RESEARCH ARTICLE

ZMYM2 inhibits NANOG-mediated reprogramming [version 1; peer review: 2 approved, 1 approved with reservations]

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Abstract

Background: NANOG is a homeodomain-containing transcription factor which forms one of the hubs in the pluripotency network and plays a key role in the reprogramming of somatic cells and epiblast stem cells to naive pluripotency. Studies have found that NANOG has many interacting partners and some of these were shown to play a role in its ability to mediate reprogramming. In this study, we set out to analyse the effect of NANOG interactors on the reprogramming process.

Methods: Epiblast stem cells and somatic cells were reprogrammed to naive pluripotency using MEK/ERK inhibitor PD0325901, GSK3β inhibitor CHIR99021 and Leukaemia Inhibitory Factor (together termed 2i Plus LIF). Zmym2 was knocked out using the CRISPR/Cas9 system or overexpressed using the PiggyBac system. Reprogramming was quantified after ZMYM2 deletion or overexpression, in diverse reprogramming systems. In addition, embryonic stem cell self renewal was quantified in differentiation assays after ZMYM2 removal or overexpression.

Results: In this work, we identified ZMYM2/ZFP198, which physically associates with NANOG as a key negative regulator of NANOG-mediated reprogramming of both epiblast stem cells and somatic cells. In addition, ZMYM2 impairs the self renewal of embryonic stem cells and its overexpression promotes differentiation.

Conclusions: We propose that ZMYM2 curtails NANOG’s actions during the reprogramming of both somatic cells and epiblast stem cells and impedes embryonic stem cell self renewal, promoting differentiation.

Keywords
Nanog, reprogramming, Zinc finger protein, pluripotency, differentiation

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Introduction

Reprogramming is the process whereby a somatic cell is reverted back to a pluripotent state. Pluripotent cells possess the ability both to self-renew and to differentiate into cells from any of the three germ layers of the adult organism. Reprogramming can be carried out by overexpressing only four factors in somatic cells: Oct4, Klf4, Sox2 and cMyc. Together, these factors reset the transcriptional and epigenetic state of the cell to those of a pluripotent cell. Much work has been carried out on factors which can execute or promote this transition. These include many members of the pluripotency-associated transcription factor network\(^1\). \textit{Nanog} is a homeodomain-containing transcription factor which constitutes one of these key factors.

\textit{Nanog} was first discovered for its ability to promote embryonic stem cell (ESC) self-renewal in the absence of LIF and for its association with the pluripotent state as opposed to somatic identities\(^2\). \textit{Nanog} is also essential for the establishment of the pluripotent naïve epiblast\(^3\). Thus, \textit{Nanog} plays a central role in the promotion of the pluripotent state, both \textit{in vitro} and \textit{in vivo}.

As a key hub of the pluripotency network, studies have been carried out aiming at understanding \textit{Nanog}’s mode of action. One approach was to define its interactome, which led to the identification of multiple interactors\(^4\)\(^\text{-}\)\(^12\). Some of these are chromatin modifiers that were shown to augment the ability of \textit{Nanog} to mediate reprogramming. These include the NuRD complex\(^13\) and the TET family proteins\(^14\). Importantly, we still do not know if most of the identified interactors play a role, either positive or negative, in the mechanism of action of \textit{Nanog}. In order to address this, we set out to analyse the effect of additional \textit{Nanog} interactors on \textit{Nanog}-mediated reprogramming. This work enabled us to identify ZMYM2/ZFP198, which physically associates with \textit{Nanog}\(^13\)\(^,\)\(^11\)\(^,\)\(^12\)\(^,\)\(^14\), as a key protein impairing \textit{Nanog}’s activity in both reprogramming and the self-renewal of naïve pluripotent stem cells.

Methods

Cell culture

Mouse ESCs, iPS cells and pre-iPS cells were cultured in Glasgow Minimum Essential Medium (GMEM; Sigma, G5154) containing 10% foetal calf serum (FCS; Life Technologies, 10091-148), 1x non-essential amino acids solution (NEAA; PAA, M11-003), 1 mM sodium pyruvate (PAA, S11-003), 0.1 mM 2-mercaptoethanol (Invitrogen, 31350-010), 2mM L-glutamine (Invitrogen, 25030024), 1x Pen/Strep (PAA, P11010) and 20 ng/mL LIF (Department of Biochemistry, University of Cambridge). This medium will hereafter be referred to as Serum Plus LIF. These cells were grown on plastic dishes which had been coated with 10 μg/mL laminin (Sigma, L6074) in PBS and washed once in PBS.

Neural stem cells (NSCs) were cultured in DMEM/F12 (Life Technologies, 11330-057) containing 27.4 mM glucose, 1x NEAA (PAA, M11-003), 1x Pen/Strep (PAA, P11010), 4 mM HEPES (Life Technologies, 15630-049), 0.011% Bovine serum albumin, 1x N2 (WT/MRC SCI, University of Cambridge), 1x B27 (Life Technologies, 17504-044), 0.1 mM 2-mercaptoethanol (Invitrogen 31350-010), 10 ng/mL of epidermal growth factor (EGF; Peprotech, 315-09) and 20 ng/mL fibroblast growth factor 2 (FGF2; WT/MRC SCI, University of Cambridge). They were cultured on plastic dishes which had been coated for at least 3 h with 10 μg/mL laminin (Sigma, L2020) in PBS and washed once in PBS.

Cell lines

Oct4 reporter EpiSCs and NSCs were used as previously detailed and contained an Oct4-GFP-IRES-puro reporter transgene in which enhanced green fluorescent protein (eGFP) is expressed under the control of Oct4 (Pou5f1) regulatory elements\(^15\)\(^,\)\(^16\). \textit{Nanog}-GFP-IRES-puro reporter NSCs were also used as previously generated and these contained GFP inserted heterozygously into the AUG start codon of one endogenous \textit{Nanog} allele\(^15\)\(^,\)\(^17\). \textit{Nanog}\(^-\) pre-iPSCs had been previously generated in the lab by the retroviral transduction of \textit{Nanog}\(^-\) NSCs isolated from E12.5 forebrain with \textit{Oct4}, \textit{Klf4} and \textit{cMyc}\(^10\). E14tg2a ESCs were used for all self-renewal assays\(^18\).

siRNA transfection

FlexiTube siRNA solutions (Qiagen) were used to knock down expression of the following genes: Znym2 (GS76007), Zfp281 (GS226442) and Nrithb1 (GS11614). All Star negative control siRNA was also used (1027281). Transfection was carried out with Lipofectamine RNAi Max (Life Technologies, 13778030). Medium was changed to medium containing MEK/ERK inhibitor PD0325901, GSK3β inhibitor CHIR99021 (WT/MRC SCI, University of Cambridge) and Leukaemia Inhibitory Factor (Department of Biochemistry, University of Cambridge) (together termed 2i Plus LIF)\(^9\) with Penicillin/Streptomycin 24 h after transfection and the cells were allowed to reprogram for 12 days. Green colonies, resulting from the expression of a Oct4-GFP reporter\(^11\)\(^,\)\(^13\)\(^,\)\(^20\), were monitored using a Leica epifluorescent DMI4000 microscope at 488nm as a readout of reprogramming efficiency.

Measurement of pluripotency-associated gene expression by qPCR

Total RNA was extracted from cells using an RNeasy mini kit (Qiagen, 74106), with DNase treatment (Qiagen, 79254). cDNA synthesis was performed using the Superscript III kit (Life Technologies, 11330-057), 1X N2 (WT/MRC SCI, University of Cambridge), 1X B27 (Life Technologies, 17504-044), 2mM L-glutamine (Invitrogen, 25030024), 1X Pen/Strep, 0.1 mM 2-mercaptoethanol (Invitrogen 31350-010).

Epiblast stem cells (EpiSCs) were cultured in N2B27-containing medium supplemented with 12.5 ng/mL FGF2 (WT/MRC SCI, University of Cambridge) and 20 ng/mL Activin A (WT/MRC SCI, University of Cambridge). They were grown on dishes which had been coated with 10 μg/mL Human recombinant fibronectin (Millipore FC010) in PBS for 30 min at room temperature.
Technologies, 11752-250) in accordance with the manufacturer’s protocol. RT-qPCR was carried out in microAmp qPCR plates (Life Technologies, 434690) on a StepOne Plus Real-Time PCR machine (Applied Biosystems) using TaqMan Fast Universal MasterMix (Applied Biosystems, 4352042) and expression levels were calculated by ΔCt to Gapdh. Mean expression levels were determined by averaging triplicate wells. TaqMan amplification was performed as follows: 2 min at 50 °C, 20 sec at 95 °C, (1 sec at 95 °C, 20 sec at 60 °C) x 40. Probes used are presented in Table 1.

Reprogramming neural stem cells and mouse embryonic fibroblasts

Retroviral reprogramming vectors (pMXs-Oct4 (13366), pMXs-Klf4 (13370), pMXs-Sox2 (13367) and pMXs-cMyc (13375)) were obtained from the Addgene repository. PLAT-E cells were transfected with these using FuGene (Promega E2311). The medium containing retroviral particles was collected from the PLAT-E cells and filtered through a 0.45 μm filter. Neural stem cells (NSCs) were transduced with retroviral (r) Oct4, cMyc and Klf4 whereas MEFs were transduced with these and rSox2. 4μg/ml polybrene (Sigma Aldrich, TR-1003) was added for transduction.

24 h after transduction, the virus-containing medium was aspirated from the NSCs or MEFs and replaced with the cells’ respective media. Four days after transduction, the medium was replaced with Serum-containing medium supplemented with Leukaemia Inhibitory Factor (Serum Plus LIF). The cells slowly became more proliferative and acquired pre-iPS cell-like morphology. If the pre-iPS were being reprogrammed in the same well, the medium was switched to medium containing MEK/ERK inhibitor PD0325901, GSK3β inhibitor CHIR99021 (WT/MRC SCI, University of Cambridge) and Leukaemia Inhibitory Factor (Department of Biochemistry, University of Cambridge) (together termed 2i Plus LIF) 4 days after the application of Serum Plus LIF (8 days after retroviral transduction).

Cells were then stably transfected with PiggyBac (PB) Nanog transgenes, and subjected to transient transfection with siRNA before reprogramming in 2i Plus LIF. Oct4- or Nanog-GFP+ colonies were counted 12 days later (see cell line section for details).

Zmym2 overexpression

Overexpression vectors were generated using Gateway cloning (Invitrogen) and PiggyBac vectors. Cells were transfected using Lipofectamine 2000 (ThermoFisher Scientific 11668019) and selected for 14 days with either hygromycin or blasticidin (WT/MRC SCI, University of Cambridge).

CRISPR/Cas9 generation of Zmym2-/- EpiSC and NSC cells

A double-stranded break was induced 108 amino acids after the start codon of Zmym2, inducing frameshift mutations in both alleles. Pools of clones were screened by T7 assay, which involves the annealing of PCR products from the edited locus to PCR products from the WT locus. These double stranded fragments are then digested with T7 endonuclease, which cuts the imperfectly annealed strands. These cut products can then be visualised using agarose gel electrophoresis. Single transfected cells were then sorted and analysed for ZMYM2 knockout by Western Blotting (see Table 2 for antibodies used). Clones were selected which had no intact Zmym2 alleles and were stably transfected with Nanog or Zmym2 transgenes or both, or their corresponding empty vector transgenes.

Antibodies

Self-renewal assay for ESCs. Zmym2 was stably overexpressed in ESCs or knocked out by CRISPR/Cas9 as detailed above. These cells were plated alongside Empty Vector (EV) controls in Serum-containing medium with or without LIF for 6 days. Alkaline-phosphatase staining (Sigma, 86R-1KT) was carried out and colonies were scored by both morphology and alkaline phosphatase staining.

Table 1. Probes used for qPCR.

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Table 2. Antibodies used for western blots.

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Transcriptome analysis
mRNA was extracted with a RNeasy kit (Qiagen, 74106), with DNAse treatment (Qiagen, 79254). It was quantified using Agilent Bioanalyzer Nano Chips (Agilent Technologies). Depletion of ribosomal RNA was performed on 2-5 μg of total RNA using the Ribo-Zero rRNA Removal Kit (Illumina) and libraries were produced from 10-100ng of ribosomal-depleted RNA using NextFlex Rapid Directional RNA-seq Kit (5138-07; Bioo Scientific), a Biorad C1000 thermocycler, and standard Illumina primers. Cycling conditions were as follows: 30 min at 37°C, 2 min at 98°C, (30 sec at 98°C, 30 sec at 65°C, 60 sec at 72°C) x 12, 4 min at 72°C. Libraries were pooled in equimolar quantities and sequenced on the HiSeq4000 platform (Illumina), using V4 chemistry.

RNA-seq reads were adaptor-trimmed with TrimGalore (version 0.3.7) and mapped to the mouse reference genome (GRCm38/mm10) with TopHat2 (version 2.2.3). Strand-specific read counts were obtained with featureCounts (version 1.4.5). Transcript counts were normalised, and the statistical significance of differential expression between samples was assessed using the R Bioconductor DESeq2 (version 1.4.5) package. Transcript counts normalized by DESeq2 size factors were subsequently normalized by their length.

Blastocyst injection and animal husbandry
Chimeras were generated from mouse strain 129 (agouti coat color) iPSCs by standard microinjection methodology at the Wellcome Trust/MRC Cambridge Stem Cell Institute. Briefly, host blastocysts of strain C57BL/6 (black coat colour) were injected at E4.5, followed by gestation in pseudo-pregnant recipient females25. These females were 6–10 weeks old and 25–30g. The resulting chimeras were then bred with WT mice and the pups analysed by coat colour for contribution of the iPSC-derived cells. The use of animals in this project was approved by the Animal Welfare and Ethical Review Body for the University of Cambridge (Procedure Project Licenses P76777883 and 80/2597). Mice were housed in individual ventilated cages with up to 5 animals per cage. Stud males were individually caged and females were housed in groups, with wood chips and mouse bedding plugs on the cage floor. The mouse facility was a barrier facility with 10 hours darkness and 14 hours light per day. The temperature was maintained at 22 °C. Food and water were provided ad libitum. Cages contained environmental enrichment for the mice, including wooden blocks and perspex houses. All animals were checked on a daily basis by trained animal house staff, but there are no welfare issues expected from the embryo transfer procedure, which is performed routinely by the dedicated transgenic facility manager. Every effort was made to reduce the numbers of animals used and the stress or discomfort caused to animals in this study. The final assay result is coat colour of the pups, and did not involve any invasive or stressful procedures. Further details regarding the mice used are presented in Table 3.

Results

Zmym2 impairs Nanog-mediated reprogramming in EpiSCs
In this study, we aimed to characterise potential regulators of Nanog’s activity during reprogramming. We compared NANOG interactomes11,12 and selected ZMYM2 and NR0B1 as candidates of interest due to these being high confidence interactors. ZFP281 was selected as a control, as knocking it down had been previously demonstrated to increase Nanog-mediated reprogramming efficiency10. In order to address whether these factors impact Nanog-induced reprogramming, Nanog-overexpressing EpiSCs, which reprogram at low efficiency9,20, were transiently transfected with siRNA against the target genes of interest (Figure 1A). The medium was then swapped to medium containing the MEK/ERK inhibitor PD0325901 and the GSK3β inhibitor CHIR990219. This medium will hereafter be referred to as (2i) plus LIF medium (2i Plus LIF). This medium promotes reprogramming9. These cells express enhanced green fluorescent protein (eGFP), under the control of Oct4 (Pou5f1) regulatory elements, making the cells GFP when fully reprogrammed to naïve pluripotency11,14. As a readout of reprogramming efficiency, Oct4-GFP+ colonies were counted 12 days after the application of 2i Plus LIF.

Nr0b1 knockdown (KD) did not alter reprogramming efficiency. Zfp281 KD increased Nanog mediated reprogramming efficiency, consistent with a previous report24. Interestingly, reprogramming efficiency was robustly increased by Zmym2 KD (Figure 1A). Zmym2 transcript and protein levels were reduced by all four siRNAs by qPCR and by Western blot (Figure 1B and 1C respectively). 48h after transfection. This contrasts with the action of many other NANOG interactors as activators of reprogramming11,13 and suggests that Zmym2 impedes Nanog-mediated reprogramming. The iPSCs generated after Zmym2 KD were characterised and had gene expression profiles consistent with the acquisition of naïve pluripotency (Figure 1D) and upon injection into C57Bl6 mouse host blastocysts, chimeras were produced (Figure 1E). Zmym2 KD iPSCs also exhibited germline competence (Figure 1F). This indicates faithful iPSC reprogramming following Zmym2 KD.

Table 3. Details of mice used for chimera contribution assay. Zmym2 KD or OE iPSCs were injected into E4.5 C57Bl6 host blastocysts. The resulting embryos were implanted into pseudopregnant females and the pups analysed for iPSC contribution by coat colour. M:male, F:female, GLT: germline transmission.

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**Figure 1. Zmym2 is a repressor of Nanog-mediated epiblast stem cell (EpiSC) reprogramming.**

**a.** 2000 EpiSCs were transiently transfected with siRNA against the indicated targets and reprogrammed by Nanog overexpression in 2i Plus LIF. Oct4-GFP⁺ colonies were scored on day 12. Data represent the mean number of Oct4-GFP⁺ colonies from two replicates +/- SEM. Example colonies are shown in the inset panel.

**b.** RT-qPCR analysis of EpiSC lines 48h after Zmym2KD. Data are the mean normalized expression level from 3 technical replicates +/- SD.

**c.** Western Blot of EpiSC lines 48h after Zmym2KD with alpha tubulin shown as a loading control.

**d.** Gene expression analysis of the parent EpiSCs and resulting iPSCs by qPCR. Data are the mean normalized expression levels from 3 technical replicates +/- SD.

**e.** iPSCs which emerged from Zmym2KD EpiSCs were injected into C57Bl6 blastocysts to generate chimeras, which can be seen from their coat colour (brown fur conferred by iPSC contribution).

**f.** Germine transmission of the iPSCs (brown pup from iPSCs shown with its chimera mother and black father).

**g.** Western Blot of lines of empty vector (EV) and NANOG-overexpressing (Nanog) EpiSCs with alpha tubulin shown as a loading control.

**h.** GFP⁺ colony count of EV- and Nanog-overexpressing EpiSCs when reprogrammed in the presence of siRNA against Zmym2 or control siRNA. Data represent the mean +/- SEM of two independent experiments.
Given that these EpiSCs overexpressed Nanog to promote reprogramming, we then investigated whether Zmym2KD is sufficient to reprogram EpiSCs in the absence of any transgenic reprogramming factors. In order to address this, EpiSCs overexpressing Nanog or a corresponding empty vector (EV) transgene (Figure 1G) were transfected with siRNA against Zmym2 and transferred to reprogramming conditions. Zmym2KD had a pronounced positive effect on Nanog-induced reprogramming but a minimal effect on the reprogramming of EV EpiSCs (Figure 1H). Therefore, Zmym2KD relies on the exogenous expression of Nanog in order to robustly enhance reprogramming.

Zmym2 impairs Nanog-mediated somatic cell reprogramming

All experiments described so far had been carried out in EpiSCs. We used reprogramming intermediates generated from neural stem cells (NSCs) through retroviral expression of Oct4, Klf4 and cMyc, to address whether Zmym2 might also inhibit Nanog-induced reprogramming in a somatic cell context. These cells were stably transduced with a PiggyBac (PB) Nanog transgene and subjected to transient transfection with either control or Zmym2 siRNA. In keeping with the results obtained in EpiSCs, Zmym2KD increased somatic cell reprogramming more than two-fold (Figure 2A).

To ascertain whether Zmym2KD could reprogram somatic cells in the absence of Nanog, the same experiment was carried out in Nanog−/− somatic cells. These were stably transduced with a rescue Nanog transgene or a corresponding EV transgene, and reprogrammed after control KD or Zmym2KD. As seen in Figure 2B, Zmym2KD also enhanced Nanog-mediated reprogramming in neural stem cell derived reprogramming intermediates. Zmym2KD was not sufficient to overcome the requirement for Nanog in somatic cell reprogramming, though we confirmed that it enhances Nanog-mediated reprogramming in this context.

As Zmym2KD increases Nanog-mediated reprogramming efficiency, we decided to carry out the converse experiment and investigate whether Zmym2 overexpression could impair reprogramming. Four lines of EpiSCs were generated which stably overexpressed either Nanog, Zmym2, or both (Figure 2C), and these were induced to reprogram by transfer to 2i Plus LIF medium. As expected, Nanog overexpression resulted in efficient EpiSC reprogramming while Zmym2 overexpression alone had no reprogramming activity (Figure 2D). However, when Zmym2 overexpression was combined with Nanog overexpression, it reduced reprogramming efficiency 8-fold relative to Nanog alone (Figure 2D). To test this result in an independent cell system mouse embryonic fibroblast (MEF)-derived reprogramming intermediates expressing retroviral Oct4, Klf4, cMyc and Sox2 and a Nanog transgene were transfected with either Empty Vector (EV) or a Zmym2 expression cassette (Figure 2E). Nanog alone led to highly efficient complete reprogramming (Figure 2F, G, H) whereas the addition of Zmym2 completely prevented reprogramming.

In order to investigate the effect of Zmym2 loss in reprogramming, Zmym2−/− EpiSCs were generated by CRISPR/Cas9-mediated mutagenesis (Figure 3A). WT and Zmym2−/− EpiSCs were then stably transfected with Nanog or Zmym2 or both (Figure 3A) and allowed to reprogram. Similar to previous results, Zmym2 overexpression decreased Nanog-mediated reprogramming in wild type cells (Figure 3B). In agreement with KD experiments, Zmym2 knockout increased Nanog-induced reprogramming by about 4-fold (Figure 3B). This effect was rescued by the addition of transgenic Zmym2 (Figure 3B). WT and Zmym2+/− iPSCs were indistinguishable by gene expression analysis of pluripotency-associated markers (Figure 3C).

To verify this result in an independent cell system, WT or Zmym2−/− NSCs were also generated by CRISPR/Cas9-mediated mutagenesis (Figure 3D). They were then retrovirally transduced with Oct4, Klf4 and cMyc and allowed to reprogram. As in EpiSCs, Zmym2−/− NSCs reprogrammed with much higher efficiency than their WT counterparts (Figure 3E, F). Both WT and Zmym2+/− iPSCs had gene expression profiles similar to those of control ESCs, demonstrating complete reprogramming (Figure 3G). Both WT and Zmym2−/− NSC lines were then stably transfected with Nanog, Zmym2, or both, to create a rescue system for reprogramming (Figure 3D). Again, Zmym2 knockout increased Nanog-induced reprogramming by 3-fold (Figure 3H), whereas its overexpression eliminated the enhancement of reprogramming by Nanog (Figure 3H).

Zmym2 reduces ESC self-renewal

Zfp281 is known to enable Nanog autorepression as so we tested whether Zmym2 levels had any effect on Nanog transcript levels. Neither KD nor overexpression lines had any change in Nanog transcript or protein levels (Figure 1B, 1C, Figure 2C, Figure 3C, 3G), suggesting that Zmym2 does not act through the regulation of Nanog expression.

Nanog was first discovered for its role in the self-renewal of ESCs. As ZMYM2 is a NANOG interactor, we hypothesised that it might also inhibit Nanog’s self-renewal-promoting capacity. In order to address this, Zmym2 was stably transduced into ESCs. These cells were plated alongside EV controls (Figure 4A) in Serum-containing medium with LIF for 6 days, to maintain pluripotency in some cells while allowing others to differentiate. Alkaline-phosphatase staining was carried out and colonies were scored. Zmym2-overexpressing ESCs exhibited greater spontaneous differentiation than control ESCs (Figure 4B, C).

In order to investigate whether Zmym2KO impedes ESC differentiation, both Zmym2 alleles were knocked out using the CRISPR/Cas9 system as previously described. ZMYM2−/− cells and FLAG-tagged Zmym2 rescue cells were generated (Figure 4D). These lines were plated alongside the parental WT ESC line in the absence of LIF for 6 days, alkaline phosphatase stained and colonies were scored by morphology. Zmym2KO increased the proportion of undifferentiated colonies (Figure 4E). This was rescued by transgenic Zmym2 expression. This is in agreement with a recently published Cas9 ESC differentiation screen which demonstrated that Zmym2KO ESCs resist differentiation.

To address the global effects of Zmym2 loss on the transcriptome, mRNA from Zmym2 KO, WT and overexpressing ESCs (Figure 5A, B) were subjected to mRNA-Seq after culture in
Figure 2. Zymym2 inhibits somatic cell reprogramming in a Nanog-dependent manner. a, Neural stem cells (NSCs) were reprogrammed with retroviral Oct4, Klf4 and cMyc and with constitutive transgenic Nanog expression in 2i Plus LIF in the presence of Zymym2 or control siRNA. Colony count per 75,000 plates NSCs. Average of three independent experiments. **** p<0.0005 by Student’s T-test. b, Average GFP+ colony count per 10,000 Nanog+ pre-iPSCs reprogrammed in the presence of Zymym2 or control KD, two replicates +/-SEM. c, Gene expression analysis of EpiSC lines stably overexpressing Nanog, Zymym2, or both by RT-qPCR. d, Average GFP+ colony count on D12 after 2i Plus LIF application per 25,000 plated EpiSCs, three replicates. e, Gene expression analysis by qPCR of MEF-derived pre-iPSCs stably overexpressing Nanog+EV or Nanog+Zymym2, with ESC control. f, Fluorescence and brightfield images of Oct4-GFP+ colonies on D12. g, Average GFP+ colony count on D12 per 50,000 plates pre-iPSCs, three replicates. h, Alkaline phosphatase staining on D12. Reprogramming counts are shown as mean +/-SEM. qPCR quantifications are shown as mean of three technical replicates +/-SD, normalised to GAPDH transcript levels.
Figure 3. Zmym2 knockout enhances Nanog-mediated reprogramming in epiblast stem cells (EpiSCs) and somatic cells. 

a. Western blot analysis of starting populations of EpiSCs. Both alleles of Zmym2 were disrupted with the CRISPR/Cas9 system. Nanog, Zmym2 or both were stably overexpressed.

b. Average GFP+ colony count of reprogrammed EpiSCs on D12 for Zmym2−/− and WT EpiSCs, per 50,000 cells plated, three replicates.

c. Gene expression analysis of the resulting iPSCs shows them to be faithfully reprogrammed, in contrast to the starting population of EpiSCs. Clustal Omega multiple sequence alignment of KO clones with the WT sequence and reverse complement sequencing traces of Zmym2 KO EpiSCs and NSCs.

d. Western blot analysis of starting populations of NSCs. Both alleles of Zmym2 were disrupted with the CRISPR/Cas9 system. Nanog, Zmym2 or both were stably overexpressed.

e. Average iPSC colony count after reprogramming of Zmym2−/− and WT NSCs per 75,000 cells plated.

f. Fluorescence and brightfield images of iPSCs generated from Zmym2−/− and WT NSCs after retroviral Oct4, Klf4 and cMyc overexpression and exposure to 2i Plus LIF. Scale bar 500μm.

g. Gene expression analysis of the resulting WT and Zmym2−/− iPSCs by qPCR. NSCs were stably transfected with Nanog, Zmym2 or both and reprogrammed with retroviral Oct4, Klf4 and cMyc in 2i Plus LIF. Average colony count for Zmym2−/− and WT NSCs per 75,000 cells plated, three replicates. Reprogramming counts are shown as mean +/- SEM. qPCR quantifications are shown as mean of three technical replicates +/- SD, normalised to Gapdh transcript levels.
Figure 4. Zmym2 inhibits embryonic stem cell (ESC) self-renewal. a. Western blot analysis of ESCs stably overexpressing Zmym2 or a corresponding EV transgene. 
b. Brightfield images of alkaline phosphatase stained plates of EV- or Zmym2-overexpressing ESCs after 6 days in medium containing either serum or Serum+LIF. Scale bar 500μm. 
c. Scores of undifferentiated colonies on D6 after plating in Serum or Serum Plus LIF. Mean of 3 replicates +/-SEM. 
d. Zmym2 was disrupted using CRISPR/Cas9. Clones were stably transfected with Zmym2 or a corresponding EV transgene. Western blot of two resulting ESC clones; the knockout clone was sequenced and used for further experiments. 
e. Scores of undifferentiated colonies on D6 after plating WT, Zmym2−/− and Zmym2 rescued lines in Serum or Serum Plus LIF indicate that Zmym2 deletion may impede differentiation in the absence of LIF. Mean of 3 replicates +/- SEM.
Figure 5. Zmym2 overexpression correlates with the mis-expression of early lineage markers. a. Brightfield images of Cas9-generated Zmym2+/− (Figure 4D), WT and Zmym2 overexpressing embryonic stem cells (ESC) in Serum Plus LIF-containing medium. b. Western blot analysis of these ESC lines. c. RNA Seq analysis was performed on the lines shows an upregulation in the transcript levels of early lineage specifiers of trophectoderm (TE), ectoderm and EpiSCs (EpiSC/Ecto), mesoderm (meso) and endoderm (endo) and a corresponding decrease in the transcript levels of inner cell mass (ICM) markers in Zmym2 overexpressing ESCs. Conversely, Zmym2−/− ESCs have lower transcript levels of early lineage markers.

Serum Plus LIF-containing medium. Zmym2 overexpressing cells had higher transcript levels of many lineage specifiers than control cells including early ectodermal, mesodermal and endodermal markers, as well as trophectodermal markers, after normalisation to housekeeping genes. In addition, they had reduced transcript levels of a number of pluripotency-associated genes. Conversely, Zmym2KO cells had reduced expression of differentiation markers. In conclusion, Zmym2 inhibits reprogramming and promotes differentiation. It has a global effect on the transcriptome of ESCs, increasing the transcription of differentiation-associated genes and reducing pluripotency-associated transcripts.

Discussion
In this work, we show that Zmym2 represents a significant barrier to Nanog-mediated reprogramming. We observed consistent results using gain and loss of function assays in many different reprogramming systems, including EpiSCs, fibroblasts and neural stem cells. This corroborates results obtained by other groups working on RNAi in human cell reprogramming. Therefore, ZMYM2 may play a similar role in the control of NANOG in mouse and human.

We also observe that Zmym2 promotes embryonic stem cell differentiation. This has also been reported in a Cas9 screen for differentiation-promoting factors. Future work could examine whether the absence of Zmym2 impacts mouse development and elucidate its role in vivo.

In conclusion, this work has elucidated the key role of Zmym2 as a barrier to reprogramming and a differentiation-promoting transcription factor. This is particularly interesting as many previous studies of Nanog’s mechanism of action have identified positive regulators of its activity. We have shown both more effective reprogramming and less differentiation upon removal of Zmym2 demonstrating how the tight control of NANOg by its binding partners exerts a directive influence on cell identity transitions, both entering and exiting the pluripotent state.

Data availability
Underlying data
RNASEq data from WT, Zmym2 knockout- and Zmym2 overexpressing- E14tg2a mouse embryonic stem cells. Accession number GSE130317: http://identifiers.org/geo:GSE130317


This project contains the following underlying data:

- 1c.pdf (x-ray films for Western blot in Figure 1c)
References


Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Version 1

Reviewer Report 02 August 2019
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Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, China

This manuscript proves that Zmym2 acts as a repressor in Nanog-mediated reprogramming through gain and loss of function assays. The novelty of this manuscript is moderate, and there are some problems that needs to be improved.

Main points:
1. The information in the introduction is too simple and incomplete. There are lots of proteins in NANOG interactome, please give more detailed reasons why the author chose the ZMYM2 as the research object. Also, the nature and function of ZMYM2 that has been studied so far could be introduced.

2. In the section of “Zmym2 reduces ESC self-renewal”, the author said that “Neither KD nor overexpression lines had any change in Nanog transcript or protein levels”, however, as is shown in Figure 2e, when Zmym2 is overexpressed, the transcript level of Nanog also increases, which seems that the expression of Nanog is regulated by Zmym2.

3. In the discussion part, could you please give some possible mechanism about why Zmym2 have a pronounced positive effect on Nanog-mediated reprogramming but a minimal effect on reprogramming of no-Nanog-mediated?

4. It seems that the authors were not serious enough when making figures. The detail of this criticism would be listed in the “minor points” section.

Minor points:
1. It's a bit confusing to emphasize that NANOG is a "homeodomain-containing" transcription factor in both the abstract and the first paragraph of the introduction.

2. The method of reprogramming epiblast stem cell is missing.
3. Font size is expected to be 8-10 pt in all figures. For example, the fonts are too large in Figure 4d while the ones are too small in Figure 3.

4. The upper half part of error bar of the “ESCs” is missing in Figure 1d.

5. The meaning of “Pool” should be explained in the figure legend of Figure 1.

6. What dose “Oct4_” mean in Figure 2c and 3g?

7. It’s inaccurate to regard neural stem cells as somatic cells in the title of Figure 3.

8. “Zmym2” is expected to be italic in all figures.

9. In the result of western blot, the “alpha tublin” should be written as “α-TUBLIN”.

10. The P value should be provided in every bar graph.

**Is the work clearly and accurately presented and does it cite the current literature?**
Partly

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**
Partly

**Are all the source data underlying the results available to ensure full reproducibility?**
Partly

**Are the conclusions drawn adequately supported by the results?**
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Pluripotency, Stem Cells, Epigenetic, 3D Chromatin.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 17 July 2019

https://doi.org/10.21956/wellcomeopenres.16645.r35873
This thorough study investigates a role for Zmym2 in Nanog-mediated reprogramming, and in ESC differentiation. By using RNA knock-down, gene knock-out and over-expression in a series of bespoke reprogramming tests, and a standard ESC self-renewal assay, the authors have comprehensively demonstrated that Zmym2 antagonises Nanog-driven reprogramming and promotes the loss of pluripotency in ESCs. This novel finding significantly extends the previously reported observation that Zmym2 physically interacts closely with the Nanog protein in ESCs.

Nanog is a member of a group of ancillary pluripotency-associated factors that are not essential in all circumstances, and may vary in their importance in embryo stem cells of different species. Given the significant protein sequence divergence of Nanog outside the core DNA binding domain, future studies may investigate what regions of Nanog (or partners) interact with Zmym2, and the degree to which this interaction is conserved in Nanog-expressing cell types, and between species.

However, questions that might be answered using the available data and included directly in this present report, are:


2. Similarly, does Zmym2 affect self-renewal/differentiation signalling in ESCs? The relevant data presented in Figure 5 shows striking induction of Cdx2 and Brachyury as well as BMP2. Since Cdx2 and Brachyury are known targets for Wnt/b-catenin signalling, is there evidence of Zmym2 upregulation of this pathway. Or is their induction mediated via expression of BMP2, and accompanied by upregulation of other BMP signalling/targets?

Other points for consideration are:

- The introduction/discussion would benefit from mentioning background information on Zmym2, as well as its pattern of expression during early differentiation (upregulation during EpiSC formation?). Could there be any significance to Zmym2/FGFR association and Nanog function?

- Clarification in the first part of the results that the "control" ZFP281 is a Nanog interacting protein.

- Minor typo - Figure legend 2 “75,000 plates NSCs” - “plated”.

Is the work clearly and accurately presented and does it cite the current literature? Yes

Is the study design appropriate and is the work technically sound? Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Stem cell biology, cell signalling.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 21 June 2019

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I think this is a very good and interesting paper that addresses an important issue in stem cell biology. Some years ago, NANOG was identified as a “gateway to pluripotency”, so how its activity is regulated during reprogramming is of considerable interest. Here the authors investigate the activity of a transcription factor called ZMYM2 that they have identified as a component of the NANOG interactome, and they show that it inhibits NANOG activity both during reprogramming and self-renewal. Other studies, including work by the same lab, have demonstrated the augmentation of NANOG activity by components of the interactome; this is the first to my knowledge to show an inhibitory activity. Interestingly, ZMYM2 does not inhibit NANOG expression, so its effects must be biochemical, and future studies to elucidate these biochemical activities will also be of considerable interest in the field. The studies are thorough, well executed and convincing. For example, the authors use several different reprogramming contexts, including epiSC, fibroblasts and neural stem cells to demonstrate the inhibitory effects of ZMYM2 on reprogramming. They also use a combination of knockout and overexpression experiments, which clearly demonstrate complementary results that reinforce the paper’s conclusions. Their data also show that ZMYM2 inhibits self-renewal by promoting differentiation, suggesting it may play an interesting role in cell-specification in vivo. In all this paper is certainly worthy of indexing and it will be of interest to the field. I think a couple of minor issues should be addressed.

First, the second line of the Introduction states that pluripotent cells possess the ability to self-renew. However, this is only true of pluripotent stem cells maintained in culture. The pluripotent cells in an
embryo do not necessarily self-renew, particularly in non-mammalian systems where the number of pluripotent cells increases by cleavage rather than expansion of an epiblast.

In Figure 4d the difference between the mutant and a knockout ESC lines should be made clearer. The different patterns of ZMYM2 in the two lines should also be explained in the text. Figure 4e does not show ESC in serum plus LIF.

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Developmental biology, stem cell biology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.