Oct4 redox sensitivity potentiates iPSC reprogramming

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Abstract

The transcription factor Oct4/Pou5f1 is a key component of the regulatory circuitry governing pluripotency. Oct4 is also widely used to generate induced pluripotent stem cells (iPSCs) from somatic cells. These observations provide compelling rationale to understand Oct4’s functions. Here we used domain swapping and mutagenesis to compare Oct4’s reprogramming activity with the paralog Oct1/Pou2f1, identifying a DNA binding domain cysteine residue (Cys48) as a key determinant of both reprogramming and differentiation. In combination with the Oct4 N-terminus, Oct1^S48C is sufficient to confer strong reprogramming activity. Conversely, Oct4^C48S strongly reduces reprogramming potential. We find that Oct4^C48S confers DNA binding sensitivity to oxidative stress. Further, C48S sensitizes the protein to oxidative stress-mediated ubiquitylation and degradation. Engineering a Pou5f1^C48S point mutation in mouse embryonic stem cells (ESCs) has little effect on undifferentiated cells, but upon retinoic acid (RA)-mediated differentiation causes retention of Oct4 expression, decreased proliferation and increased apoptosis. Pou5f1^C48S ESCs also contribute poorly to adult somatic tissues. Collectively, the data support a model in which Oct4 redox sensing serves as a positive reprogramming determinant during one or more steps promoted by Oct4 downregulation during iPSC generation.

Keywords Oct4 (Pou5f1); Oct1 (Pou2f1); induced pluripotent stem cells (iPSCs); oxidative stress; ubiquitylation
**Introduction**

The early mammalian embryo contains undifferentiated, pluripotent cells capable of generating all embryonic tissue lineages. Cultured embryonic stem cells (ESCs) are derived from these cells and have similar capabilities (Abranches et al., 2009). The transcription factor Oct4/Pou5f1 governs the establishment of pluripotent, undifferentiated inner cell mass and epiblast cells, as well as primordial germ cells, germline progenitor cells and ESCs (Morey et al., 2015). Both ESC differentiation in culture and blastocyst implantation in animals are followed by Oct4 loss (Nichols et al., 1998). Together with transcription factors such as Nanog and Sox2, Oct4 forms a network of pluripotency master transcriptional regulators that bind their own and each other’s genes to reinforce expression (Boyer et al., 2005).

Oct4 is also widely used to generate induced pluripotent stem cells (iPSCs) from somatic cells (Li et al., 2011; Takahashi and Yamanaka, 2006). Reprogramming methods that omit Oct4 nevertheless rely on activation of the endogenous Pou5f1 (Oct4) gene (Gao et al., 2013; Heng et al., 2010). These findings pinpoint Oct4 as a central reprogramming component and place priority on understanding its functions.

Reprogramming strategies that increase efficiency (Costa et al., 2013; Esteban et al., 2010; Rais et al., 2013; Soufi et al., 2012; Vierbuchen and Wernig, 2012), or improve the quality of iPSC clones (Chen et al., 2015; Velychko et al., 2019; Yuan et al., 2011) reveal difficult trade-offs: efficiently generating iPSC ethat are of sub-optimal quality, or inefficiently generating iPSCs with improved developmental potential (Buganim et al., 2014; Chen et al., 2015; Velychko et al., 2019).

Pluripotent cells co-express Oct4 together with the widely expressed prototypic POU transcription factor Oct1/Pou2f1 (Okamoto et al., 1990; Perovanovic et al., 2023; Rosner et al., 1990; Shen et al., 2017; Suzuki et al., 1990). The two proteins have similar DNA binding domains and recognize the same 8-bp 5’ATGCAAAT3’ consensus octamer sequence as well as sequence variants (Tantin, 2013). The two proteins also associate with many of the same target genes (Ferraris et al., 2011; Perovanovic et al., 2023).

Nevertheless, it is established that Oct1 as well as other mammalian Oct4 paralogs do not function in reprogramming (Feng et al., 2009; Takahashi and Yamanaka, 2006). In contrast, Sox2 and Klf4 can be
replaced by their close paralogs while c-Myc can be omitted (Nakagawa et al., 2008; Shi et al., 2008). Structure/function studies comparing Oct4 with its paralogs have identified Oct4 transcriptional determinants (Esch et al., 2013; Jerabek et al., 2017; Jin et al., 2016). An example is three residues present in the Oct4 DNA binding domain but absent in Oct6/Pou3f1 needed to form Sox2 heterodimers. Introducing these residues, plus the Oct4 N- and C-termini, results in efficient reprogramming (Jerabek et al., 2017). However, several Oct4 paralogs including Oct1 contain these residues but cannot reprogram, indicating the presence of additional reprogramming determinants present in Oct4 (and likely Oct6) but absent in Oct1.

Recent studies show that removal of Oct4 from reprogramming cocktails is highly inefficient, requires higher-than-typical levels of the other factors, and only works with target cells of high mitotic potential, but results in qualitatively superior iPSC clones (An et al., 2019; Velychko et al., 2019). The counterproductive activities of Oct4 are likely caused by transient promotion of counterproductive off-target gene expression (Velychko et al., 2019). The rare iPSCs derived in the absence of Oct4 have enhanced developmental potential. For example, iPSCs reprogrammed without Oct4 are better able to generate viable mice in a single generation using tetraploid complementation. A logical extension of these findings is that transient down-regulation of counterproductive Oct4 activities can aid reprogramming.

Here, we identify a new, central Oct4 reprogramming determinant – a single DNA binding domain cysteine residue (Cys48), that accounts for much of its differential reprogramming potential vis-à-vis Oct1. A serine residue is present in Oct1 at this position. In the presence of the Oct4 N-terminus, changing the Oct1 serine to cysteine at this position is sufficient to confer levels of reprogramming activity comparable to wild-type Oct4. Conversely, mutating this cysteine to serine in the context of Oct4 reduces reprogramming efficiency by over 50%. Mechanistically, this cysteine confers DNA binding hypersensitivity to oxidative stress, and induces Oct4 protein ubiquitylation via a redox regulation mechanism. We then generated CRISPR-edited Oct4C48S embryonic stem cell (ESC) lines, which are phenotypically normal until differentiated. Upon differentiation, multiple edited ESCs abnormally retain
Oct4 expression, proliferate less and undergo programmed cell death at higher rates compared to their unedited parental controls. When injected into preimplantation blastocysts, these cells contribute poorly to the development of viable adult mice. Cumulatively, our findings identify a redox-sensitive Oct4 residue that restrains protein function, but nevertheless functions positively in reprogramming.
Results

Oct4 vs. Oct1 differential reprogramming potential localizes to the DNA binding domain

The POU-specific and POU-homeodomain (POUS and POUH) DNA binding subdomains of Oct1 and Oct4 are similar, with 71% and 59% identity in mouse, respectively (Fig.1A). In contrast, the N- and C-termini, and the linker between the subdomains, are highly divergent (Fig.1B). The human proteins are structured similarly. Because the DNA binding domains are similar, we can standardize the amino acid numbering by referring to the first amino acid of the POUS DNA binding domain as residue 1 (Fig.1B).

To identify Oct4 determinants important for reprogramming, we generated Oct4/Oct1 chimeric constructs containing the distinct domains. Because introducing different domains and epitopes can confound the analyses with protein-specific antibodies, we included C-terminal FLAG-tags on Oct4, Oct1 and all chimeric proteins to track protein expression. Unlike an N-terminal FLAG tag (Supplemental Fig.1), engineering a C-terminal tag results in reprogramming efficiency similar to untagged Oct4 (Fig.1C). All subsequent constructs therefore bore C-terminal tags. In addition, prior work comparing Oct4 and another reprogramming-incompetent POU transcription factor, Oct6, identified a lysine residue within the POUS DNA binding subdomain (K40, of the Oct4 DNA binding domain, N320 in full-length Oct1) as being important for reprogramming (Jin et al., 2016). We therefore mutated N40 of the Oct1 DNA binding domain to lysine in some constructs (Fig.1D-F).

To measure reprogramming efficiency, we transduce primary mouse embryonic fibroblasts (MEFs) expressing GFP under the control of the endogenous Pou5f1 gene (Lengner et al., 2007; Shakya et al., 2015; Shen et al., 2018) with mSTEMCCA lentiviruses, which express all four Yamanaka factors from an EF-1A promoter (Sommer et al., 2009) (Fig.1B). Substituting Oct1 for Oct4 eliminated all reprogramming potential (Fig.1D,E). The other reprogramming factors – Sox2, Klf4 and c-Myc – can mediate reprogramming without Oct4 (An et al., 2019; Velychko et al., 2019), however reprogramming is inefficient. The results are therefore consistent with the original reprogramming findings showing that
Oct4 is essential for conventional reprogramming (Takahashi and Yamanaka, 2006), and set a useful baseline for making domain-swaps and mutations to assess reprogramming potency.

We then replaced the Oct1 N- and C-termini with that of Oct4, and mutated asparagine 40 of the Oct1 DNA binding domain to the lysine present in Oct4. Prior work (Jin et al., 2016) showed that the Oct4 lysine at this position (Fig.1A) is important for Oct4 reprogramming. Of these constructs, only fusion of the Oct4 N-terminus generated detectable colony-forming potential, at ~5% the level of WT Oct4 (Fig.1D,E). These findings are consistent with work establishing the importance of the Oct4 N-terminus (Boija et al., 2018). Similar data were obtained at multiple timepoints (not shown), indicating that this result does not simply reflect kinetic differences in reprogramming. Anti-FLAG immunoblotting showed equivalent expression (Fig.1F). Viral titers and expression of Klf4 from the mSTEMCCA vector were also similar (not shown). Because a construct with both the Oct4 N- and C-termini displayed only ~5% efficiency (Fig.1D,E), we conclude that determinants within the Oct4 DNA-binding domain confer the bulk of reprogramming potential.

**Oct4 vs. Oct1 differential reprogramming potential concentrates in a DNA binding domain cysteine**

The POU DNA binding domain is comprised of two subdomains, connected by a peptide linker, that make separate sequence-specific contacts with DNA (Tantin, 2013). These domains are termed POU$_S$ and POU$_H$ (Fig.1A,B). We systematically mutagenized DNA binding domain amino acid residues that differ between Oct1 and Oct4. We focused on possible Oct4 post-translationally modifiable residues: Lys, Ser, Thr and Cys. This analysis identified 14 possible amino acid substitutions, not including the aforementioned position lysine 40 (Fig.1A and not shown). Work from others has shown that Oct6 is capable of reprogramming when modified to enable Sox2 dimerization (Kim et al., 2020). Filtering the data using conservation between Oct4 and Oct6 resulted in just six residues. We replaced the Oct1 residues at these positions with the corresponding Oct4 residue in the context of the “4N” construct, which contains the Oct4 N-terminus. We reasoned that because this construct already has low but measurable activity, it
would be primed to show differences with single amino acid substitutions. Using this method, we found that a single serine to cysteine substitution at position 48 in POU5 (Fig.1A, red box) conferred the majority of the differential Oct4 activity, increasing activity >7-fold over the construct containing the Oct4 N-terminus alone (Fig.2A,B). We term this the “1-4All” construct. iPSC colonies formed under these conditions were robust morphologically and expressed GFP from the Pou5f1 locus at levels comparable to cells reprogrammed with Oct4 (Supplemental Fig.2). The remaining activity relative to Oct1 appeared to be concentrated in the linker domain, as a construct containing the Oct4 N-terminus, Cys48 and the Oct4 linker (4N-S48C-4L) had 100% activity. The Oct4 linker has been shown to be involved in reprogramming via selective interactions with different cofactors (Esch et al., 2013; Han et al., 2022). The differences were maintained over time, with the 1-4All construct consistently generating ~60% activity over multiple days, indicating that the differences were qualitative and not simply a function of different colony formation rates (Supplemental Fig.3).

Replacing the Oct4 Cys with Ser at this position reduced activity by 60% (Fig.2A,B), indicating that more than half of Oct4’s differential reprogramming activity vis-à-vis Oct1 is lost by changing this residue. The converse experiment, replacing Oct1 S48 with cysteine, resulted in no reprogramming activity (Fig.2A,B, Oct1-S48C), indicating that Cys48 is important but not sufficient, and highlighting its combined importance with the Oct4 N-terminus. The chimeric proteins were equivalently expressed (Fig.2C). These results indicate that in the context of the Oct4 N-terminus, a cysteine residue at position 48 confers robust reprogramming activity. In the co-crystal structure of the Oct1 DNA binding domain bound to DNA (Klemm et al., 1994), Ser48 hydrogen bonds with the DNA backbone (Fig.2D). In a recent Oct4:Sox2:nucleosome structure (Michael et al., 2020), the Cys48 sulfhydryl also allows for hydrogen bonding. Predictably, mutation of Cys48 to glycine greatly diminishes Oct4 DNA binding in vitro (Marsboom et al., 2016). Oct6, which can reprogram when changes are made to POUH allowing for Sox2 dimerization (Jerabek et al., 2017), contains a cysteine at this position (Fig.2E). These structural studies
along with our findings suggest that Cys48 has minimal effects on DNA binding vis-à-vis a serine (but not glycine) at this position, but nevertheless plays a role in reprogramming.

**Oct4 Cys48 is redox-sensitive and mediates degradation in response to oxidative stress**

Oct4 is known to be sensitive to oxidative stress (Guo et al., 2004; Lickteig et al., 1996; Marsboom et al., 2016) in a manner reversed by thioredoxin (Guo et al., 2004), implicating cysteine thiols in redox sensitivity. We reproduced this result (Supplemental Fig.4), which with the results above suggested that Cys48 may potentiate reprogramming through a mechanism involving redox sensitivity. We measured wild-type and cysteine 48 mutant Oct4 DNA binding using electrophoretic mobility shift assays (EMSA) with Cy5-labeled canonical octamer element containing DNA (Ferraris et al., 2011). To measure redox sensitivity, we used methods similar to Guo et al. (Guo et al., 2004) in which Oct4 is pre-treated with the oxidizing agent diamide prior to addition of the DNA probe.

We studied Oct4 Cys48 oxidation using a system with lysates from 293T cells transfected with WT or C48S mutant Oct4. 293T cells do not express Oct4, but do express endogenous Oct1 as an internal control (Fig.3A, lanes 2-3). Oct4 bound to octamer DNA specifically (Fig.3A, lanes 7-8) and was hypersensitive to diamide treatment (lanes 9-11). Oct4C48S bound DNA equivalently (lane 13) and also conferred resistance to oxidation, in particular at higher concentrations (lanes 14-16). In contrast, little sensitivity was observed with Oct1. Quantification from three independent replicate experiments is shown in Fig.3B. The recombinant Oct4 contained a C-terminal FLAG tag, allowing for purification (Supplemental Fig.5). Similar relative resistance to oxidative inhibition of DNA binding was observed using purified C48S recombinant Oct4 protein (Fig.3C). Quantification from three independent replicates is shown in Fig.3D. We conclude that Oct4 cysteine 48 sensitizes Oct4 to oxidative inhibition of DNA binding in vitro.

Oct4 oxidation has been linked to its degradation (Marsboom et al., 2016). In studying recombinant Oct4 expressed in 293T cells, we noted in over-exposed immunoblots the presence of
multiple slow-migrating high molecular weight forms of wild-type Oct4. These high-molecular weight forms were largely missing with C48S mutant protein (Fig.3E, lanes 2-3). These were increased by hydrogen peroxide treatment specifically using wild-type protein (lanes 4-5), indicating that they are increased by oxidative stress in a manner that requires cysteine 48. They were also specifically increased using the proteasome inhibitor MG-132, strongly suggesting that the high-molecular weight forms are ubiquitylated species (lanes 6-7). Interestingly, the combination of both H2O2 and MG-132 increased the intensity of the banding pattern over that observed using either treatment individually (lanes 8-9). These treatments alone and in combination did not affect short-term viability of the cells (Supplemental Fig.6).

To identify the nature of the ubiquitylated Oct4 species, we purified Oct4 expressed from 293T cells using a C-terminal streptavidin tag, and subjected the isolated proteins to immunoblotting using antibodies specific to different ubiquitin linked chains. Analysis using antibodies to pan linked Ub chains revealed strong diminution with Oct4C48S as expected (Fig.3F, FK2). Strong diminution was observed with K48 linkage-specific polyubiquitin antibodies (Fig.3F), while K63 linkage-specific antibodies however showed more modest differences compared to total Oct4 high-molecular weight bands (Supplemental Fig.7). We conclude that cysteine 48 promotes the degradation of Oct4 in response to oxidative stress largely via catalysis of K48-linked polyubiquitin chains.

**ESCs with endogenous Oct4C48S mutation appear normal in a pluripotent state but differentiate abnormally**

The ability of cysteine at position 48 to promote pluripotency, and the negative impact of Oct4 C48S mutation on iPSC generation, suggested that sensitivity to oxidative stress mediated by Cys48 helps potentiate reprogramming. To test if Oct4 oxidative stress sensitivity also regulates forward developmental steps, we studied the role of Cys48 in ESC differentiation. Using CRISPR, we generated ESCs with a single-base missense mutation that converts Cys48 to Ser (see Methods). Two silent point mutations were also engineered in the gRNA to monitor targeting. Two low-passage parent ESC lines
were chosen for targeting, one on a pure C57BL/6 background and one on a mixed BL6/129 background. Multiple independent clones were generated for each line. Homozygous mutation was confirmed by HRMA (not shown) and resequencing (Fig.4A). Tested clones were confirmed to be karyotypically normal and wild-type for *Tp53* (not shown). Undifferentiated ESCs were phenotypically identical to the parent clone in terms of Oct4 expression and morphology (Fig.4B,C), as well as maintenance of pluripotency and proliferative capacity (not shown). Cells were then differentiated in vitro using retinoic acid (RA) to assess Oct4 expression, morphology and cell counts. Oct4 protein expression was abnormally retained early during differentiation, mostly at day 2 (Fig.4D, lanes 3 and 7). Quantification from multiple experiments revealed that the effect was transient, with a >2.8-fold increase of Oct4 at day 3 that was lost by day 4 (Fig.4E). These results were recapitulated using a second clone from the same parental ESC line (Supplemental Fig.8A,B), as well as a C48S clone from a different 129×C57BL/6 ESC line, G4-N, which differentiates more slowly and shows even greater differences (Supplemental Fig.8C). RA-mediated differentiation also revealed a significant depletion of cells later in the differentiation timecourse that was retained through at least 12 days (Fig.4F,G).

The decreased cell numbers observed during differentiation of C48S-edited cells with RA could be due to decreased proliferation, increased death or some combination of the two. In order to investigate the nature of the effect on differentiation more closely, we studied proliferation using cell trace violet and TUNEL staining to look at genomic DNA fragmentation during apoptosis. Adherent, viable differentiating C48S cells showed increased apoptosis at day 2 (Supplemental Fig.9) and decreased proliferation at days 3 and 4 (Fig.4H) These results indicate that C48S-edited ESCs are phenotypically normal in the pluripotent state, but differentiate abnormally in assays that necessitate Oct4 silencing.

**Oct4^{C48S} mutant ESCs contribute poorly to adult chimeric animals**

One essential hallmark of pluripotency is the ability to contribute to cells and tissues of the embryo (De Los Angeles et al., 2015). We conducted chimerism assays to assess the ability of CRISPR-edited ESCs
to contribute to development. We used nine lines of mutant ESCs, derived from three independent wild-type parent lines with different strain backgrounds to minimize background and clonal effects. Derived clones had karyotypes similar to parent clones, with the proportion of diploid cells ranging between 71 and 93%. Compared to the parent cells, which contributed to offspring at a rate of 10-20% (Fig.5A, chimerism ranging from 2-99%), none of the derived Oct4$^{C48S}$ mutant ESCs were able to contribute to adult mice (Fig.5). The results strongly suggest that Oct4C48S ESCs are defective in contributing to embryonic development.
Discussion

Oct4 is a central mediator of pluripotency and is widely used to generate iPSCs from somatic cells (Feng et al., 2009; Nakagawa et al., 2008; Okita et al., 2007; Takahashi et al., 2007). Together with other factors it sustains pluripotency by activating “core” targets such as Pou5f1 (which encodes Oct4), Sox2 and Nanog (Boyer et al., 2005). Oct4 also maintains silent targets, including critical developmental regulators, in a readily inducible “poised” state (Bernstein et al., 2006; Meissner et al., 2008). Yet the mechanisms by which Oct4 is itself regulated, particularly at a post-transcriptional level, are poorly defined. Here, we used Oct4/Oct1 domain-swapping to pinpoint Oct4 redox sensitivity as an important determinant in reprogramming and differentiation. Oct1 itself appears to have no role in pluripotency establishment or maintenance, but upon differentiation and Oct4 loss becomes important for guiding developmental-mediated induction of targets shared with Oct4 (Perovanovic et al., 2023).

Pluripotent cells co-express Oct4 together with the widely expressed prototypic Oct/POU factor Oct1/Pou2f1 (Shen et al., 2017). Despite having similar DNA binding domains and recognizing the same 8-bp consensus octamer sequence (5’ATGCAAAT3’) (Kang et al., 2009b; Tantin, 2013), Oct1 and other Oct4 paralogs do not function in reprogramming (Takahashi and Yamanaka, 2006). Structure/function studies comparing Oct4 with these paralogs, in particular Oct6, have identified Oct4 transcriptional determinants (Boija et al., 2018; Esch et al., 2013; Jerabek et al., 2017; Jin et al., 2016). An example is three residues (K7,T22,151S) present in the Oct4 DNA binding domain (Fig.1A) but absent in Oct6 needed to form Sox2 heterodimers. Introducing these residues plus the Oct4 N- and C-termini into Oct6 results in efficient reprogramming (Jerabek et al., 2017). However, several Oct4 paralogs including Oct1 contain these residues and readily heterodimerize with Sox2 but cannot reprogram, indicating that additional reprogramming determinants may be present in Oct4 (and Oct6) but absent in Oct1. We identify an additional central Oct4 reprogramming determinant – a single DNA binding domain cysteine residue that confers redox sensitivity of Oct4.
We found that Oct4 C48S mutation maintains DNA binding and confers resistance of oxidation-induced DNA binding inhibition in vitro, while reducing reprogramming efficiency by more than half. Cys48 is located within helix 3 (the DNA recognition helix) of the POU-specific domain (Fig.1A). This residue makes hydrogen bond contacts with the DNA phosphate backbone, predicting that any oxidation event will block DNA binding. In the context of the Oct4 N-terminus, a single S48C mutation in the Oct1 DNA binding domain is sufficient to confer reprogramming activity. This result suggests that any unique Oct4 cofactors required for reprogramming vis-à-vis Oct1 are likely to be recruited through the N-terminus. The ability of the Oct4 N-terminus to mediate liquid-liquid phase transitions (Boija et al., 2018) may also play a role. Mutation of the endogenous Oct4 Cys48 to serine does not appear to affect the undifferentiated state, but results in numerous phenotypes upon RA-mediated differentiation, including abnormal retention of Oct4 expression, decreased proliferation and increased apoptosis. Prominent roles for Oct4 in both cell survival and proliferation during post-implantation embryonic development have been documented (DeVeale et al., 2013).

The redox sensitivity of Cys thiols is known to be enhanced by immediately adjacent basic amino acid residues (Britto et al., 2002), and Cys48 is followed by a conserved Arg (Fig.1A). In Oct1, this residue is a serine, strengthening hydrogen bonding due to the increased electronegativity of the oxygen in the hydroxyl relative to the sulfur in the sulfhydryl. Redox-regulated transcription factors include Nrf2, Yap1, p53 and HIF-1 (Brigelius-Flohe and Flohe, 2011; Delaunay et al., 2000; Marinho et al., 2014). A subset of these are regulated by Cys oxidation, including disulfide bond formation (Brigelius-Flohe and Flohe, 2011; Delaunay et al., 2000; Zheng et al., 1998). Oct4 is similarly known to be sensitive to oxidative stress (Guo et al., 2004; Lickteig et al., 1996; Marsboom et al., 2016) in a manner reversed by thioredoxin (Guo et al., 2004) (Supplemental Fig.4). Relative to other Oct proteins, mouse Oct4 has unusually high cysteine content in the DNA binding domain, with two cysteines in each subdomain (Fig.1A), including cysteine 142 (Cys50 of the POU-homeodomain), which also lies in the recognition helix. Cys50 is the distinguishing feature of POU-homeodomains relative to other members of the homeodomain family.
(Kang et al., 2009b; Tantin, 2013). All other homeodomains, from bacteria to flies to mammals, have bulkier residues such as Q or K at this position that make base contacts (Tantin, 2013). Yet in Oct4, mutating Cys142 to Gly has no effect on DNA binding (Marsboom et al., 2016). In Oct1, C61A and C142A mutation does not interfere with DNA binding, but rather improves crystallization properties (Remenyi et al., 2001). These findings suggest that variable Cys oxidation interferes with uniform crystal packing.

Prior studies have also identified a link between Oct4 oxidation and degradation (Marsboom et al., 2016). In addition to mediating relative sensitivity to oxidative stress, we found that Cys48 increases protein ubiquitylation, in particular following exposure to oxidative stress or MG-132. Oct4 is known to be ubiquitylated via K63-linked chains by the Stub1 E3 Ub ligase to reduce protein half-life (Mamun et al., 2022), however we found that Oct4 marked with K48-specific linkages were more strongly affected with mutation of cysteine 48 to serine, suggesting a possibly related by distinct pathway.

Transcription factors with complex (both positive and negative) roles in reprogramming include Oct4 and Smad3 (An et al., 2019; Ruetz et al., 2017; Velychko et al., 2019). Although endogenous Oct4 must be activated and expressed as a key component of the core pluripotency network, recent studies show that removal of Oct4 from reprogramming cocktails is highly inefficient but results in qualitatively superior iPSC clones that more closely resemble a pluripotent “ground state” (An et al., 2019; Velychko et al., 2019). As a consequence, the few iPSC colonies derived in the absence of Oct4 have enhanced developmental potential. For example, iPSCs reprogrammed without Oct4 are better able to generate viable mice in a single generation using tetraploid complementation. The counter-productive activity of Oct4 is likely due to transient promotion of off-target, ectoderm-specific gene expression (Velychko et al., 2019). The studies indicate that transient down-regulation of counterproductive Oct4 activities can be an important step for efficient reprogramming. Our work identifying a role of Oct4 redox sensitivity in reprogramming may explain this process and may provide a route to generating superior iPSC reprogramming outcomes with high efficiency.
Materials and Methods

Cell culture
ESC/iPSCs were cultured as described previously (Shakya et al., 2015; Shen et al., 2018) with “2iV” supplementation: the MEK inhibitor PD03259010 (1 μM, LC laboratories) and the glycogen synthase kinase-3β (GSK3β) inhibitor CHIR99021 (3 μM, LC Laboratories). Culture medium was also supplemented with 50 μg/mL ascorbic acid (Sigma). H2O2 (Sigma) was supplied at 1 mM for 2 hr and MG-132 (Sigma) was supplied at 10 μM for 2 hr. Cell trace violet proliferation assays were conducted using a kit (Cell Trace Violet Cell Proliferation Kit for Flow Cytometry, ThermoFisher) according to the manufacturer instructions. TUNEL assays were conducted using a kit (Apo-Direct, Becton-Dickinson) according to the manufacturer instructions.

FLAG-tagged and chimeric Oct4/Oct1 constructs for iPSC generation
The “mSTEMCCA” 4-in-1 lentiviral vector expressing Oct4, Klf4, Sox2 and c-Myc was used for these studies (Somers et al., 2010). To identify determinants of reprogramming, the Oct4 cDNA cassette was replaced with c-terminally FLAG-tagged full-length mouse Oct4, mouse Oct1 or chimeric constructs. FLAG-tagged Oct4 was generated by overlap PCR using overlapping primers containing the FLAG peptide sequence: DYKDDDDK (Forward: 5’-GATTACAAGGATGACGACGATAAGGGAAGTTGCGTGAAACAGA; Reverse: 5’-CTTATCGTCGTCTTCTTTGAATCGTTTGAATGCGATGGGAGAG). The first fragment was amplified using a forward primer containing a NotI restriction enzyme site (5’-AATGAAAAAGCGCGCCCATGGCCTGGCAGACCT) and overlapping reverse primer containing the FLAG sequence. A second fragment was amplified using the reverse primer containing an Ndel restriction site (5’-GGGAATTCCATATGATGCTGTGCGGAGAG) and overlapping forward primer. Mixed first fragment and second fragment as template and amplified the whole fragment by using forward
and reverse primers containing restriction enzymes (NotI and Ndel). After purification and digestion with NotI and Ndel, the purified fragment was ligated with the mSTEMCCA vector digested by NotI and Ndel. A similar strategy was applied to generate other tagged constructs. For point mutation, a site-directed mutagenesis kit (New England Biolabs Q5 Site-Directed Mutagenesis kit) was used following manufacturer instructions. When making fusions between the different linker domains and POUH, we took care to maintain the “RKRKR” sequence at the N-terminus of Oct4 POUH, which has been shown to be important for reprogramming (Jerabek et al., 2017). Chimeric constructs between the Oct1 linker and Oct4 POUH contained the amino acid sequence N-AEGLN/ARKRKR as shown in Fig.1A.

**iPSC generation**

293T cells were transfected by polycation polyethylenimine (PEI) with mSTEMCCA encoding FLAG-tagged Oct4, Oct1 and different domain-swaps and mutants as well as the packaging plasmids (pMDLg/pRRE and pRSV-Rev) and envelope plasmid(VSV-G). Lentiviral supernatants were filtered and used to transduce passage 3 primary MEFs in which EGFP is expressed from the endogenous Pou5f1 locus (Oct4-GFP MEFs, Jackson Labs strain #008214). Cells were cultured in ESC medium (above). Unless stated otherwise, green iPSC colony emergence was quantified after 12 days using an Olympus IX-71 epifluorescence microscope.

**Immunoblotting**

Antibodies for immunoblotting were as follows: Oct4, Santa Cruz sc-5279; Oct1, Bethyl A301-716A+A301-171A; Nanog, GeneTex GTX100863; Sox2, GeneTex GTX101507; GAPDH, EMD Millipore MAB374; α-Tubulin, Santa Cruz sc-5286; β-actin, Santa Cruz sc-47778.

**Lentiviral Oct1 complementation**
The Oct1 (Pou2f1) cDNA and IRES (internal ribosomal entry site) elements were amplified and cloned together by overlap PCR. In the first PCR, primers to the 5’ end of Oct1 containing a NotI restriction site and to the 3’ end of Oct1 that contained a 5’ extension of IRES-complementary DNA were used. The sequences were: Oct1-NotI-For: 5’-AATGAAAAAAGCGGCCGCTGATGAATAATCCATCAGAAAC-3’; Oct1-Rev-IRES: 5’-TTAGGGGGGAGGGAGGATCTTCAGCTGTGCCTGGAG-3’. In the second PCR, an IRES sequence was amplified using primers to the 5’ end of the IRES containing a 5’ extension of DNA complementary of the Oct1 3’ end, and primers to the 3’ end of the IRES containing an Ndel restriction site. The sequences were: IRES-overlap-FOR: 5’-AGATCCCTCCCCCCCCCTAACGTTACTGCGCCAAGA-3’; IRES-Rev-Ndel: 5’-GGGAATTCCATATGTTGTGGCCATATTATCATCGGT-3’. The third PCR used as a template the PCR products from the first two rounds, along with the Oct1-NotI-For and IRES-Rev-Ndel primers. This process generated a DNA fragment containing an Oct1 cDNA fused to an IRES at the 3’ end, along with a NotI site at the 5’ terminus and an Ndel site at the 3’ terminus. The fragment was cloned into the optimized, self-inactivating, nonreplicative pHAGE lentiviral vector using the NotI and Ndel restriction sites. To insert a Puro cassette after the IRES, the cDNA was amplified using primers containing 5’ Ndel and 3’ ClaI restriction sites. The sequences were Puro-Ndel-For: 5’-GGAATTCATATGAGCAGTGACGAGGATC-3’; Puro-ClaI-Rev: 5’-GGTTTATCGATTCAGGCAAGGCTTGTGC-3’. Because the IRES apparently attenuated expression of the Puro resistance cassette in this vector, puromycin selection was performed at 0.75 µg/mL. To generate an empty vector control, the vector was cut with Ndel and NotI, filled in with Klenow fragment, and re-ligated.

**Electrophoretic mobility shift assay**

EMSA was performed using published methods (Kang et al., 2009a; Tantin et al., 2008) Briefly, reactions were assembled on ice in 0.6× buffer D (12 mM HEPES pH 7.9, 60 mM KCl, 0.06 mM EDTA, 12%
glycerol, all buffer reagents from Sigma) supplemented with 0.1 mM dithiothreitol (DTT, Sigma), 0.5 mM phenylmethylsulphonyl fluoride (PMSF, Sigma), 50 µg/mL poly-dI•dC (Sigma), 1 mg/mL bovine serum albumin (BSA, Sigma) and added DNA probe and lysate or recombinant protein. Diamide (Sigma) was added at final concentrations ranging from 0 to 300 µM on ice during reaction assembly. 5’Cy5-labeled double-stranded DNA probes were as follows: wild-type octamer, 5’-TGTCAATGCAAATC-3’; mutant octamer, 5’-TGTCAATGCAAGCC-3’. Reactions were incubated for 30 min at room temperature prior to gel loading. For experiments involving reversal of oxidative inhibition, thioredoxin (Sigma) was supplied at a final concentration of 0 to 60 µM after 15 min incubation with diamide, and both thioredoxin-treated and control samples were incubated for a further 15 min at 37°C prior to gel loading. Reactions were resolved by electrophoresis through 0.5 × TBE (45 mM Tris-Cl pH 8.3, 45 mM boric acid, 1 mM EDTA, all reagents from Sigma), 4% polyacrylamide gels containing 1% glycerol. Gels were run for 90 min at room temperature. DNA was visualized using a Molecular Dynamics Typhoon system.

**Recombinant Oct4 protein**

Wild-type and C48S mutant Oct4 constructs were generated bearing dual C-terminal FLAG and Twin-Strep tags. A g-block (IDT) fragment coding for the Twin-Strep and FLAG epitope tags was inserted between the NcoI and KpnI sites in the transient expression vector pACE-MAM2 (Geneva Biotech). The resultant construct was then digested with NcoI and PvuII to insert the g-block fragment coding for either wild-type or C48S mutant murine Oct4. After Sanger sequencing to confirm veracity, the plasmids were transfected into Expi293F cells (ThermoFisher Scientific) using ExpiFectamine 293 Tranfection Kit (ThermoFisher Scientific) following the manufacturer instructions. After 2 days, transfected cells were harvested by centrifugation at 4°C for 5 min at 500×g, washed with ice-cold PBS, and centrifuged again at 4°C for 5 min at 500×g. Cells were resuspended in Buffer A (20 mM HEPES pH 8.0, 1.5 mM MgCl₂, 0.5 mM phenylmethylsulphonyl fluoride (PMSF, Sigma), 50 µg/mL poly-dI•dC (Sigma), 1 mg/mL bovine serum albumin (BSA, Sigma) and added DNA probe and lysate or recombinant protein. Diamide (Sigma) was added at final concentrations ranging from 0 to 300 µM on ice during reaction assembly. 5’Cy5-labeled double-stranded DNA probes were as follows: wild-type octamer, 5’-TGTCAATGCAAATC-3’; mutant octamer, 5’-TGTCAATGCAAGCC-3’. Reactions were incubated for 30 min at room temperature prior to gel loading. For experiments involving reversal of oxidative inhibition, thioredoxin (Sigma) was supplied at a final concentration of 0 to 60 µM after 15 min incubation with diamide, and both thioredoxin-treated and control samples were incubated for a further 15 min at 37°C prior to gel loading. Reactions were resolved by electrophoresis through 0.5 × TBE (45 mM Tris-Cl pH 8.3, 45 mM boric acid, 1 mM EDTA, all reagents from Sigma), 4% polyacrylamide gels containing 1% glycerol. Gels were run for 90 min at room temperature. DNA was visualized using a Molecular Dynamics Typhoon system.
10 mM KCl, 0.25% NP-40, 0.5 mM DTT, 2x protease inhibitor cocktail (Roche), incubated for 10 min on ice, and homogenized using a Dounce homogenizer (Wheaton). The homogenate was centrifuged at 4°C for 5 min at 3,800×g). Pelleted nuclei were resuspended in Buffer C (20 mM HEPES pH 8.0, 25% glycerol, 1.5 mM MgCl₂, 420 mM KCl, 0.25% NP-40, 0.2 mM EDTA, 0.5 mM DTT, 2x protease inhibitor cocktail) and homogenized again using a Dounce homogenizer (Wheaton). Nuclei were extracted for 30 min at 4°C with end-to-end rotation, then centrifuged at 4°C for 30 min at 20,000×g. The nuclear extract was then subjected to immunoprecipitation with anti-FLAG (M2)-agarose beads (Sigma). Bead-bound proteins were eluted with 200 ng/µL 3×FLAG peptide (Sigma) by incubating at 4°C for 30 min. The protein was pure and monodisperse as verified using Coomassie blue staining and size exclusion chromatography.

Generation of Oct4C₄₈S mutant mouse ESCs

Mutant ESCs with a single-base missense mutation that converts Cys48 to Ser were generated by the University of Utah Health Sciences Center Mutation Generation and Detection Core and the University of Utah Transgenic and Gene Targeting Mouse Facilities using a templated CRISPR approach. Briefly, a gRNA was designed to target Cas9 and cutting near the target nucleotide, 5’ GGCCTCGAAGCGACAGATGG 3’, where the underlined C indicates the target nucleotide. The sgRNA was ordered as a modified synthetic sgRNA from Synthego and was complexed with Cas9 protein (IDT) to form a ribonucleoprotein (RNP) complex that was electroporated with program CG-104 into into 6×10⁴ mouse ESCs using a LONZA 4D-Nucleofector X-unit. The RNP was co-electroporated with a corresponding single-stranded oligodeoxynucleotide (ssODN) with 5’ and 3’ phosphorothioate modifications, 5’-ctcactctgtttgtcgccgcttcatgaaaggtgttcagccagaccaccatctctctagtcttcgagtgcctgtgccagctcggcttaaagacatgtgtaagcgt-3’ where lower-case denotes intronic sequence. The ssODN contained the target mutation (underlined), silent mutations in the seed region (double underlined) to block CRISPR re-cutting after incorporation of the donor and to generate a unique XbaI restriction
enzyme site for screening purposes (the sgRNA site is in bold). Incorporation of the ssODN sequence was identified via PCR and restriction enzyme digestion, and confirmed by sanger sequencing of the 709 bp region flanking the mutation. Mutant clones were generated from a pure C57BL/6 ESC line, a 129/BL6 hybrid line expressing constitutive GFP and a reporter line used to de-differentiate ESCs into a 2C-like state. Clones were tested by karyotype and any clones with <70% normal diploid content were excluded.

Chimeric mouse assays

Targeted or parental ESCs were disassociated into single cell suspension by treatment with 0.05% Trypsin (ThermoFisher). After collection by centrifugation, the cells were resuspended in injection medium consisting of DMEM with HEPES (ThermoFisher) supplemented with 10% FBS (Peak Serum) and 0.1 mM 2-mercaptoethanol (Sigma). C57B6 clones were injected into albino B6 blastocysts, while the G4N and C4 lines (and derived lines, B6:129 and 129:129 in origin, respectively) were injected into B6 blastocysts. E3.5 blastocysts were used. 10-25 ESCs were injected into each blastocyst using micromanipulators (Eppendorf) and a Leica inverted microscope. After culturing injected blastocysts in KSOM (CytoSpring), approximately 12 to 18 blastocysts were surgically transferred into the uterine horns of day 2.5 pseudopregnant mice or the oviduct of day 0.5 pseudopregnant mice. Mice were scored based on coat and eye color by members of the transgenic core facility at weaning.
Acknowledgements

We thank S. Buckley for critical reading of the manuscript. We thank C. Davey and the University of Utah Health Sciences Center Mouse Transgenic Core and Mutation Generation Core Facilities for assistance with generating CRISPR-edited ESC lines, chimerism assays and karyotyping. This work was supported by NIH/NIGMS grant (R01GM122778) to DT.

Conflict of interest

The authors declare that they have no conflicts of interest.
Figure legends

Figure 1. Oct4’s reprogramming activity vis-à-vis Oct1 maps to the DNA binding domain.

(A) Mouse Oct1 vs Oct4 DNA binding domain alignment. Asparagine 40 mutated in some constructs to lysine is shown in blue. The key Oct1 serine 48 residue (cysteine in Oct4) is shown in blue and boxed in red. (B) Schematic of the reprogramming vector with C-terminal Oct4 FLAG tag. The different domains of Oct4 and Oct1 are color coded. (C) Reprogramming activity of C-terminally tagged Oct4 relative to the untagged control. Results show an average of experiments performed in biological triplicate. Error bars show ± standard deviation. Anti-FLAG and anti-Oct4 immunoblots using tagged and untagged constructs transiently transfected into 293T cells are shown at right. (D) iPSC formation assay using target primary fibroblasts with an Oct4-GFP cassette. Reprogramming was assessed using the number of GFP-expressing iPSC colonies. Oct4 was used as a positive control and Oct1 as a negative control. All constructs bore C-terminal FLAG-tags. Experiments were performed in biological triplicate. Error bars show ± standard deviation. (E) Schematic and summary of size and activity of the constructs used in (D). The activity of Oct4 in (D) was set to 100%. C-terminal FLAG-tags are not shown for simplicity. (F) Anti-FLAG immunoblot showing expression of the different constructs when transiently transfected into 293T cells. Cells were transduced with the modified STEMCCA reprogramming vectors similar to the primary Oct4-GFP fibroblasts used in the reprogramming assays. Histone H3 is shown as a loading control.

Figure 2. Activity of Oct4 cysteine 48 in reprogramming.

(A) iPSC formation assay using Oct1-Oct4 lentiviral constructs that manipulate cysteine 4. Oct4 was used as a positive control and Oct1 as a negative control. All constructs bore C-terminal FLAG-tags. Experiments were performed in biological triplicate. Error bars show ± standard deviation. (B) Schematic and summary of size and activity of the constructs used in (A). The activity of Oct4 in (A) was set to...
100%. (C) Anti-FLAG immunoblot showing expression of the different constructs when transiently transfected into 293T cells. Cells were transduced with the modified STEMCCA reprogramming vectors similar to the primary Oct4-GFP fibroblasts used in the reprogramming assays. Histone H3 is shown as a loading control. (D) Detail from an Oct1:octamer DNA co-crystal structure (PDB ID: 1OCT) (Klemm et al., 1994) showing Ser48 making hydrogen bond contacts with the DNA backbone. (E) Paralog comparison of mouse POU protein amino acid residues corresponding to α-helix 3 of the POU8 sub-domain.

Figure 3. Oct4 cysteine 48 confers DNA sensitivity to oxidative stress and oxidative stress-induced ubiquitylation.

(A) EMSA using canonical octamer DNA and wild-type of C48S mutant full-length Oct4. 293T cells were transiently transfected with plasmids encoding C-terminally Twin-Strep-FLAG-tagged Oct4 constructs. Protein lysates were made and immediately used for EMSA with increasing amounts of diamide (see methods). The larger redox-resistant Oct1 protein that binds wild-type (WT) but not mutant (Mut) probe is highlighted with an arrow. Asterisk shows a redox sensitive band with superior binding to mutant rather than octamer DNA that is presumably nonspecific. Together these proteins serve as loading controls. (B) Quantification of Oct4 band intensity relative to no diamide, which was set to 100%, from three independent experiments. Error bars show ±standard deviation. (C) Oct4 containing a C-terminal streptavidin tag was purified from 293T cell lysates using anti-FLAG beads (see methods). Isolated protein was used in assays similar to (A). (D) Quantification of (C) and two other similar experiments. Error bars show ±standard deviation. (E) Highly exposed Oct4 immunoblot using lysates prepared from 293T cells transfected with constructs expressing C-terminally FLAG/Twin-Strep-tagged Oct4. Subsets of cells were pre-treated with H2O2 (1 mM for 2 hr), MG-132 (10 μM for 2 hr) or both. All samples contained MG-132 in the lysis buffer to prevent degradation. Histone H3 is shown as a loading control. (F) Purified wild-type and C48S mutant Oct4 was immunoblotted using antibodies against specific ubiquitin linkages. The
FK2 antibody (Enzo Life Sciences) detects pan-Ub linkages, and the antibody detecting Ub-K48-specific linkages was obtained from Cell Signaling.

**Figure 4. Oct4<sup>C48S</sup> mutant ESCs are phenotypically normal but retain Oct4 expression when differentiated.**

(A) Top: Schematic for CRISPR-mediated Pou5f1 (Oct4) mutation. A single nucleotide change (arrow) converts the wild-type cysteine to the serine present in Oct1. Asterisk depict two silent point mutations present in the guide RNA to monitor mutagenesis. Bottom: DNA sequencing trace of homozygous mutant clone. (B) An example C57BL/6 ESC clone (#46) was immunoblotted for Oct4, Sox2 and Nanog. Histone H3 was used as a loading control. (C) Example images of pluripotent colonies from a parental ESC line and derived Oct4<sup>C48S</sup> mutant ESC line (#46). Images were collected at 10× magnification. Scale bar = 0.2 mm. (D) Parent or derived Oct4<sup>C48S</sup> mutant ESCs were differentiated without feeders using retinoic acid. Lysates were collected at the indicated times and probed for Oct4 by immunoblotting. β-actin was used as a loading control. (E) Average quantified Oct4/β-actin levels from three replicate immunoblots conducted with the same ESC clone (#46). Error bars depict ±standard deviation. (F) The same cells were used to collect phase-contrast images at longer retinoic acid timepoints. Images were collected at the indicated times at 10× magnification. Scale bar = 0.2 mm. (G) Differentiating cells were trypsinized and counted using trypan blue exclusion using a hemacytometer. Cell counts were averaged with two other biological replicates (n=3) and plotted. Error bars depict ±standard deviation.

**Figure 5. Oct4<sup>C48S</sup> mutant ESCs do not contribute to adult chimeric animals.**

Three different parent (left side) or CRISPR-mutated Oct4<sup>C48S</sup> ESCs (right side) were injected into recipient blastocysts and implanted in pseudo-pregnant animals. For each line, the proportion of chimeric
offspring at weaning is shown as a pie chart. All karyotypes were confirmed to be similar to parent lines (between 72% and 92% normal).
References


Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. Nat Cell Biol 11, 197-203.


**Figure 2**

**A**

Day 12

GFP+ colonies

- 350
- 300
- 250
- 200
- 150
- 100
- 50
- 0

**B**

mSTEMCCA

- Oct4
- Klf4
- Sox2
- c-Myc

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**C**

100 kDa

70 kDa

40 kDa

FLAG

H3

**D**

3 A

Ser48

**E**

FSQTTISRFEMm Oct1/Pou2f1 (NP_035267)
FSQTTICRFEMm Oct4/Pou5f1 (NP_038661)
FSQTTISRFEMm Oct2/Pou2f2 (Q00196-1)
FSQTTICRFEMm Oct6/Pou3f1 (P21952)
FSQTTISRFEMm Oct11/Pou3f2 (XP_011240722)
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**A**

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**B**

% DNA binding activity vs. [diamide] (µM)

- WT
- C48S


**C**

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**D**

% DNA binding activity vs. [diamide] (µM)

- WT
- C48S


**E**

**F**

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Oct4

55 kDa | 40 kDa

H3

1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16

UB-Oct4

55 kDa | 40 kDa

Oct4

55 kDa | 40 kDa