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Frat Is a Phosphatidylinositol 3-Kinase/Akt-Regulated Determinant of Glycogen Synthase Kinase 3β Subcellular Localization in Pluripotent Cells

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Suppressing the activity of Gsk3β is critical for maintenance of murine pluripotent stem cells. In murine embryonic stem cells (mESCs), Gsk3β is inhibited by multiple mechanisms, including its inhibitory phosphorylation on serine 9 by protein kinase B (Akt), a major effector of the canonical phosphatidylinositol 3-kinase (PI3K) pathway. A second PI3K/Akt-regulated mechanism promotes the nuclear export of Gsk3β, thereby restricting its access to nuclear substrates such as c-myc and β-catenin. Although Gsk3β shuttles between the nucleus and cytoplasm under self-renewing conditions, its localization is primarily cytoplasmic because its rate of nuclear export exceeds its rate of nuclear import. In this report, we show that Gsk3β is exported from the nucleus in a complex with Frat. Loss of PI3K/Akt activity results in dissociation of this complex and retention of Gsk3β in the nucleus. Frat continues to shuttle between the nucleus and cytoplasm under these conditions and remains predominantly in the cytoplasm. These results indicate that Frat carries Gsk3β out of the nucleus under self-renewing conditions and that PI3K regulates this by promoting its association with Frat. These findings provide new links between PI3K/Akt signaling and regulation of Gsk3β activity by Frat, an oncogene previously shown to cooperate with Myc in tumorigenesis.

Suppression of Gsk3β activity in murine embryonic stem cells (mESCs) is required for stabilization of the pluripotent state and for maintenance of self-renewing capacity (5, 13, 14). This is achieved by the action of canonical phosphatidylinositol 3-kinase (PI3K) signaling through its key effector, protein kinase B (Akt), which negatively regulates Gsk3β by inhibitory phosphorylation of serine 9 (Gsk3β^{pS9}) (1, 3, 16). Withdrawal of leukemia inhibitory factor (LIF), the stem cell maintenance cytokine, results in decreased PI3K/Akt signaling, activation of Gsk3β, and loss of pluripotency (3). One important consequence of Gsk3β activation in mESCs is the accelerated proteolysis of c-myc, triggered by its Gsk3β-dependent phosphorylation on threonine 58 (c-myc^{pT58}). Mechanistically, decreased c-myc activity allows differentiation-specific genes such as GATA6 to be derepressed while others such as the mir-17-92 miRNA cluster are downregulated (15). Restraining the action of Gsk3β and its effect on c-myc and other nuclear substrates is therefore critical for maintenance of murine pluripotent stem cells (PSCs).

While characterizing the regulation of Gsk3β in mESCs, we previously demonstrated that it constantly shuttles between the nucleus and cytoplasm, even though it is predominantly a cytoplasmic protein (1). This is primarily due to its rate of nuclear export being greater than its rate of nuclear import. When PI3K/Akt activity declines, as it does following LIF withdrawal, Gsk3β accumulates in the nucleus independently of its catalytic activity and S9 phosphorylation status (1). Restoration of Akt activity is sufficient to reverse the nuclear accumulation of Gsk3β, restoring the shuttling mechanism previously observed in self-renewing mESCs (1). These observations place PI3K/Akt at the center of a mechanism regulating the subcellular localization of Gsk3β in mESCs. How PI3K/Akt regulates Gsk3β nuclear export, however, is not understood and is the major focus of this report.

The biological importance of Gsk3β nuclear retention in mESCs has been established by several approaches (1). These findings show that, while Gsk3β has a critical role in regulating self-renewal and pluripotency, neither its enzymatic activation nor sustained nuclear localization is individually sufficient to promote differentiation. Instead, both the activation of Gsk3β and its nuclear retention are required to sufficiently antagonize the pluripotency regulatory network. This clearly establishes that Gsk3β regulates pluripotency by targeting nuclear substrates. Some of the known substrates for Gsk3β in this scheme include c-myc (1, 3) and β-catenin (10).

Gsk3β has a well-characterized bipartite nuclear localization sequence (NLS) (12) that does not appear to be signal regulated (1). Nuclear export of Gsk3β, on the other hand, is Crm1 dependent but lacks a definable leucine-rich nuclear export signal (NES). This raises the possibility that an adaptor protein, with its own NES, carries Gsk3β out of the nucleus as part of a protein complex. Axin and Frat are Gsk3-interacting proteins that bind to overlapping sites and have Crm1-dependent nuclear export sequences (4, 6, 22). The binding of either Frat or axin to Gsk3β therefore represents one possible mechanism by which Gsk3β could be exported from the nucleus. How PI3K/Akt would regulate this process is unclear, however. In this report, we show that Frat and axin are binding partners for Gsk3β in pluripotent stem

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cells. Only Frat, however, plays a role in the nuclear export of Gsk3β. The interaction between Frat and Gsk3β occurs only in the presence of elevated PI3K/Akt activity, providing a mechanism by which self-renewal signals control the subcellular localization of Gsk3β. This mechanism has general implications with respect to how Gsk3β accesses nuclear substrates in pluripotent cells and a wide range of other cell types.

MATERIALS AND METHODS

Cell culture and reagents. mESCs were cultured in the absence of feeders on culture-grade plastic precoated with 0.2% gelatin–phosphate-buffered saline (PBS), as previously described (1). Murine ESC cultures were maintained at 37°C and 10% CO_2 and subjected to passage every 2 to 3 days as previously described (1). The Frat triple-knockout (triple-TKO) murine induced pluripotent stem cells (miPSCs) were obtained by reprogramming mouse embryonic fibroblasts (MEFs) with homozygous deletions of Frat1, -2, and -3 (Anton Berns, Netherlands Cancer Institute) as previously described (17). Frat TKO miPSCs were cultured, maintained, and subjected to passaging as described above for mESCs. MEFs were maintained at 37°C and 10% CO_2 in Dulbecco's modified Eagle medium (DMEM; Cellgro) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals), 2 mM L-glutamate (Cellgro), 1 mM sodium pyruvate (Cellgro), 0.1 mM β-mercaptoethanol (Gibco), penicillin-streptomycin (Cellgro) (100 U/ml), and LIF (ESGRO, Chemicon) (1,000 U/ml). The following reagents were used in this study: PI-103 (catalog no. 528101; Calbiochem) and leptomycin B (LB) (catalog no. 431050; Calbiochem).

RNA isolation and RT-PCR. Total RNA from cells was isolated using an RNeasy kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using approximately 1 μg of RNA and an iScript cDNA synthesis kit (Bio-Rad) following the protocol recommended by the manufacturer. The PCR conditions and sequences of primers for reverse transcriptase PCR (RT-PCR) were as previously described (18).

Antibodies, immunoblotting, immunostaining, and immunoprecipitation. Antibodies used in this study were as follows: mouse anti-Gsk3β (catalog no. 610202; BD Biosciences), mouse anti-Cdk2 (catalog no. SC-6248; Santa Cruz), mouse antihemagglutinin (anti-HA) (catalog no. 2367; Cell Signaling), rabbit antiaxin (catalog no. SC-14029; Santa Cruz), and rabbit anti-Flag (catalog no. F7425; Sigma). Western blot analysis and immunostaining were performed as previously described (1). Proteins were immunoprecipitated from approximately 600 μg of whole-cell lysate prepared as previously described (1) and adjusted to a concentration of 1 mg per ml. Whole-cell lysates were precleared by adding 40 μl of protein A/G Plus-agarose (catalog no. SC-2003; Santa Cruz) at 4°C for 1 h. All incubations were performed with end-over-end tumbling. Primary antibody was added to the precleared lysate at a dilution of 1:100 and incubated overnight at 4°C. A 40-μl volume of protein A/G Plus-agarose was then added, and samples were incubated for an additional 4 h at 4°C. Samples were then centrifuged for 30 s at 100 × *g*, the supernatant was removed, and the remaining protein A/G Plus-agarose was subjected to four wash steps with lysis buffer. To detect immunoprecipitated proteins, a washed protein A/G Plus-agarose/antibody mix was boiled in 50 μl of sodium dodecyl sulfate (SDS) and subjected to Western blot analysis.

Site-directed mutagenesis and plasmid construction. All site-directed mutagenesis was performed using a QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions and as previously described (1). Gsk3β mutations were generated using pBS.Gsk3β_{HA} or pBS.Gsk3β_{HA}^{S9A} as a template, while the Frat1 mutant was generated using pBS.Frat1$_{Flag}$ as a template. Following site-directed mutagenesis, the Frat1$_{Flag}^{\Delta NES}$ mutant was removed from pBS.KS$^+$ and ligated back into pCAGineomycin. All of the resulting mutations were verified by sequencing performed on both DNA strands. Mutagenic primers were designed according to the primer design guidelines laid out specifically for the QuikChange II site-directed mutagenesis kit and are available upon request.

Gsk3β_{HA} and Gsk3β_{HA}^{S9A} expression constructs have been described previously (1). pCAG-Frat1$_{Flag}$ was generated by digesting the 2× N-terminal Flag-tagged Frat1 from pcDNA-Frat1$_{Flag}$ (a gift from Trevor Dale) before ligation into the XhoI-NotI sites of pCAGineomycin was performed. pBS.Frat1$_{Flag}$ was constructed by digesting Flag-Frat1 from pCAG-Flag.Frat1 before ligation into the EcoRI-NotI sites of pBS.KS$^+$ was performed. Using pcDNA-Frat1$_{Flag}$ as a template, myr-Frat1$_{Flag}$ was generated using PCR to attach a myristoylation sequence to the N terminus of Frat1 and a 2× Flag tag to its C terminus immediately preceding the stop codon. The PCR product was then digested and ligated into pBS.KS$^+$. Myr-Frat$_{Flag}$ was then digested from the XhoI-NotI sites of pBS.KS$^+$ and ligated into a similarly digested pCAGipuromycin. The following primers were used in the PCR: the forward primer was 5′-CAC ACA GAA TTC AGC CGC CAC C ATG GGG AGC AGC AAG AGC AAG CCC AAG TCT AGA CCC ATG CCT TGC CGG AGG GAG and the reverse primer was 5′-CAC ACA GCG GCC GC TTA CTT GTC ATC GTC GTC CTT GTA GTC CTT GTC ATC GTC GTC CTT GTA GTC GGG GCT GCC AGG GAC AAG AAG GTC-3′. Details of the exact PCR conditions are available upon request.

Alkaline phosphatase assays, transfections, and qRT-PCR analysis. Cells were washed with PBS and then assayed for alkaline phosphatase activity using Vector Red alkaline phosphatase substrate kit I (Vector Labs) according to the manufacturer's instructions. For transfections, mESCs were seeded at a density of 1×10^4 cells/cm^2 on gelatin-coated dishes. Transfection of mESCs was carried out 24 h later using Lipofectamine 2000 reagent (Invitrogen) per the manufacturer's instructions except that 10 μg of DNA was used per 6-well dish and 2 μg of DNA in a 4-well slide was used for transfections. To generate clonal cell lines stably expressing the gene of interest, transfected mESCs were subjected to passages the following day onto a 10-cm-diameter gelatinized tissue culture dish and grown in the presence of puromycin (1 μg/ml) for ~7 days or neomycin (200 μg/ml) for ~14 days. After selection, individual colonies were picked and subcultured further. For transient transfections, mESCs were grown and transfected as described above, with the exception that antibiotic selection was not applied. qRT-PCR was performed using TaqMan assays (Applied Biosystems) on a iCycler (Bio-Rad), according to the manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) assays were performed in triplicate, normalized to *Gapdh*, and analyzed according to the ΔΔCT threshold cycle method. Data are representative of the results of multiple experiments.

RESULTS

Gsk3β mutants that fail to bind Frat accumulate in the nucleus of mESCs. The general shuttling mechanism for Gsk3β, previously defined by us (1), is shown in Fig. 1A and illustrates that, in the presence of elevated PI3K/Akt signaling, the rate of Gskβ nuclear export exceeds the rate of nuclear import. To establish whether Frat or axin or both contribute to the cytoplasmic-nuclear shuttling of Gsk3β in mESCs, we first determined the effects of Gsk3β mutants that are defective in Frat and/or axin binding. Multiple reports have identified several residues in Gsk3β that, when mutated, disrupt axin and/or Frat binding (6, 7). Two of these mutants were assayed for their effect on the binding of axin and Frat to Gsk3β in mESCs (Q206E and K85M) (Fig. 1B). As there are no antibodies commercially available that can reliably detect endogenous Frat protein, antibodies recognizing exogenously expressed epitope-tagged Frat were used (11). Immunoprecipitation of Frat1$_{Flag}$ showed that, while it forms a complex with wild-type Gsk3β, it does not form stable complexes with Q206E and K85M mutants (Fig. 1C). Immunoprecipitation of Gsk3β with HA antibody confirmed that Frat binding to Gsk3β was disrupted in both mutants, as expected (6) (Fig. 1D). Axin binding, however, was disrupted in the K85M mutant but not the Q206E mutant (Fig. 1D). Levels of Frat1 were constant under each

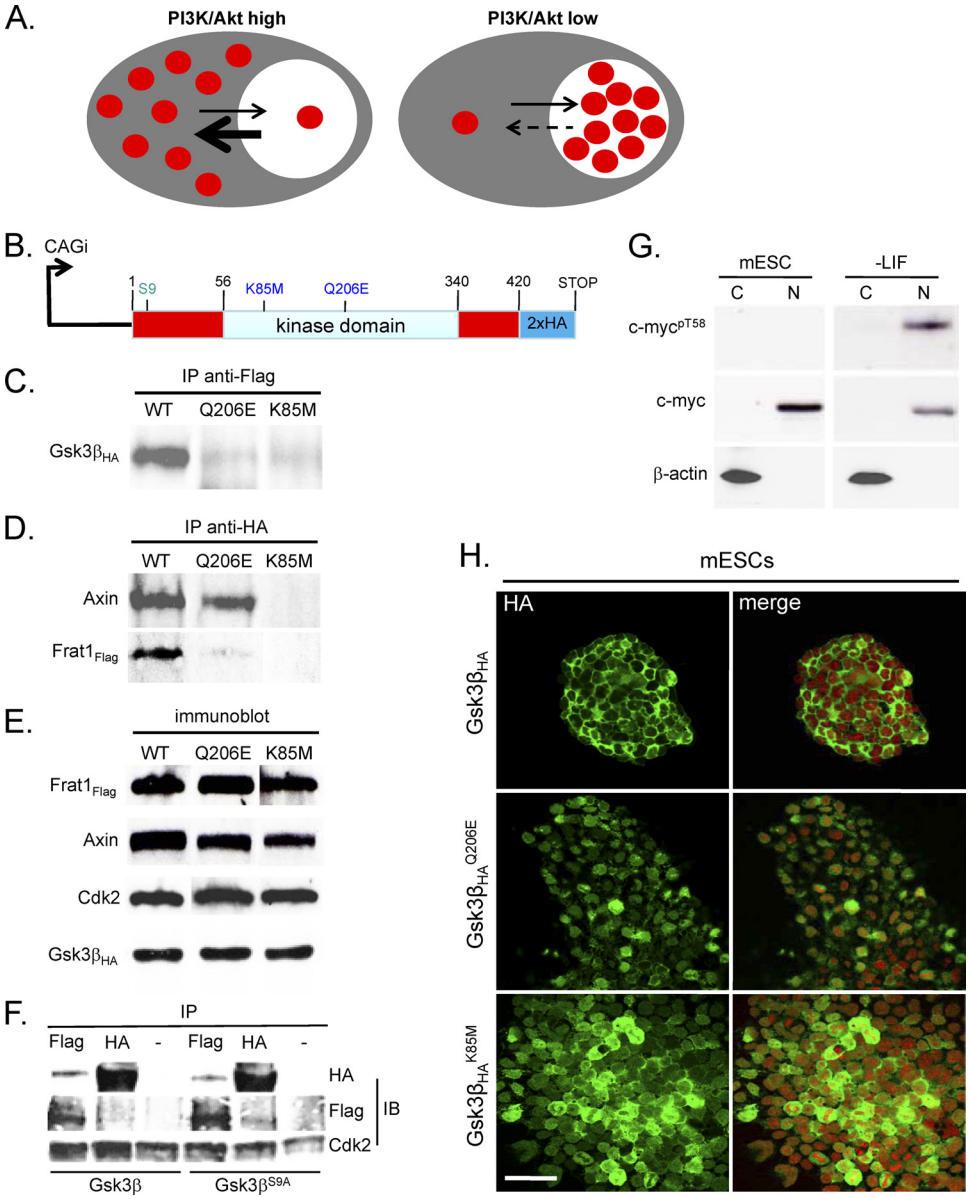

FIG 1 Gsk3β mutants unable to bind Frat accumulate in the nucleus. (A) Diagram depicting our previous findings (1), showing the role PI3K/Akt signaling in regulating the localization of Gsk3β by controlling its rate of nuclear export. (B) Schematic of HA-tagged Gsk3β showing key regulatory residues, including the positions of mutations used to disrupt axin and/or Frat binding. (C) Whole-cell lysates from mESCs that stably express $Gsk3\beta_{HA}^{Q206E}$, $Gsk3\beta_{HA}^{K85M}$, or wild-type (WT) $Gsk3\beta_{HA}$ with $Frat_{Flag}$ were subjected to immunoprecipitation using an anti-Flag antibody. Immunoprecipitates were then subjected to Western blot analysis by probing for $Gsk3\beta_{HA}$ with anti-HA antibody. (D) Using cell lysates from the experiment described for panel C, HA-tagged wild-type and mutant (Q206E and K85M) Gsk3β were immunoprecipitated using an anti-HA antibody before being subjected to Western blot analysis by probing with antibodies as indicated. (E) A 20-μg volume of the whole-cell lysate used in the immunoprecipitation reactions was subjected to Western blot analysis by probing with antibodies as indicated. (F) Reciprocal immunoprecipitation-immunoblot assays evaluating the ability of HA-tagged Gsk3β/$Gsk3\beta^{pS9A}$ to form complexes with Flag-tagged Frat1 in whole-cell lysates (400 μg of total protein) prepared from mESCs. The whole-cell lysate (20 μg) was probed with antibodies for Cdk2 to demonstrate that the levels of input protein going into each immunoprecipitation were comparable. (G) mESCs were maintained in LIF medium (+LIF), or LIF was withdrawn for 24 h. Nuclear and cytoplasmic protein fractions were then prepared, and equivalent amounts were analyzed by immunoblot analysis. Blots were then probed with antibodies for β-actin, c-myc, and $c\text{-}myc^{pT58}$. (H) mESC lines from the experiment described in the panel A legend were subjected to immunostaining as indicated. DNA was visualized with TO-PRO-3. Images were obtained by confocal imaging. Bar, 50 μm.

of the sets of conditions tested (Fig. 1E), ruling out variations in Frat1 protein levels as an explanation for these observations. The results of binding of Frat to the wild-type and constitutively active S9A versions of Gsk3β were indistinguishable, as determined by immunoprecipitation-immunoblot assays (Fig. 1F). These results indicate that correct localization of Gsk3β under self-renewing conditions correlates with its ability to bind Frat independently of its enzymatic activity. Subcellular fractionation experiments were used to confirm that c-myc, the Gsk3β substrate, is phosphorylated on T58 in the nucleus following LIF withdrawal (Fig. 1G). This is consistent with previous immunofluorescence localization data showing that accumulation of active Gsk3β in the nucleus

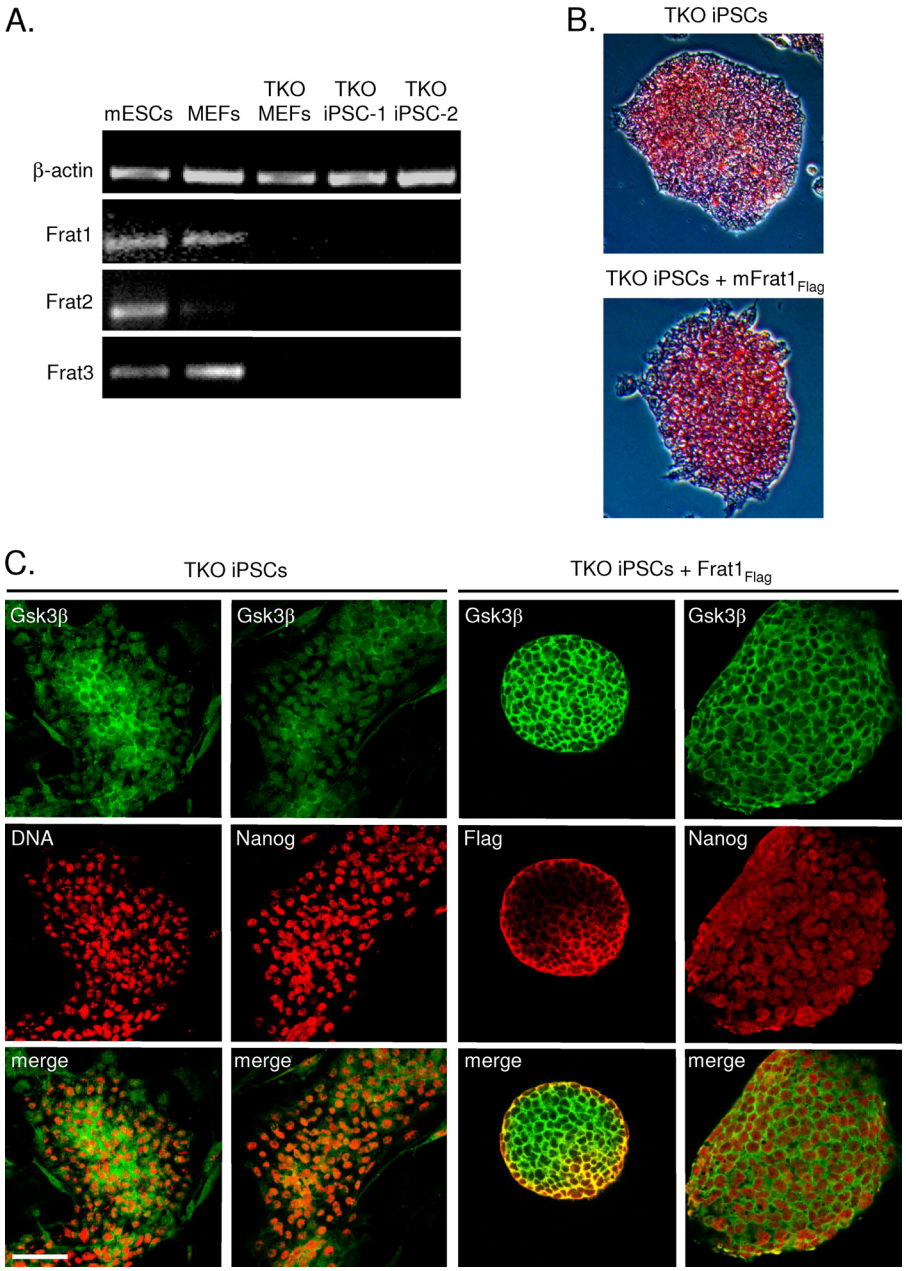

FIG 2 Frat is required for the nuclear export of Gsk3β. (A) mRNA levels from R1 mESCs, MEFs, FRAT1, -2, and -3 triple-knockout (TKO) MEFs, and two individual FRAT1, -2, and -3 TKO iPSCs (TKO iPSC-1 and TKO iPSC-2) were analyzed by RT-PCR using primers designed against Frat1, Frat2, Frat3, and β-actin. (B) The TKO iPS cell line described in the panel A legend and TKO iPSCs stably expressing Flag-tagged Frat1 were grown in the presence of LIF for approximately 3 days before being analyzed by alkaline phosphatase staining. (C) The TKO iPS cell line described in the panel A legend and TKO iPSCs stably expressing Flag-tagged Frat1, grown as previously described, were fixed and then immunostained for antibodies as indicated. DNA was visualized with TO-PRO-3. Images were obtained by confocal imaging. Bar, 50 μm.

promotes c-myc T58 phosphorylation and degradation (1). When the localization of both Gsk3β mutants was assessed in mESCs, the Q206E and K85M mutants both mislocalized to the nucleus (Fig. 1H).

Frat is required for nuclear export of Gsk3β in mESCs. To validate the previous results, we generated iPSCs from mouse embryo fibroblasts (17) carrying a triple knockout (TKO) for all three FRAT genes (FRAT1, -2, and -3). TKO iPSCs did not produce transcripts of Frat1, -2, and -3 (Fig. 2A) and exhibited characteristics typical of murine pluripotent cells, including a uniform, dome-shaped colony morphology, Nanog expression, and robust alkaline phosphatase activity (Fig. 2B and C). Unlike the situation in wild-type mESCs that express all three Frat family members, Gsk3β localized primarily to the nucleus in TKO iPSCs (Fig. 2C). Moreover, when Frat1 was ectopically expressed in TKO iPSCs, the localization of Gsk3β reverted to being primarily cytoplasmic (Fig. 2C). This indicates that Frat is a key determinant of Gsk3β subcellular localization. Despite accumulating in the nucleus,

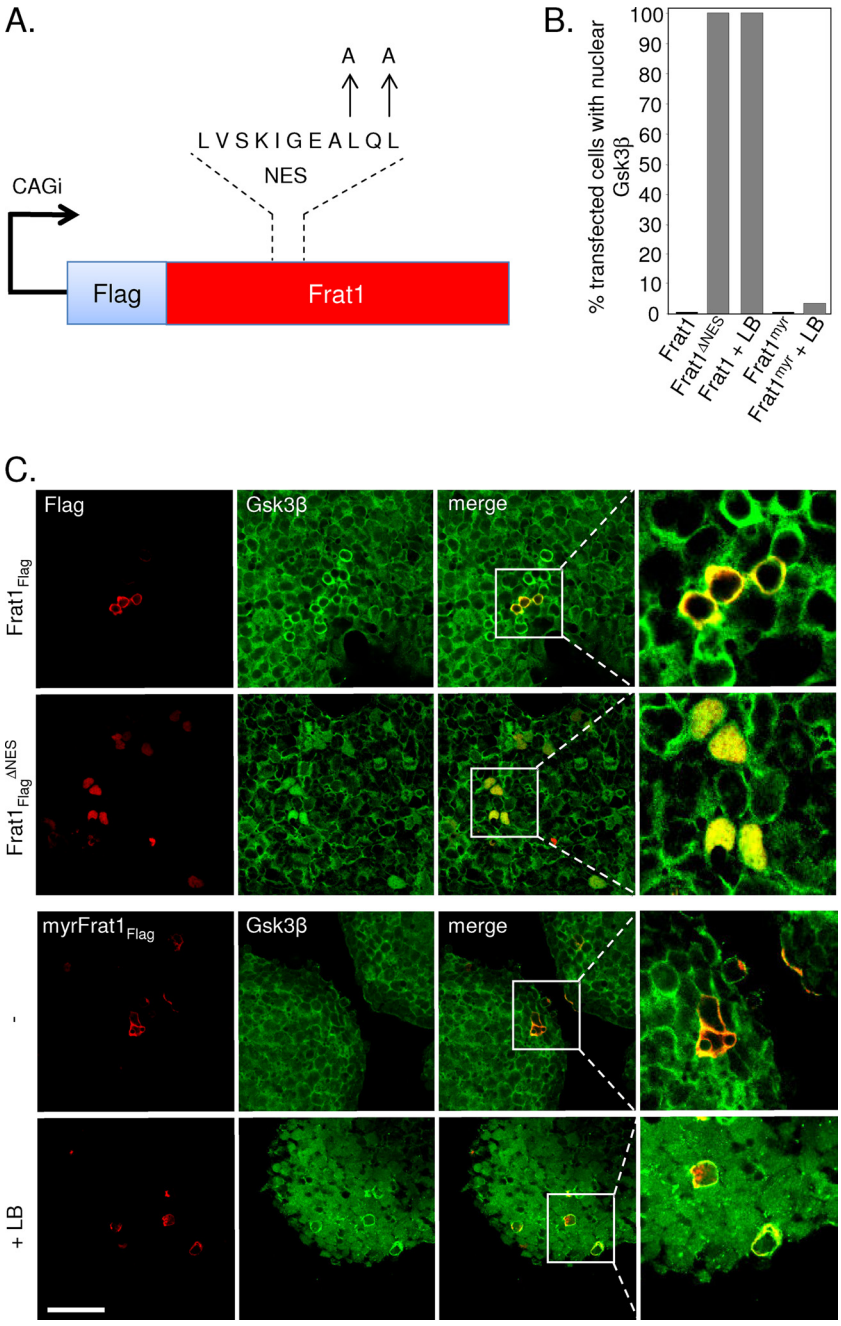

FIG 3 Disruption of Frat nuclear export results in the nuclear accumulation of Gsk3β. (A) Schematic of the expression vector pCAGineo carrying an N-terminal 2× Flag-tagged Frat1 with mutations designed to disrupt the NES ($Frat1_{Flag}^{\Delta NES}$). (B) Graphical representation of the percentage of Gsk3β localized to nucleus of cells expressing $Frat1_{Flag}$, $Frat1_{Flag}^{\Delta NES}$, $Frat1_{Flag}$ plus LB, myr-$Frat1_{Flag}$, or myr-$Frat1_{Flag}$ plus LB. The localization of Gsk3β was considered cytoplasmic or nuclear when >90% was localized either to the cytoplasm or nucleus, respectively. (C) Upper panels: R1 mESCs were transiently transfected with pCAG-$Frat1_{Flag}^{\Delta NES}$ or pCAG-$Frat1_{Flag}$ and then immunostained with antibodies as indicated 48 h later. Lower panels: R1 mESCs were transiently transfected with pCAG-myr-$Frat1_{Flag}$ and then either left untreated or treated with LB (110 nM for 6 h) 48 h later before immunostaining for antibodies as indicated was performed. Images were obtained by confocal imaging. Bar, 50 μm.

phosphorylation of nuclear substrates such as c-myc (T58) was independent of Frat status (data not shown), consistent with previous observations that sustained PI3K/Akt signaling suppresses the activity of nuclear Gsk3β (1).

To establish that nuclear export of Frat is critical for correct Gsk3β localization, we expressed a form of Frat1 carrying a mutated NES in mESCs (Fig. 3A) (6). Although expression of wild-type Frat1 had no effect on Gsk3β localization, the NES mutant form of Frat prevented the nuclear export of endogenous Gsk3β, resulting in its nuclear retention (>95% of cells; Fig. 3B and C). The ability of Frat to be exported from the nucleus is therefore critical for cytoplasmic accumulation of Gsk3β in mESCs. Next,

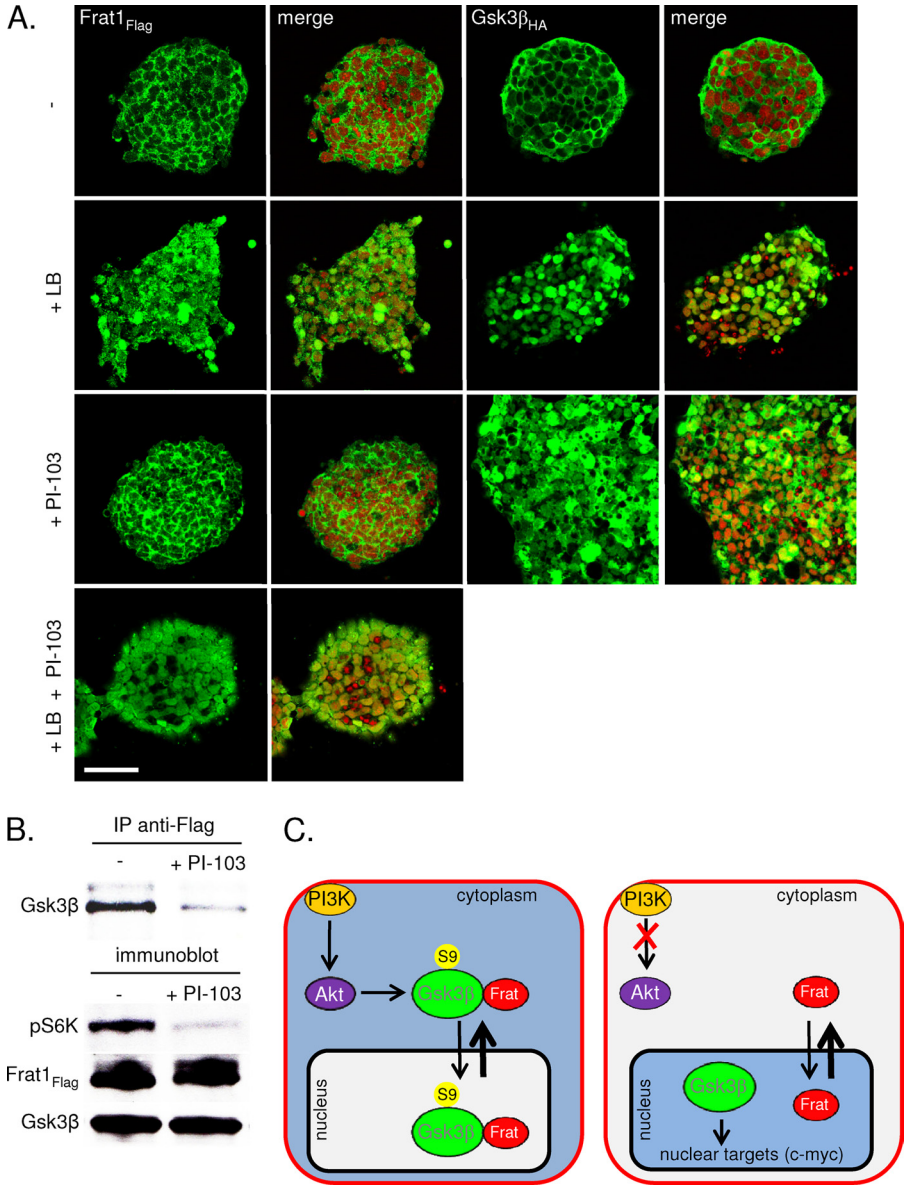

FIG 4 Inhibition of PI3K/Akt dissociates Gsk3β from Frat, resulting in the nuclear accumulation of Gsk3β. (A) R1 mESCs stably expressing $Frat1_{Flag}$ and $Gsk3\beta_{HA}$ were left untreated (−), treated with LB (110 nM for 6 h), or treated with PI-103 (10 μm for 12 h) and then fixed and immunostained with antibodies as indicated. In addition, Frat localization was examined in cells treated with PI-103 before being treated with LB, 6 h prior to immunostaining. DNA was visualized with TO-PRO-3. Images were obtained by confocal imaging. Bar, 50 μm. (B) Upper panel: whole-cell lysates from R1 mESCs stably expressing $Frat1_{Flag}$ were subjected to immunoprecipitation using an anti-Flag antibody. Immunoprecipitates were then subjected to Western blot analysis by probing for antibodies as indicated. Lower panel: 20 μg of the whole-cell lysate used in the immunoprecipitation reactions was subjected to Western blot analysis by probing with antibodies as indicated. (C) Model depicting the results of this report. Under conditions in which PI3K/Akt signaling is active, Gsk3β and Frat are found in a complex. Frat is required for the nuclear export of Gsk3β. When PI3K/Akt signaling declines, Frat dissociates from Gsk3β and, although it continues to shuttle, Gsk3β remains in the nucleus.

we asked whether Gsk3β can still shuttle in a cell where Frat1 does not. This was tested by expressing a plasma membrane-anchored form of Frat (myristoylated Frat1) in mESCs and asking where Gsk3β localizes and whether it still shuttles between the cytoplasm and nucleus. Expression of myr-$Frat1_{Flag}$ had no effect on the cytoplasmic localization of Gsk3β in mESCs, but this did not establish whether Gsk3β retained the ability to shuttle. When leptomycin B was added to myr-$Frat1_{Flag}$-expressing cells, Gsk3β remained in the cytoplasm, indicating that it no longer shuttled (Fig. 3B). The ability of Gsk3β to shuttle is therefore dependent on the ability of Frat to do the same and is consistent with a model where Frat is required to carry Gsk3β out of the nucleus as part of a protein complex.

PI3K signaling is required for Frat to complex with Gsk3β in mESCs. Immunostaining of mESCs revealed that Frat1 and Gsk3β colocalized to the cytoplasm in mESCs (Fig. 4A). Addition of leptomycin B trapped both proteins in the nucleus, consistent with their being part of a complex that shuttles in and out of the

nucleus (Fig. 4A). Addition of the PI3K inhibitor PI-103, however, resulted in the accumulation of nuclear Gsk3β, as described previously (Fig. 4A). Frat, however, remained primarily in the cytoplasm, indicating that its shuttling is PI3K independent and that its nuclear export exceeds import under all conditions tested (Fig. 4A). Addition of leptomycin B to PI-103-treated cells resulted in both Gsk3β and Frat being trapped in the nucleus, indicating that Frat still shuttles in the absence of binding to Gsk3β (Fig. 4A). The ability of PI-103 to uncouple the localization of Gsk3β to Frat suggested that the two proteins were no longer in a complex together. When this issue was addressed by coimmunoprecipitation experiments, PI-103 was shown to disrupt the interaction between Gsk3β and Frat, consistent with the immunofluorescence data (Fig. 4A and B). As expected, PI-103 decreased the phosphorylation of S6K, a downstream target of the PI3K pathway. No changes in Frat or Gsk3β expression were observed as a consequence of PI3K inhibition, ruling out the possibility that changes in protein levels account for the loss of interaction (Fig. 4B). In sum, these data indicate that Frat shuttles between the nucleus and cytoplasm in mESCs and serves to carry Gsk3β out of the nucleus when they are in a complex together, under conditions in which PI3K/Akt is active. Loss of PI3K/Akt activity results in the two proteins dissociating, leaving Frat to continue shuttling while Gsk3β remains trapped in the nucleus (Fig. 4C).

Nuclear localization and enzymatic activation of Gsk3β are required to promote ESC differentiation. We previously showed that constitutive nuclear targeting of Gsk3β is insufficient to promote differentiation of mESCs or to increase the phosphorylation of nuclear substrates such as c-myc (1). On the surface, this seems paradoxical, but it was shown that under self-renewing conditions, nuclearly targeted Gsk3β remains inactive due its sustained inhibition by PI3K/Akt. This previous observation is consistent with maintenance of pluripotency under conditions where Gsk3β^{Q206E} localizes to the nucleus (see Fig. 5A and B). When an S9A mutation was introduced in conjunction with Q206E, however, cells differentiated, as shown by a decrease in the percentage of alkaline phosphatase-positive colonies (Fig. 5A and B) and by increases in the numbers of endoderm markers such as Gata6 and FoxA2 (Fig. 5C). The Nanog pluripotency marker was almost completely lost in the Q206E/S9A Gsk3β mutant, indicating that retention of active Gsk3β in the nucleus antagonizes pluripotency.

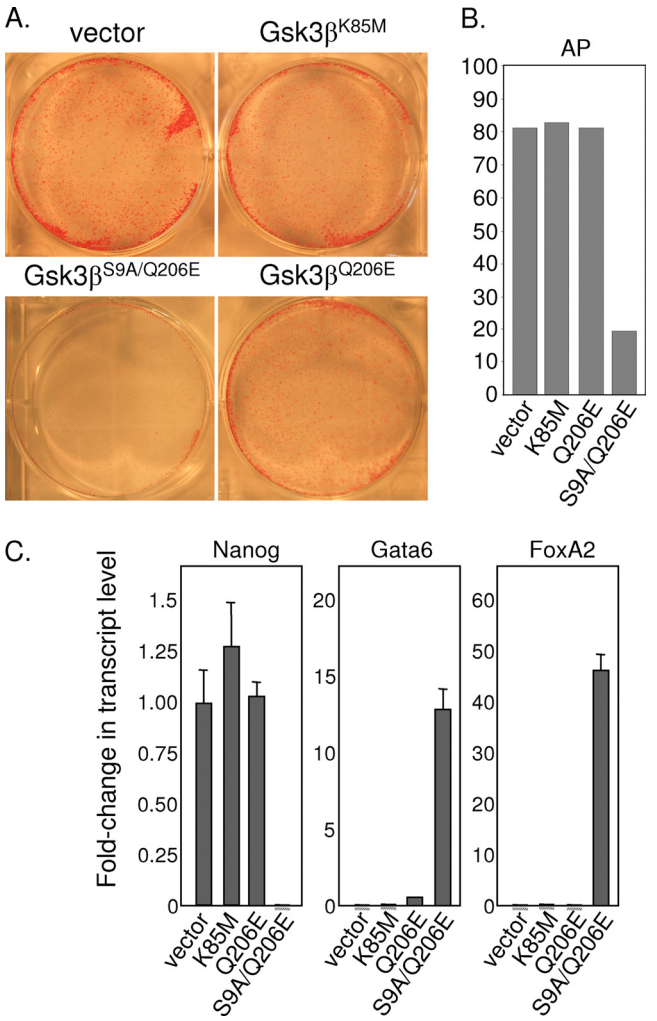

FIG 5 Enforced expression of active Gsk3β incapable of binding Frat disrupts mESC self-renewal. (A) R1 mESCs were transiently transfected with vector alone (pCAGipuro) or the equivalent vector expressing Gsk3β^{K85M}, Gsk3β^{Q206E}, or Gsk3$\beta^{S9A,Q206E}$. At 24 h posttransfection, cells were selected with puromycin for 4 days and self-renewal capacity was then analyzed by alkaline phosphatase (AP) staining. (B) Graphical representation of the percentages of colonies positive (+) or negative (−) for AP staining determined in experiments performed in duplicate. (C) qRT-PCR analysis of Nanog, Gata6, and FoxA2 transcripts following transfection of R1 mESCs with vector or with the indicated Gsk3β expression constructs. Assays were performed in triplicate; data shown represent means ± standard deviations of the results.

DISCUSSION

Gsk3β is an important effector of PI3K/Akt and Wnt signaling but is regulated within these separate pathways by distinct mechanisms (21). In mESCs, most Gsk3 activity is coupled to the canonical PI3K/Akt pathway (1), where it serves to target nuclear substrates such as c-myc (3). More recently, β-catenin has been identified as another nuclear substrate for Gsk3β in mESCs, where it maintains the pluripotent state by a TCF- and Wnt-independent mechanism (10). Understanding how Gsk3 regulates nuclear targets in mESCs is therefore essential to understand fundamental aspects of pluripotency and cell fate commitment.

Our previous work showed that there are two requirements that must be met for Gsk3β to promote differentiation (1). First, Gsk3β must be dephosphorylated on S9, rendering it catalytically active. This activity, however, is insufficient for Gsk3β to promote differentiation. The insufficiency was reconciled by the discovery of a second level of regulation where Gsk3β must accumulate in the nucleus so that it can target nuclear substrates (1). The validity of this general principle is reinforced by results presented in this report, most notably by the maintenance of pluripotency observed under conditions in which the non-Frat binding, nuclear Gsk3β^{Q206E} mutant is expressed. Combining the Q206E mutation with S9A, however, activated nuclear Gsk3β and promoted differentiation. Both levels of regulation are under the control of PI3K and Akt, which together are critical for maintaining pluripotency. In this report, we characterize the mechanism underpinning the shuttling of Gsk3β in mESCs and how this mechanism is regulated by PI3K/Akt.

The primary aim of this work was to define mechanisms regulating nuclear shuttling of Gsk3β by PI3K/Akt in mESCs and miPSCs. Our model (see Fig. 4C) indicates that Gsk3β shuttling is dependent on its binding to Frat. Gsk3β mutants incapable of

binding Frat localize to the nucleus, consistent with Gsk3β having its own bipartite NLS (6, 12). Loss of Frat binding, however, impacts at the level of nuclear export. Since Gsk3β has no NES of its own, it relies on Frat to carry it out of the nucleus in a Crm1-dependent manner. After Frat and Gsk3β dissociate under conditions of low PI3K activity, Frat continues to shuttle in a manner indistinguishable from that of its association with Gsk3β but, importantly, in a PI3K/Akt-independent manner. Although Frat can be trapped in the nucleus by treatment with leptomycin B, indicating that it shuttles between the nucleus and cytoplasm, its rate of nuclear export exceeds that of its import. The imbalances in rates of Frat nuclear import versus export are not understood.

Frat TKO ESCs were not available for this study, so we generated pluripotent iPSCs from the TKO mouse line. Gsk3β nuclear cytoplasmic shuttling and PI3K/Akt regulation are indistinguishable between mESCs and miPSCs, validating this general approach. Although we show that Frat1 can rescue the Gsk3β localization defect in TKO cells, it is unclear whether Frat2 and Frat3 can also perform this function, even though Frat proteins perform overlapping and redundant roles (9). Although axin can bind Gsk3β and is expressed in mESCs, it was unable to rescue the nuclear accumulation of Gsk3β mutants incapable of binding Frat. Therefore, axin does not appear to be involved in this aspect of Gsk3β regulation.

Frat proteins are members of a family of oncoproteins that cooperate with c-myc in lymphomagenesis (8, 20). The mechanism by which Frat cooperates with Gsk3β, however, is unclear. Previously, Frat proteins were believed to regulate canonical Wnt signaling as part of vertebrate development, but more recent data indicate they are dispensable (18). Other roles outside this pathway, including Gsk3-independent roles for Frat in the targeting of Jun N-terminal protein kinase (JNK) and Ap-1 for activation, have been recently demonstrated (19). Our studies identified a novel PI3K/Akt-regulated role for Frat that targets the pool of Gsk3β regulated by canonical PI3K/Akt signaling. Previously, the Frat-Gsk3 connection was established as part of Wnt signaling, but since there are multiple pools of Gsk3β in vertebrate cells (21), it is perhaps not surprising that Frat reaches across to regulate Gsk3β through alternate mechanisms. Our work provides the first demonstration that Frat regulates a pool of Gsk3β primarily under the control of PI3K/Akt signaling. Since Frat restricts the access of Gsk3β to nuclear substrates such as c-myc, this may explain how Frat and c-myc cooperate in lymphomagenesis. In this scenario, absence of nuclear retention may contribute to c-myc stability, enhancing its oncogenic properties.

Despite the clear demonstration of a role for Frat in Gsk3 nuclear-cytoplasmic shuttling, the physiological significance of this is unclear, at least in pluripotent cells. Two potential functions can be tentatively ruled out, however. First, Frat does not seem to be required for Gsk3 to target its nuclear substrates, since the S9A/Q206E mutant promotes differentiation presumably by targeting substrates such as c-myc and Nanog. The issue of whether Frat directs Gsk3 for Akt-dependent regulation is less clear, but that it does so is unlikely, since the Q206E mutant is regulated on S9 independently of Frat binding. The general role of Frat, however, could lie in a more subtle mode of regulation that manifests in cell types where PI3K/Akt signaling strength varies. In this scenario, the kinetics of Gsk3 nuclear export in cells with intermediate levels of PI3K/Akt activity would be reduced and so net nuclear retention time would increase. This is consistent with our previous observations indicating that, although Gsk3 shuttles in all cell types, its distribution between the nucleus and cytoplasm varies considerably (see reference 1), indicating that nuclear retention time varies between different cell types. In concert with this, dampened PI3K/Akt activity would also impact S9 phosphorylation kinetics, especially if it is regulated by a balance between phosphatase and kinase activity. A consequence of this would be a slight elevation in the activity of nuclear Gsk3 that would serve to modulate levels of nuclear substrates as required by the cell. In the case of pluripotent cells, high PI3K/Akt activity is required to maintain high levels of Myc and Nanog.

The shuttling of Gsk3β between nucleus and cytoplasm clearly requires elevated PI3K/Akt signaling. One important aspect of this is that PI3K/Akt is necessary for Gsk3β and Frat to form stable complexes. The exact mechanism by which PI3K/Akt controls this interaction is unclear, however. Regulation by phosphorylation on S9 is ruled out, since shuttling is independent of the S9 phosphorylation status and catalytic activity of Gsk3β (1). Mass spectrometry analysis of Gsk3β immunoprecipitates also failed to detect any new PI3K/Akt-regulated sites (M. Bechard and S. Dalton, unpublished data). One possibility is that Akt directly phosphorylates Frat or another interacting protein, such as 14-3-3, to promote the binding of Gsk3β to Frat and the nuclear export machinery (2). The shuttling of Gsk3β is a common feature to all cells, raising the possibility that the Frat-mediated mechanism identified in mESCs is common to a wide variety of cell types. Future work should resolve this issue.

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