Glycogen Synthase Kinase-3 Inhibition Enhances Translation of Pluripotency-Associated Transcription Factors to Contribute to Maintenance of Mouse Embryonic Stem Cell Self-Renewal

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Abstract

Maintenance of embryonic stem cell (ESC) self-renewal and pluripotency are controlled by extrinsic factors, molecular signaling pathways and transcriptional regulators. While many of the key players have been studied in depth, how the molecular signals interact with transcription factors of the pluripotency network to regulate their action remains less well understood. Inhibition of glycogen synthase kinase 3 (Gsk-3) has been implicated in the maintenance of mouse ESC pluripotency, although there is contradictory data on its role, with enhancement of cell survival and metabolism, stabilization of c-Myc and activation of Wnt signalling proposed as potential mechanisms. We have discovered that suppression of Gsk-3 activity leads to enhanced protein levels of key transcriptional regulators of the pluripotency network, notably Nanog, Tbx3 and c-Myc. Protein stability was unchanged following Gsk-3 inhibition, although interestingly, Nanog and Tbx3 proteins were found to have half-lives of 1–3 h, while that of Oct4 protein was longer, at 6 h. We demonstrate that the effects on protein levels seen following inhibition of Gsk-3 are due to both enhanced de novo synthesis of Nanog protein and increases in the proportion of Nanog and Tbx3 RNAs bound to polysomes, findings consistent with Gsk-3 regulating translation of these factors. These effects were not due to changes in regulators of general translation initiation machinery nor mediated via the 5’ or 3’ UTR sequences of Nanog alone. The data we present provide both new conceptual insights into the mechanisms regulated by Gsk-3 that may contribute to ESC self-renewal and, importantly, establish control of protein translation as an additional mechanism involved in modulation of ESC pluripotency.
key to maintaining pluripotency [21] and the finding that Oct4, Sox2 and Nanog bind to many of the same promoter sequences has led to the proposal that they form a regulatory network which reinforces pluripotency [22,23]. Other transcription factors also contribute to the maintenance of the ESC state and include Tbx3, which is regulated by LIF and Fgf3-dependent pathways [24,25] and c-Myc, ectopic expression of which can relieve the need for LIF/STAT3 signaling [7]. The nuclear receptor Esrrb can also contribute to maintenance of the ESC state and include Tbx3, which is regulated by LIF and Fgf3-dependent pathways [24,25]. Despite this knowledge, we still lack a detailed understanding of how the molecular signals implicated in control of self-renewal interact with the intrinsic network of pluripotency-associated transcription factors, in large part due to the focus on transcriptional regulation.

The dynamic transcriptional control of Nanog and other ESC-expressed factors such as Rex1 and Esrrb has been reported [19,28], implying that in a pluripotent state ESCs are primed to respond to environmental signals, whether that signal promotes pluripotency or differentiation. However, transcriptional changes can be slow in comparison to post-transcriptional mechanisms so, conceptually, regulation of protein, rather than RNA levels would endow ESCs with the ability to respond rapidly to changes in the environment. Intriguingly, very little is currently known about the dynamics of pluripotency transcription factor protein expression and the regulatory mechanisms involved. Here, we demonstrate that a key regulator of mouse ESC pluripotency, Gsk-3, controls the protein levels of key members of the pluripotency network of transcription factors by post-transcriptional mechanisms. Acute inhibition of Gsk-3 led to up-regulation of protein expression of Nanog and Tbx3. At early stages following Gsk-3 inhibition enhanced protein synthesis was observed, which preceded increases in transcription. Furthermore, inhibition of Gsk-3 increased the proportion of Nanog and Tbx3 transcripts associated with polyribosomes, consistent with enhanced translation. By demonstrating that control of protein translation by Gsk-3-dependent signaling regulates levels of key transcription factors, our results provide new conceptual insight into the mechanisms contributing to ESC pluripotency.

**Materials and Methods**

**Embryonic Stem Cell Culture**

The mESC lines, E14tg2a [29], Nanog-TNG [19], Gsk-3 DKO and Gsk-3 WT [17] were cultured on tissue culture dishes coated with 0.1% (v/v) gelatin with Knock-Out (KO) Dulbecco’s modified Eagle medium (In vitrogen) in the presence of 1000 units/ml murine LIF (ESGRO; Chemicon) as previously described [11]. The Nanog reporter mESC line, referred to as Nd-ESC, was derived from E14tg2a ES cells and contains a BAC transgene in which the short-lived fluorescent protein VNP (Venus) has been placed under the control of Nanog regulatory regions. The generation and characterisation of this cell line are described in detail elsewhere [30]. The Nd-ESC reporter line was routinely grown in Glasgow Modified Eagle Medium (GMEM, Invitrogen), supplemented with 10% (v/v) fetal bovine serum (FBS) (ESGRO; Chemicon) and 2 ng/ml LIF. For time-course analyses with Gsk-3 inhibitors, ESCs were cultured in GMEM supplemented with 1% (v/v) ES-tested Fetal Bovine Serum (FBS) (HyClone, ThermoFisher) and 1000 U/ml LIF or in defined N2B27 medium supplemented with 1000 U/ml LIF (Chemicon) and 10 ng/ml BMP4 (Stemgen). Defined N2B27 medium consists of 1 volume DMEM F-12 medium: 1 volume Neurobasal medium (Invitrogen) supplemented with N2 and B27 supplements (Invitrogen), 0.0125% (v/v) Monothioglycerol (Sigma), 50 mg/ml bovine serum albumin (BSA) (Sigma), 2 mM Glutamine (Invitrogen), as previously described [12,31]. Gsk-3 inhibitors used were CHIR99021 (Axon MedChem) and 1 m [14]. mESCs were also cultured in N2B27 in the absence of LIF and BMP4 and the presence of 3 m CHIR99021 and 1 m PD0325901 (MEK inhibitor; Axon MedChem) ‘2i conditions’, as previously described [15].

**Measurement of ESC Growth**

Cells were plated at 2 x 10^5 cells/cm^2 onto 5 cm dishes in GMEM supplemented with FBS and LIF, N2B27 supplemented with LIF and BMP4 or N2B27 with DMSO (Control), 3 m Gsk-3 inhibitor (CHIR) or 1 m MEK inhibitor (PD0325901) for 3 days. Dishes were counted in triplicate using Trypan Blue dye.

**Time-lapse Confocal Microscopy, Flow Cytometry and Cell Sorting**

For time-lapse confocal microscopy, 24 h after plating in serum plus LIF Nanog-TNG mESCs [19] were equilibrated for 1 h at 37°C on the confocal microscope prior to addition of 3 m CHIR99021, or DMSO as a control. Cell populations were imaged by time-lapse for 24 hours, with pictures taken every 10 minutes. 10 random fields, each containing approximately 15 colonies, were recorded for each condition. Fluorescence-activated cell sorting, performed with a FACS Aria instrument (Becton Dickinson) was used to sort Nanog-VNP-low populations of Nd-ESCs. Live cells were gated based on forward and side scatter. Immediately after sorting, cell viability was determined and Nanog-VNP-low cells were plated at 3 x 10^4 cells/cm^2 onto gelatin-coated dishes in the presence of serum and LIF, and incubated in the presence of 3 m CHIR99021 or DMSO (as a control). Cells were followed for 24 h, during which cell morphology and the percentage of Nanog-VNP positive cells were assessed (time points –0, 4, 8, 12 and 24 h). E14tg2a cells were used as a negative control, to obtain the positive gate region. Nanog-VNP analyses were performed on a FACS Calibur flow cytometer (Becton Dickinson).

**Assessment of Protein Stability and Re-synthesis**

To assess protein stability, ESCs were cultured in GMEM supplemented with LIF and serum or N2B27 supplemented with BMP4 and LIF overnight before adding 10 mg/ml of cycloheximide. Protein extracts were obtained after 0, 1, 3 and 6 hours of cycloheximide addition using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) following the Manufacturer’s instructions. Protein concentrations were measured using the Bio-Rad protein assay reagent following the Manufacturer’s instructions. To investigate protein re-synthesis, cells were cultured as above, treated with 10 mg/ml cycloheximide for 4 hours after which cells were washed extensively before addition of fresh medium free of cycloheximide. Protein extracts were obtained using the NE-PER nuclear and cytoplasmic extraction reagent (Pierce).

**SDS-PAGE and Immunoblotting**

Cell lysates were separated by SDS-PAGE using 10 and 12% polyacrylamide gels and transferred to nitrocellulose membranes by immunoblotting in semi-dry transfer buffer as described previously [32]. Blots were probed with the following antibodies using the concentrations stated: 1:2000 anti-Oct4 (Santa Cruz Biotechnology; sc-9801), 1:1000 anti-Nanog (Abcam; Ab80892); 1:1000 anti-c-Myc (Cell signalling, 9402), 1:750 anti-Tbx3 (Santa Cruz Biotechnology; sc17871) and 1:15000 anti-Gapdh (Ambion;...
AM4300). Goat anti-rabbit, rabbit anti-goat or goat anti-mouse antibodies conjugated to horseradish peroxidase (DAKO) were used at 1:20,000 and blots were developed using Chemiglow (GRF/AlphaInnotech) or ECL Prima (GE Healthcare) according to the manufacturer’s directions. Blots were stripped and reprobed as previously described [32].

Polysomal Fractionation and RNA Isolation
ESCs grown in GMEM supplemented with LIF and serum were treated as required and polysomal fractionation carried out by differential sucrose density centrifugation as described in [33], with minor amendments. Briefly, prior to cell lysis, 150 μg/ml cycloheximide was added for 15 minutes to stop ribosome movement. Cells were then lysed using buffer described previously [32] supplemented with 2 mM DTT, 150 μg/ml cycloheximide and 80 U/ml RNAsin. Cell lysates were centrifuged for 3 minutes at 4°C and 6000 rpm to remove the nuclei and the supernatant transferred to new tubes and re-centrifuged at 4°C and 16,000 rpm for 5–10 minutes to remove mitochondria and membrane particles. Supernatant from equivalent cell numbers for each sample was loaded into a sucrose gradient column made in DEPC-treated water with 10 mM Tris-HCl pH 7.5, 140 mM NaCl, 1.5 mM MgCl2 and 10% (w/v) l-EDTA (stock 0.5 M) and 10 μM proteinase K (stock 100 mg/ml), mixed immediately and incubated at 37°C for 30 minutes. After incubation, 200 ng yeast tRNA was added to assist with precipitation. Samples were extracted using phenol:chloroform (1:1) and RNA precipitated from the aqueous phase. Washed and dried RNA pellets were resuspended in 20 μl DEPC-treated water. OD at 260 nm was measured to determine polysomal distribution and equal volumes of individual fractions were either pooled to generate monosomal (fractions 1–7) and polysomal (fractions 8–16) samples or analysed as individual fractions. LiCl precipitation was performed on the pooled fractions.

Preparation of Total RNA
Total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturers instructions. To remove contaminating genomic DNA, RNA samples were treated with RQ1 DNase. 1 μg of RNA was incubated with 1 U DNase (Promega) in DNase buffer (400 mM Tris-HCl pH8.0, 100 mM MgSO4, 10 mM CaCl2) (Promega) at 37°C for 30 minutes. DNase was heat inactivated at 65°C for 10 minutes with 1 μl DNase stop solution (20 mM EDTA pH8.0). Reverse transcription was carried out as previously described [34].

Quantitative RT-PCR
qRT-PCR was performed using the LightCycler™ 1.5 system (Roche) as previously described [34]. Gene specific amplification was verified by performing melt-curve analysis after 40 amplification cycles. PCR efficiencies of both the target and the reference genes were calculated and the relative values for calibrator-normalised target gene expression determined using Lightcycler software (v4.0). Primer sequences used are shown in Table S1. Transcript levels were normalised to β-actin and either a 2-way ANOVA followed by Bonferroni post-hoc test or two-tailed Students t-test were used to check statistical significance between samples.

Bioinformatic Analysis of Nanog 5’ and 3’ UTR Sequences
Potential IRES sequences were identified by searching for polypyrimidine tracts (≥10) and then analysing for sequence motifs e.g. CCTCC, often found within such tracts in functional IRES. Potential upstream open reading frames (uORFs) were identified by looking for a start codon (AUG, or alternatives) in the 5’ UTR, an in-frame stop codon before the end of the coding sequence and a length of at least 9 nucleotides. All analyses were performed using in-house Perl scripts. AU-rich elements (ARE sites), that bind proteins such as TTP, HuR, and AUF1 to stabilise/destabilise miRNA, were searched for using AREst 6 [http://rna.tbi.univie.ac.at/cgi-bin/AREsite.cgi].

Cloning of Nanog 5’ and 3’ UTR Sequences and Luciferase Reporter Assays
The 215bp 5’ UTR region of mouse Nanog gene (RefSeq NM_02802816), was amplified with the following primers (Nanog 5’UTR forward primer sequence with Nhel restriction site underlined - 5’ AATAGGCTAGCTGCTATCCGGTTTTGACCCCGTTGG 3’ and Nanog 5’ UTR reverse primer 2 sequence with XhoI restriction site underlined - 5’ ATAACAATGCAGTGTCAGGTGTAATGCGGAAGTGA 3’) and cloned into Nhel/XhoI restricted pGL3’ [35,36] to generate pGL3’-Nanog-5’ UTR. 222bp (R1) or 1048bp (R2) segments of the 3’ UTR region of mouse Nanog gene (RefSeq NM_02802816) were amplified with the following primers (Nanog 3’UTR forward primer sequence with SpeI site underlined – 5’ ATATAACGTGACTTACGCAACATCTGGGCT 3’ and Nanog 3’ UTR reverse primer 1 5’ TTATAAGATTT9GACTGCTTTCCGGAAGGTCTC 3’ and Nanog 3’ UTR reverse primer 2 5’ TTATAGAATTT9GACTGCTTTCCGGAAGGTCTC 3’ to generate 222 bp segment (R1) or reverse primer 2, to generate 1048 bp segment (R2), with EcoRl site underlined) and cloned into SpeI/EcoRl restricted pGL3-MCS [35,36] to generate pGL3-Nanog-3’-UTR-R1 and R2 constructs. All constructs were verified by DNA sequencing. Mouse E14 ESCs were transiently transfected with 2 μg of either parental vector (pGL3’ or pGL3-MCS) or test vector (pGL3’-Nanog-5’ UTR, pGL3-Nanog-3’-UTR R1 or R2) along with 0.04 μg of Renilla plasmid using Lipofectamine 2000 as previously described [14]. 24 h after transfection cells were treated with DMSO (vehicle control) or Gsk-3 inhibitor 1 μM and after a further 4–16 h cell extracts were prepared and dual luciferase assays performed as previously described [14].

Results
Gsk-3 Inhibition Regulates Expression of Pluripotency-associated Transcription Factor Proteins
Based on the range of roles reported for Gsk-3 in regulation of mouse ESC self-renewal and pluripotency [14,15,16,21,22,29,30,31,32] and the gap in our understanding of how signaling pathways interact with the intrinsic network of ESC pluripotency transcription factors, we decided to examine the impact of inhibition of Gsk-3 on protein levels of key regulators of ESC pluripotency. Protein and RNA levels for the transcription factors Nanog, Tbx3, Oct4 and c-Myc were measured in wild-type E14 ESCs cultured with Gsk-3 inhibitors (CHIR99021 [15] or 1 μM [14]) and in ESCs derived from this line in which all four alleles of Gsk-3 have been knocked out [117], Gsk-3 DKO ESCs. Previous studies have used various culture conditions to maintain mouse ESCs, commonly DMEM supplemented with serum and LIF or serum-free N2B27 supplemented with LIF and BMP4, so we felt it was important to analyse the effects of Gsk-3 inhibition under these different conditions. As shown in Fig 1A(i) and B(i) Gsk-3-inhibitor-treated WT ESCs and Gsk-3 DKO exhibited...
Regulation of Nanog and Tbx3 by Gsk-3 in mES Cells

Gsk-3 Inhibition does not Alter the Stability of Nanog, Tbx3, Oct4 or c-Myc Proteins

The data presented above raise the possibility that following Gsk-3 inhibition post-transcriptional mechanisms contribute to the increases in protein levels of Nanog, Tbx3 and c-Myc observed. The dynamics of protein expression of these factors has not been previously investigated in ESCs, although Gsk-3 is known to regulate ß-catenin [43] and c-Myc protein stability [7]. Therefore, we examined whether increased protein stability accounted for the increased levels of Nanog, Tbx3 and c-Myc proteins observed following Gsk-3 inhibition. Protein half-lives were estimated by blocking new protein synthesis with cycloheximide and monitoring protein levels over time. In each case, protein levels were normalised relative to Gapdh and levels at t = 0 were set at 100% to enable direct comparison of half-lives in different samples, irrespective of initial relative expression. The results demonstrate that inhibition of Gsk-3 does not appear to consistently affect stability of any of the proteins studied in either N2B27 supplemented with LIF and BMP4 (shown in Figure 4) or in serum plus LIF (see Figure S2). However, that it remains formally possible that in order for Gsk-3 inhibition to extend the protein half-lives of these factors new protein synthesis is required. Interestingly, these data reveal previously undocumented differences in the protein half-lives of these transcription factors. Nanog had the shortest half-life, estimated to be approximately 1 hour; Tbx3 had a half-life of between 1–3 h, c-Myc a half-life of 2–3 h and Oct4 a half-life of 6 h or more. These findings suggest that Nanog, Tbx3 and c-Myc protein levels may be able to change rapidly in response to different stimuli.

Gsk-3 - a Possible Role in Regulating Translation of Nanog?

Our finding that Nanog and Tbx3 proteins have much shorter half-lives than Oct4 protein raises the possibility that dynamic changes in protein levels allow for rapid responses to environmental signals. However, the stability of Nanog and Tbx3 was not influenced by inhibition of Gsk-3 meaning an alternate mechanism must account for the enhancement in protein levels of these transcription factors observed. General translation has been reported to be low in undifferentiated mouse ESCs and increases as cells differentiate, suggesting that regulation of translation may play a role in controlling stem cell fate [33]. Direct control of protein levels of key transcription factors via translational regulation would represent a new mode by which signalling pathways interact with components of the network of pluripotency transcriptional regulators. Therefore, we decided to investigate a possible role for Gsk-3 in translational regulation of Nanog.

To examine effects of Gsk-3 on de novo protein synthesis of Nanog, we performed a series of protein re-synthesis experiments. Protein synthesis was halted by addition of cycloheximide and re-synthesis initiated by its removal, extensive washing and addition of fresh medium. Increases in Nanog protein levels were observed as early as 1–2 hours after CHX washout in the Gsk-3 DKO ESCs, whereas in WT ESCs, Nanog protein recovery was not observed even after 4 hours (Fig 5A (i) and (ii)). Nanog RNA levels in the same samples did not significantly increase even 3 h after CHX washout, compared to CHX-treated (Fig 5A (iii)). Results demonstrating a similar trend were obtained when this experiment was conducted in serum-free N2B27 medium in the presence of BMP4 and LIF (Fig 5B). In no situation were changes in levels of Oct4 observed and our results are consistent with Nanog protein re-synthesis in Gsk-3 DKO ESCs occurring without corresponding increases in Nanog RNA levels. Treatment of WT ESCs with Gsk-
3 inhibitor 1 m also led to enhanced re-synthesis of Nanog protein (shown in Figure S3). In these latter analyses we noted that β-catenin levels do not decline significantly following 4 h treatment with cycloheximide, whereas Nanog levels do. In contrast, after a 16 h recovery phase in the absence of Gsk-3 inhibition, Nanog levels have approximately doubled, while β-catenin levels have approximately halved (compared to levels after cycloheximide treatment). These data demonstrate a disconnection between Nanog and β-catenin levels.

Next, we sought to confirm these data using an approach that did not rely on the use of cycloheximide. To achieve this we used an mESC reporter line in which the coding sequence for the fluorescent protein VNP (Venus) had been inserted into the endogenous Nanog locus (Nd-ESCs; [30]). In this cell line, VNP expression is under the control of endogenous sequences that influence Nanog expression. Furthermore, VNP has a half-life of 2–3 h, which is very similar that of Nanog, making this an ideal reporter for our studies. Flow cytometry was used to sort a population of Nanog:VNP cells with low levels of VNP (Nanog) expression (see Figure S4). These cells were immediately plated into media containing serum and LIF, in the presence or absence of Gsk-3 inhibitor, and the reappearance of VNP expression monitored over a period of 24 h. Images of the replated cells are shown in Figure S4. As shown in Figure 3C, the proportion of VNP positive cells is increased in the presence of Gsk-3 inhibitor, when compared to control cells, while cell viability was maintained at over 95% in each condition. The increase in the proportion of Nanog:VNP positive cells was significant from 8 h following re-plating of the Nanog:VNP-low population, consistent with Gsk-3 inhibition enhancing protein resynthesis. Thus, based on two alternate approaches, our results are consistent with Gsk-3 playing a role in regulating translation of Nanog.

Gsk-3 Inhibition Increases Association of Nanog RNA with Polyrribosomes

Our data demonstrate that the increase in Nanog protein observed between 1 and 3 h in Gsk-3 DKO ESCs following release of CHX block does not directly correlate with increases in Nanog RNA. However, at later time points, for example 24 h following addition of 1 m, we have observed modest increases in Nanog RNA (Fig. 1A, B (iii)). These findings suggest that while Gsk-3 may contribute to regulation of Nanog transcription at later time points, at early stages following Gsk-3 inhibition, Gsk-3’s main role may be in regulating Nanog translation. To further investigate a possible role for Gsk-3 in regulating translation of Nanog, and other pluripotency-associated transcription factors, we examined association of their RNAs with polysomes. RNAs associated with polysomes are more actively translated hence a shift in the proportion of RNA encoding a particular protein to the polysomal fraction is consistent with enhanced translation of that protein [44]. Monosomal and polysomal-enriched fractions of RNA were generated by differential sucrose density centrifugation. We analysed the distribution of Nanog, Tbx3 and Oct 4 RNAs across the entire gradient (see Figure 6A) and we also pooled fractions into generate monosomal and polysomal samples, allowing us to perform statistical analyses (see Figure 6B). As can be seen in Figure 6A, inhibition of Gsk-3 leads to an increase in Nanog RNA associated with polysomes, with two populations of Nanog RNAs clearly detected. In contrast, only very low levels of Oct4 RNA were detected in the polysomal fractions and these did not appear to alter upon inhibition of Gsk-3. Tbx3 RNAs also showed an increase in the polysomal fractions following inhibition of Gsk-3. When we analysed the distribution within the pooled monosomal and polysomal fractions, increases of between 30 and 45% in the proportion of Nanog and Tbx3 RNAs bound to polysomes were observed after 4 and 8 h treatment of WT ESCs with the Gsk-3 inhibitor 1 m and in Gsk-3 DKO ESCs and these were statistically significant (Figure 6B). On the other hand, no significant differences were observed in the distribution of Oct4 and CyclinD1 RNAs between polysomal and monosomal fractions. In the case of c-Myc a significant difference was only observed in Gsk-3 DKO ESCs, suggesting sustained inhibition of Gsk-3 is needed to increase loading of c-Myc RNA to polysomes. The increase in the proportion of Nanog RNA associated with the polyribosomes in 1m-treated and Gsk-3 DKO ESC is consistent with an increase in Nanog translation following inhibition of Gsk-3 activity and correlates with enhancement in de novo protein synthesis.

Inhibition of Gsk-3 in Mouse ESCs does not Affect Regulators of General Translation Initiation Machinery

Next, we were interested to investigate the mechanism mediating the effects of Gsk-3 inhibition on Nanog translation. A previous report has suggested that general translation rates are increased as mouse ESCs differentiate [33], indicating that translational control may be involved in regulation of ESC fate and self-renewal. Our studies have demonstrated that Gsk-3 inhibition increases the expression of pluripotency-associated transcription factors Nanog and Tbx3 and that their RNAs appear to be more actively translated. Therefore, we investigated the effect that Gsk-3 inhibition has on key regulators of translation initiation in somatic cells. The activity of the initiation factor eukaryotic translation factor 2 (eIF2) and its guanine nucleotide exchange factor, eIF2Bε, are regulated by phosphorylation. Gsk-3, downstream of PI3K, phosphorylates Ser 539 of eIF2Bε resulting in its inactivation [45,46], while phosphorylation of Ser51 on eIF2α prevents binding of eIF2Bε to eIF2α. Thus, decreased phosphorylation of both sites would be consistent with increased...
translation initiation of most mRNAs [47,48]. We examined the levels of phosphorylation of Ser539 on eIF2B and Ser51 on eIF2α in WT ESCs grown in the presence and absence of 1m and in Gsk-3 DKO ESCs. Phosphorylation of neither of these regulatory sites was altered following perturbation of Gsk-3 activity (Fig 7A), suggesting general translation initiation events are not significantly affected, consistent with our polysomal data where only a subset of pluripotency factors are targeted by Gsk-3, e.g. Nanog and Tbx3.

Another pathway that regulates protein synthesis is the mTor pathway. Gsk-3 can inhibit the mTor pathway by phosphorylating TSC2 at Ser1337 and Ser1341 leading to TSC2 activation and the subsequent inhibition of Rheb and mTor activity [49]. A key downstream effector of mTor is S6K1, which can regulate translation initiation, elongation and ribosome biogenesis and was, therefore, of particular interest. Surprisingly, we discovered that levels of S6K1 T389 phosphorylation, the site phosphorylated by mTor, were reduced following treatment of ESCs with 1 m or CHIR and in Gsk-3 DKO ESCs (Fig 7B). This effect appears selective as 1 m had no effect on T389 phosphorylation in Gsk-3 DKO ESCs (see Figure S5). We also studied changes in T389 phosphorylation in cells grown with CHIR or 1 m overnight in comparison with PD alone (Fig 7C). Consistent with the results shown in Fig 7B, in the presence of Gsk-3 inhibitors, levels of phosphorylation of S6K1 at T389 were reduced. Furthermore, when CHIR was added to cells that had been treated for 16 h with PD alone, phosphorylation of T389 declined rapidly. Taken together, these results suggest that inhibition of Gsk-3 in ESCs does not lead to general increases in translation. This supports our polysomal distribution data, where proportions of Oct4 and CyclinD1 are unaffected, arguing against a general enhancement in translation and in favour of selective action of Gsk-3 in controlling protein levels of a subset of factors involved in regulation of pluripotency.

Given that Gsk-3 inhibition does not appear to be acting via regulation of the general translation initiation machinery, we were interested to examine whether the 5′ or 3′ untranslated regions of Nanog are responsible for mediating the effects of Gsk-3 inhibition. Our bioinformatic analyses demonstrate that the 215 base pair 5′ UTR region of the mouse Nanog gene contains three pyrimidine tracts, which represent potential internal ribosomal entry sites, and one potential upstream open reading frame (uORF) with an ATG start codon, shown in Figure 7D(i). A number of uORFs with non-ATG start codons were also identified (spanning bases 10–99, 18–32, 39–113, 62–91, 153–176 and 166–219). The 1048 bp 3′ UTR region of the mouse Nanog gene contained only weakly conserved ARE sites and poorly

Figure 2. Gsk-3 inhibition or ablation can maintain the expression of pluripotent markers upon LIF withdrawal. (A) WT and Gsk-3 DKO ESCs were grown in serum-containing medium in the presence or absence of LIF and WT ESCs were also cultured in the absence of LIF in medium supplemented with 2 μM 1 m. Protein and RNA were extracted at the times indicated. (i) Immunoblotting of 15 μg of nuclear protein was performed with the indicated antibodies. (ii) Antibody signals were quantified and values normalised to Gapdh. A value of 1 was given to normalised protein levels in WT+LIF 24 h sample and values for other samples were related to these. The data show relative protein levels and are the average and SD of duplicate experiments. (iii) Quantitative RT-PCR was carried out and a value of 1 was given to normalised RNA levels in WT ESCs +LIF at 24 h and other samples related to these. The data show relative gene expression levels and are the average and S.E.M of quadruplicate samples. (B) (i) E14tg2a ESCs were grown for 24 hours in basal N2B27 medium without LIF or BMP4 in the presence or absence of PD (1 μM), CHIR (3 μM, 1 m (2 μM), CHIR plus PD or 1 m plus PD. Immunoblotting was performed with the antibodies specified. (ii) ESCs were grown for 16 h in basal N2B27 with 1 μM PD before either 3 μM CHIR or vehicle were added for 4, 24 and 48 hrs. Samples were immunoblotted with the antibodies indicated.

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Discussion

The data we present here provide new insight into the mechanisms regulated by Gsk-3 in ESCs. We show that inhibition or ablation of Gsk-3 activity enhances the protein levels of the pluripotency-associated transcription factors Nanog, Tbx3 and c-Myc. The stability of these proteins were not altered, but rather we demonstrate, using two approaches, that the de novo synthesis of Nanog protein is increased following Gsk-3 inhibition. We also report corresponding increases in polyribosome association of Nanog and Tbx3 RNAs following Gsk-3 inhibition, consistent with enhanced translation. Based on our data, we propose that Gsk-3 is involved in the regulation of dynamically expressed pluripotency-associated factors in mouse ESCs, including Nanog and Tbx3, by altering their translation. This represents a novel additional mechanism by which Gsk-3 may contribute to modulation of ESC self-renewal and highlights the role that control of translation can play in fine-tuning protein levels to reinforce the network of pluripotency factors. Furthermore, our study provides additional evidence regarding the coupling of molecular signals to transcriptional control in ESCs.

A number of possible mechanisms have been proposed to account for the ability of Gsk-3 inhibition to enhance self-renewal of mouse ESCs, including promotion of ESC survival and metabolism [15], stabilisation of c-Myc [42,50] and activation of Wnt signalling [13,50,51,52,53,54]. This latter aspect had proven to be somewhat controversial, but several recent reports support that inhibition of Gsk-3 leads to enhancement of β-catenin levels, which binds to Tcf3 thereby relieving the repressive effects of Tcf3 on expression of genes of the pluripotency network [38,39], including Esrrb [27]. Our study suggests an alternative additional mechanism by which Gsk-3 may contribute to modulation of ESC self-renewal and reveals how the signals known to impact on control of pluripotency can influence the transcriptional machinery. While Gsk-3 inhibition did not, in our analyses, appear to alter the mechanisms regulated by Gsk-3 in ESCs. We show that inhibition or ablation of Gsk-3 activity enhances the protein levels of the pluripotency-associated transcription factors Nanog, Tbx3 and c-Myc. The stability of these proteins were not altered, but rather we demonstrate, using two approaches, that the de novo synthesis of Nanog protein is increased following Gsk-3 inhibition. We also report corresponding increases in polyribosome association of Nanog and Tbx3 RNAs following Gsk-3 inhibition, consistent with enhanced translation. Based on our data, we propose that Gsk-3 is involved in the regulation of dynamically expressed pluripotency-associated factors in mouse ESCs, including Nanog and Tbx3, by altering their translation. This represents a novel additional mechanism by which Gsk-3 may contribute to modulation of ESC self-renewal and highlights the role that control of translation can play in fine-tuning protein levels to reinforce the network of pluripotency factors. Furthermore, our study provides additional evidence regarding the coupling of molecular signals to transcriptional control in ESCs.

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Previous reports have suggested that Nanog binds to its own promoter, enhancing its transcription [23,55], although in contrast a very recent report suggests that Nanog is auto-repressive [56]. How then, do our data relate to these somewhat conflicting findings? We propose that the increased levels of Nanog protein, caused by increased translation as a result of Gsk-3 inhibition, could contribute to the fine-tuning of Nanog protein levels in response to the external environment, providing a means for more rapid and dynamic regulation of pluripotency factors than allowed for by transcriptional changes alone, a concept not previously considered [57]. While the focus of this study has been on the early events arising as a result of Gsk-3 inhibition, this mechanism could contribute to sustaining ESC pluripotency whenever Gsk-3 inhibition is invoked. Indeed, there are many examples where short-term signalling events, e.g. receptor phosphorylation, have significant effects on cellular responses occurring hours later.

In our study we were interested to determine the mechanism underlying the ability of Gsk-3 to modulate protein translation. Interestingly, when we investigated whether inhibition of Gsk-3 in ESCs led to a change in regulators controlling general (CAP-dependent) initiation of translation, no alteration in phosphorylation of either Ser51 on eIF2α or Ser 539 on eIF2Bc phosphorylation were observed. These results suggest that general translation initiation is not perturbed following Gsk-3 inhibition in ESCs, consistent with the fact that only a subset of pluripotency factors are targeted by Gsk-3, e.g., Nanog and Tbx3. These results are interesting in view of the fact that general translation has been reported to be lower in undifferentiated ESCs, increasing following differentiation into embryoid bodies [33]. We also discovered that mTor activity did not appear to increase following Gsk-3 inhibition and unexpectedly phosphorylation of T389 on S6K1 declined. The effect of Gsk-3 inhibition on levels of T389 S6K1 phosphorylation were consistently observed following treatment with structurally unrelated Gsk-3 inhibitors (1 m and CHIR) and also evident in Gsk-3 null ESCs. One possible explanation for this observation is that it has recently been reported that Gsk-3-phosphorylates S371 of S6K1 [58]. S371 phosphorylation of S6K1 is essential for phosphorylation of T389 [59] so it is possible that the decrease in T389 phosphorylation we observe is due to inhibition of Gsk-3-mediated phosphorylation of S371. Further studies are required to examine this in detail and determine any functional significance in mouse ESCs.

Figure 4. Gsk-3 inhibition does not alter Nanog, Tbx3, Oct4 or c-Myc protein stability. (A) E14tg2a WT ESCs and (B) WT and Gsk-3 DKO ESCs were grown in the presence of N2B27 plus LIF and BMP4 for 24 h. In (A) 1 m (2 μM) or vehicle alone control (CTL) were added to the cells for this 24 h period. All samples were then incubated with cycloheximide (CHX) to halt protein synthesis for the times indicated. (i) Protein samples were extracted after 0, 1, 3 and 6 hours of CHX treatment and immunoblotting performed with the indicated antibodies. (ii) Antibody signals were quantified and protein levels normalised to Gapdh. In each case a value of 100% was given to the t = 0 samples to enable direct comparisons to be made. The data are the average relative protein expression levels and S.E.M of triplicate experiments.

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Nanog and Tbx3 are, at least partially, selective. Translation of specific RNA transcripts, without an increase in general translation or in conditions where the CAP-dependent translation is compromised, can occur via a number of different mechanisms including IRES-based translation, control by miRNAs or via RNA binding protein-mediated mechanisms. Our bioinformatic studies predicted that the 5' UTR of Nanog contains three possible IRES and a potential in-frame upstream open reading frame, so we were interested to investigate whether it mediated the effects of Gsk-3 inhibition observed. Our functional analyses show that while the Nanog 5' UTR enhances reporter gene expression, by itself it does not confer responsive to Gsk-3 inhibition. Our further analyses demonstrate a lack of conservation in the Nanog 5' UTR sequences across a range of species. We also discovered that

Figure 5. Gsk-3 inhibition or ablation increases Nanog protein synthesis in the absence of increased transcription. WT and Gsk-3 DKO ESCs were incubated in (A) GMEM supplemented with LIF and serum or (B) N2B27 medium supplemented with LIF and BMP4 prior to addition of 10 mg/ml CHX for 4 hours to halt protein synthesis. CHX was then washed out and fresh media (A, GMEM plus LIF plus serum and B, N2B27 plus LIF and BMP4) added back. (i) & (ii) Protein and (iii) RNA were extracted between 1 and 4 hours after CHX washout. (i) Immunoblotting was performed with the antibodies indicated. (ii) Antibody signals were quantified and normalised to Gapdh. A value of 1 was given to CHX-treated samples. The data are the average relative protein expression levels and S.D. of triplicate experiments. (C) Nanog reporter Nd ESCs were sorted by flow cytometry and cells with low to no VNP expression collected and grown in GMEM supplemented with LIF and serum in the presence or absence of DMSO (controls) or in the presence of 3 µM CHIR99021. The data shown are the average Nanog:VNP expression levels (relative to DMSO control) and standard deviation of at least 3 biological replicates. All p-values were calculated using a two-tailed distribution, two-sample equal variance t-test (** p-value <0.01; *** p-value <0.0001).
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Figure 6. Association of Nanog and Tbx3 RNAs with polysomes increases upon inhibition of Gsk3. WT ESCs grown in the absence (WT) or presence of 2 µM 1 m (1 m) and Gsk-3 DKO ESCs (DKO) were cultured in serum supplemented with LIF for 4 and 8 hours before extracting cell lysates. Sedimentation through sucrose gradients was used to separate the polysomal-enriched fractions from the monosomal fractions. The levels of RNA bound to polysomal or monosomal fractions were measured using quantitative RT-PCR, with the primers shown in Table S1. In each case gene expression was normalized relative to β-actin levels. A. Expression of (i) Nanog, (ii) Tbx3 and (iii) Oct4 in individual fractions across the sedimentation gradient. A representative experiment is shown in each case (4 h time points for (i) and (ii) and 8 h time point for (iii)). Values show expression relative to β-actin. B. Gene expression in pooled monosomal and polysomal fractions. Values show the proportion of RNA bound to the polysome fraction (Bound/Total RNA). The data are the average and S.E.M of three independent experiments run in duplicate for the 8-hour time point and the average and S.E.M of two independent experiments run in duplicate for the 4-hour time point. *, p<0.05; **, p<0.01, ***, p<0.005 (Student's t-test).
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Nanog 3’UTR sequences do not appear to independently mediate the effects of Gsk-3 inhibition, thus, it may be that a combination of 5’ and 3’ UTR sequences are required. Another alternate possibility is that ß-catenin mediates the effects of Gsk-3 inhibition, given its reported role in maintaining ESC self-renewal [37,38,50,52,60,61]. Interestingly, while the levels of nuclear ß-catenin reflect the enhanced re-synthesis of Nanog following 1 m treatment (Figure S3), the changes in the levels of Nanog and ß-catenin protein do not correlate well following either short-term cycloheximide treatment or a 16 h recover phase in the absence of Gsk-3 inhibition, suggesting the effects on Nanog protein levels are at least partially ß-catenin-independent. In future studies it will be interesting to examine the ß-catenin-independence/dependence in greater detail.

In this study we present evidence supporting a role for Gsk-3-dependent regulation of translation of selected pluripotency-associated transcription factors that show dynamic protein turnover. We propose that this represents a novel additional mechanism that may contribute to modulation of ESC pluripotency by enabling rapid responses to changes in signals, leading to alteration of protein levels that act to reinforce the network of pluripotency-associated transcription factors. This study highlights the importance of interrogating the control of protein dynamics and translational regulation to gain further insight into the mechanisms contributing to the control of ESC fate.

Supporting Information

Figure S1 Colony morphology of E14 mouse ESCs cultured in N2B27 medium in the different conditions indicated. (TIF)

Figure S2 Gsk-3 inhibition does not alter Nanog, Tbx3, Oct4 or c-Myc protein stability in the presence of serum and LIF. ESCs were grown in GMEM plus serum plus LIF. (A) E14tg2a WT ESCs were pre-incubated for 24 h without (CTL) or with 2 μM 1 m. Cells were harvested at 4, 8 and 16 h and Firefly luciferase activity determined and normalised relative to Renilla luciferase activity. Luciferase activity is expressed as fold-change over pGL3’ (i) or pGL3-MCS (ii) controls (DMSO) for a given time point. Data are the average and S.E.M. of three independent experiments. doi:10.1371/journal.pone.0060148.g007

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indicated antibodies. (ii) Antibody signals were quantified and protein levels normalised to Gapdh. In each case a value of 100% was given to the untreated (t = 0) samples to enable direct comparisons to be made. The data are the average and S.E.M. of triplicate experiments.

(EPS)

Figure S3 Gsk-3 inhibition increases Nanog protein synthesis. E14tg2a mouse ESCs were incubated with cycloheximide (CHX) for 4 h to halt protein synthesis or left untreated (UT) as a control. Cells were washed thoroughly to remove CHX and fresh media supplemented with serum and LIF and vehicle (DMSO/D) or 2 μM 1 m (1 m) added back. Cells were harvested and protein extracts prepared at the times indicated after CHX wash-out. (ii) Immunoblotting was performed with the antibodies indicated. (iii) Nanog and β-catenin expression levels normalised to Gapdh and expressed relative to the UT control are shown following the 16 h treatment. The values are the average and S.E.M. from 3 independent experiments.

(TIF)

Figure S4 Gsk-3 inhibition increases Nanog protein synthesis in Nd ESCs. Nd ESCs were sorted and the VNP-low sub-population collected and incubated in GMEM supplemented with LIF and serum in the presence or absence of DMSO (controls) or in the presence of 3 μM CHIR99201. (A) Representative bright field images of Nd ESCs at 4 h and 24 h after plating. Scale-bar = 100 μm. (B) Representative dot blots for Nanog:VNP expression at 4 h and 24 h after plating. VNP-low FACS sorted populations of Nd ESCs were plated in the conditions indicated and reporter expression measured after 4 and 24 h. At 4 h no differences were observed, while at 24 h a statistically significant increase in the number of Nanog:VNP positive cells was observed. (C) Relative Nanog:VNP expression after plating of low-Nanog sorted population. In the presence of CHIR99201, Nanog:VNP expression increases approximately 30-fold relative to time 0 h, while in DMSO only a 10-fold increase is observed. No differences were observed between GMEM supplemented with LIF and serum in the presence or absence of DMSO. The data are the average relative Nanog:VNP expression levels (relative to time 0 h) and Standard Deviations of at least 3 biological replicates. E14tg2a cells were used as a negative control, to obtain the positive gate region. All p-values were calculated using a two-tailed distribution, two-sample equal variance t-test. For 8, 12 and 24 h time points p<0.05.

(TIFF)

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