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Original Research

Ubiquitin E3 Ligase FBXO9 Regulates Pluripotency by Targeting DPPA5 for Ubiquitylation and Degradation

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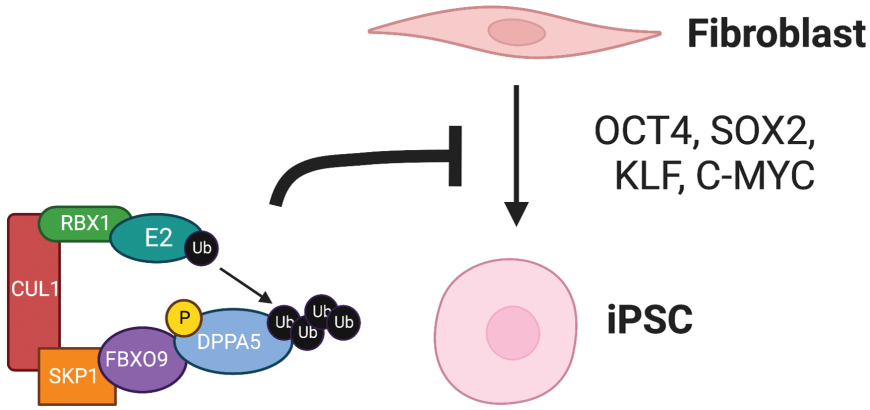
Abstract

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have unique characteristics where they can both contribute to all three germ layers in vivo and self-renewal indefinitely in vitro. Post-translational modifications of proteins, particularly by the ubiquitin proteasome system (UPS), control cell pluripotency, self-renewal, and differentiation. A significant number of UPS members (mainly ubiquitin ligases) regulate pluripotency and influence ESC differentiation with key elements of the ESC pluripotency network (including the "master" regulators NANOG and OCT4) being controlled by ubiquitination. To further understand the role of the UPS in pluripotency, we performed an RNAi screen during induction of cellular reprogramming and have identified FBXO9 as a novel regulator of pluripotency associated protein DPPA5. Our findings indicate that *FBXO9* silencing facilitates the induction of pluripotency through decreased proteasomal degradation of DPPA5. These findings identify FBXO9 as a key regulator of pluripotency.

Key words: ubiquitination; DPPA5; pluripotency; E3 ligase

Graphical Abstract



Significance Statement

This research identified a novel role of regulating pluripotency by ubiquitin E3 ligase FBXO9 through ubiquitination of pluripotency associated protein DPPA5.

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Introduction

Cellular reprogramming requires several changes to molecular pathways to initiate new transcriptional programs, increase proliferation, and alter a cell's epigenetic program. It was previously shown that pluripotent stem cells, including both embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC), have increased proteasomal activity compared to differentiated populations, suggesting an additional layer to the molecular mechanisms required for cellular reprogramming and to maintain their pluripotent state.[1] The reprogramming factors OCT4, KLF4, and c-MYC are targeted for degradation in self-renewing ESCs, suggesting a role for the ubiquitin proteasome system (UPS) to maintain pluripotency; however, the ubiquitin E3 ligases responsible for degradation of a number of proteins associated with pluripotency are unknown.[2] Interestingly, SOX2, a key reprogramming factor, is not regulated by the UPS, suggesting that specific proteins are targeted by the UPS to regulate their protein abundance.[2]

The UPS coordinates the degradation of proteins globally and compartmentally within a cell and is a key regulatory mechanism for various cellular processes, including proliferation and differentiation.[3] The two of the main components of this system are the ubiquitin E3 ligases that determine substrate specificity and the 26S proteasome responsible for protein degradation. Of particular interest are the E3 ligases: the component of the UPS responsible for substrate recognition and determination of which proteins receive a K48-linked poly-ubiquitination tag marking them for degradation. There are over 600 known E3 ligases, each of which recognizes a specific set of substrates, while the other UPS enzymes combined, E1 and E2, total <50.[4] Ubiquitin E3 ligases can be classified as RING-finger, HECT-domain, or RBR based on their domains and mode of transferring ubiquitin onto their target substrates.[5-8] The largest family of E3 ligases, the SKP1-CUL1-FBOX (SCF) family, is named for the various components that compose the core of the complex.[9,10] SKP1 and CUL1 act as scaffolding proteins to bring the ubiquitin-binding RING-finger protein, RBX, in proximity with the substrate recognition FBOX protein component.[11] One characteristic shared by most FBOX proteins is the need for a phosphodegron. A phosphodegron is a specific amino acid sequence, typically containing a serine or threonine, whose phosphorylation permits recognition by the E3 ligase preceding ubiquitination for proteasomal degradation. Examples are the DSGxxS degron motif for β-TrCP1 (FBXW1) and TPxxS degron motif for FBXW7 (LLP**T**P**P**L**S** is the c-MYC the degron motif).[12,13] There are 69 distinct FBOX proteins that interact with SKP1 via their FBOX domain and with substrate proteins through a variety of substrate-recognition domains.[14,15] FBXW7 targets c-MYC for degradation, and silencing of *Fbxw7* promotes cellular reprogramming.[2] We identified differentially ubiquitinated proteins in self-renewing vs. differentiated ESC populations as well as ubiquitinated proteins in iPSCs and mouse embryonic fibroblasts (MEFs). Among the peptides that were overrepresented in self-renewing ESC and iPSCs were several known regulators of pluripotency, including NANOG, OCT4 (POU5f1), DPPA5, p53, c-MYC, DAX1 (NR0B1), and ZFP42.[2]

Inhibition of the proteasome during reprogramming prevents the generation of iPSC confirming that the UPS plays a key role in cellular reprogramming.[2] Here we show that pluripotency factors OCT4, c-MYC, DAX1 (NR0B1), and KLF4 are targeted for proteasomal degradation during reprogramming. Mutating identified ubiquitinated lysine residues of DAX1 to arginine increases efficiency of reprogramming to a pluripotent state. To identify ubiquitin E3 ligases that inhibit cellular reprogramming, by potentially targeting pluripotency associated proteins, we performed an shRNA screen and revealed novel regulators of reprogramming. We previously showed that inhibition of suppressor of cytokine signaling 3 (SOCS3), which has been found in ubiquitin ligase complexes, inhibits ESC differentiation,[2,16] which corresponds to what we show here, that inhibition of *Socs3* enhances the generation of iPSC from MEFs. In addition, we identified a novel E3 ligase, FBXO9, that plays an important role in pluripotency. Our findings show that FBXO9 targets the protein developmental pluripotency associated 5 (DPPA5) for degradation and inhibition of *Fbxo9* leads to an enhanced in cellular reprogramming. Together, these findings define a new mechanism in the induction and maintenance of pluripotency through FBXO9 regulation of DPPA5.

Methods

Cell Culture and Screen

"Reprogrammable" mouse model was crossed to an OCT4-GFP mouse and MEFs were isolated as previously described.[17] For shRNA screen, 6 pools of retroviral ubiquitin E3 ligase shRNA libraries were used (Supplementary Table S1).[25] Reprogrammable MEFs were grown to 50% confluency in 15 cm^2 dish and treated with concentrated retrovirus of one shRNA pool per dish in duplicate with 8 μg/mL polybrene. Media was replaced after 48 hours with ESC media containing 2 μg/mL doxycycline (Dox) and leukemia inhibitory factor (LIF) then replaced every other day for 10 days. Dox was removed for the last 4 days of cultures. For each pool, one dish of cells was harvested 48 hours post retroviral transduction as day 0 input. At day 14, cells were sorted for GFP^+ followed by genomic extraction and deep sequencing as described previously.[43]

For shRNA silencing during reprogramming, reprogrammable MEFs were plated with concentrated pLMP-GFP retrovirus in the presence of 8 μg/mL polybrene. The 48 hours post infection GFP^+ cells were sorted and plated on mitomycin C treated MEFs, and media was replaced with ESC media containing 2 μg/mL Dox and LIF, then replaced every other day for 10 days, where Dox was removed for the last 4 days of cultures. At day 14, ESC like colonies were enumerated following alkaline phosphatase (AP) staining (Millipore Sigma). SSEA-1 expression was followed by flow cytometry analysis at given time points. Cells were stained for 1 hour in 3% FBS in PBS.

All mice were housed in a pathogen-free facility at the University of Nebraska Medical Center. Procedures performed were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center in accordance with NIH guidelines.

Generation of ESC Lines

Complementary DNAs (cDNAs) for *Socs3*, and *Fbxo9*, were cloned in frame into the modified and Tet-operated vector pBS31, with N-terminal Strep-TagII/Flag (SF) tandem tags[44] (kind gift of Dr. M Stafedlt, NYU School of Medicine). shRNA was cloned in pCol-TGM.[45] Fifty micrograms of resulting

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vectors and 25 µg pCAGs-FLPe were nucleoporated (Amaxa) into KH2 ESCs engineered to carry an M2rtTA transactivator in the ROSA26 locus, ensuring inducible expression of the tagged cDNA or shRNA.[26,46] ESCs were selected with 140 µg/mL hygromycin for 7 days and expression validated following 48 hours of doxycycline treatment.

Immunofluorescence

ESCs were cultured in 96-well black plates (Corning). Cells were fixed with 4% paraformaldehyde (BD Biosciences) for 10 min and permeabilized for 10 min using 0.1% Triton X-100 (Sigma-Aldrich). Wells were blocked with 3% goat serum (Invitrogen) and stained with primary antibodies overnight. Secondary antibody staining was performed with Alexa488- or 594-conjugated antibodies (Invitrogen), Alexa594-conjugated Phalloidin (Invitrogen), and DAPI.

Cloning, Western Blot Analysis, and Immunoprecipitation

DAX1 and DPPA5 were PCR amplified from ESC cDNA and cloned into pMIGR1 or pCDNA plasmids, respectively. For mutagenesis, plasmids were subjected to site-directed mutagenesis using the New England Biolabs Q5 Site-Directed Mutagenesis Kit (E0554S) to introduce lysine to arginine mutations. Reactions were performed per manufacturers' protocol.

For western blot analysis, samples were lysed in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA) containing 1× Halt Protease and Phosphatase Inhibitor Cocktail (ThermoFisher, Waltham, MA, USA) and 10 mM *N*-ethylmaleimide (NEM). Membranes were blocked in 5% milk. All protein quantification was determined utilizing relative densitometry calculated with ImageJ Software. Quantification is reflective of relative fold change as a ratio of each protein band relative to our loading control, β-actin. For transient HEK293T transfection, cells were transfected with respective plasmids, cultured for 48 hours, collected, and lysed as described above. Protein lysates were incubated with Anti-HA (Sigma) or Anti-Flag (Sigma) beads overnight at 4 °C with gentle rocking, beads were washed with lysis buffer five times, and eluted by boiling the beads in 1x laemmli buffer.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was harvested using QIAGEN RNeasy Kit (Qiagen, Hilden, Germany). Following RNA extraction, cDNA synthesis was performed using High-Capacity RNA-to-cDNA Kit (ThermoFisher). qRT-PCR was carried out on equal concentrations of cDNA using iTaq Universal SYBR Green Supermix.

Proteomics

For global proteome quantification, ESCs were cultured in duplicate following 72 hours with Dox, and protein was isolated as described above. Samples were prepared and TMT labeled per manufacturer's protocol (ThermoScientific TMT10plex Mass Tag Labeling Kits). Following TMT labeling, acetonitrile was removed by speedvac, and samples were resuspended in 0.1% trifluoroacetic acid (TFA). Sample cleanup with C18 tips was performed per manufacturer's protocol (Pierce). Sample concentrations were re-quantified (Pierce Quantitative Colorimetric Peptide Assay kit) and then combined in equal concentration. Samples were then fractionated using ThermoScientific high pH reverse-phase fractionation kit following manufacturer's protocol. Resulting fractions were speedvac to dryness and resuspended in 0.1% formic acid for mass spectrometry (MS) analysis. Data will be made available via ProteomeXchange.

For IP followed by mass spectrometry, cells were lysed, and IP was performed overnight at 4 °C using anti-StrepII followed by IP with anti-Flag. Samples were washed five times with lysis buffer prior to analysis by MS.

In Vitro Ubiquitination Assay

Plasmids containing the DAX1 or DPPA5 gene were isolated and subjected to site-directed mutagenesis using the New England Biolabs Q5 Site-Directed Mutagenesis Kit (E0554S) to introduce lysine to arginine mutations. Reactions were performed per manufacturer's protocol. HEK293T cells were transfected with plasmids encoding Flag-FBXO9; Flag-ΔFBXO9; Flag-FBXO9 ΔTPR; HA-DPPA5; HA-DPPA5 K9,16R; HA-DPPA5 K35R; and HA-DPPA5 K103,109R. Forty-eight hours post transfection, cells were immunopurified from the whole cell extracts using Anti-HA (Sigma) or Anti-Flag (Sigma) beads overnight at 4 °C. The immunopurified E3 ligase (0.5 µg) proteins were incubated with immunopurified 0.5 µg substrate, E1 (Boston Biochem), E2-UbcH5a (500 ng, Boston Biochem), ubiquitin (0.5 µg, Boston Biochem), and in the presence or absence of activated ATP (10 mM). Ubiquitylation reactions were performed in assay buffer (100 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, 25 mM Tris-Cl (pH 7.5)) and incubated at 30 °C for 2 hours. The reactions were stopped with 2× laemmli buffer (10 minutes at 95 °C), resolved on SDS-PAGE gels, and analyzed by Western blot.

Statistical and Data Analysis

Heatmap was generated using published datasets through morpheus online software (https://software.broadinstitute.org/morpheus/).[17] All experiments were performed in triplicate unless noted and statistical analyses were performed using paired two-tailed Student's *t*-test assuming experimental samples of equal variance. $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$, $^{****}P \leq .0001$.

Results

Key Pluripotency Factors Are Targeted by the UPS During Reprogramming

Pluripotent stem cells have high levels of proteasomal activity and show a marked decrease in activity during lineage differentiation.[1] Expression of the components that make up the 26S proteasome show low expression in mouse embryonic fibroblasts (MEF) and upon expression of reprogramming factors (OCT4, KLF4, SOX2, and c-MYC (OKSM)), cells poised to reprogram identified by the expression of pluripotency associated cell surface marker SSEA1 (SSEA1^{+}), upregulate the proteasome components (Figure 1A).[17] Inhibition of the proteasome with a low dose (1 µM) of the proteasome inhibitor MG132 completely inhibited cellular reprogramming with limited impact on cell survival, further suggesting that increased proteasomal activity is essential for cellular reprogramming.[2] Previous studies have demonstrated a key role of protein ubiquitination of pluripotency factors in cell maintenance and differentiation; however, less is known about the role of ubiquitination during reprogramming. Utilizing reprogrammable MEFs,

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we inhibited the proteasome with MG132 for 3 hours and isolated SSEA1+ cells poised to reprogram.[18] SSEA1+ cells showed less accumulation of reprogramming factors (OCT4, KLF4, and c-MYC) than SSEA1- MEF cells that express THY1+ and are not undergoing cellular reprograming (referred to as THY1+ from this point on), indicating that ubiquitination of exogenously expressed reprogramming factors may inhibit cellular reprogramming (Figure 1B). We found that not only proteins expressed through the reprogramming cassette were targeted for ubiquitylation, but additional pluripotency associated proteins, including DAX1, had a higher accumulation of protein following proteasome inhibition (Figure 1B). Notably, not all reprogramming factors and pluripotency associated proteins are targeted for degradation by the proteasome. Reprogramming factor SOX2 showed no protein accumulation in SSEA1+ or THY1+ cells (Figure 1B). Here we show that during cellular reprogramming, pluripotency proteins accumulate following proteasome inhibition in THY1+ cells compared to SSEA1+ cells poised to reprogram, suggesting increased degradation of these proteins in cells not undergoing cellular reprogramming. These findings are interesting considering that pluripotent cells have high levels of proteasomal activity in comparison to THY1+ cells at day 6 post induction of reprogramming and suggests that proteasomal activity does not always correlate to the rate of protein degradation of specific proteins and that cellular context plays an important role.

Ubiquitin diGLY proteomics in both ESC and IPSC identified several ubiquitinated proteins associated with pluripotency, including NANOG, OCT4, KLF4, cMYC, DPPA5, and DAX1.[2] NANOG, KLF4, cMYC, and OCT4 have been previously shown to be regulated by ubiquitination; however, DPPA5 and DAX1 have not been validated.[12,19-22] DAX1 has been shown to be a key regulator in the pluripotent transcriptional network.[23] Through immunoprecipitation of KGG-motif peptides followed by mass spectrometry in ESC, we identified 5 lysine residues of DAX1 were modified by ubiquitylation (K251, K266, K272, K384, and K400) in ESC (Figure 1C).[2] Overexpression of DAX1 led to increased efficiency of cellular reprogramming, which was attenuated by expression of DAX1 with K272R mutation, whereas K400R

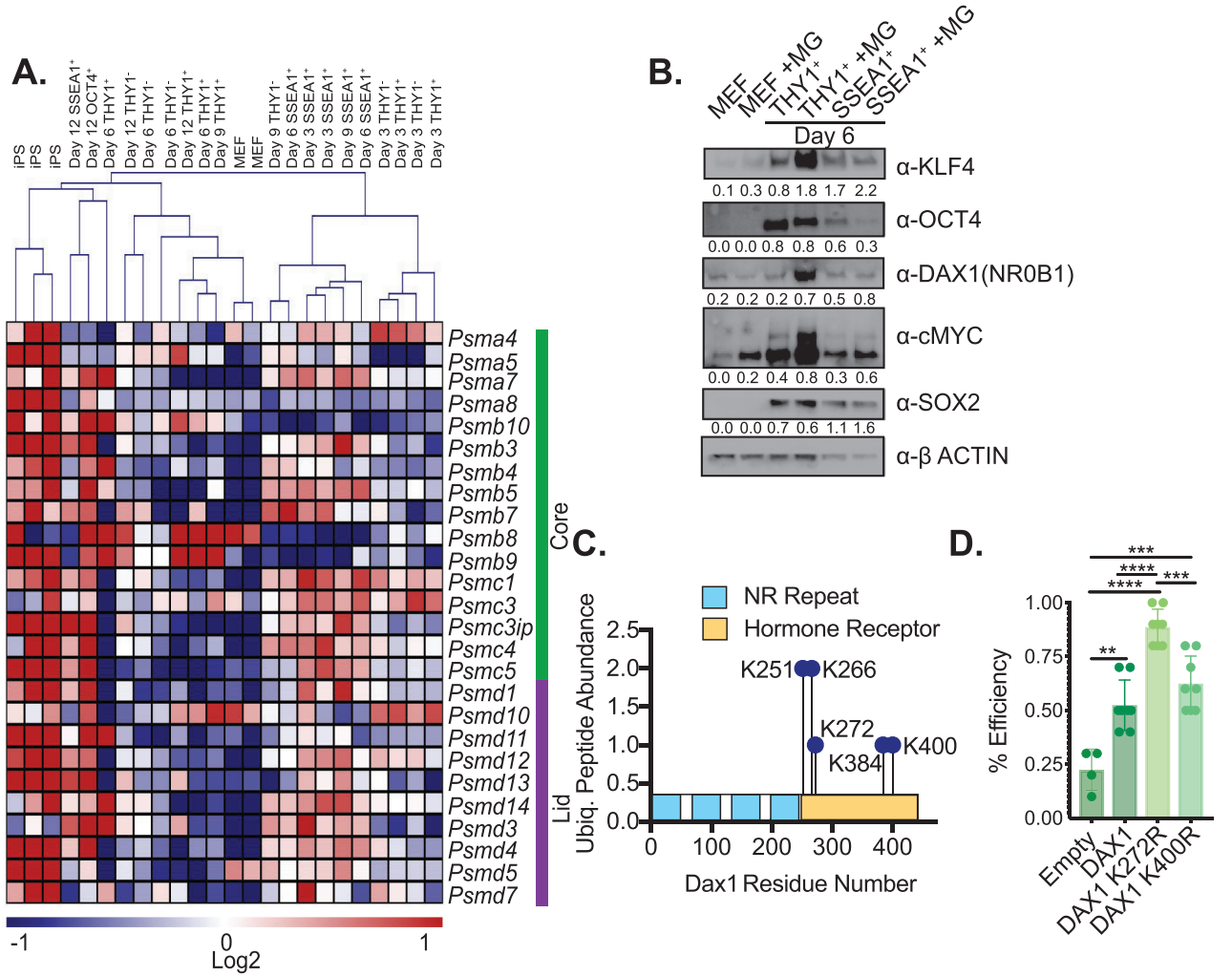

Figure 1. Ubiquitination of pluripotency factors inhibits induced pluripotency. (**A**) Heat-map illustrating differentially expressed proteasome members during reprogramming (log2). (Data from GSE42475). (**B**) Reprogrammable MEFs following 6 days with Dox were treated with either DMSO or 10 μM MG132 for 3 hours, separated by magnetic selection of SSEA1, and analyzed by western blot. Numbers below lanes indicate expression value normalized to actin quantified by ImageJ. (**C**) Abundance of NR0B1 lysine residues identified by K-ε-GG immunoprecipitation followed by mass spectrometry. (**D**) Reprogramming efficiency of OKSM MEFs expressing wild-type Dax1 or Dax1 with lysine to arginine mutations enumerated for AP+ ES-like colonies at day 14 post Dox induction of reprogramming. $N = 3$ for all experiments; (**$P \leq .01$, ***$P \leq .001$, ****$P \leq .0001$).

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showed no significant increase compared to overexpression of wild-type DAX1 suggesting loss of ubiquitination at K272 promotes stabilization and protein expression of DAX1 (Figure 1D). These studies suggest that the ubiquitination of key pluripotency factors inhibits cellular reprogramming.

RNAi screen during cellular reprogramming.

To elucidate key ubiquitin E3 ligases that may target pluripotency factors and thereby inhibit cellular reprogramming, we set up a high-throughput screen (Figure 2A). For this study, we utilized MEFs isolated from "reprogrammable mice" with

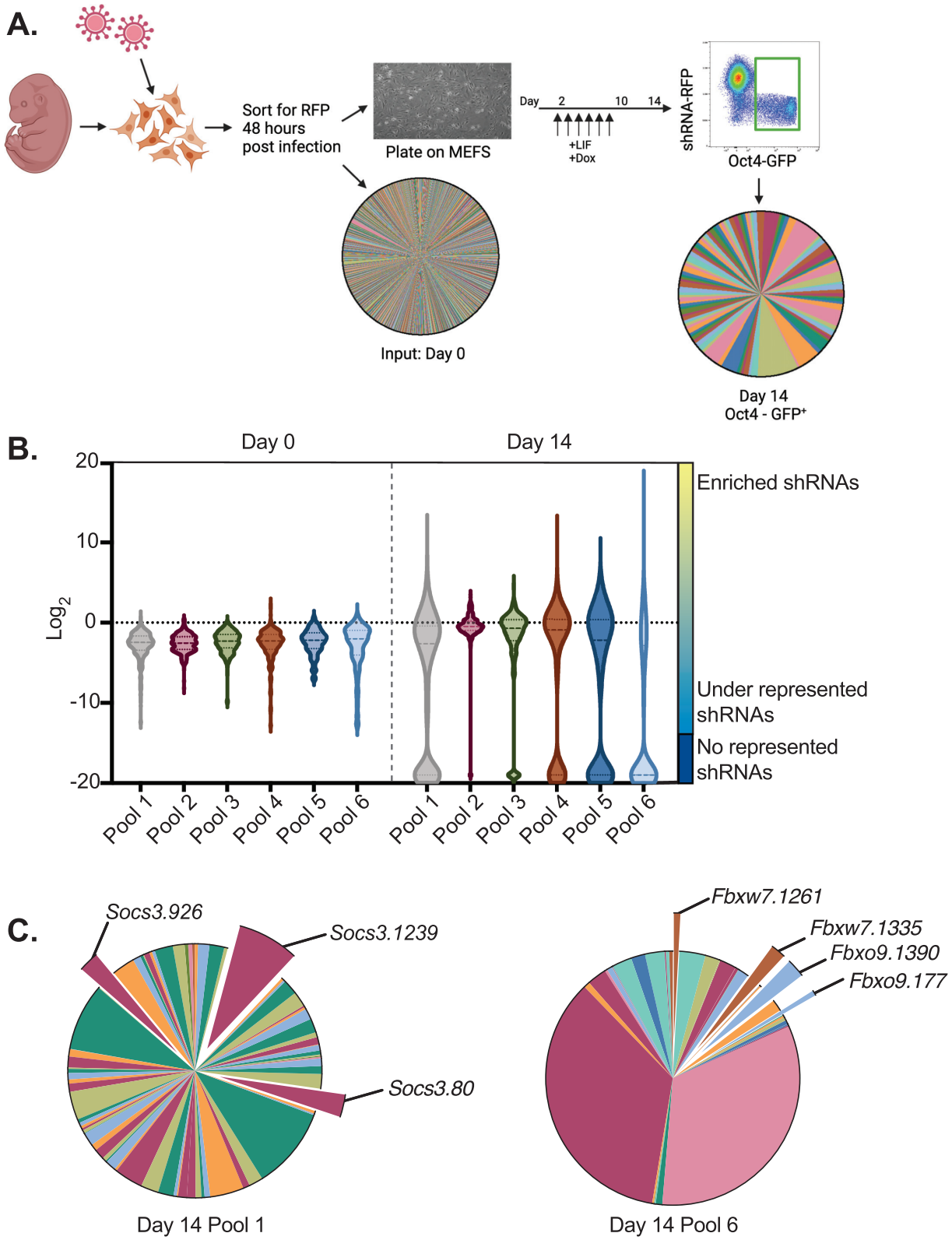

Figure 2. Representation of shRNAs in reprogrammed cells. (**A**) Schematic of shRNA screen during reprogramming. (**B**) Representation of pools at days 0 and 14 normalized to total number of reads per pool. (**C**) Pie graph of day 14 for pool 1 and pool 6.

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inducible expression of reprogramming factors *Oct4*, *Sox2*, *c-Myc*, and *Klf4* (OKSM) crossed to an *Oct4*-IRES-eGFP fusion protein reporter mouse.[18,24] We utilized a Doxycycline (Dox) inducible retroviral shRNA library of ~700 genes, including E2 conjugating enzymes, ubiquitin E3 ligases, and predicted ubiquitin E3 ligases.[25] These genes were split into 6 pools containing ~500 shRNAs, with ~5 shRNAs targeting each gene, for a total of 2,382 shRNAs. The shRNA was expressed in tandem to a fluorescent reporter (mCherry), which allowed for the isolation of shRNA expressing cells prior to inducing reprogramming with Dox. Fourteen days following reprogramming induction, through the addition of Dox and leukemia inhibitory factor (LIF), OCT4-GFP$^+$ cells, which represent cells that underwent reprogramming to a pluripotent state, were isolated. From the OCT4-GFP$^+$ cells, along with cells collected at Day 0, we isolated genomic DNA for high-throughput sequencing of the shRNA region. Day 0 MEFs represented the initial input and representation of each shRNA (Figure 2A).

Sequencing revealed that in the 2,382 shRNAs screened, which corresponded to ~475 genes, ~110 genes were overrepresented in the OCT4-GFP population compared to input at day 0 (Figure 2B, Supplementary Table S1). Genes considered over-represented at day 14 were represented by a minimum of two shRNAs. In the population of shRNAs overrepresented in the OCT4-GFP$^+$ population compared to the day 0 input, we identified 2 shRNAs against *Fbxw7* and 3 shRNAs against *Socs3* (Figure 2C). *Fbxw7* and *Socs3* were previously identified in our ESC siRNA screens to inhibit ESC differentiation when silenced.[2]

To validate the genes identified in the shRNA screen, we silenced *Socs3* with each of the shRNAs individually during cellular reprogramming and found that a decrease in *Socs3* expression enhanced reprogramming (Supplementary Fig. S1A). In addition, we generated a locus-specific, inducible ESC line expressing StrepII/Flag tagged-*Socs3* using the FRT-FlpE recombinase-mediated strategy.[26] Overexpression of SOCS3 in ESCs leads to differentiation and loss of the pluripotent state, characterized by decreased *Nanog* RNA expression as well as loss of ESC-like morphology (Supplementary Fig. S1B, S1C). Although SOCS proteins have been shown to recruit substrates to ubiquitin E3 ligase complexes, their primary role is the regulation of cytokine signaling. SOCS3 is a regulator of the JAK/STAT pathway, an important downstream target of LIF, which is essential in maintaining pluripotency in vitro for mouse ESC.[27,28] Overexpression of SOCS3 leads to the loss of STAT3 phosphorylation and its downstream transcription factor targets, OCT4 and NANOG (Supplementary Fig. S1D). Taken together, these data indicate that in the context of cellular reprogramming, SOCS3 does not function through ubiquitin E3 ligase activity, but instead inhibits the phosphorylation and activation of STAT3 required for cellular reprogramming of mouse cells.

Fbxo9 silencing enhances generation of iPSC

In the population of shRNAs overrepresented in the OCT4-GFP$^+$ population compared to the day 0 input, we identified two shRNAs against FBOX protein, FBXO9 (Figure 2D). RNA expression of *Fbxo9* shows no change following induction of reprogramming factors in OKSM MEFs at days 3, 6, and 9; however, ESCs expressed high levels of *Fbxo9* (Figure 3A). qRT-PCR primers to target the substrate recognition domain of FBXO9 in exon 4 that is required for ubiquitination of its substrate suggests decreased FBXO9 expression correlates with decreased ubiquitination activity. To validate *Fbxo9* shRNAs identified in the shRNA screen, we silenced *Fbxo9* during cellular reprogramming utilizing OKSM inducible MEFS. Silencing of *Fbxo9* led to increased SSEA1$^+$ cells by day 6 and a ~2-fold increase in iPSC generation (Figure 3B, 3C). To determine that the mechanistic importance of *Fbxo9* was specific to reprogramming, we also silenced this gene in wild-type MEFS. We found no changes in the cells' proliferation rate, cell cycle, or apoptosis (Data not shown). These results further establish that members of the UPS, including FBXO9, play a key role in cellular reprogramming, and further analysis of the identified ligases should help unravel molecular mechanisms underlying the induction of pluripotency.

Identification of pluripotent-specific FBXO9 interacting partners

We utilized proteomic approaches to identify interacting partners of FBXO9 and its potential ubiquitination substrates. To identify potential pluripotency-specific substrates of FBXO9, we generated a locus-specific, inducible ESC line expressing tagged-*Fbxo9* using the FRT-FlpE recombinase-mediated strategy. *Fbxo9* was cloned in tandem with StrepII/Flag tags downstream of a TRE, and the resulting vector was electroporated into the KH2 ESC cell line that was previously engineered with a constitutively active rTta as well as FRT sites for locus-specific targeting of tagged *Fbxo9*

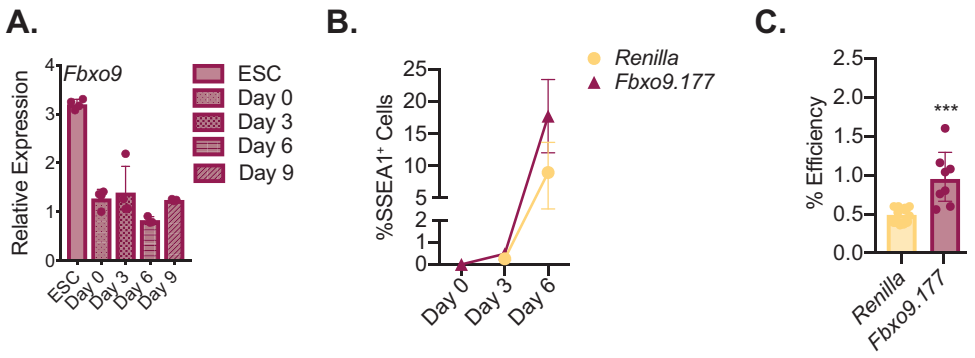

Figure 3. *Fbxo9* silencing enhances cellular reprogramming. (**A**) Relative expression of *Fbxo9* in ESCs and OKSM MEFS induced to reprogram at days 0, 3, 6, and 9 by qRT-PCR. (**B**) Plot of SSEA$^+$ cells by flowcytometry following silencing of *Fbxo9*. (**C**) Reprogramming efficiency of OKSM MEFs expressing shRNAs against *NTC*, or *Fbxo9* to generate Oct4-GFP$^+$ ES-like colonies. Efficiency = Oct4-GFP$^+$ ES-like colonies/total number of OKSM MEFs plated at day 0. Colonies were enumerated at day 14. $N = 8$. ****$P \leq .0001$.

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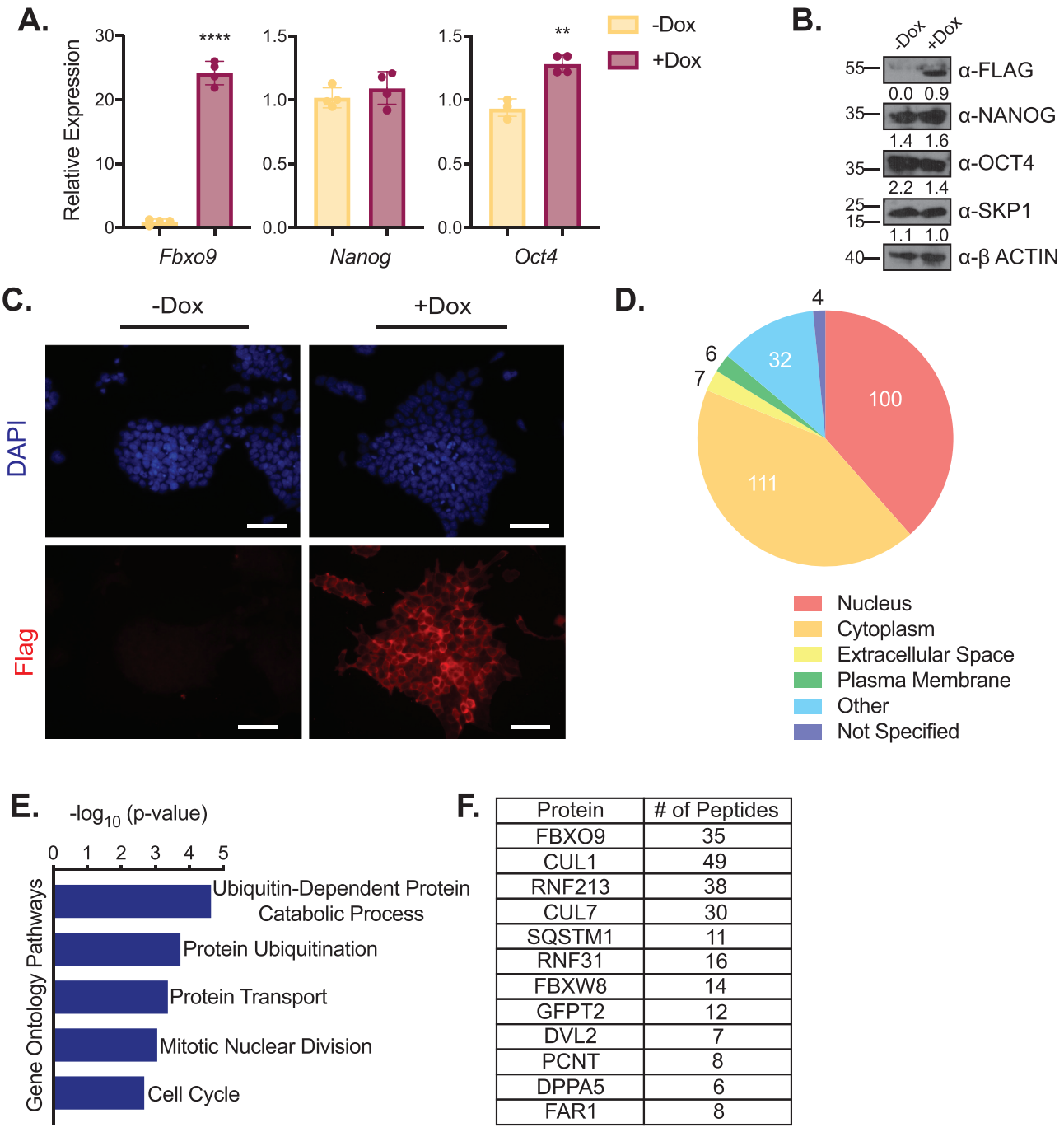
[26]. Overexpression in ESC leads to minimal alterations in pluripotency factors NANOG and OCT4 at both the RNA and protein levels (Fig. 4A-4C). The engineered inducible ESC line was then used to identify novel substrates and interacting proteins by tandem immunoprecipitation (IP) (Strep/Flag tags) in combination with mass spectrometry (MS). These studies identified ~277 unique interacting proteins, with ~110 found in the cytoplasmic compartment where the FBXO9 protein mainly localizes (Fig. 4C-4D, Supplementary Table S3). Most proteins identified were associated with protein ubiquitination, and as expected members of the SCF complex, CUL1 and SKP1 (Figure 4E-F). Interestingly, the top 12 interacting proteins in the cytoplasm included pluripotency-specific protein DPPA5 with 5 unique peptides, and proteins associated with early development including DVL2 with 7 unique peptides.



F.

Protein	# of Peptides
FBXO9	35
CUL1	49
RNF213	38
CUL7	30
SQSTM1	11
RNF31	16
FBXW8	14
GFPT2	12
DVL2	7
PCNT	8
DPPA5	6
FAR1	8

Figure 4. Overexpression of *Fbxo9* does not alter ESC. Flag-Strep tagged *Fbxo9* was targeted to the *Col1a1*-locus of KH2 ES cells carrying rTta in the ROSA locus. Expression was induced in ESC for 4 days with Doxycycline (Dox), and assayed for **A** relative expression of *Fbxo9*, *Nanog*, and *Oct4* by qRT-PCR, (**B**) western blot, and (**C**) immunofluorescence, scale = 100μm. FBXO9 was IP by Flag-Strep beads from ESC treated with Dox. IP was followed by mass spectrometry. (**D**) Cellular compartments of proteins identified. (**E**) Gene ontology analysis showing pathways known to be associated with interacting proteins using DAVID bioinformatics database. (**F**) Top 12 interacting proteins with number of unique peptides identified. $^{**}P \leq .01$, $^{****}P \leq .0001$.

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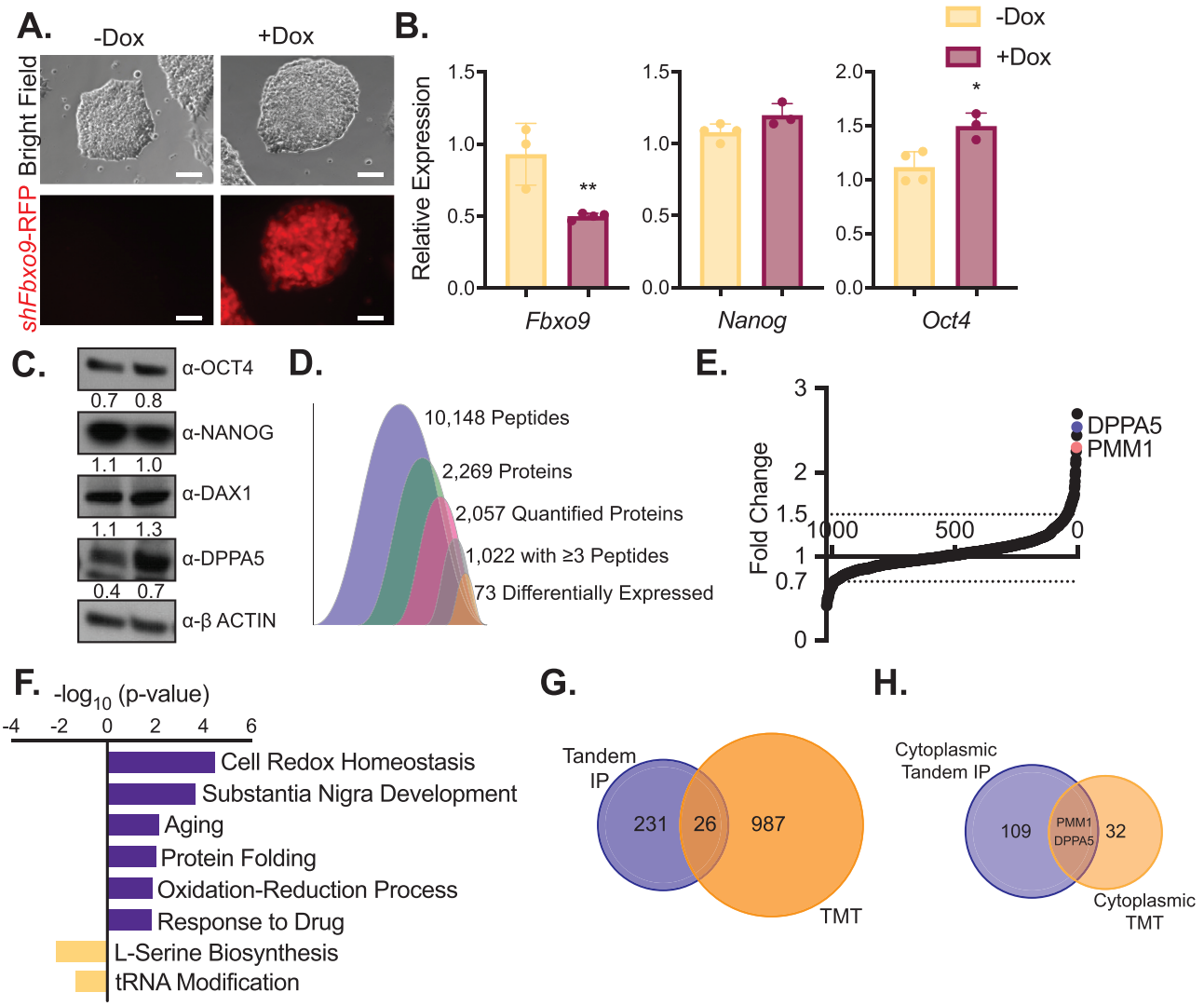

Figure 5. Silencing of *Fbxo9* increased levels of DPPA5 protein. shRNA against *Fbxo9* in tandem with RFP was targeted to the *Col1a1*-locus of KH2 ES cells carrying rTta in the ROSA locus. Expression of shRNA was induced in ESC for 4 days with Doxycycline (Dox) and assayed for (**A**) morphology and RFP expression, scale = 100 μm, (**B**) relative expression of *Fbxo9*, *Nanog*, and *Oct4* by qRT-PCR, and (**C**) western blot. Protein was isolated from *shFbxo9* and *shNTC* ESC and labeled with TMT tags followed by mass spectrometry. (**D**) Schematic showing the number of peptides, proteins, and differentially expressed proteins identified from MS analysis. (**E**) Dot plot showing fold change of expressed proteins from *shFbxo9* compared to *shNTC* cells. (**F**) Gene ontology analysis showing pathways known to be associated with differentially expressed proteins using DAVID bioinformatics database. (**G**) Venn diagram of combined TMT MS, and IP/MS. (**H**) Venn diagram of combined TMT MS and IP/MS for cytoplasmic proteins. (*$P \leq .05$, **$P \leq .01$).

Silencing Fbxo9 maintains pluripotency

To further elucidate the role of FBXO9 in pluripotency and define pluripotent-specific substrates, we generated an ESC line that has inducible expression with dox of an shRNA against *Fbxo9*. Utilizing FRT-FlpE recombinase-mediated strategy, we inserted an shRNA against *Fbxo9* in the Col1A locus in tandem with RFP. Silencing *Fbxo9* led to tight round ESC colonies suggesting decreased differentiation of cells on the outer rim of the colonies (Figure 5A). Expression of the shRNA leads to a 50% decrease in the *Fbxo9* transcript with an increase in *Oct4* RNA expression but little change at the protein level (Figure 5B-C). To determine changes at the proteomic level, we performed tandem mass tag (TMT) MS, which allowed us to quantitatively identify changes in protein abundance following the expression of an shRNA against *Fbxo9* compared to non-targeted control (*shNTC*). Here we identified 2,269 total proteins, with 73 proteins significantly differentially expressed following the silencing of *Fbxo9*, including proteins associated with cell redox homeostasis, aging, and protein folding (Figure 5D-5F, Supplementary Table S4). To identify potential substrates of FBXO9, we compared the interacting proteins identified by MS in Figure 4 to the proteins identified in the quantitative TMT MS in Figure 5 and identified 26 proteins found in both MS datasets (Figure 5G). Of the 26 proteins, only 2 proteins, PMM1 and DPPA5, were found in the cytoplasmic compartment, which is where FBXO9 localizes (Figure 5H). The combination of mass spectrometry approaches identified two potential substrates of FBXO9 in pluripotent cells.

FBXO9 targets DPPA5 for ubiquitination and proteasomal degradation

We previously treated ESCs with the proteasome inhibitor MG132 to determine proteins targeted by the UPS in pluripotent cells.[2] In the mass spectrometry, we identified that DPPA5 was targeted by the proteasome and identified

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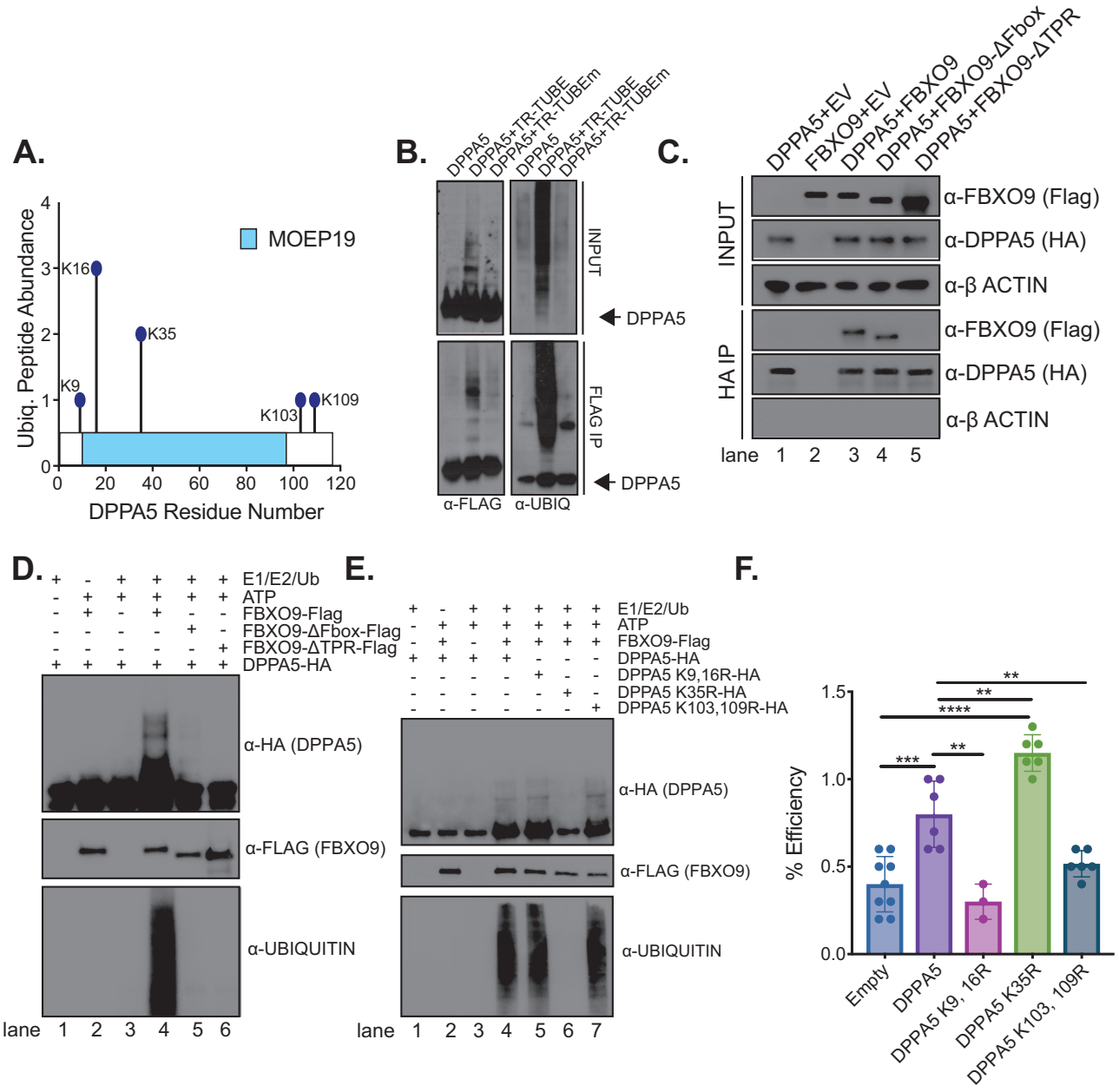

Figure 6. FBXO9 ubiquitinates DPPA5 for degradation inhibiting cellular reprogramming. (**A**) Lysine residues identified by K-ε-GG IP followed by mass spectrometry. (**B**) Western blot of ubiquitin IP of HEK293T cells transiently transfected with DPPA and TR-TUBE or mutant TR-TUBE. (**C**) Western blot of HA IP in HEK293T cells transiently transfected with plasmids expressing HA-tagged DPPA5 and/or Flag-tagged FBXO9/FBXO9$^{\Delta FBOX}$/FBXO9$^{\Delta TPR}$. (**D**) In vitro ubiquitylation of DPPA5 was performed in the presence of E1, E2, and ubiquitin (Ub) after IP with anti-Flag/anti-HA. Samples were analyzed by western blot with the indicated antibodies. (**E**) In vitro ubiquitylation of DPPA5 with lysine to arginine mutations was performed in the presence of E1, E2, and ubiquitin (Ub) after IP with anti-Flag/anti-HA. (**F**) Reprogramming efficiency of OKSM MEFs expressing wild-type DPPA5 or DPPA5 with lysine to arginine mutations enumerated for AP$^+$ ESC-like colonies at day 14 post Dox induction of reprogramming. n = 3-6 for all experiments (**$P \leq .01$, ***$P \leq .001$, ****$P \leq .0001$).

specific lysine residues ubiquitinated; however, PMM1 was not identified in the mass spectrometry (Figure 6A). In order to validate ubiquitination of DPPA5, we utilized trypsin-resistant ubiquitin binding entity (TR-TUBE) plasmid that protects polyubiquitin chains.[29,30] We transfected 293T cells with flag-tagged DPPA along with an empty control plasmid, TR-TUBE plasmid tagged with HA, or TR-TUBE mutant plasmid tagged with HA that lacks ubiquitin binding, which confirmed DPPA5 is polyubiquitinated (Figure 6B). To confirm the interaction of DPPA5 and FBXO9, we transiently expressed DPPA5 tagged with HA with either wild-type FBXO9, FBOX domain deleted FBXO9 (FBXO9$^{\Delta Fbox}$) that cannot bind to the SCF complex, or TPR domain deleted FBXO9 (FBXO9$^{\Delta TPR}$) that cannot bind FBXO9 substrates, all tagged with Flag, in HEK293T cells and immunoprecipitated Flag or HA. We found DPPA5 interacted with both full-length (lane 3) and FBXO9$^{\Delta Fbox}$ (lane 4) suggesting a direct interaction with FBXO9, but lost interaction with FBXO9$^{\Delta TPR}$ (lane 5) (Figure 6C). Finally, we reconstituted the ubiquitination of DPPA5 in vitro. Immunopurified FBXO9 (lane 4), but not

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FBXO9$^{\Delta Fbox}$ (lane 5) or FBXO9$^{\Delta TPR}$ (lane 6) ubiquitinated DPPA5 in vitro (Figure 6D). To identify the lysine residues of DPPA5 required for polyubiquitination, we mutated K9, K16, K35, K103, and K109 to arginine (Figure 6A). To determine the lysine required for polyubiquitination of DPPA5 by FBXO9, we performed an in vitro ubiquitination assay. Immunopurified DPPA5 K35R (lane 6) led to the loss of ubiquitination in vitro, whereas lysine K9, K16, K103, and K109 were not required for polyubiquitination (Figure 6E). Overexpression of DPPA5 lead to increased efficiency of cellular reprogramming, which was attenuated by expression of DPPA5 with a K35R mutation (Figure 6F). Together, these results demonstrate that FBXO9 directly mediates the ubiquitylation and degradation of DPPA5 at K35 in pluripotent cells and during cellular reprogramming.

Discussion

Although in recent years a number of molecular pathways have been identified to be roadblocks in cellular reprogramming, and the efficiency of reprogramming can be near 100% with depletion of MBD3 in mouse cells, the molecular changes during reprogramming are not fully understood.[31] The UPS plays a significant role in regulating pluripotency, and these studies begin to unravel the ubiquitin-related molecular mechanisms that regulate pluripotency induction. During cellular reprogramming, cells go through intermediates that are poised to become iPSCs; however, a large population of these cells fail to reprogram. In MEFs, the cellular kinetics can be followed by cell surface marker expression, where SSEA1 expressing cells have a high efficiency of reprogramming and cells that maintain THY1 expression fail to generate iPSCs.[17,18] We find that along with many other molecular changes to the cells, cells poised to reprogram have increased expression of proteasomal components. Interestingly, during reprogramming, we find that cells that are THY1^{+} have increased accumulation of OCT4, KLF4, and c-MYC following proteasome inhibition, suggesting that degradation of proteins by the proteasome are what inhibits reprogramming. This is true for both exogenously expressed reprogramming factors OCT4, KLF4, and c-MYC, and endogenous pluripotent-associated proteins, including DAX1.

Utilizing shRNA screens during reprogramming, we identified several ubiquitin E3 ligases that could regulate cellular reprogramming. Although *Socs3*, in the context of pluripotency, does not function as a ubiquitin E3 ligase, it does regulate cellular reprogramming in mice through inhibition of the JAK/STAT pathway.[27,28] As part of the UPS, an F-box protein, FBXO9, serves as a substrate-recognizing component of the SKP1/CUL1/FBOX (SCF) ubiquitination complex. Two substrates, TEL2 and TTI1, have been identified as target substrates for FBXO9, and FBXO9 expression was found to promote cell survival and proliferation of multiple myeloma cells through the degradation of TEL2 and TTI1.[32] Here we find that FBXO9 targets pluripotency associated protein DPPA5 for degradation by the UPS. Interestingly, *Fbxo9* is expressed higher in ESCs than in MEFs during cellular reprogramming, suggesting that it is required to maintain self-renewal in ESCs; however, during reprogramming it inhibits induction of pluripotency through degrading DPPA5. These findings suggest distinct roles for FBXO9 in during cellular reprogramming and in maintenance of pluripotent stem cells. Previous studies by Qian et al have demonstrated that DPPA5 overexpression increases NANOG protein expression, and NANOG plays a key role in transcriptional regulation of pluripotency factors, where a balance of pluripotency factors is required to maintain pluripotency.[33] This is distinct from MEFs during reprogramming where FBXO9 is expressed lower than in ESC; however, NANOG is not expressed, suggesting that during reprogramming although low expression inhibits reprogramming by ubiquitinating DPPA5 potentially influencing induction of NANOG protein expression.[34] Although DPPA5 is expressed in the early embryo, germ cells, and pluripotent cells, it is not required for embryonic development in the mouse.[35-37]

The dynamic reversibility of the ubiquitin modification (by kinases, phosphatases, E3 ligases, and de-ubiquitinases), the recent development of both general proteasome inhibitors,[38,39] and specific antagonists of E3 ligase function open the potential of future UPS pharmacological manipulation allowing for more efficiently induced pluripotency and targeted differentiation towards pre-determined lineages.[40-42] In the long run, these advances will facilitate the translation of pluripotency mechanisms identified here to human cells to optimize iPSCs for future cell therapy and disease modeling.

Author Contributions

S.A.S., K.K.D., and S.M.B. conceived and designed the experiments. R.W.H., S.A.S., K.K.D., H.C.P., C.B.W., M.C., K.J.W., and S.M.B performed experiments and analysis. S.A.S., K.K.D. and S.M.B. wrote the manuscript. G.G. provided technical and/or material support. All authors reviewed the manuscript before submission.

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Conflict of Interest

The authors declared no potential conflicts of interest.

Data Availability

Data are available via ProteomeXchange.

Supplementary Material

Supplementary material is available at *Stem Cells* online.

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