LETTER

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NANOG–dependent function of TET1 and TET2 in establishment of pluripotency

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Molecular control of the pluripotent state is thought to reside in a core circuitry of master transcription factors including the homeodomain-containing protein NANOG[1,2], which has an essential role in establishing ground state pluripotency during somatic cell reprogramming[3,4]. Whereas the genomic occupancy of NANOG has been extensively investigated, comparatively little is known about NANOG-associated proteins[5] and their contribution to the NANOG-mediated reprogramming process. Using enhanced purification techniques and a stringent computational algorithm, we identify 27 high-confidence protein interaction partners of NANOG in mouse embryonic stem cells. These consist of 19 previously unknown partners of NANOG that have not been reported before, including the ten-eleven translocation (TET) family methylcytosine hydroxylase TET1. We confirm physical association of NANOG with TET1, and demonstrate that TET1, in synergy with NANOG, enhances the efficiency of reprogramming. We also find physical association and reprogramming synergy of TET2 with NANOG, and demonstrate that knockdown of TET2 abolishes the reprogramming synergy of NANOG with a catalytically deficient mutant of TET1. These results indicate that the physical interaction between NANOG and TET1/TET2 proteins facilitates reprogramming in a manner that is dependent on the catalytic activity of TET1/TET2. TET1 and NANOG co-occupy genomic loci of genes associated with both maintenance of pluripotency and lineage commitment in embryonic stem cells, and TET1 binding is reduced upon NANOG depletion. Co-expression of NANOG and TET1 increases 5-hydroxymethylcytosine levels at the top-ranked common target loci *Esrrb* and *Oct4* (also called *Pou5f1*), resulting in priming of their expression before reprogramming to naive pluripotency. We propose that TET1 is recruited by NANOG to enhance the expression of a subset of key reprogramming target genes. These results provide an insight into the reprogramming mechanism of NANOG and uncover a new role for 5-methylcytosine hydroxylases in the establishment of naive pluripotency.

We expanded the NANOG interactome in mouse embryonic stem cells using an improved affinity purification and mass spectrometry (AP-MS) strategy[6,7] similar to that described previously[8], combined with an interactomics analysis (see Supplementary Information). This analysis identified 27 high-confidence interaction partners of NANOG (Fig. 1a, b, Supplementary Figs 1–4 and Supplementary Tables 1 and 2). Notable among the 19 previously unknown interaction partners of NANOG was the methylcytosine hydroxylase TET1[9,10] (Fig. 1b). Specific association of TET1 with NANOG was detected in all five affinity purification runs of three independent affinity purifications (Supplementary Fig. 5b and Supplementary Table 2), and the interaction between NANOG and TET1 was further confirmed by immunoprecipitation and co-immunoprecipitation (Fig. 1c and Supplementary Fig. 5c, d). Whereas NANOG clearly associates with TET1 in embryonic stem cells, there also exists TET1-free NANOG protein as shown by immunodepleting TET1 in embryonic stem cells (Supplementary Fig. 5e). Notably, among the 27 high-confidence interaction partners of NANOG, at least 5 (NACC1, SGOL2, QSER1, HDAC2 and OCT4) were also associated with TET1 by co-immunoprecipitation and/or immunoprecipitation-mass spectrometry experiments (Supplementary Fig. 5f–h). Expression of TET1, like that of NANOG, is upregulated during reprogramming to pluripotency (Supplementary Fig. 6a). Because NANOG is a critical determinant during establishment of pluripotency[3,11], we investigated whether TET1 may also be required for efficient nuclear reprogramming. Indeed, RNA interference-mediated inhibition of TET1 during reprogramming reduced generation of induced pluripotent stem (iPS) cells from mouse embryonic fibroblasts (Supplementary Fig. 6b–g and Supplementary Fig. 7). The requirement of TET1 for efficient reprogramming was confirmed using *Tet1*$^{-/-}$ embryonic stem cells[12] in an independent, heterokaryon-based reprogramming system[13] (Supplementary Fig. 8).

The physical association of TET1 with NANOG prompted us to consider whether TET1 may modulate NANOG function in establishing pluripotency. NANOG and TET1 are only minimally expressed in reprogramming intermediates resulting from retroviral infection of neural stem cells with the reprogramming factors OCT4, KLF4 and c-Myc (rOKM) (Fig. 2a). We addressed whether NANOG-mediated reprogramming of these cells requires functional contribution of TET1. A clonal line of reprogramming intermediates was transfected with a PiggyBac NANOG transgene followed by addition of short interfering RNA (siRNA) against *Tet1* (Supplementary Fig. 9a, b). Downregulation of TET1 reduced NANOG reprogramming efficiency by 26-fold compared with the non-targeting control (Fig. 2b and Supplementary Fig. 9c), indicating that TET1 and/or its associated catalytic activity may be a limiting factor for reprogramming by NANOG.

We then asked if ectopic TET1 expression could enhance NANOG reprogramming activity. Neural stem cells + rOKM were transfected with PiggyBac vectors expressing NANOG, TET1 or TET1 bearing two mutations in the catalytic domain (TET1(H1671Y, D1673A), hereafter TET1Mut)[10] (Supplementary Fig. 10a). Individual expression of wild-type TET1 (TET1WT) or TET1Mut did not have a significant effect on generation of green fluorescent protein (GFP)-positive colonies in cells containing a *GFP* gene driven by *Oct4* regulatory sequences (Fig. 2c and Supplementary Fig. 10b, c). In contrast, NANOG expression enhanced the generation of iPS cell colonies by more than tenfold (Fig. 2c and Supplementary Fig. 10b, c), in accordance with previous studies[11,14]. Importantly, NANOG-mediated reprogramming efficiency was further augmented by up to fourfold in the

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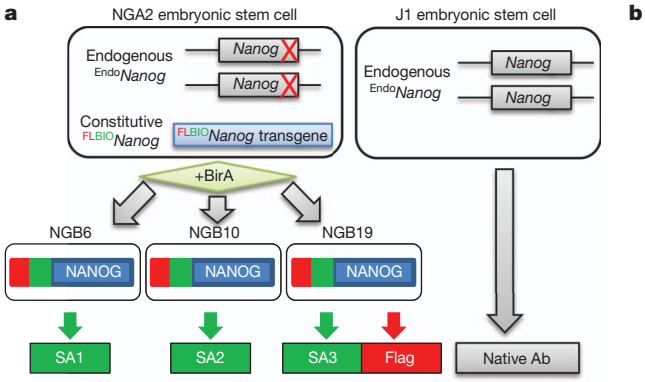

b

Preys	CCP Score
NANOG	23.7
ZFP609	23.2
MKI67	21.1
EMSY	20.3
NACC1	18
JMJD1C	16.5
BRCA2	16.1
TET1	15.9
NCOR2	15.7
SGOL2	15.6
HNRNPM	15
QSER1	12.9
MGA	12.7
BPTF	12.4
GATAD2A	12.3
ARID3B	11.9
RBM14	11.8
HDAC2	11.6
SKIV2L2	10.4
OCT4	9.7
LMNB1	9.4
MTA2	9.4
RFC1	9.3
TRRAP	9
SALL4	8.9
MTA3	8.6
SMC3	8.5
WDR18	8.5

Figure 1 | Identification of TET1 as a novel partner of NANOG. **a**, Schematic depiction of embryonic stem cells expressing NANOG with Flag (FL) and Biotin (BIO) tags (left), and NANOG antibody (Ab)-based affinity purification (right). SA1–SA3, three independent streptavidin pull-down experiments. **b**, List of 27 preys identified as true interactors ordered by combined cumulative probability (CCP) score. Candidates shaded in green are the ones whose interaction with NANOG has been validated previously[5,25,26] or in this study by immunoprecipitation/co-immunoprecipitation. Two previously identified NANOG partners, DAX1 and ZFP281[5], were identified by mass spectrometry (Supplementary Fig. 5a), but not selected as high-confidence interactors using our stringent criteria. **c**, Validation of NANOG–TET1 interaction by immunoprecipitation/co-immunoprecipitation in embryonic stem (left) and HEK293T (right) cells. The asterisk indicates the presence of TET1 in input that can be visualized under longer exposure (Supplementary Fig. 5c). IP, immunoprecipitation; WB, western blot.

presence of TET1WT transgene, a synergistic effect that is highly reproducible (Fig. 2c and Supplementary Fig. 10b–e). A similar reprogramming synergy was also observed for the combination of NANOG with TET1Mut (Fig. 2c and Supplementary Fig. 10b–e). iPS cells derived with NANOG and either TET1WT or TET1Mut transgenes contributed to the germ lineage and live-born chimaeras following blastocyst injection (Fig. 2d, e). Together, our data show that NANOG and TET1 enhance the efficiency of somatic cell reprogramming in a cooperative manner. This conclusion was corroborated in mouse embryonic fibroblasts, where the combined action of NANOG and TET1WT increased reprogramming efficiency by up to 16-fold (Supplementary Fig. 11).

To explore the molecular mechanism underlying the NANOG–TET1 partnership during reprogramming, we quantified global 5-hydroxymethylcytosine (5hmC) levels[15]. As expected, 5hmC levels were increased upon TET1WT but not TET1Mut expression in neural stem cells + rOKM (Fig. 3a, left). Unexpectedly, co-expression of NANOG and either TET1WT or TET1Mut resulted in increased 5hmC levels (Fig. 3a, left). These results indicate that NANOG can potentiate 5hmC modifications by its association with TET1, and that transcriptional activation of endogenous TET1 and/or its paralogue TET2 may compensate for the lack of catalytic activity of TET1Mut during reprogramming with NANOG. Indeed, TET2 was upregulated by NANOG and TET1WT or TET1Mut, and its expression levels follow a very similar trend to that of 5hmC/C levels (Fig. 3a and Supplementary Fig. 10f, g). TET2 was identified in two out of three independent affinity purifications in our NANOG interactomics study (Supplementary Fig. 12a, b), and physical association of TET2 with NANOG was confirmed by immunoprecipitation/co-immunoprecipitation (Supplementary Fig. 12c, d). TET2 was recently found to contribute to an epigenetic program that directs subsequent transcriptional induction at the pluripotency loci *Nanog* and *Esrrb* during the early stage of somatic cell reprogramming[16]. TET1 and TET2 share the common carboxy-terminal catalytic domain but are divergent in their amino termini for a CXXC DNA-binding domain, which renders TET2 functionally similar to a truncated form of TET1, TET1C (Fig. 3b). We investigated whether the catalytic activity of TET1 is sufficient to enhance NANOG-mediated reprogramming. Indeed, TET1C acts together with NANOG to enhance reprogramming (Fig. 3c) and retains its physical association with NANOG (Fig. 3d). Not surprisingly, we also observed reprogramming synergy between NANOG and TET2 (Fig. 3c and Supplementary Fig. 13). TET1 and TET2 function is redundant in the context of NANOG-induced reprogramming, as exogenously expressing both TET enzymes together with NANOG does not enhance somatic cell reprogramming beyond expressing NANOG with either individual enzyme (Fig. 3c).

Given that endogenous TET2 was upregulated in the presence of NANOG and TET1Mut (Fig. 3a right and Supplementary Fig. 10f), and that TET2 synergizes with NANOG during reprogramming (Fig. 3c), we tested whether knockdown of *Tet2* could abrogate the reprogramming synergy of NANOG and TET1. Indeed, siRNAs directed against *Tet2* diminished the reprogramming synergy of NANOG and TET1Mut, but did not affect that of NANOG and TET1WT (Fig. 3e, f). This result confirms that TET2 activation compensates for the lack of catalytic activity of TET1Mut during reprogramming with NANOG. Together, our results demonstrate that neither TET1 nor TET2 is sufficient for the induction of pluripotency (Fig. 2c, Supplementary Figs 10 and 11, and Fig. 3c), but either enzyme can partner with NANOG to enhance reprogramming of somatic cells to naive pluripotency.

We compared deposited chromatin immunoprecipitation coupled with DNA sequencing (ChIP-Seq) data for both NANOG[17,18] and TET1[19,20] and found a statistically significant overlap between NANOG and TET1 binding sites in the mouse embryonic stem cell genome ($P < 2 \times 10^{-4}$, permutation test) (Fig. 4a and Supplementary Table 3). Gene ontology analysis revealed that genes with roles in 'multicellular organismal development' and 'positive regulation of transcription from Pol II promoter' are enriched in the common

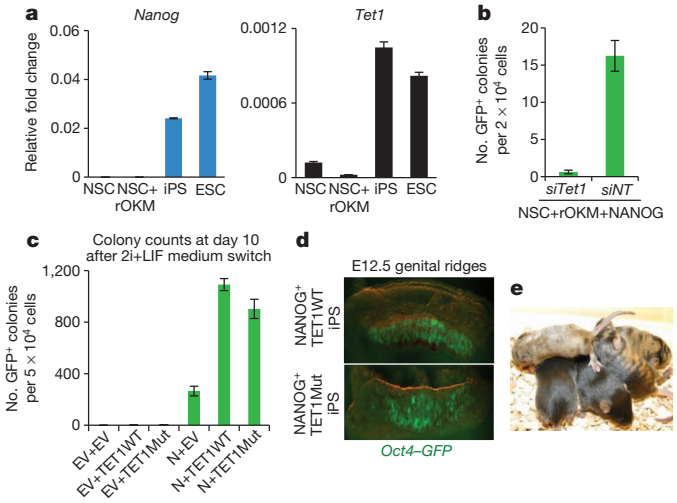

Figure 2 | Synergy between NANOG and TET1 during reprogramming. **a**, NANOG and TET1 are specifically expressed in pluripotent cells. ESC, embryonic stem cells; NSC, adult neural stem cells; +rOKM, transduced with retroviral *Oct4*, *Klf4* and *c-Myc* transgenes. **b**, Knockdown of TET1 compromises reprogramming activity of a constitutive NANOG transgene in reprogramming intermediates. *siNT*, non-targeting siRNA control; *siTet1*, siRNA against *Tet1*. Sequences for quantitative PCR primers and siRNA targets are listed in Supplementary Table 5. **c**, Both wild-type and mutant TET1 enhance NANOG-dependent reprogramming. Quantification of the number of iPS colonies at day 10 of 2i+LIF (dual inhibition of mitogen-activated protein kinase signalling (PD0325901, 1 μM) and glycogen synthase kinase-3 (GSK3) (CHIR99021, 3 μM) with leukaemia inhibitory factor) treatment is shown in Supplementary Fig. 10b. EV, empty vector; N, NANOG. **d**, **e**, Contribution of iPS cells generated with NANOG and TET1WT (top) or TET1Mut (bottom) transgenes to the germline at E12.5 (**d**) and live-born chimaeras (**e**). *Oct4-GFP*, *GFP* gene driven by *Oct4* regulatory sequences. Error bars indicate standard deviation ($n = 3$).

targets (Supplementary Fig. 14). We ranked the common target genes of NANOG and TET1 based on the number of overlapping ChIP-Seq peaks in four studies (Fig. 4b and Supplementary Table 4). Among the common targets with the highest number of overlapping NANOG and TET1 peaks was *Esrrb* (Fig. 4b). To investigate whether NANOG may be required to direct TET1 to shared target genes, we used embryonic stem cells containing an inducible *Nanog* transgene in a *Nanog*$^{-/-}$ background[21] (Fig. 4c, d). Loss of NANOG expression reduced TET1 binding to a number of common targets, including *Esrrb* (Fig. 4e, f and Supplementary Fig. 15). NANOG-dependent binding of TET1 to the *Esrrb* locus seems to be independent of OCT4, as OCT4 is not present at the same genomic location (Fig. 4e). Thus, NANOG is responsible for the recruitment of TET1 to a subset of shared genomic loci that are implicated in both the regulation of pluripotency (for example, *Esrrb*) and lineage commitment (for example, *Pax6*). Such NANOG-dependent target binding of TET1 is highlighted by the fact that the truncated form of TET1 lacking the CXXC DNA binding domain (that is, TET1C) maintains its physical interaction and reprogramming synergy with NANOG (Fig. 3b–d).

Because the functional synergy between NANOG and TET enzymes was dependent on catalytic activity, we examined 5hmC levels at NANOG/TET1 peaks in mouse embryonic stem cells. A recent study reported 5hmC enrichment at promoter-distal NANOG binding sites in human embryonic stem cells[22] (Supplementary Fig. 16a). In contrast, we observed an inverse correlation between 5hmC and NANOG/TET1 binding at actively expressed target genes in mouse embryonic stem cells (Supplementary Fig. 16). This led us to consider whether 5hmC may be transiently deposited to common NANOG/TET1 targets before the establishment of pluripotency, that is, during *in vitro* reprogramming when NANOG is required[3]. We focused on target gene regulation of *Esrrb* and *Oct4*, two key pluripotency genes that are among top ranked common targets of NANOG and TET1 (Fig. 4b). Significantly, we observed expression priming of both *Esrrb* and *Oct4* by combined expression of NANOG with TET1WT, TET1Mut, TET1C or TET2 in reprogramming intermediates of two

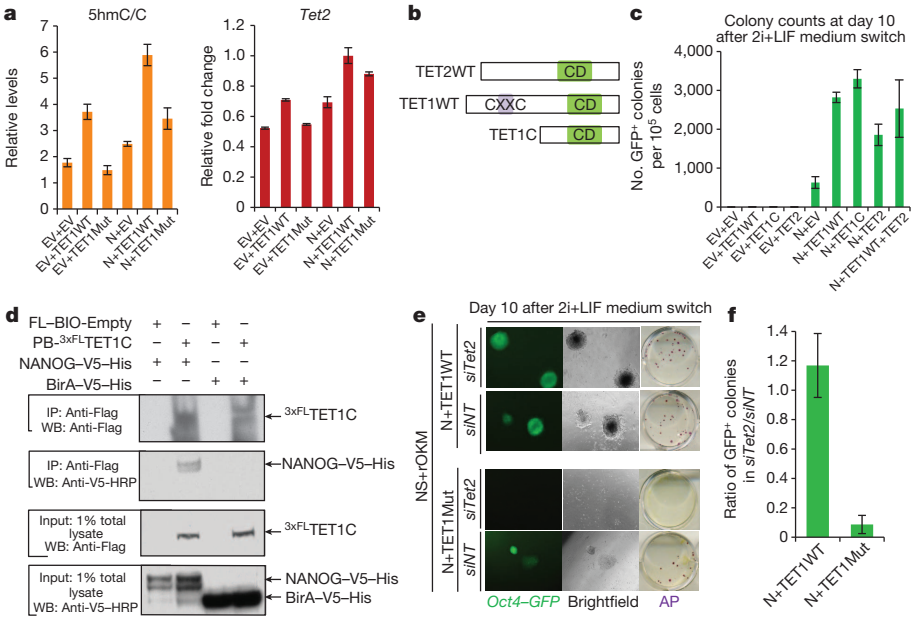

Figure 3 | Synergy between NANOG and TET1/TET2 during reprogramming is dependent upon catalytic activity of TET1/TET2. **a**, Measurement of global levels of 5hmC relative to unmodified C (left, 5hmC/C) and TET2 expression (right) in reprogramming intermediates transfected with PiggyBac (PB) transgenes. **b**, Schematic depiction of wild-type TET1 and TET2 (TET1WT and TET2WT, respectively), and the truncated TET1 mutant (TET1C). Note the absence of a CXXC DNA binding domain in TET2WT and TET1C proteins. CD, catalytic domain. **c**, Quantification of GFP$^+$ iPS colonies. **d**, Physical association of NANOG with TET1C. Co-immunoprecipitation was performed in HEK293T cells. HRP, horseradish peroxidase. **e**, TET2 knockdown (*siTet2*) reduces reprogramming efficiency in intermediate cells transgenic for NANOG+TET1Mut compared to NANOG+TET1WT. Non-targeting siRNA (*siNT*) serves as a control. **f**, Quantification of the number of iPS colonies in (**e**). Error bars indicate standard deviation ($n = 3$).

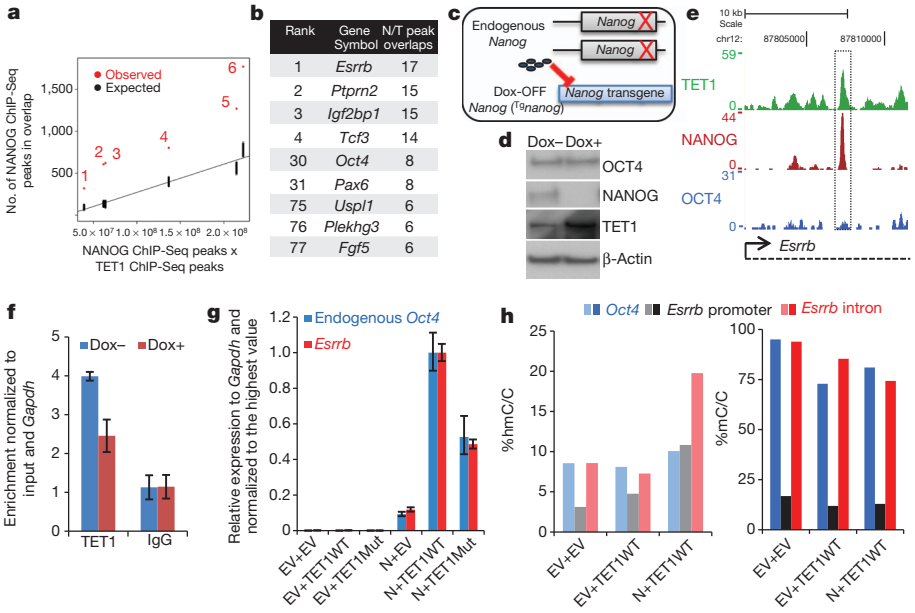

Figure 4 | Mechanism and genome-wide significance of the NANOG–TET1 interaction. **a**, Scatterplot showing the observed against expected overlap in genomic binding sites of NANOG and TET1 according to comparisons performed in Supplementary Table 3. **b**, Ranked list of common targets of NANOG and TET1 based on the comparisons in Supplementary Table 3. **c**, Schematic representation of embryonic stem cells harbouring a doxycycline (Dox)-suppressible NANOG transgene in a *Nanog*$^{-/-}$ genetic background[21]. **d**, Western blot analysis of OCT4, NANOG, and TET1 expression in NgcKO embryonic stem cells treated with (+) or without (−) Dox. **e**, Overlapping peaks of TET1 and NANOG from ChIP-Seq studies[17–19] in the *Esrrb* locus. **f**, Relative enrichment of TET1 in the absence (-) and presence (+) of Dox in the *Esrrb* genomic locus as shown in (**e**). **g**, Transcriptional priming of *Esrrb* and *Oct4* by NANOG and TET1 in reprogramming intermediates. **h**, Relative enrichment of 5hmC and 5mC in the *Esrrb* and *Oct4* loci. Error bars indicate standard deviation ($n = 3$). Primers used for glucosylated hydroxymethyl-sensitive qPCR are listed in Supplementary Table 6.

independent cellular systems (Fig. 4g and Supplementary Figs 11g and 13b). More importantly, we detected increased 5hmC and decreased 5-methylcytosine (5mC) levels at these loci when NANOG is co-expressed with TET1 (Fig. 4h). Thus, NANOG and TET1 act before the transition to naive pluripotency by inducing local transcriptional changes in shared target genes that are critically involved in the regulation of pluripotency.

In summary, we identified 5mC hydroxylases TET1 and TET2 as novel interaction partners of NANOG. TET1/TET2 and NANOG synergistically enhance the efficiency of reprogramming and this phenotype is dependent on the hydroxylation of 5mC to 5hmC during somatic cell reprogramming. This study thus provides mechanistic insight into how NANOG establishes pluripotency, demonstrating that interactions between NANOG and epigenetic regulators fine-tune induced pluripotency. Future experimental work is needed to delineate the precise composition of NANOG–TET1/TET2 protein complexes, and the contribution of other interaction partners to the reprogramming mechanism described herein. Our work supports an emerging view that TET proteins can overcome epigenetic roadblocks during reprogramming and transdifferentiation[16,23].

METHODS SUMMARY

Affinity purification coupled with mass spectrometry (AP-MS). Nuclear extraction and affinity purification of Flag–biotin–NANOG-associated protein complexes were performed as previously described[5], with several modifications as described[7]. Three biological replicates were performed for streptavidin agarose-based affinity purification and one each for Flag and NANOG antibody-based affinity purifications. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used by the Taplin Biological Mass Spectrometry Facility at Harvard Medical School to sequence and identify NANOG affinity purification samples.

Reprogramming assays. To investigate the consequences of NANOG and TET1 co-expression during reprogramming, adult neural stem cells were infected with pMX-based retroviral reprogramming factors[24]. Cultures were switched to embryonic stem cell medium (serum/leukaemia inhibitory factor (LIF)) at day 3 post-transduction. A clonal line of proliferative, *Oct4*-GFP negative cells (reprogramming intermediates) was transfected using nucleofection (Amaxa) with various combinations of NANOG and TET1 PiggyBac transgenes. Selection for stable transgene expression was applied to transfectants for a minimum of 12 days and maintained until medium switch to 2i (dual inhibition of mitogen-activated protein kinase signalling (PD0325901, 1 μM) and glycogen synthase kinase-3 (GSK3) (CHIR99021, 3 μM))+LIF. Puromycin selection for an *Oct4-GFP*-IRES-*puro* reporter transgene was applied from day 6 of 2i+LIF treatment. GFP-positive colonies were scored at day 10. Similar reprogramming assays were applied to *Nanog-GFP*-IRES-*puro* reporter mouse embryonic fibroblasts with modifications described in Supplementary Information.

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Supplementary Information is available in the online version of the paper.

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Author Contributions J.C.R.S. and J.W. conceived the project, designed the experiments, prepared and approved the manuscript. T.W.T. designed and performed experiments and wrote the manuscript draft. Y.C., J.D., F.F., M.F. and A.S. designed and performed experiments and prepared the manuscript. T.A.H. designed and performed experiments. P.V.S. performed interactomics data analysis. M.L. provided technical assistance. S.Di. provided bioinformatic analysis. S.Da., D.N.L., Z.L. and M.X. contributed to the reagents. W.R. designed experiments and contributed to the reagents. J.C.R.S. and J.W. are equal senior and corresponding authors.

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