Biochimica et Biophysica Acta 1853 (2015) 3279–3293

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

DPF2 regulates OCT4 protein level and nuclear distribution

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ARTICLE INFO

Article history:
Received 29 May 2015
Received in revised form 28 August 2015
Accepted 21 September 2015
Available online 28 September 2015

Keywords:
OCT4
DPF2
PHD finger
E3
Ubiquitination

ABSTRACT

The amount of transcription factor OCT4 is strictly regulated. A tight regulation of OCT4 levels is crucial for mammalian embryonic development and oncogenesis. However, the mechanisms underlying regulation of OCT4 protein expression and nuclear distribution are largely unknown. Here, we report that DPF2, a plant homeodomain (PHD) finger protein, is upregulated during H9 cell differentiation induced by retinoic acid. Endogenous interaction between DPF2 and OCT4 in P19 cells was revealed by an immunoprecipitation assay. GST-pull down assay proved that OCT4 protein in H9 cells and recombinant OCT4 can precipitate with DPF2 *in vitro*. *In vitro* ubiquitination assay demonstrated DPF2 might serve as an E3 ligase. Knock down of *dpf2* using siRNA increased OCT4 protein level and stability in P19 cells. *DPF2* siRNAs also up-regulates OCT4 but not NANOG in H9 cells. However, RA fails to downregulates OCT4 protein level in cells infected by lenitviruses containing *DPF2* siRNA. Moreover, overexpression of both DPF2 and OCT4 in 293 cells proved the DPF2–OCT4 interaction. DPF2 but not PHD2 mutant DPF2 enhanced ubiquitination and degradation of OCT4 in 293 cells co-expressed DPF2 and OCT4. Both wild type DPF2 and PHD2 mutant DPF2 redistributes nuclear OCT4 without affecting DPF2–OCT4 interaction. Further analysis indicated that DPF2 decreases monomeric and mono-ubiquitinated OCT4, assembles poly-ubiquitin chains on OCT4 mainly through Ub–K48 linkage. These findings contribute to an understanding of how OCT4 protein level and nuclear distribution is regulated by its associated protein.

1. Introduction

OCT4 plays crucial roles in maintaining stem cell pluripotency [1–3]. A critical amount of OCT4 is required to sustain embryonic stem cell (ESC) self-renewal and multilineage differentiation capacity [4]. OCT4 is required for generating induced pluripotent stem cells (iPSCs) [5–7]. OCT4 alone is sufficient to generate iPSCs with the aid of small molecules [8,9]. Moreover, OCT4 also plays roles in oncogenesis and may be a potential target for drug therapy of cancer [10–13]. Spatially, OCT4 subnuclear distribution is related to oncogenesis [14,15]. Recent report also showed that OCT4 nucleocytoplasmic dynamics is involved in cell reprogramming and self-renewal of ESCs [16]. In addition, nuclear localization of OCT4 is required for its transactivating activity [17]. Therefore, understanding regulation of OCT4 protein level and intracellular localization may contribute to manipulating OCT4 relevant function and further application.

The ubiquitin (Ub) proteasome system (UPS) is a proteolytic system that regulates protein levels through ubiquitination of its targeted proteins [18,19]. Ubiquitination is a process whereby a small protein, Ub, conjugates its target protein. Mono-ubiquitination occurs through isopeptide bond between the ε-amino group of the lysine (Lys, K) side chain in target protein and the C-terminal glycine (Gly76) residue on Ub, which is achieved through enzymatic cascade activation by E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligase) enzymes. The addition of one or more Ub moieties to the first Ub on the target substrates results in di- or poly-ubiquitination [20]. Poly-ubiquitination, instead of mono-ubiquitination, is targeted for proteasomal degradation [21]. UPS thus plays crucial roles in many cellular processes [18], including maintaining pluripotency of stem cells and determining developmental potency of various adult stem cells, through substrate ubiquitination [22]. Recent report indicated that RNF2, an E3, interacts with OCT4 and functions in maintaining stem cell pluripotency [23]. WWP2, another E3, also interacts with OCT4 and regulates OCT4 protein level in human ESCs [24]. While fusion of a single Ub to OCT4 inactivates its transcriptional activity [25], poly-ubiquitination of OCT4 decreases its level [24]. Specifically, K48-linked poly-Ub chains instead of K63-linked

Abbreviations: ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; PHD, plant homeodomain; Ub, ubiquitin; UPS, ubiquitin proteasome system; E3, ubiquitin ligase; RING, really interesting new gene; GST, glutathione S-transferase; RA, retinoic acid; WT, wild type; IP, Immunoprecipitation; IB, immunoblotting; IF, immunofluorescence; siRNA, small interfering RNA; CHX, cycloheximide

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http://dx.doi.org/10.1016/j.bbamcr.2015.09.029
0167-4889/

poly-Ub chains are considered to be a proteasomal degradation signal [18,19]. In UPS, E3s interact directly with substrate proteins and thus determine the substrate levels [18]. E3s also play crucial roles in translocation of the related substrates [26,27]. Two main types of E3s, homologous to the E6AP carboxyl terminus (HECT) and really interesting new gene (RING) finger families of E3s, have been identified [28,29]. HECT domain receives Ub from the E2 through a thiol linkage to a conserved Cysteine and transfers it to substrate. The RING finger marks the vast majority of E3s that play crucial roles in protein degradation and chelate also two $Zn2^{+}$ ions through a conserved set of cysteines and histidines arranged in a Cysteine-3-Histidine-Cysteine-4(C3HC4) pattern [28,29].

DPF2, also named ubi–d4/requiem (REQU), interacts with OCT4 or a protein complex containing OCT4 *in vivo* [30–32]. It contains double plant homeodomain (PHD) fingers and is involved in cell apoptosis [33,34] and also noncanonical NF-kappaB transcriptional activation and its associated oncogenic activity [35]. PHD fingers are zinc-binding motifs that function in DNA binding, chromatin organization and protein–protein interaction [36,37]. However, the exact function of PHD fingers remains elusive. Typical PHD fingers contain a Cysteine-4-Histidine-Cysteine-3(C4HC3) consensus that coordinates two $Zn2^{+}$ ions in a crossbrace topology [38], which is similar with RING fingers [28]. Therefore, PHD finger is considered as one subgroup of RING-related E3s [28]. Indeed, more and more studies discovered that PHD finger proteins function in ubiquitination and degradation of target proteins [39–44], suggesting PHD finger proteins might have shared an E3 activity. We therefore want to check if DPF2 directly interacts with and functions in OCT4 stability and intracellular localization.

In this study, we report that all-trans retinoic acid (RA) treatment leads to down-regulation of OCT4 protein accompanied by up-regulation of DPF2 in H9 cells. DPF2 interacts with OCT4 and serves as an E3 ligase. *dpf2* siRNA increased OCT4 protein level and stability in P19 cells. *DPF2* siRNA also up-regulates expression of OCT4 but not NANOG in H9 cells. However, RA induced differentiation downregulates OCT4 protein level in cells infected by lenitviruses containing control siRNA but not *DPF2* siRNA. Overexpression of DPF2 changes OCT4 subnuclear distribution and increases OCT4 ubiquitination and degradation, and all of these processes depend on the second PHD finger of DPF2. Further analysis indicated that DPF2 decreases monomeric and mono-ubiquitinated OCT4, assembles poly-ubiquitin chains on OCT4 mainly through Ub–K48 linkage.

2. Materials and methods

2.1. Plasmids

The OCT4 plasmid previously described was provided as a gift from Dr. Yun Qiu [6]. The wild-type (WT) DPF2 cDNA was constructed by subcloning a PCR product amplified using primers 5′- CGGAATTCCCATGGAAGATGGCGGCTGTGGTGGAG-3′ and 5′-A CGCGTCGACCAAGAGGAGTTCTGGTTCTGGTAGA-3′ from the pCMV-SPORT6-DPF2 (Open Biosystems, Huntsville, AL) vector and was then cloned into pGEX-5X-1 and pEGFP-N1 vectors via EcoRI/SalI sites. Wild type RNF2 cDNA was amplified by PCR using primers 5′- CGGGATCCCGATGGCAATGTCTCAGGCTGTGCAGAC-3′ and 5′-ACGCGTCGACCGTTTGTGCTCCTTTGTAGGTGCGTAA-3′ from the pDNR-LIB-RNF2 (Open Biosystems, Huntsville, AL) vector and was then inserted into the pGEX-5X-1 vector via the BamHI/SalI sites. pFLAG–CMV–6C–DPF2 was created by excising DPF2 via EcoRI/Sal sites from pEGFP–N1–DPF2 and cloned into the pFLAG–CMV–6C vector. The following point mutations in the gene were introduced using site-directed mutagenesis (Stratagene, La Jolla, CA): a PHD2 mutant DPF2, DPF2 (M), was created using primers 5′-GATCGTGGCTACGCCATGTACGCGTTAACCCCGTCCATG-3′ and 5′-CATGGACGGGGTTAACGCGTACATGGCGTAGCCACGATC- 3′. A 6His-tagged Ub, a lysine null mutant Ub, and a single-lysine-containing mutant Ub were provided as kind gifts from Dr. Yun Qiu. Plasmids encoding FLAG-tagged Ub and HA-tagged Ub were provided as kind gifts from Dr. Yihong Ye. A pET28a-OCT4 plasmid was provided as a kind gift from Dr. Ying-jie Wang.

2.2. Cell culture

P19 cell was cultured as previously described [45]. HeLa and 293 culturing was previously described, respectively [46,47]. Briefly, cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin and glutamine under 5% CO_2 in a humidified incubator. Cells were transfected with plasmid DNA by using calcium phosphate precipitation or Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Human ESC line, H9, was maintained on Matrigel (BD Bioscience, Bedford, MA) in mTeSR medium (Stem Cell Technologies, Vancouver, BC, Canada), as previously described [48,49].

2.3. GST-pull down assay

Glutathione S-transferase (GST) fusions were expressed in log phase *Escherichia coli* BL-21 (DE3) (Novagen, Madison, WI) that were grown overnight at room temperature and induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 1 h. Bacterial pellets were resuspended in sonication buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100, 5 mM DTT, 2 mM phenylmethylsulfonyl fluoride) and lysed by probe sonication, using 4 ml of sonication buffer per 100 ml of bacterial culture. The sonicated lysate was clarified by centrifugation at 4 °C for 15 min at 14,000 rpm, aliquoted, and stored at −70 °C. The GST fusion proteins were purified with glutathione-Sepharose beads. His-tagged human OCT4 was expressed in *Escherichia coli* BL-21 and purified using Ni-NTA agarose (QIAGEN, Hilden, Germany) as described previously [50]. H9 cells were treated with MG132 (Calbiochem) at 20 μM for 4 h and lysed in NP-40 lysis buffer (150 mM NaCl, 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.2% NP-40). GST-DPF2 and/or GST-RNF2 immobilized on glutathione-Sepharose beads were incubated with H9 cell lysates or recombinant His-tagged OCT4 overnight at 4 °C. After washing with NP-40 lysis buffer, bead-associated proteins were immunoblotted for OCT4. IB was performed following the previously published protocol [51].

2.4. Immunoprecipitation (IP) and immunoblotting (IB)

Immunoprecipitation under native condition was performed as previously described [51]. Briefly, cells were harvested 18–20 h after transfection and lysed in RIPA buffer (50 mM Tris/HCl, PH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride) with or without 20 μM MG132. Lysates were incubated with 2–3 μg of primary antibody and 40 μl of protein A (Zymed) or Protein G (Roche Diagnostics, Indianapolis, USA) Agarose overnight at 4 °C. Beads were then washed three times in wash buffer containing 50 mM TrisHCl (pH 7.5), 100 mM NaCl, and 0.5% Triton X-100 before processing for IB as previously described [51]. For immunoprecipitation (IP) under denatured condition, 10% of the harvested cells were lysed with RIPA lysis buffer and kept for input. The other cells were lysed with buffer containing 1% SDS, 15 U/ml DNAse, 5 mM EDTA, 10 mM DTT, heated for 5 min at 95 °C, and then diluted with 9 volumes of RIPA lysis buffer. After incubation on ice for 5 min, the lysates were processed for IP followed by IB assay as previously described [51]. Antibodies used for IB are as follows: rabbit anti-DPF2 (1:5000, Lifespan Bioscience, Seattle, WA, USA), monoclonal rabbit anti-OCT4 (1:3000, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-DPF2 (1:500, Proteintech), rabbit anti-BIP (1:1000, Cell Signaling Technology, Danvers, MA, USA), mouse anti-VCP (1:5000), rabbit anti-GAPDH (1:5000), mouse anti-OCT4 (1:1000,