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PIAS Proteins as Repressors of Oct4 Function

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The POU domain transcription factor Oct4 plays essential functions in the maintenance of pluripotent embryonic and germ cells of mammals. Molecular mechanisms of Oct4 action remain poorly understood. To isolate modulators of Oct4 activity, we performed a yeast two-hybrid screen with the Oct4 POU domain as a bait and isolated PIASy as an Oct4-interacting protein. Oct4 and PIASy interact in vivo via their POU domain and SAP-domain-containing N terminus, respectively. PIASy does not enhance Oct4 sumoylation but acts as a potent inhibitor of Oct4-mediated transcriptional activation, sequestering Oct4 protein from the vicinity of Cajal bodies and splicing speckles to the nuclear periphery. These modes of PIASy action are uncoupled from its sumoylation activity. Other PIAS family members, PIAS1 and PIAS3, can also interact with Oct4 in vivo and target Oct4 to the nuclear periphery, depending on cellular context. We propose that Oct4 inhibition, mediated by this new class of transcriptional partners, might be instrumental during mammalian development.

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Introduction

Development of multicellular organisms is characterized by an intricate series of genetic and epigenetic events that generate the complex adult body from a single cell, the zygote. A refined and sophisticated regulatory network that is established during embryogenesis relies on a relatively small number of transcriptional regulators. The diversity in transcriptional control is achieved through a complex network of combinatorial protein–protein and protein–DNA interactions affecting the stability and subcellular and subnuclear localization of these transcriptional regulators. The primary structure of cis-regulatory DNA elements, superimposed by their epigenetic status, defines the composition and architecture of the transcriptional activation complexes that ultimately control gene expression in the appropriate temporospatial context of the developing organism.

The POU domain transcription factor Oct4 (also called Oct3, Oct3/4, and Pou5f1) cloned 17 years ago¹⁻³ has a unique place in the array of transcriptional regulators because it is an indispensable component of the core regulatory circuitry controlling the self-renewal and maintenance of the undifferentiated state of early pluripotent cells within the epiblast, the primordial germ cells (PGCs), as well as of the cultured counterparts of these embryonic cell types, most notably the embryonic stem (ES) cells.⁴⁻¹⁰ Significant effort was put into defining the regulatory network operated by Oct4, as well as dissecting the molecular mechanism of its action. Increasing evidence suggests that Oct4 does not activate transcription of target genes alone but requires DNA-dependent heterodimerization with another DNA-binding transcription factor, the HMG-box protein Sox2. These two transcription factors are found to cooperatively bind regulatory

Abbreviations used: SUMO, small ubiquitin-related modifier; PIAS, protein inhibitor of activated STAT; PGC, primordial germ cell; ES, embryonic stem; MT, Myc epitope; tk, thymidine kinase; TE, trophectoderm; ICM, inner cell mass.
regions of a number of target genes, including their own promoters. The recruitment of another class of interacting partners, either DNA-binding transcription regulators or viral coactivators, have also been described for Oct4. In addition, a DNA site-dependent differential recruitment of transcriptional cofactors by distinct types of dimers has been described for the POU domain factors Ptf1, Oct1, and Oct2, however, a biological role of Oct4 dimerization has not been well characterized. Finally, no negative coregulators of Oct4 function have been reported to date.

The family of PIAS proteins, initially described as protein inhibitors of activated STATs, comprise at least five genes and/or splice variants (PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy). These proteins share the conserved N-terminal SAP domain, which confers the interaction with both nuclear matrix-associated DNA sequences and the central RING-like domain that is required for E3 small ubiquitin-related modifier (SUMO) ligase activity. PIAS proteins indeed have been reported to promote sumoylation of a number of protein targets, affecting their stability, localization, and activity, and hence are involved in diverse cellular processes. However, the major function of PIAS family member proteins seems to modulate the activity of transcription factors, which is frequently uncoupled from their sumoylating function.

It is currently believed that PIAS proteins operate through relocalization of target proteins to different nuclear compartments. For instance, PIASy targets the Wnt/β-catenin pathway mediator LEF1 to PML nuclear bodies, resulting in the repression of LEF1 activity. Although PIASy can markedly stimulate sumoylation of LEF1, this modification is not required either for the repression or for the relocalization.

Another PIAS family member, PIAS1, has been shown to relocalize the homeobox transcription factor Msx1 to the nuclear periphery, allowing Msx1 to engage and repress MyoD and Myf5 gene promoters. Again, this event does not require the PIAS1-dependent sumoylation of Msx1.

In this paper we report a novel class of Oct4 partners belonging to the PIAS family of transcriptional repressors and SUMO modifying enzymes: PIASy, PIAS1, and PIAS3. These proteins were found to associate with Oct4 in vivo and alter its subnuclear localization in cell-type-specific fashion. As exemplified with PIASy, this relocalization of Oct4 correlates with a repression of Oct4-mediated transcription activation. We outline a developmental process in which PIAS-mediated repression might have a biologically significant readout.

Results

Isolation of PIASy as an Oct4 interacting partner

The transcription factor Oct4 is known to associate with various cellular proteins that modulate its trans-activating potential. To further gain insight into the mechanism of Oct4 action, we searched for additional Oct4 interacting proteins. To this end, we carried out a yeast two-hybrid screen with the POU domain of Oct4 (POU4) fused to the GAL4 DNA-binding domain as bait. We chose the POU domain of Oct4 for the screen because it mediates all reported to date interactions of POU-domain family members with other proteins. Two of the identified positive clones contained an open reading frame encoding the first 144 N-terminal amino acids (aa) of PIASy, including the so-called SAP domain, fused to an unrelated 19-aa sequence that had no matches in protein databases. The relative stringency of Oct4 interactions with the PIASy-related clones was estimated by liquid β-galactosidase assay and compared with the interactions between Oct4 and randomly picked yeast clones, as well as between Oct4 and recently reported Oct4 partner, Cdx2 (Fig. 1a). The results of the yeast two-hybrid assay imply that PIASy and Oct4 interact via their SAP domain-containing N terminus and POU domain, respectively.

PIASy and Oct4 interact in vivo

To confirm the Oct4/PIASy interaction in the context of the full-length proteins, Myc epitope (MT)-tagged Oct4 and T7 epitope-tagged PIASy...
were analyzed by coimmunoprecipitation assay in transfected 293T cells. Towards this end, we coimmunoprecipitated T7-PIASy with antibodies directed against MT (Fig. 1b). The coimmunoprecipitated PIASy was detected by immunoblot analysis with anti-T7 antibody. The interaction between full-length Oct4 and PIASy proteins was additionally confirmed by a reciprocal coimmunoprecipitation assay (see below).

Next we examined the interaction of endogenous Oct4 and PIASy. First, we found that PIASy is expressed in ES cells at a relatively high level compared to COS7 cells (Fig. 1c). Subsequently, we were able to coimmunoprecipitate the endogenous
Oct4 from ES cell lysate, using anti-PIASy antibody (Fig. 1d), suggesting that these two proteins interact in vivo in the context of physiological expression levels.

Because PIASy is a member of a highly conserved protein family that additionally comprises PIAS1, PIAS3, and PIASxa/β, we assessed whether Oct4 can interact with other family members as well. Plasmids expressing different PIAS proteins tagged with the FLAG epitope were transfected into 293T cells, alone or together with MT-Oct4. Coimmunoprecipitation with anti-MT antibody and anti-FLAG immunoblot analysis resulted in the detection of PIAS1 and PIAS3, but not of PIASxa and β (Fig. 1e).

PIASy does not act as SUMO ligase for Oct4

As PIASy was originally described as an E3 SUMO ligase, we examined whether it can sumoylate Oct4. First, we performed a coimmunoprecipitation with T7 antibody from 293T cells transfected with T7-PIASy or its sumoylation-deficient mutant (RING mut), MT-Oct4, and FLAG-SUMO1/2. In the presence of both wild-type and RING mut PIASy, no obvious high molecular weight bands emerged that would correspond to SUMO-modified Oct4 protein (Fig. 2a). Inspection of the amino acid sequence of Oct4 identified three potential SUMO acceptor lysines (K118, K215, and K244) that conform to the consensus ΨKXE. However, we found that the mutation of these lysines to arginines in Oct4 affected neither its covalent modifications nor interaction with PIASy (Fig. 2b). Therefore, we conclude that first, PIASy is unlikely to serve as E3 SUMO ligase for Oct4 and second, that PIASy-Oct4 interaction does not depend on SUMO modification of Oct4.

To enhance the visualization of possible SUMO-modified Oct4 species, we performed coimmunoprecipitations with MT antibody from 293T cells cotransfected with MT-Oct4, Ubc9, FLAG-SUMO, and T7-PIASy. Using FLAG antibody, we detected SUMO-modified Oct4 species on immunoblot (Fig. 2c). In the presence of Ubc9 and the absence of PIASy, several SUMO-coupled Oct4 species were detected as a ladder above 100 kDa, characteristic of an oligomerized SUMO modification of Oct4, Oct4-(SUMO)ₙ (Fig. 2c). Note that the Oct4-(SUMO)ₙ bands were not formed in the absence of Ubc9 (Fig. 2c, lane 1). The K118R Oct4 mutants showed quite reduced Oct4-(SUMO)ₙ species, suggesting that this lysine, which is located in the N terminus just outside the POU domain of Oct4, serves as the amino acid to which SUMO molecules are covalently linked. It is surprising that the addition of PIASy markedly reduced the formation Oct4-(SUMO)ₙ bands (Fig. 2c, lane 3). Taken together, these data imply that Oct4 can be SUMO-modified at K118 by the E2 conjugating Ubc9 and that this modification is inhibited by PIASy. Possible molecular mechanisms of this inhibition will be discussed below.

PIASy inhibits Oct4-mediated transcriptional activation in 293 cells

Oct4 trans-activating potential is modulated through homo- and heterodimerization with other POU family members, as well as through association with transcriptional coactivators and DNA-binding transcription factors of diverse families. Therefore, we set out to examine whether interaction with PIASy in vivo affects the Oct4-mediated transcriptional readout. To this end, we performed transient transfection of 293 cells with luciferase reporter constructs containing known Oct4-binding DNA motifs that were hexamerized and cloned upstream of the minimal thymidine kinase (tk) promoter. The MORE and PORED motifs bind Oct4 dimer in two distinct conformations,18–20 the OCT motif represents the classical octamer site, and the Fgf4 motif is a bipartite DNA element that binds and thereby mediates trans-activation of the Fgf4 gene by an Oct4/Sox2 heterodimer in pluripotent cells.11,12 In line with previous reports, Oct4 shows transcriptional activation either alone (on 6×MORE-, 6×PORE-, and 6×OCT-luc reporters) or in conjunction with Sox2 protein (on 6×Fgf4-luc reporter). On each reporter construct tested, the Oct4- or Oct4/Sox2-mediated activation was significantly attenuated in the presence of either PIASy or PIASy RING mut (Fig. 3a). These data suggest that PIASy acts as a capable transcriptional repressor of Oct4 and that this activity is not coupled to its E3 SUMO ligase activity. Consistent with the latter conclusion, the sumoylation-deficient Oct4 mutant K118R was similar to its wild-type counterpart in terms of both transcriptional activation and response to PIASy (data not shown). Taken together, these observations suggest that SUMO modification is dispensable not only for PIASy-mediated repression but also for the trans-activation capacity of Oct4 itself.

Fig. 2. PIASy does not enhance SUMO modification of Oct4. (a) Coimmunoprecipitation of MT-Oct4 from lysates of 293T cells transfected as indicated above the panels, using anti-T7 antibodies (upper panel). SUMO1- or SUMO2-encoding plasmids were included in lanes 4 and 5 to facilitate corresponding modification in target proteins. No pronounced anti-MT antibody reactive bands, corresponding to SUMO-modified Oct4 species, could be detected either in anti-T7 precipitated (upper panel) or in input lysates (middle panel). Detectable amounts of MT-Oct4 were coprecipitated in the absence of T7-PIASy (asterisk, lane 2), possibly due to a nonspecific binding to the beads; however, these amounts were notably higher when T7-PIASy was present (lanes 3–6). In addition, PIASy mutant lacking a SUMO ligase activity (RING mut) could also coimmunoprecipitate MT-Oct4 (lane 6). (b) Oct4 proteins mutated from lysine to arginine in the potential sumoylation sites coimmunoprecipitate with T7-PIASy protein, revealed by anti-MT immunoblot. Again, some amounts of MT-Oct4 could coimmunoprecipitate in the absence of T7-PIASy (asterisk, lane 6). (c) FLAG-SUMO-modified Oct4 species, designated as MT-Oct4-(SUMO)ₙ, were first enriched from lysates of 293T cells using anti-MT antibody-conjugated beads from 293T cells transfected as indicated, then detected on immunoblot with anti-FLAG antibody.
As tested using the 6×MORE-luc reporter, the ability to repress Oct4-mediated transcriptional activation in 293 cells is restricted to PIASy, as the other known members of the PIAS family, including the established Oct4-interacting PIAS1 and PIAS3 (see above), are neutral towards Oct4 (Fig. 3b).

**Fig. 2** (legend on previous page)
These data imply that the ability of a PIAS protein to bind Oct4 in vivo is not sufficient to suppress Oct4-dependent activation.

PIAS proteins target Oct4 to the nuclear periphery in a cell-type-specific fashion

We next assessed whether and how Oct4 subnuclear localization is affected in the presence of PIAS proteins. Oct4 protein has been previously reported to associate, in human oocytes, with splicing speckles and Cajal bodies. Accordingly, HeLa cells transfected with MT-Oct4-expressing plasmid showed Oct4 localization within or on the periphery of these nuclear organelles, revealed by indirect colabeling with anti-Myc tag, SC35, and coilin antibodies (data not shown). A technical prerequisite for achieving this fine subnuclear immunostaining was the use of TPS buffer-based fixation protocol (see Materials and Methods).

A marked relocalization of Oct4 from the Cajal bodies and splicing speckles to the nuclear periphery was observed in the presence of PIASy in HeLa...
In addition, we observed that Oct4 subnuclear localization was uncoupled from its SUMO modification. First, the sumoylation-deficient K118R Oct4 mutant still localized adjacent to splicing speckles and Cajal bodies (data not shown) and was targeted to nuclear periphery by PIASy (Fig. 4d). Second, PIASy RING mutant was still capable of sequestering Oct4 in the nuclear periphery (data not shown).

We next assessed the ability of all known PIAS family members to affect subnuclear distribution of Oct4 protein in different cellular contexts. First, introduced Oct4 was again found to be associated with nuclear organelles, presumably the splicing speckles and Cajal bodies, across all tested cell types (Fig. 5a, g, and m). In the embryonic kidney 293 cells, PIASy and PIAS3 showed a significant degree of colocalization with Oct4 (Fig. 5b and d), whereas PIASxα and PIASxβ did not (Fig. 5e and f), consistent with the distinct abilities of these PIASes to bind Oct4 in vivo (Fig. 1e). It is somewhat surprising that PIAS1 showed very limited degree of colocalization with Oct4 in 293 cells (Fig. 5c), even though this PIAS, along with PIASy and PIAS3, has been shown to bind Oct4 in 293T cells (Fig. 1e). More important, only PIASy was able to target Oct4 to the nuclear periphery in 293 cells (Fig. 5b), while the other PIAS family members failed to do so (Fig. 5c–f). This directly correlates with the selective ability of PIASy to suppress Oct4-mediated transcriptional activation in these cells (Fig. 3b).

In addition to PIASy, PIAS1 and PIAS3 also showed the ability to sequester Oct4 in the nuclear periphery in C2C12 myoblast cells (Fig. 5 h–j). Despite being able to do so, PIAS1 itself remained associated with some nuclear organelles, mostly outside the nuclear periphery (Fig. 5i). The unusual behavior of this PIAS family member will be discussed below.

In HeLa cells, PIASy, PIAS1, and PIAS3 readily colocalized with Oct4; however, as in 293 cells, only PIASy was capable of sequestering Oct4 in the

Fig. 4. PIASy alters subnuclear localization of Oct4 in HeLa cells. Oct4 protein (green), detected with anti-MT antibody, is located on the periphery of splicing speckles (a) and Cajal bodies (b). The two nuclear organelles were revealed with anti-SC35 and anti-coilin antibodies (red), respectively. Cotransfected PIASy (red), detected with anti-T7 antibody, targets both wild-type Oct4 (c) and SUMOylation-deficient Oct4-K118A mutant (d) to the nuclear periphery, as revealed using confocal microscopy.

PIAS Proteins Inhibit Oct4

MT-Oct4

αMT

Hoechst

merge

αSC35

αCoilin

αT7

MT-Oct4 + T7-PIASy

MT-Oct4 K118R + T7-PIASy

αT7
Fig. 5. Differential Oct4-targeting activities of PIAS family members in various cellular contexts. The 293, C2C12, and HeLa cells (denoted above the panels) were transfected with plasmids indicated on the left and stained for Oct4 (green) and PIAS proteins (red): PIASy with anti-T7, and PIAS1, PIAS3, and PIASxα/β with anti-FLAG antibodies. Note that PIASy can sequester Oct4 in the nuclear periphery across all three cell types (b, h, n), whereas PIAS1 and PIAS3 can do this only in C2C12 cells (i, j, compare to c, d and o, p). PIASxα and β exhibit neither colocalization nor the alteration of Oct4 subnuclear localization (e, f, k, l, q, r). The images were collected using a confocal microscope.
nuclear periphery (Fig. 5n–p). As in two other cell types, PIAS\textsubscript{x\textalpha} and \textbeta, neither colocalized nor altered Oct4 subnuclear distribution (Fig. 5e and f, k and l, q and r).

In sum, these data demonstrate that the members of the PIAS family exhibit differential activities towards Oct4 protein: PIAS\textsubscript{x\textalpha} and PIAS\textsubscript{x\textbeta} are always neutral towards Oct4, consistent with their inability to bind Oct4 \textit{in vivo}, whereas PIAS\textsubscript{y} is capable of dramatically relocating Oct4 to the nuclear periphery across all three tested cell types. However, PIAS\textsubscript{1} and PIAS\textsubscript{3} can also exhibit such an activity in C2C12 cell context, indicative of redundant functions of PIAS\textsubscript{y}, PIAS\textsubscript{1}, and PIAS\textsubscript{3} towards

**Fig. 6.** Reciprocal expression of PIAS\textsubscript{y} and Oct4 proteins in mouse blastocyst. (a) Two early mouse blastocysts and one late morula embryo in the upper right corner (all at 3.5 dpc) stained for PIAS\textsubscript{y} (green), Oct4 (red), and Cdx2 (yellow) proteins and analyzed by confocal microscopy. Green and red channels were merged in the third column (merge r+g). Note that PIAS\textsubscript{y} is frequently expressed at higher levels in the nuclei of the trophectoderm (TE) and TE precursors (TEp), both marked by Cdx2 expression, whereas the expression is slightly diminished in the inner cell mass of the blastocysts and its precursors of the late morula (ICMp). (b) Optical section though the TE of a 3.5-dpc blastocyst (Cdx2\textsuperscript{+}), showing a colocalization PIAS\textsubscript{y} and Oct4 proteins at a higher magnification. (c) Expanded blastocysts at 4.5 dpc stained as above except that primary anti-PIAS\textsubscript{y} antibody was omitted.
modulating Oct4 activity during mammalian development in certain cell types.

PIASy and Oct4 in preimplantation mouse development

We next looked at preimplantation development, when PIASy-mediated restriction of Oct4 function could be of biological significance. Oct4 expression during this developmental period has been well studied: zygotic Oct4 expression starts at 4 to 8-cell stage and continues in all cells throughout morula [2.5 days postcoitum (dpc)] and early blastocyst stage (3.5 dpc). By expanded blastocyst stage (4.5 dpc), Oct4 expression becomes restricted to the epiblast and primitive endoderm, being excluded from the trophectoderm (TE). However, in contrast to the above HeLa cell results, no obvious sequestration of Oct4 in the nuclear periphery was observed (Fig. 6a). We further took a closer look at subnuclear distribution of PIASy and Oct4 proteins at 3.5 dpc, that is, during the time window where their expression domains overlap in the TE. The two proteins seemed to colocalize in the nuclei of TE cells. However, in contrast to the above HeLa cell results, no obvious sequestration of Oct4 in the nuclear periphery was seen in the nuclei of TE cells (Fig. 6b). A reason for that could be different fixation/permeabilization protocols used for cultured cells and embryos. The TPS buffer-based method (see Materials and Methods), which allowed one to resolve fine subnuclear structures in HeLa cells, did not produce a satisfactory signal in preimplantation embryos, for both PIASy and Oct4 proteins. Thus, it remains to be defined whether PIASy-mediated Oct4 targeting to the nuclear periphery depends in vivo.

By the expanded blastocyst stage (4.5 dpc), PIASy seems to be expressed predominantly in the TE (Fig. 6c). Therefore, during the transition from early to late blastocyst stage, Oct4 and PIASy expression domains become segregated: Oct4 protein is restricted to the epiblast and primitive endoderm and is excluded from the TE, whereas PIASy is restricted to the TE. The reciprocal pattern of expression of the two proteins is consistent with the idea that PIASy may play a restrictive function towards Oct4 activity during preimplantation development.

Discussion

Oct4 is an important transcriptional regulator required for the maintenance of early pluripotent cell types of mammalian embryo, PGCs, and ES cells.1-16 Several transcriptional modulators of Oct4 function have been described in the past.10-17 However, no negative transcriptional coregulators have been reported to date. Here we show that some members of the PIAS family, most notably PIASy, represent a novel class of transcriptional modulators of Oct4 function that act to suppress Oct4-mediated transcriptional activation through a sequestration of the protein in the nuclear periphery. In line with previous reports,24,25 this repressive PIASy function is uncoupled from its E3 SUMO ligase activity.

Genetic ablation of Oct4 function leads to preimplantation lethality due to a differentiation of the ICM cells into trophectoderm.7 According to the proposed model, which suggests that PIASy inhibits Oct4 function in the TE, a loss of PIASy should lead to an increased Oct4 activity in the TE of blastocyst. However, PIASy-deficient embryos and mice do not manifest any obvious developmental defects,29,30 suggesting that PIASy-mediated inhibition of Oct4 is either not detrimental for the TE or is obscured by a redundant function of coexpressed PIAS family members. In support of the latter possibility, our data suggest that first, PIAS3 is coexpressed with PIASy in the TE and is mostly absent from the epiblast and primitive endoderm (Supplementary Fig. 1) and second, PIASy, PIAS1, and PIAS3 are redundant in targeting Oct4 to the nuclear periphery in C2C12 cells (Fig. 5).

Contrary to PIASy, PIAS1- and PIAS3-mediated targeting of Oct4 to the nuclear periphery depends on the cellular context. The cell-type-specific engagement of Oct4 by these PIAS family members might be of importance, considering that Oct4 is expressed and, in some cases, was shown to play essential functions in different cell types, such as the epiblast, PGCs, and spermatogonial stem cells.7,9,31,32

There is a notable difference in the mode of PIAS1 action towards Oct4 in C2C12 cells compared to PIASy and PIAS3. Despite being able to promote Oct4 relocalization to the nuclear periphery, PIAS1 itself is not enriched in this nuclear compartment (Fig. 5). This observation is especially surprising considering that PIAS1 is retained together with its interacting partner, Msx1, in the nuclear periphery of the same type of cells.25 On the other hand, PIAS1 and Oct4 show a substantial degree of colocalization in C2C12 cells examined shortly after transfection (see Supplementary Fig. 2), suggesting a transient association between the two proteins. Molecular aspects and functional significance of such an intriguing behavior of this PIAS family member need further investigation.

Although dispensable for PIASy-mediated Oct4 repression, the sumoylation of Oct4 might have an important readout in other processes, for example, in the regulation of Oct4 stability. However, our data suggest that PIASy does not serve as an E3 SUMO ligase for Oct4. Moreover, it appears that PIASy inhibits K118 Oct4 sumoylation promoted by the E2 SUMO conjugating enzyme Ubc9 (Fig. 2c). To our
knowledge, this is the first example of an inhibitory function of PIASy in sumoylation, although it cannot be excluded that this effect is due to the overexpression of PIASy. On the other hand, Ubc9 has been shown to sumoylate some protein targets without the help of E3 SUMO ligases. \(^{33-35}\) Ubc9, likewise PIASy, was also isolated in our two-hybrid screen as a protein interacting with the POU domain of Oct4 (data not shown). Therefore, it is possible that the mechanism by which PIASy inhibits Ubc9-dependent sumoylation involves a displacement of Ubc9 from the complex with the POU domain. The participation of two other E3 SUMO ligases, RanBP2 and P2c, \(^{36-37}\) in Oct4 sumoylation cannot be ruled out as well.

Dissecting regulatory mechanisms underlying Oct4 function is of a high priority because this knowledge will eventually help to outline basic principles of pluripotency and to manipulate this cellular state. Members of the PIAS family might represent important molecular components of regulatory network controlling pluripotent cell fate via the modulation of Oct4 function.

### Materials and Methods

#### Plasmids

To construct the pCS2-MT-Oct4 and pCS2-Ubc9 plasmid, an Ncol–EcoRI 1.3-kb fragment of Oct4 cDNA and PCR-amplified Ubc9 were cloned in pCS2-MT and pCS2 vector, respectively (D. Turner, R. Rupp, and H. Weintraub). The K118R, K215R, K244R, and K118/K244R Oct4 mutant-encoding plasmids were derived from the pCS2-MT-Oct4 by PCR-based site-directed mutagenesis. The pBD-GAL4-POU4 plasmid was made by inserting PCR-amplified POU domain-encoding part of Oct4 (127-282 aa) at EcoRI–Xmal of pBD-GAL4/C Am (Stratagene). The 0.95-kb Cdx2 cDNA was excised from pCX-GR–cdx2ires-Hyg plasmid \(^{38}\) with Ncol–XhoI and cloned into pACT2 (Clontech), resulting in pACT2-Cdx2 control plasmid. For 6×G4-luc, the oligonucleotide 5′-AACTTGGCAAT-3′ and Sox2-binding motifs (CTTTGTT) from A. Hecht). The 6×OCT-luc, 6×MORE-luc, 6×PORED-luc, 6×Fgf4-luc, the oligonucleotide 5′-ATTCTGGCAAT-3′ and Sox2-binding motifs (CTTTGTT) from A. Hecht).

#### Yeast two-hybrid screening

Mouse 11-day embryo MATCHMAKER cDNA Library (Clontech) cloned in pACT2 vector and pretransformed into yeast strain Y187 was screened by mating it to the yeast strain AH109 carrying the pBD-GAL4–POU4 bait vector. In a control assay, the Y187 strain carried the pACT2-Cdx2 plasmid in place of the library. Colony-lift filter and liquid culture assay was carried out according to the manufacturer’s guidelines (Clontech).

### Coimmunoprecipitation

293T cells transfected by calcium phosphate method or non-transfected R1 ES and COS7 cells were washed in phosphate-buffered saline (PBS), then lysed by sonication in icecold CoIP buffer (50 mM Tris–HCl at pH 7.5, 100 mM NaCl, 15 mM EGTA, 0.1% Triton X-100, a protease inhibitor mix, 1 mM NaF, and 0.4 mM sodium orthovanadate). The lysates were cleared of cell debris by centrifugation at 16,000 g for 15 min at 4 °C. Equivalent amounts of total protein were preincubated by incubation with a 1:1 mix of protein A- and protein G-conjugated beads (Sigma) and immunoprecipitated overnight at 4 °C with either 1 μg of mouse anti-T7 (Novagen) or rat anti-PIASy supernatant \(^{24}\) (1:10), followed by 2-h incubation at 4 °C with protein A+G beads (25 μl) or with rabbit anti-Myc antibody conjugated with agarose (25 μl, Sigma). Beads were washed several times with CoIP buffer, boiled in 1× SDS loading buffer, and the supernatants were loaded onto SDS-PAGE. Western blots were carried out by the ECL detection method (Amersham-Pharacmia), using peroxidase-conjugated rabbit anti-Myc or mouse anti-FLAG tag antibodies (1:5,000, Sigma), or using mouse anti-T7 (1:10,000, Novagen), mouse anti-Oct4 (1:500, Santa Cruz), followed by peroxidase-conjugated goat anti-mouse F(ab)\(^2\) (1:10,000, Jackson ImmunoResearch).

### Transient transfections

293T cells were transfected in 24-well tissue culture plates using SuperFect reagent (Qiagen). The total amount of DNA per well was equalized with a carrier plasmid. After 24–36 h, cells were washed in PBS and lysed directly in wells in Glo-Lysis buffer (Promega). Approximately one-tenth of the crude extract was used to measure the luciferase and β-galactosidase activities (Bright-Glo and β-gal assays, Promega).

### Immunostaining

HeLa, C2C12, and 293 cells were plated on LabTek II chambered coverslips (Nunc) and transfected the next day with the SuperFect (Qiagen) or Lipofectamine 2000 (Invitrogen). After 24 h, the cells were fixed with 1% paraformaldehyde in the TPS buffer (10 mM Tris–HCl, pH 8.0, 13.5 mM KCl, 20 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100) at room temperature for 10 min. Preimplantation mouse embryos were flushed from superovulated females, fixed in 2% paraformaldehyde in PBS for 10 min, and permeabilized with 0.25% Triton X-100 for 8 min at room temperature. The cells and embryos were subsequently blocked in 2% bovine serum albumin, 1% heat-inactivated normal sheep serum in TPS or PBS for 30 min, and incubated for 2–5 h at room temperature in the same blocking solution plus 0.1% Tween 20 with the following primary antibodies: rabbit anti-Myc tag (1:500, Sigma), mouse anti-T7 tag (1:1000, Novagen), mouse anti-FLAG-M2 (1:250, Sigma), rat anti-PIASy supernatant (1:2; Ref. 24), mouse anti-Oct4 (1:50, Santa Cruz), rabbit anti-coilin (1:400; see Ref. 28), mouse anti-SC35 (1:10; see Ref. 28), rabbit anti-Cdx2 (1:100, gift of Felix Beck), and rabbit anti-PIAS3 (1:25, Santa Cruz). Secondary antibodies used mouse anti-T7 tag (1:1000, Novagen), mouse anti-FLAG tag antibodies, mouse anti-SC35 (1:100, Santa Cruz), and goat anti-mouse F(ab)\(^2\) fragment (1:10,000, Jackson ImmunoResearch).

### Yeast two-hybrid screening

Mouse 11-day embryo MATCHMAKER cDNA Library (Clontech) cloned in pACT2 vector and pretransformed into yeast strain Y187 was screened by mating it to the yeast strain AH109 carrying the pBD-GAL4–POU4 bait vector. In a control assay, the Y187 strain carried the pACT2-Cdx2 plasmid in place of the library. Colony-lift filter and liquid culture assay was carried out according to the manufacturer’s guidelines (Clontech).
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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2007.09.081

References


