hES cells were trypsinized and MEF-depleted by plating on gelatin-coated plates for 45 minutes. $1 – 4 \times 10^6$ cells were resuspended in 0.5 ml PBS followed by the addition of 0.5 ml of 100% ice-cold ethanol to the cells in a drop-wise manner while vortexing. After incubation for a minimum of 20 minutes on ice, cells were harvested by centrifugation (1000 rpm for 5–7 minutes) and the ethanol was decanted. Finally, 1 ml of Propidium Iodide-RNAse solution [(final concentrations 100 μg/ml PI (Molecular Probes) + 10 μg/ml RNAse Type I-A (CONCERT, Invitrogen) in PBS)] was added to the cells. After 30 minutes of incubation, the samples were analyzed by flow-cytometry by using BD-LSRII and FACSDiva. FlowJo analysis was used to determine the relative percentage of cells in different stages of the cell cycle using the Dean-Jett-Fox model.

Mitotic cell assessment by immunofluorescence

Cells were washed with PBS and fixed for 30 min with a 4% paraformaldehyde solution. Cells were then blocked using 5% donkey serum and stained with the following antibodies: anti-Oct-4 (1:200, Santa Cruz Biotechnology), anti-Human Nuclear Antigen (1:100, Millipore), anti-Phospho-histone H3 (1:100, Millipore). Appropriate secondary antibodies produced in donkey and fluorescently conjugated were acquired from Molecular Probes and used at a 1:300 dilution. Incubations with primary antibodies were done overnight at 4°C. Secondary antibodies were incubated for 1 hr at room temperature or overnight at 4°C. Cell nuclei were stained with DAPI for 20 minutes at room temperature. Image-based quantification was done using a Cellomics system.

Teratoma Formation Assay

3×10^6 to 1×10^7 hES cells overexpressing Nanog, GFP, or Vgll4 were resuspended in hES-grade Matrigel (BD) and injected under the kidney capsule of SCID-Beige mice. 30–45 days later, mice were sacrificed and the teratoma was isolated. Tissues were embedded in paraffin, sectioned, and stained with haematoxylin and eosin.

Gene Set Enrichment Analysis

GSEA was carried out using the Broad Institute GSEA analysis program (http://www.broadinstitute.org/gsea/index.jsp). Vgll4 microarray expression data was compared against WT controls (in quadruplicates) using 1000 gene set permutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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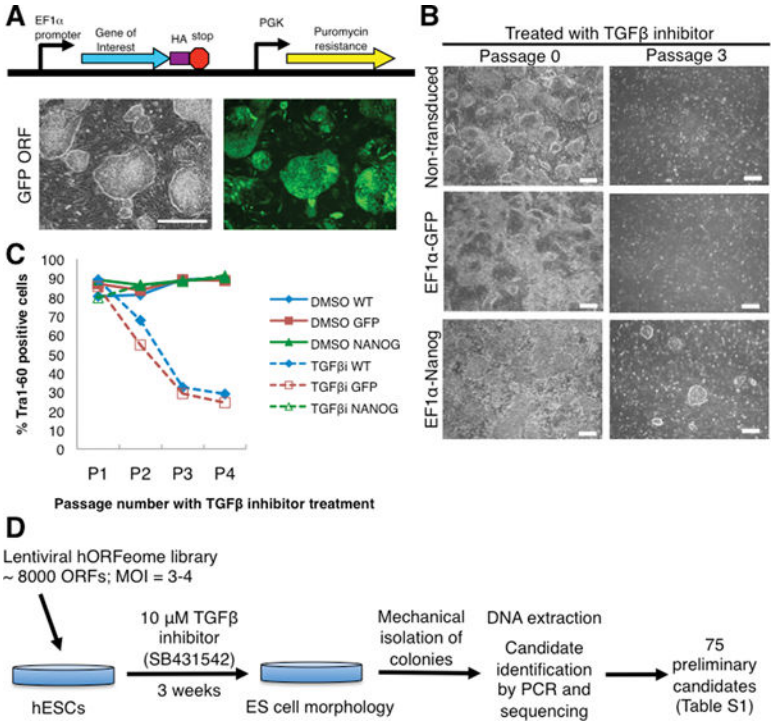

Figure 1. Strategy for the identification of novel regulators of hESC maintenance

A) The hORFeome library is contained in a Gateway-adapted pHAGE lentiviral vector (top). The expression of the hemagglutinin (HA)-tagged ORF is driven by an EF1α (elongation factor 1 alpha) promoter, and a puromycin resistance gene driven by a PGK (phosphoglycerate kinase) promoter facilitates selection of transduced cells. Primer-binding sites are indicated by black arrows. The system was validated by transducing hESCs with a GFP ORF and selecting with 2 μg/ml of puromycin two days after transduction (bottom). Bar = 500 μm. B) Validation of screening conditions. Cells transduced with a Nanog ORF were used as a positive control, cells transduced with a GFP ORF and wildtype cells were used as negative controls. Negative controls lost the characteristic hESC morphology after three weeks or 3 passages of TGFβ inhibitor (TGFβi) treatment. Bars = 500 μm. C) Cells transduced with GFP and treated with TGFβi for three weeks downregulate Tra1–60 whereas NANOG-transduced cells do not. D) hESCs transduced with lentiviruses containing approximately 8000 genes were subjected to three weeks of treatment with 10μM TGFβ inhibitor. Colonies with characteristic ES cell morphology after treatment were isolated manually and their DNA was sequenced to identify 75 preliminary candidates (Supplementary Information Table S1). MOI=Multiplicity of infection.

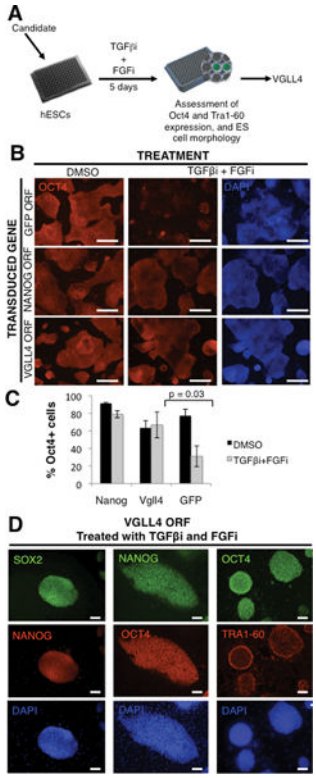

Figure 2. Verification of primary hits using a secondary verification assay

A) Preliminary candidates were verified by 5 days of treatment of transduced cells with with 20μM FGF inhibitor (FGFi) in addition to 10μM TGFβi. Following treatment, colony morphology and expression of hESC markers OCT4 and TRA 1–60 were analyzed. B) Cells transduced with VGLL4 or control genes were treated with vehicle control or with the inhibitor mix for 5 days and stained for OCT4 at the end of the treatment. Bars = 500 μm. C) Quantification of $OCT4^{+}$ cells after treatment using Cellomics image acquisition and software. Error bars represent the standard deviation and p-values were obtained using a Student's T-test. D) VGLL4-transduced cells treated with the inhibitor combination and stained for several hES cell-specific markers. Bars = 100 μm.

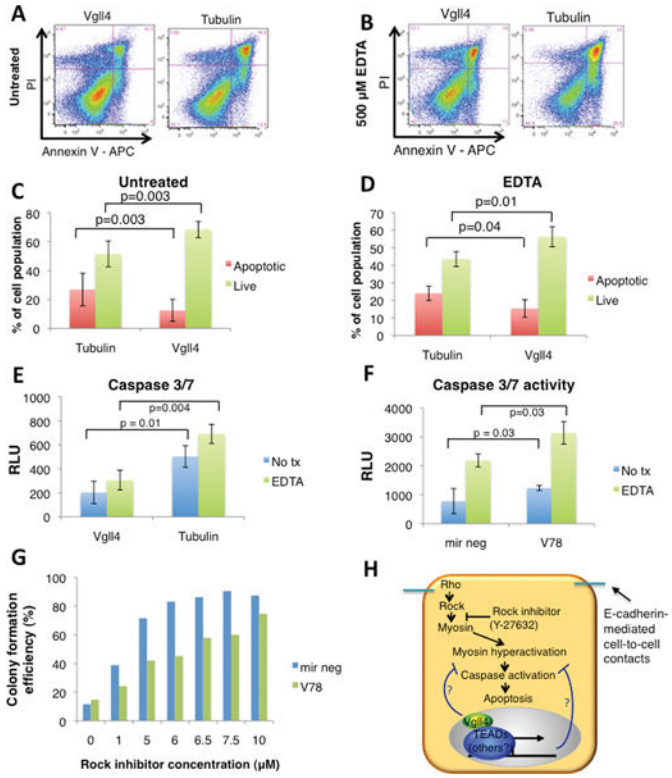

Figure 3. VGLL4 promotes survival and prevents apoptosis of hES cells through modulation of Caspase activation

A–D) AnnexinV and PI staining of VGLL4 or control hESCs maintained in self-renewal conditions (A) or treated with 500 μM EDTA (B) for 12–18 hours and analyzed by FACS. Live cells are APC^{-}, PI^{-}; apoptotic cells are APC^{+}, PI^{-}, and dead cells are PI^{+}. C, D) Flow cytometry data of cells processed as in (A, B) from 6 independent experiments. Error bars represent the standard deviation and the p-values were obtained with an unpaired Student's T-test. E) Caspase 3/7 activity for VGLL4 and control hESCs after treatment with self-renewal or dissociation conditions (500 μM EDTA) using an activity-dependent caspase luminescence assay. Relative luminescence units (RLU) represent the signal after subtracting the background signal. Error bars represent the standard deviation of triplicates. P-values were obtained using an unpaired Student's T-test. F) Increased caspase activity upon VGLL4 knockdown. Caspase 3/7 activity for scrambled control (mir neg) and VGLL4 knockdown (V78) in self-renewal and dissociation conditions measured as described in (E). Error bars represent the standard deviation from triplicate samples. P-values were calculated using an unpaired Student's T-test. G) Colony formation efficiency for 100 cells/well treated with the indicated concentration of Rock inhibitor. H) Model for mode of action of VGLL4. Rho signaling is activated upon disruption of E-cadherin cell-to-cell contacts resulting in apoptosis. VGLL4 could inhibit cell death by modulating the expression of apoptosis and cytoskeleton genes. Alternatively, VGLL4 could have a role in the cytoplasm independent from its role as a co-transcriptional regulator in the nucleus.

Table 1

Functional networks with a significant response to Vgll4 overexpression in hES cells

#	Networks	p-value (downregulated genes)
1	Cell adhesion: Integrins signaling to Beta-catenin	2.576E-05
2	Cytoskeleton biogenesis	4.156E-05
3	Cartilage development	3.824E-04
4	Blood coagulation through the Calpain system	7.398E-04
5	Ossification through BMPs	9.927E-04
6	Transmission of nerve impulse through Ephrin receptors	2.327E-03
7	Apoptosis: LIGHT (Tumor Necrosis Factor Superfamily Member 14), Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (Apo-2), CD27BP, Fas Ligand, TWEAK (Tumor Necrosis Factor Superfamily Member 14), Tumor Necrosis Factor-alpha signaling	2.584E-03
8	Cell adhesion: Plasminogen Activator Inhibitor 1 signaling, Peroxisome Proliferator-Activated Receptor-beta (delta), Retinoid X Receptor-alpha regulation	2.584E-03
9	Apoptosis: Necrosis Growth Factor, Tumor Necrosis Factor signaling, NF-?B regulation	2.864E-03
10	Cell adhesion: Signaling to Myosin Light Chain Phosphatase via Integrins	3.493E-03