FEBS Letters

FEBS PRESS science publishing by scientists

The pluripotency transcription factor OCT4 represses heme oxygenase-1 gene expression

María Victoria Petrone[1], Ayelén Toro[1], Camila Vazquez Echegaray[1], Marcos Gabriel Francia[1], Claudia Solari[1], Maria Soledad Cosentino[1], Elba Vazquez[2,3] and Alejandra Guberman[3,4]

1 Instituto de Química Biológica de la Facultad de Ciencias Exactas y Naturales (IQUIBICEN), CONICET-UBA, Buenos Aires, Argentina
2 Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales Universidad de Buenos Aires, Buenos Aires, Argentina
3 IQUIBICEN, CONICET-UBA, Buenos Aires, Argentina
4 Departamento de Fisiología y Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales Universidad de Buenos Aires, Argentina

Correspondence
A. Guberman, Intendente Guiraldes 2160, Ciudad Universitaria, Pab. 2, 4to piso, QB-71, C1428EGA, Buenos Aires, Argentina
Tel: +54 115285 8683
E-mail: algub@qb.fcen.uba.ar

(Received 14 April 2021, revised 17 May 2021, accepted 23 May 2021, available online 28 June 2021)

doi:10.1002/1873-3468.14138

Edited by Ned Mantei

In embryonic stem (ES) cells, oxidative stress control is crucial for genomic stability, self-renewal, and cell differentiation. Heme oxygenase-1 (HO-1) is a key player of the antioxidant system and is also involved in stem cell differentiation and pluripotency acquisition. We found that the HO-1 gene is expressed in ES cells and induced after promoting differentiation. Moreover, downregulation of the pluripotency transcription factor (TF) OCT4 increased HO-1 mRNA levels in ES cells, and analysis of ChIP-seq public data revealed that this TF binds to the HO-1 gene locus in pluripotent cells. Finally, ectopic expression of OCT4 in heterologous systems repressed a reporter carrying the HO-1 gene promoter and the endogenous gene. Hence, this work highlights the connection between pluripotency and redox homeostasis.

Keywords: gene modulation; heme oxygenase-1; OCT4; pluripotency transcription factors; pluripotent stem cells; transcription

Genomic stability is safeguarded by the concerted action of a highly active stress defense system and DNA repair mechanisms that minimize spontaneous mutation frequency. Particularly in embryonic stem (ES) cells, which can differentiate into any cell type of the adult organism including the germline, these mechanisms are highly active, since maintaining genomic stability is crucial for proper development. Moreover, embryonic stem cells (ES cells) that accumulate mutations are generally eliminated by induction of differentiation or apoptosis [1]. Reactive oxygen species (ROS) are the major source of DNA damage, not only when they increase as a consequence of oxidative stress but also under nonstressed physiological conditions. In this way, pluripotent stem (PS) cells are highly efficient in their antioxidant defense, a property that progressively diminishes during differentiation [1–3]. On the other hand, ROS also play an important role in signal transduction [4]. Particularly, their function in the physiological regulation of crucial developmental processes, such as differentiation and apoptosis, has been widely described [5]. Due to the dual role of ROS in cell damage and normal cell functions, the proper balance of these molecules is rigorously controlled in PS cells and in the course of differentiation [5].

A relevant component of this cellular defense system is heme oxygenase (HO), the enzyme that catabolizes cellular heme to biliverdin, carbon monoxide, and free iron [6]. The isoform heme oxygenase-1 (HO-1), encoded by the *Hmox1* gene, is strongly upregulated

Abbreviations
dox, doxycycline; ES cells, embryonic stem cells; HO-1, heme oxygenase-1; IF, immunofluorescence; MEF, mouse embryonic fibroblast; shRNA, short hairpin RNA; WB, western blot.

during stress and is considered one of the most sensitive and reliable indicators of cellular oxidative stress [7]. Besides its classical antioxidant function, HO-1 is also known for its pro-angiogenic [8] and anti-inflammatory [9] activities during embryogenesis, as a crucial factor for fetal growth [10], and as a regulator of cell cycle progression [11]. Even though this protein is expressed in most tissues, its subcellular localization has been mainly studied in the context of oxidative stress and cancer [12,13]. Due to HO-1 localization at the membrane of the smooth endoplasmic reticulum, its canonical enzymatic activity takes place in the cytoplasm. However, this protein was also detected in other cellular compartments including caveolae [14], mitochondria [15], and the nucleus [16,17]. It has been suggested that HO-1 is involved in signal transduction and regulates the function of certain transcription factors (TFs) acting independently of its enzymatic activity, particularly in the contexts mentioned above [17–19]. It has also been reported that HO-1 interacts with NRF2, the main TF that induces *Hmox1*, promoting its stabilization and thus enhancing the antioxidant cell defense [20].

In the context of stem cells and development, HO-1 has been implicated in embryogenesis and differentiation [11]. It was found that HO-1 influences cell differentiation both positively and negatively, depending on the cell type [21]. Particularly, HO-1 has been reported to upregulate a mesodermal gene expression profile during ES cell differentiation through embryoid-body formation [22] and was also found to be involved in differentiation of ES cells into functional cardiac cells [23]. In line with these observations, it has been reported that knockout PS cells for *Hmox1* displayed attenuated spontaneous cardiac differentiation. Interestingly, it has been suggested that HO-1 is required for efficient reprogramming [24], and critical for induced pluripotent stem (iPS) cell survival and differentiation, since HO-1-depleted cells have increased susceptibility toward exiting the pluripotent state and are more prone to oxidative stress-induced cell death [25].

We have previously reported evidence of a regulatory relationship between components of the antioxidant cell system and pluripotency TFs in ES cells. We found that OCT4, SOX2, and NANOG, essential TFs for self-renewal and pluripotency maintenance [26,27], regulate the expression of relevant genes involved in oxidative stress defense, specifically by inducing superoxide dismutases 1 and 2 (Sod) genes [28,29], and by transcriptionally repressing glutathione reductase (Gsr) [30].

Remarkably, although HO-1 relevance in PS cells is emerging, the regulation of *Hmox1* expression has not yet been studied in this cellular context. In this work, we have explored HO-1 gene regulation in ES cells and in complementary heterologous systems and found that the pluripotency TF OCT4 negatively modulates *Hmox1* expression, providing further insights to the connection between pluripotency and redox homeostasis.

Materials and methods

Cell culture conditions and differentiation protocol

The W4 mouse ES cell line was provided by the Rockefeller University Core Facility and was cultured in DMEM containing 2 mM Glutamax, 100 mM MEM NEAA, 0,1 mM 2-mercaptoethanol, 100 $U{\cdot}mL^{-1}$ penicillin, and 100 $\mu g{\cdot}mL^{-1}$ streptomycin (Gibco), supplemented with 15% FBS (Gibco, Carlsbad, CA, USA), LIF, 1 μM PD0325901 (Tocris, Bristol, UK), and 3 μM CHIR99021 (Tocris). Cells were maintained on 0.1% gelatin-coated dishes and grown at 37 °C in a 5% CO_2 (v/v) incubator. Standard ES cell culture conditions require LIF [31], which is sufficient to maintain pluripotency, and may include the '2i' inhibitor set [32] CHIR99021 (CHIR) and PD 325901 (PD). LIF and 2i withdrawal is commonly used to promote nondirected differentiation. The differentiation protocol was performed as previously described [28,33–35].

The doxycycline (dox)-inducible NIH/3T3 cell line expressing YPet-tagged OCT4 (NIH/3T3 YPet-OCT4) was generated in this work. A detailed generation procedure, the control, and setting of the induction conditions of this cell line are described in Supporting Information. NIH/3T3 and NIH/3T3 YPet-OCT4 cell lines were cultured in DMEM supplemented with 10% FBS (Internegocios S.A.) and antibiotics.

mRNA and protein analysis

mRNA levels were analyzed by RT-qPCR, and proteins were studied by immunofluorescence (IF) and/or western blot (WB) as previously described [36] with minor modifications detailed below.

RT- qPCR

Total RNA was extracted with Trizol (Thermo Fisher Scientific, Waltham, MA, USA) and reverse-transcribed using MMLV reverse transcriptase (Thermo Scientific) and Random Primers (Invitrogen, Carlsbad, CA, USA). Quantitative PCR (qPCR) amplification of DNA was performed using FastStart SYBR Green Master (Roche, Basilea, Switzerland) and specific primers in a LightCycler 480 real-time PCR system. Primer efficiency and N_0 values were determined by LinReg software [37], and gene expression was normalized to the geometric mean of Gapdh and Pgk1

housekeeping genes N_0, for each condition. Primers were designed using PRIMER3 software and are listed in Supporting Information. All experiments were performed in three biological replicates, with two technical replicates for each condition.

Immunostaining

Cells were fixed by incubation with 4% paraformaldehyde for 15 min, and then, they were permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked with 1% normal goat serum (Sigma) in PBS-Tween 0.1% for 1 h. Primary antibodies in blocking solution were added to the samples, incubated at 4 °C overnight, and washed three times in PBS-Tween 0.1% for 5 min. The incubation with secondary antibodies and DAPI (Sigma) prepared in blocking solution was performed at room temperature for 1 h. Samples were washed as described above and imaged in an Olympus (Shinjuku, Tokyo, Japan) IX71 or FV1000 microscope. All the antibodies used are listed in Supporting Information.

Western blot analysis

Proteins were collected from cell lysates with RIPA buffer, run in 12% SDS/polyacrylamide gel electrophoresis, and transferred to PVDF membranes (Amersham). Membranes were blocked for 1 h at room temperature in TBS-T containing 1% BSA, and primary antibodies were incubated overnight at 4 °C in blocking solution. Secondary antibodies were incubated at room temperature for 1 h. Membranes were revealed with ECL Prime Western Blotting Detection (GE Healthcare, Chicago, IL, USA) in a G-Box System (Syngene, Cambridge, UK). All the antibodies used are listed in Supporting Information.

Luciferase activity assay

NIH/3T3 cells were cotransfected in 24-well plates with 300 ng of pHO-1-Luc reporter, and 0 (basal) or 200 ng of pMXs-Oct4 (Addgene, Watertown, MA, USA). To normalize transfection efficiency, 20 ng of pRL-TK reporter (Promega, Madison, WI, USA), which encodes the *Renilla reniformis* luciferase driven by the TK promoter, was also included in all conditions. When necessary, the total amount of plasmid DNA was adjusted with nonspecific DNA. Transfection and luciferase assays were carried out as previously described [28,29,38].

Downregulation of TFs by shRNA approach

W4 ES cells cultured in standard medium on gelatin-coated p60 plates were transfected with 3 μg pLKO.1-Puro-derived vectors (Sigma), expressing short hairpin RNA (shRNA) targeting Nanog (SHCLND-XM_132755), Oct4 (SHCLND-NM_013633), Sox2 (SHCLND-NM_011443), or eGFP (SHC005), which was used as control shRNA. Transfection, selection, and mRNA expression analyses were carried out as previously described [28,39].

Statistics and data analysis

Experimental results are presented as mean ± standard error of the mean (SEM). Statistical comparisons were performed using randomized block design ANOVA for at least three biological replicates using INFOSTAT statistical software [40]. In all cases, residuals fitted the normal distribution as assessed by the Shapiro–Wilks test and homogeneity of variance using the Levene test. When necessary, Tukey's test was used for comparisons between means. P values < 0.05 were considered significant.

Results

To explore *Hmox1* regulation in ES cells, we first analyzed HO-1 mRNA levels in undifferentiated ES cells (D0) and 4 days after differentiation induction by LIF/2i withdrawal (D4; Fig. 1A). As shown in Fig. 1, HO-1 mRNA and protein were detectable in undifferentiated ES cells and showed an increase in their levels after differentiation (Fig. 1B). Moreover, IF images also revealed increased HO-1 after 4 days of differentiation and showed that this protein localizes both in the cytoplasm and in the nucleus (Fig. 1C).

Since the pluripotency TFs OCT4, SOX2 and NANOG promote the expression of pluripotency genes, repress lineage-associated genes, and decrease in activity during differentiation [26,27], we wondered whether they could regulate *Hmox1* expression. To explore this possibility, we downregulated these TFs by transfection of specific shRNAs and analyzed the effect on HO-1 mRNA levels (Fig. 2A). All shRNA encoding vectors have been previously used [28–30,38,39,41] and were highly effective in silencing the respective TF (Fig. 2B). As shown in Fig. 2C, we found that HO-1 mRNA levels were significantly increased in ES cells transfected with shOct4, compared with the control cells transfected with shGFP. On the contrary, the transfection of shRNA against Nanog or Sox2 did not significantly affect HO-1 mRNA levels. These results indicate that Oct4 downregulation induced *Hmox1* transcription, suggesting that this TF could be involved in the repression of this gene in ES cells.

To further study the putative effect of OCT4 on *Hmox1* expression, we analyzed the sequence of this gene locus and found eight putative binding sites for

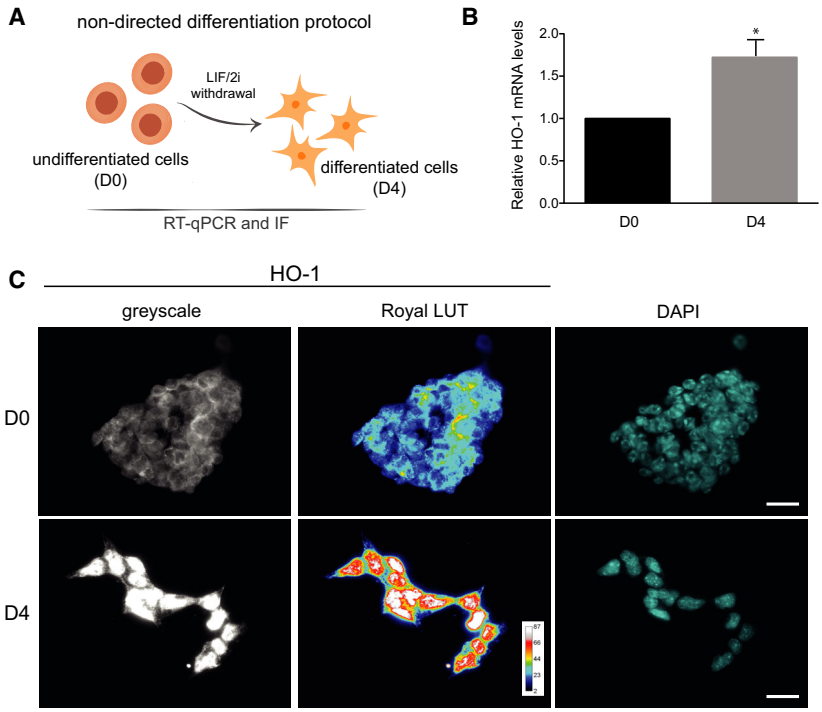

Fig. 1. The HO-1 gene is expressed in ES cells and induced during differentiation. (A) Schematic diagram of the experimental design. ES cells were cultured under standard conditions in the presence of LIF and 2i (D0) or induced to differentiate by LIF/2i withdrawal for 4 days (D4). (B) HO-1 mRNA levels were analyzed by RT-qPCR, normalized to housekeeping genes, and referred to the control condition (D0). Results are shown as mean ± SEM of three independent replicates. Asterisk indicates significant differences analyzed by randomized block design ANOVA ($P < 0.05$). (C) HO-1 was visualized by IF. Representative images from three independent experiments. HO-1 signal is shown in grayscale and pseudocolor (Royal LUT). Nuclei were visualized by DAPI staining (cyan). Scale bars: 20 μm.

this TF in a region spanning 5 Kbp upstream of the transcription start site (TSS; Fig. 3A). Relevantly, the analysis of available public data from high-throughput ChIP-seq experiments [42,43] with the ChIP Atlas data mining platform [44] revealed that OCT4 binds to two of the putative binding sites from this region of *Hmox1* in ES cells and iPS cells (Fig. 3B). These results suggest that this TF could repress HO-1 expression in ES cells by interacting with *Hmox1* locus. To explore this possibility, we exploited a heterologous system in which endogenous Oct4 expression is undetectable, the NIH/3T3 mouse embryonic fibroblast (MEF) cell line [28,29,38,45]. We first evaluated the responsiveness to OCT4 of a HO-1 reporter (pHO-1-Luc) containing a 3 Kbp fragment of the *Hmox1* promoter by a transactivation assay in the NIH/3T3 cell line (Fig. 4A). As shown in Fig. 4B, and in line with our previous results, OCT4 repressed luciferase expression, strengthening the evidence that this TF could have a negative effect on HO-1 gene expression.

Based on these results, we decided to evaluate the effect of OCT4 on the expression of the endogenous gene. For this purpose, we generated an NIH/3T3 dox-inducible cell line which expresses the fusion protein YPet-OCT4 under the control of a Tet-on regulatory element. Since, as mentioned, the NIH/3T3 cells do not express OCT4, this new cell line, NIH/3T3 YPet-OCT4, expresses this TF only as a fusion with the YPet fluorescent protein after treatment with dox (Fig. 5A), providing a valuable tool for our purpose. The NIH/3T3 YPet-OCT4 cell line was generated as described in Supporting Information and Fig. S1A, and the conditions for YPet-OCT4 induction were established (Fig. S1B). The generated cell line presented a normal cell cycle distribution (Fig. S1C) and expressed YPet-OCT4 after dox treatment (Fig. S1D). Moreover, OCT4 was not detected by IF in cells that were not treated with dox, confirming the absence, or at least the undetectable levels, of both endogenous OCT4 and YPet-OCT4 in these cells growing in basal conditions (Fig. S1D). Additionally, it has been previously verified that fusion to YPet does not affect OCT4 nuclear localization and function [45,46]. Then, we studied *Hmox1* expression, at mRNA and protein levels, in this heterologous system under dox induction. As shown in Fig. 5, NIH/3T3 YPet-OCT4 cell line expresses HO-1 in basal conditions. As expected, HO-1 mRNA and protein levels were greatly reduced after YPet-OCT4 induction with dox (Fig. 5B–D), agreeing with the results shown above. Importantly, dox treatment did not modify HO-1 protein levels in the NIH/3T3 parental cell line (Fig. 5C), demonstrating that the effect observed on HO-1 levels was due to YPet-OCT4 induction and not to an unspecific effect of dox treatment. Overall, our observations demonstrate a novel repressive effect of OCT4 on *Hmox1* gene expression.

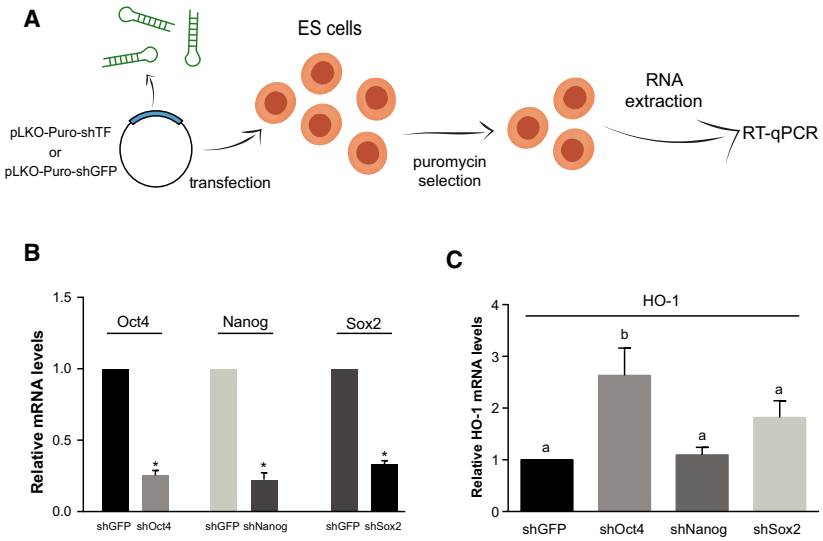

Fig. 2. HO-1 mRNA levels are increased in ES cells transfected with shRNA targeting Oct4. (A) Schematic diagram of the experimental design. ES cells were transfected with pLKO.1-puro-derived vectors targeting Oct4, Nanog or Sox2 (shOct4, shNanog, or shSox2, respectively), or eGFP (shGFP, control). Transfected cells were selected with puromycin, and RNA was extracted after 48 h. The mRNA levels of Oct4, Nanog, and Sox2 (B) and HO-1 (C) were analyzed by RT-qPCR. Gene expression was normalized to the geometrical mean of Gapdh and Pgk1 mRNA levels and referred to the control condition. The labels under each bar indicate the shRNA transfected in each case. Results are shown as mean ± SEM of three independent experiments. Asterisks indicate significant differences with respect to the control condition, and different letters indicate differences among treatments, both analyzed by randomized block design ANOVA ($P < 0.05$).

Discussion

The connection between pluripotency and the stress defense cell system highlights the central role of redox homeostasis in PS cell identity and in cell fate commitment. Furthermore, ROS balance has been shown to impact cell differentiation [5,47]. However, the relationship between pluripotency TFs and the components of the oxidative stress defense systems remains poorly explored. We have previously found that OCT4, SOX2, and NANOG regulate the expression of Sod1, Sod2, and Gsr [28–30], revealing a regulatory link between pluripotency TFs and the antioxidant system. In this work, we found that the pluripotency TF OCT4 represses the *Hmox1* gene, demonstrating a meaningful connection between the pluripotency core and this system.

Hmox1 regulation has been mainly studied in the context of oxidative stress [7,48] and cancer cell models and tumors [19,49,50]. It was found to be overexpressed in several types of tumors, suggesting that HO-1 promotes cell proliferation and survival in specific tumor environments [49,51,52]. However, contrary results have attributed an antiproliferative effect to HO-1 in other types of cancer [53–56]. These findings suggest that HO-1 function and expression are highly dependent on the cellular context.

Particularly in stem cells, the requirement of HO-1 for differentiation emerged in the last decade, but its precise role remains uncovered. HO-1 was shown to be involved in neurogenesis regulation, since it is downregulated in mesenchymal stem cells during neural differentiation [57] and along the terminal maturation of astroglial cells [58]. HO-1 was also found to attenuate hematopoietic stem cell differentiation [59] and to promote osteoblast stem cell differentiation [60]. Additionally, enhanced HO-1 activity inhibits the differentiation of murine muscle precursors [61]. On the other hand, ES cells that do not express HO-1 show higher levels of mesodermal and smooth muscle cell markers during embryoid-body differentiation, suggesting that HO-1 could be regulating the expression of specific genes [22]. Moreover, HO-1 activity contributes to the differentiation and maturation of ES cells into cardiomyocytes [23] and HO-1 knockout PS cells show impaired differentiation toward cardiac lineage [24]. On the contrary, it was suggested that HO-1 protects ES cells against spontaneous differentiation [25], on the basis that OCT4 levels were lower in HO-1 knockout iPS cells compared to wild type iPS and ES cells, and that the products of HO-1 enzymatic activity, bilirubin, and CO, rescue the accelerated loss of OCT4. In agreement with an HO-1 pro-stemness role, it was demonstrated that it is also required for

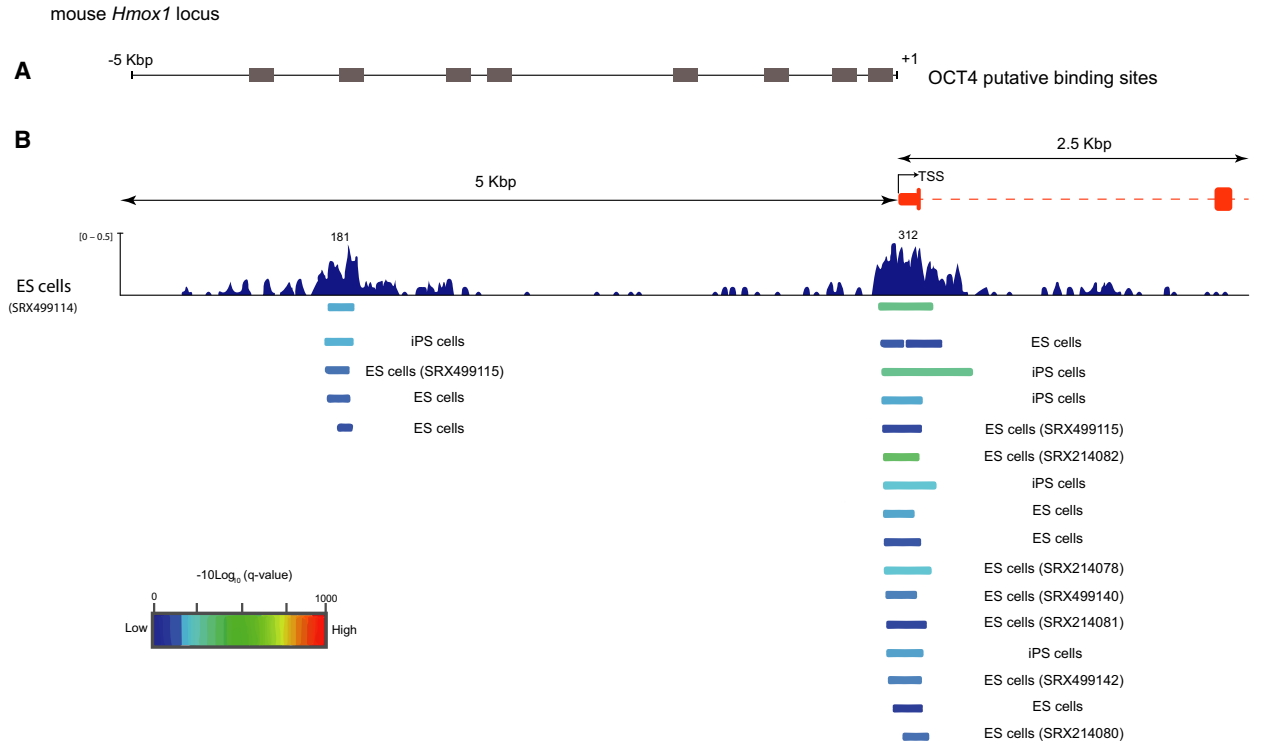

Fig. 3. Oct4 binds to the *Hmox1* genomic *locus*. (A) A region of the mouse *Hmox1 locus* was analyzed using MatInspector software. Putative OCT4 binding sites are indicated with gray boxes. (B) To explore OCT4 binding to the *Hmox1 locus*, public data from ChIP-seq experiments performed in pluripotent stem cells were analyzed with the ChIP Atlas tool (Chip Atlas database: http://chip-atlas.org) [44]. A representative enrichment profile (reads per million) of OCT4 is shown. The y-axis indicates the number of reads normalized to total mapped reads and the black numbers above the major peaks of the significant ChIP-seq reads. Each of the colored boxes corresponds to a different experiment and shows the genomic regions with significant ChIP-seq reads. Color scale bar indicates the number of significant reads. The cell type (ES cells or iPS cells) and the corresponding accession number of each dataset are indicated. Data were visualized using the Integrative Genomics Viewer (IGV) software [80]. TSS,+1: transcription start site.

efficient pluripotency acquisition [24] and is critical for the survival and differentiation of iPS cells [25]. This evidence gives rise to a conundrum regarding the mechanisms associated with the multifaceted features of HO-1 function.

Remarkably, the fact that HO-1 is expressed in ES cells agrees with its role in the oxidative stress defense system in PS cells. On the other hand, the increase in its expression during differentiation and our finding that it is repressed by OCT4, whose levels and function decrease along differentiation, reinforce the hypothesis that HO-1 might be relevant for this process. We have previously performed a bioinformatic analysis with public data from high-throughput microarrays and RNA-sequencing experiments and found that HO-1 mRNA levels increase in nondirected and in multiple different types of directed differentiation protocols; however, we have also found that HO-1 mRNA levels decrease in differentiation directed to the endodermal lineage [62]. This is consistent with the fact that basal HO-1 expression is high in some cellular types and low in others [62,63]. It is worth mentioning that, in this work, we have analyzed an early time point of a nondirected differentiation protocol that displays a relatively homogeneous cell population in which there are no terminally differentiated cells yet. Further research is required to identify the nature of the HO-1 role in certain differentiation processes. One possibility is that HO-1 induces the exit from the pluripotent state; the other, and not mutually exclusive, is that this protein is required for the execution of specific transcriptional programs that promote specific differentiation processes. Thus, to allow cell differentiation, *Hmox1* transcriptional repression by OCT4 must be released, and this takes place only when the functionality of this pluripotency TF decreases. We have previously reported that even though the levels of this TF do not drop for the first 3–4 days of differentiation, its subnuclear distribution and dynamical interaction with chromatin do modify

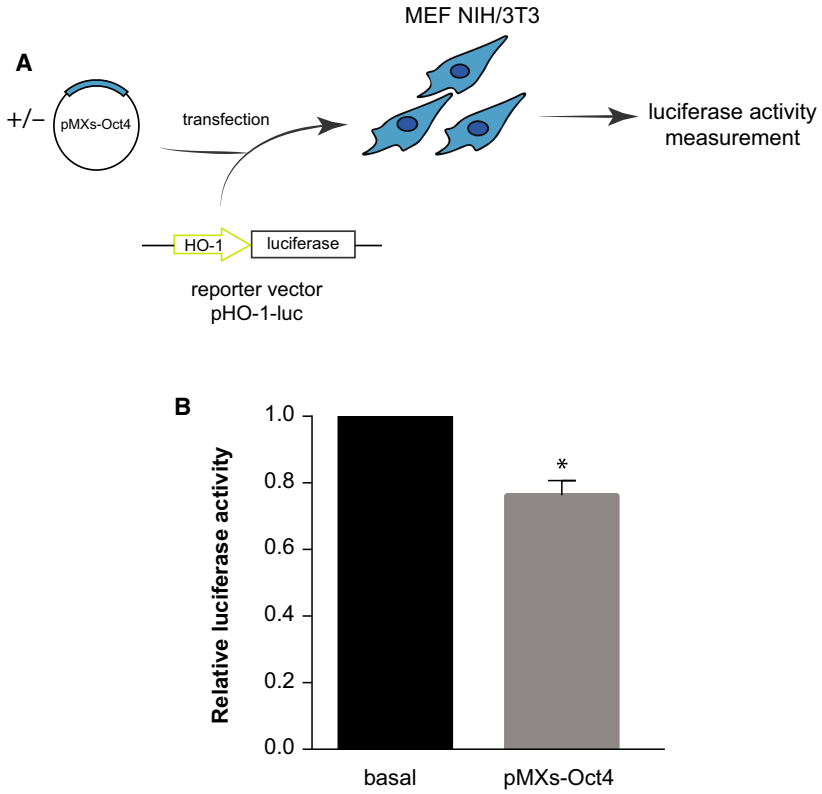

Fig. 4. OCT4 represses pHO-1-Luc reporter. (A) Schematic diagram of the experimental design. MEF NIH/3T3 cells were transfected with pHO-1-Luc and pMXs-Oct4 or none (basal) and luciferase activity was analyzed as described in Material and Methods. (B) Values were normalized to *Renilla's* luciferase and referred to the basal condition. Results are shown as mean ± SEM of three independent experiments. Asterisk indicates statistically significant differences between treatments analyzed by randomized block design ANOVA ($P < 0.05$).

at these early stages of the process [64], most probably impacting on its function.

OCT4 could be exerting its repressive effect on HO-1 gene expression through interaction with other transcriptional regulators that affect chromatin architecture, such as histone and nucleic acid modifiers. Interestingly, analysis of public ChIP-seq data sets showed that components of repressive complexes bind to *Hmox1* locus in undifferentiated ES cells [62]. For example, we have found components of the polycomb repressive complex (PRC) like EZH2 and SUZ12 [65] and the components of the nuclear remodeling and histone deacetylation complex (NuRD) Chd4 and Mbd3 [66], among others. Interestingly, we did not find these proteins bound to *Hmox1* locus in data sets from ES cells subjected to differentiation protocols [62]. Moreover, we have found histone and DNA modifications typically associated with repressive chromatin, particularly H3K27me3, H3K4me1, and 5-mC, in undifferentiated ES cells, and permissive marks, specifically H3K4me3 and H3K27Ac, in stem cells that have been subjected to differentiation [62]. Remarkably, OCT4 interactome analysis in ES cells has revealed that this TF physically interacts with proteins associated with gene repression, such as Sin3a, Chd4, and Mbd3 [67,68]. Moreover, proteins from the PRC act cooperatively with OCT4 in gene regulation [69,70]. Since we have found that several of these proteins bind to the *Hmox1* locus in ES cells [62], we speculate that it might be possible that OCT4-mediated repression of HO-1 gene expression could involve the recruitment of some of these transcriptional regulators.

Furthermore, the low levels of HO-1 under normal conditions are a consequence of *Hmox1* gene repression by BACH1 [63]. This TF modulates, in a heme level-dependent manner, the availability of *Hmox1* enhancers to its well-known inductor, NRF2, most likely by assembling a repressive multiprotein complex [63]. Notably, one of the *Hmox1* enhancers reported to be regulated by BACH1 and NRF2 [63] is located in the same region where OCT4 bounds, around 4 Kbp upstream the TSS (Fig. 3). Interestingly, BACH1, besides that it has been reported to be highly expressed in mouse embryos [71] and to inhibit some differentiation processes [72], was recently found to interact with OCT4, SOX2, and NANOG in mouse and human ES cells [73,74]. This interaction stabilizes these pluripotency TFs contributing to stem cell identity maintenance [74]. Additionally, BACH1 recruits members of the PRC2, including the above named EZH2 and SUZ12, to regulatory regions of mesendodermal genes,

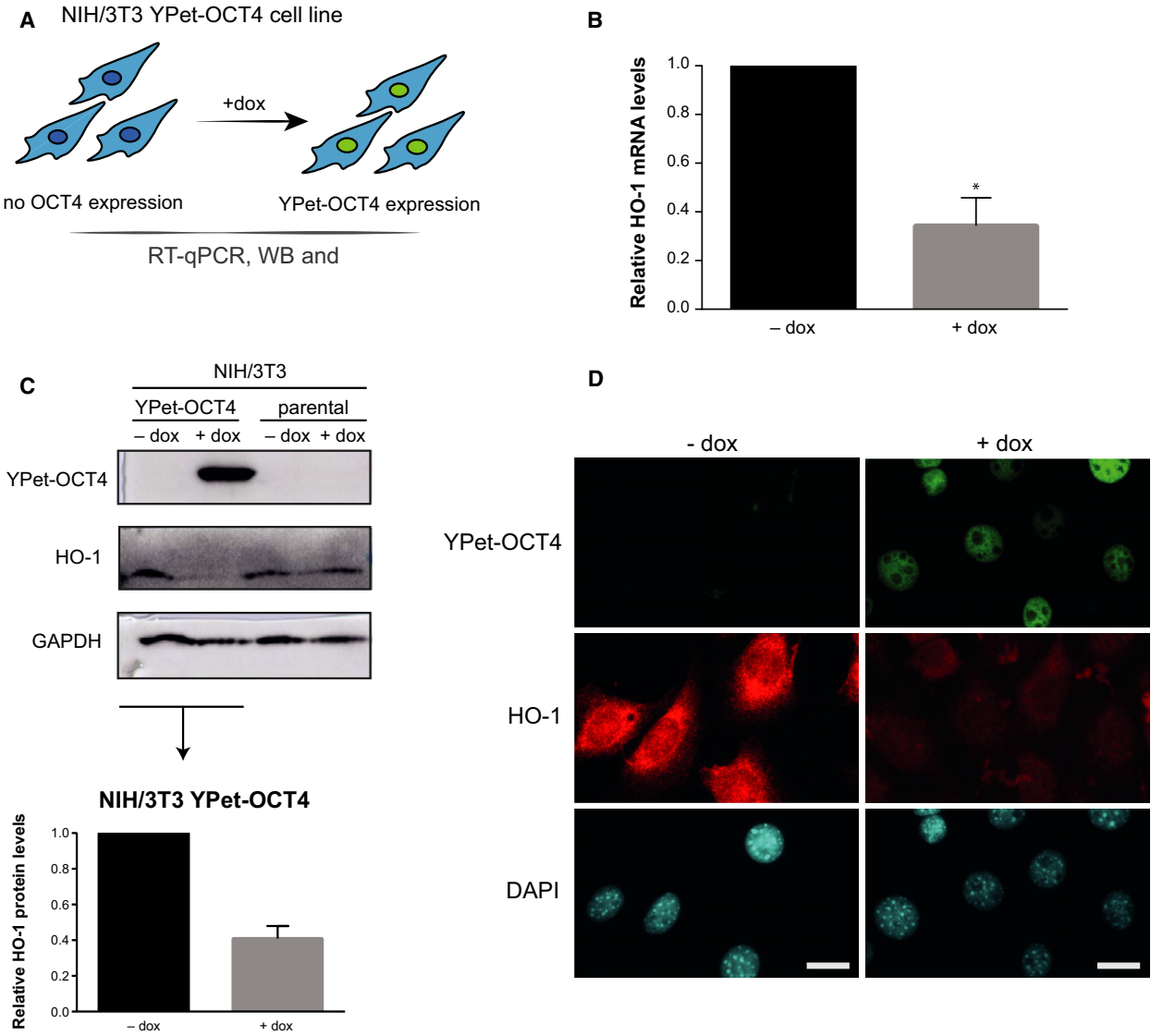

Fig. 5. YPet-OCT4 represses HO-1 gene expression in a heterologous system. (A) Schematic diagram of the experimental design. MEF NIH/3T3 YPet-OCT4 cells were induced to express YPet-OCT4 by incubation with 2.5 μg·mL^{-1} dox for 48 h, and then, HO-1 expression was studied and compared to untreated cells (control). (B) mRNA levels of HO-1 were analyzed by RT-qPCR, normalized to housekeeping genes, and referred to the control condition (-dox). Results are shown as mean ± SEM of three independent replicates. Asterisk indicates significant differences analyzed by randomized block design ANOVA ($P < 0.05$). (C) YPet-OCT4 and HO-1 expression was analyzed by WB in NIH/3T3 YPet-OCT4 cells and in the parental cell line, treated or not with dox, as indicated. Representative immunoblots revealed with antibodies against OCT4, HO-1, or GAPDH (loading control). Full-size blots are shown in Supporting Information. (D) YPet-OCT4 was visualized by fluorescence microscopy (green), HO-1 was visualized by IF (red), and chromatin was stained with DAPI (cyan) in NIH/3T3 YPet-OCT4 cells treated or not with dox as indicated. The figure shows representative images from three experiments. Scale bars: 20 μm.

promoting the trimethylation of H3K27 and hence repressing mesendodermal gene expression [74]. Remarkably, as we have mentioned above, OCT4 cooperates with these PCR2 components, which we have found to be bound to the *Hmox1* promoter in undifferentiated ES cells along with the aforementioned chromatin repressive mark. Along with the reported role of HO-1 during differentiation, this evidence raises the possibility that the repression of *Hmox1* by OCT4 that we have demonstrated in this work might occur in cooperation with BACH1. Further research is required to explore these hypotheses.

Finally, regarding the HO-1 role in ES cell differentiation, it could be explained by its classic enzymatic activity and/or due to a different yet unknown and noncanonical function. A nuclear function for this

protein has been suggested previously, mainly in the context of stress conditions [17,20,75–78]; however, to date it has not been elucidated. Interestingly, it was recently found that nuclear HO-1 affects the accumulation of G4 structures, which are supposed to influence DNA replication, gene transcription, and translation, in some specific types of stem cells [79]. In this work, as well as previously [62], we have detected HO-1 in the nucleus of ES cells and, remarkably, this signal seems to increase during differentiation, suggesting that HO-1 has a nuclear function relevant to this process which would be worth exploring.

In conclusion, we have discovered that OCT4 represses HO-1 gene expression, further highlighting the connection between pluripotency and the regulation of key components of the stress defense system. The requirement of HO-1 for specific differentiation processes and for efficient reprogramming along with its nuclear localization and its repression by a pluripotency TF, strongly invites further exploration of novel HO-1 functions relevant to such different processes in ES cells. Unraveling key pluripotency TF-mediated gene regulation most certainly enriches our understanding of fundamental properties of stem cells.

Acknowledgement

This work was supported by Universidad de Buenos Aires (20020160100025BA to AG) and Agencia Nacional de Promoción Científica y Tecnológica (PICT 2016-1366 to EV). We would like to thank Dr. Omar Coso and Dr. David Suter for kindly providing pHO-1-Luc and pLVTRE3G YPet-Oct4 vectors, respectively. We would also like to thank Dr. Javier Cotignola for training us in the use and interpretation of databases.

Author contributions

MVP and AG conceived the experiments and wrote the manuscript. MVP performed most of the experiments, analyzed the data and performed the statistical analyses. AT conducted some of the experiments. CVE, CS, MGF, AT, and MSC contributed with experimental work, data interpretation and discussion. EV contributed to the design of the study and discussion. All authors have read and approved the manuscript.

Data accessibility

The data the support the findings of this study are available from the corresponding author upon reasonable request.

References

1 Saretzki G, Walter T, Atkinson S, Passos JF, Bareth B, Keith WN, Stewart R, Hoare S, Stojkovic M, Armstrong L *et al.* (2008) Downregulation of multiple stress defense mechanisms during differentiation of human embryonic stem cells. *Stem Cells* **26**, 455–464.
2 Guo YL, Chakraborty S, Rajan SS, Wang R and Huang F (2010) Effects of oxidative stress on mouse embryonic stem cell proliferation, apoptosis, senescence, and self-renewal. *Stem Cells Dev* **19**, 1321–1331.
3 Saretzki G, Armstrong L, Leake A, Lako M and von Zglinicki T (2004) Stress defense in murine embryonic stem cells is superior to that of various differentiated murine cells. *Stem Cells* **22**, 962–971.
4 Finkel T (2011) Signal transduction by reactive oxygen species. *J Cell Biol* **194**, 7–15.
5 Bigarella CL, Liang R and Ghaffari S (2014) Stem cells and the impact of ROS signaling. *Development* **141**, 4206–4218.
6 Tenhunen R, Marver HS and Schmid R (1968) The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc Natl Acad Sci USA* **61**, 748–755.
7 Poss KD and Tonegawa S (1997) Reduced stress defense in heme oxygenase 1-deficient cells. *Proc Natl Acad Sci USA* **94**, 10925–10930.
8 Wong RJ, Zhao H and Stevenson DK (2012) A deficiency in haem oxygenase-1 induces foetal growth restriction by placental vasculature defects. *Acta Paediatr Int J Paediatr* **101**, 827–834.
9 Trigona WL, Porter CM, Arcidiacono JAH, Majumdar AS and Bloom EDAT. (2007) Could heme-oxygenase-1 have a role in modulating the recipient immune response to embryonic stem cells? *Antioxid Redox Signal* **9**, 751–756.
10 Kreiser D, Nguyen X, Wong R, Seidman D, Stevenson D, Quan S, Abraham N and Dennery PA (2002) Heme oxygenase-1 modulates fetal growth in the rat. *Lab Invest* **82**, 687–692.
11 Kozakowska M, Szade K, Dulak J and Jozkowicz A (2014) Role of heme oxygenase-1 in postnatal differentiation of stem cells: a possible cross-talk with MicroRNAs. *Antioxidants Redox Signal* **20**, 1827–1850.
12 Dennery PA (2014) Signaling function of heme oxygenase proteins. *Antioxidants Redox Signal* **20**, 1743–1753.
13 Mascaró M, Alonso EN, Alonso EG, Lacunza E, Curino AC and Facchinetti MM (2021) Nuclear localization of heme oxygenase-1 in pathophysiological conditions: does it explain the dual role in cancer? *Antioxidants* **10**, 1–15.
14 Jung N, Kim HP, Kim B, Cha SH, Kim GA, Ha H, Na YE and Cha Y (2003) Evidence for heme

oxygenase-1 association with caveolin-1 and -2 in mouse mesangial cells. *IUBMB Life* **55**, 525–532.
15 Slebos DJ, Ryter SW, Van Der Toorn M, Liu F, Guo F, Baty CJ, Karlsson JM, Watkins SC, Kim HP, Wang X *et al.* (2007) Mitochondrial localization and function of heme oxygenase-1 in cigarette smoke-induced cell death. *Am J Respir Cell Mol Biol* **36**, 409–417.
16 Sacca P, Meiss R, Casas G, Mazza O, Calvo JC, Navone N and Vazquez E (2007) Nuclear translocation of haeme oxygenase-1 is associated to prostate cancer. *Br J Cancer* **97**, 1683–1689.
17 Lin Q, Weis S, Yang G, Weng YH, Helston R, Rish K, Smith A, Bordner J, Polte T, Gaunitz F *et al.* (2007) Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. *J Biol Chem* **282**, 20621–20633.
18 Elguero B, Gueron G, Giudice J, Toscani MA, De Luca P, Zalazar F, Coluccio-Leskow F, Meiss R, Navone N, De Siervi A *et al.* (2012) Unveiling the association of STAT3 and HO-1 in prostate cancer: role beyond heme degradation. *Neoplasia* **14**, 1043–1056.
19 Medina MV, Sapochnik D, Garcia Solá M and Coso O (2020) Regulation of the expression of heme oxygenase-1: signal transduction, gene promoter activation, and beyond. *Antioxidants Redox Signal* **32**, 1033–1044.
20 Biswas C, Shah N, Muthu M, La P, Fernando AP, Sengupta S, Yang G and Dennery PA (2014) Nuclear heme oxygenase-1 (HO-1) modulates subcellular distribution and activation of Nrf2, impacting metabolic and anti-oxidant defenses. *J Biol Chem* **289**, 26882–26894.
21 Grochot-Przeczek A, Dulak J and Jozkowicz A (2012) Heme oxygenase-1: non-canonical roles in physiology and pathology. *Clin Sci* **122**, 93–103.
22 Lai Y-LL, Lin C-YY, Jiang W-CC, Ho Y-CC, Chen C-HH and Yet S-FF (2018) Loss of heme oxygenase-1 accelerates mesodermal gene expressions during embryoid body development from mouse embryonic stem cells. *Redox Biol* **15**, 51–61.
23 Suliman HB, Zobi F and Piantadosi CA (2016) Heme oxygenase-1/carbon monoxide system and embryonic stem cell differentiation and maturation into cardiomyocytes. *Antioxidants Redox Signal* **24**, 345–360.
24 Stepniewski J, Pacholczak T, Skrzypczyk A, Ciesla M, Szade A, Szade K, Bidanel R, Langrzyk A, Grochowski R, Vandermeeren F *et al.* (2018) Heme oxygenase-1 affects generation and spontaneous cardiac differentiation of induced pluripotent stem cells. *IUBMB Life* **70**, 129–142.
25 Lin CY, Peng CY, Huang TT, Wu ML, Lai YL, Peng DH, Chen PF, Chen HF, Yen BL, Wu KK *et al.* (2012) Exacerbation of oxidative stress-induced cell death and differentiation in induced pluripotent stem cells lacking heme oxygenase-1. *Stem Cells Dev* **21**, 1675–1687.
26 Loh Y-H, Wu Q, Chew J-L, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J *et al.* (2006) The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet* **38**, 431–440.
27 Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG *et al.* (2005) Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* **122**, 947–956.
28 Solari C, Echegaray CV, Cosentino MS, Petrone MV, Waisman A, Luzzani C, Francia M, Villodre E, Lenz G, Miriuka S *et al.* (2015) Manganese superoxide dismutase gene expression is induced by Nanog and Oct4, essential pluripotent stem cells' transcription factors. *PLoS ONE* **10**, e0144336.
29 Solari C, Petrone MV, Vazquez Echegaray C, Cosentino MS, Waisman A, Francia M, Barañao L, Miriuka S and Guberman A (2018) Superoxide dismutase 1 expression is modulated by the core pluripotency transcription factors Oct4, Sox2 and Nanog in embryonic stem cells. *Mech Dev* **154**, 116–121.
30 Solari C, Petrone MV, Toro A, Vazquez Echegaray C, Cosentino MS, Waisman A, Francia M, Barañao L, Miriuka S and Guberman A (2019) The pluripotency transcription factor Nanog represses glutathione reductase gene expression in mouse embryonic stem cells. *BMC Res Notes* **12**, 1–7.
31 Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M and Rogers D (1988) Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**, 688–690.
32 Ying Q-L, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P and Smith A (2008) The ground state of embryonic stem cell self-renewal. *Nature* **453**, 519–523.
33 Losino N, Luzzani C, Solari C, Boffi J, Tisserand ML, Sevlever G, Barañao L and Guberman A (2011) Maintenance of murine embryonic stem cells' self-renewal and pluripotency with increase in proliferation rate by a bovine granulosa cell line-conditioned medium. *Stem Cells Dev* **20**, 1439–1449.
34 Luzzani C, Solari C, Losino N, Ariel W, Romorini L, Bluguermann C, Sevlever G, Barañao L, Miriuka S and Guberman A (2011) Modulation of chromatin modifying factors' gene expression in embryonic and induced pluripotent stem cells. *Biochem Biophys Res Commun* **410**, 816–822.
35 Losino N, Waisman A, Solari C, Luzzani C, Espinosa DF, Sassone A, Muro AF, Miriuka S, Sevlever G, Barañao L *et al.* (2013) EDA-containing fibronectin

increases proliferation of embryonic stem cells. *PLoS ONE* **8**, e80681.
36 Waisman A, Vazquez Echegaray C, Solari C, Cosentino MS, Martyn I, Deglincerti A, Ozair MZ, Ruzo A, Barañao L, Miriuka S *et al.* (2017) Inhibition of cell division and DNA replication impair mouse-naïve pluripotency exit. *J Mol Biol* **429**, 2802–2815.
37 Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, van den Hoff MJB and Moorman AFM (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res* **37**, e45.
38 Solari C, Echegaray CV, Luzzani C, Cosentino MS, Waisman A, Petrone MV, Francia M, Sassone A, Canizo J, Sevlever G *et al.* (2016) Protein arginine Methyltransferase 8 gene is expressed in pluripotent stem cells and its expression is modulated by the transcription factor Sox2. *Biochem Biophys Res Comm* **473**, 194–199.
39 Cosentino MS, Oses C, Vázquez Echegaray C, Solari C, Waisman A, Álvarez Y, Petrone MV, Francia M, Schultz M, Sevlever G *et al.* (2019) Kat6b modulates Oct4 and nanog binding to chromatin in embryonic stem cells and is required for efficient neural differentiation. *J Mol Biol* **431**, 1148–1159.
40 Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L and Tablada MRCW (2014) Infostat - Statistical software. Grupo InfoStat FCA, Universidad Nacional de Córdoba, Argentina.
41 Villodre ES, Felipe KB, Oyama MZ, de Oliveira FH, da Lopez PLC, Solari C, Sevlever G, Guberman A and Lenz G (2019) Silencing of the transcription factors Oct4, Sox2, Klf4, c-Myc or Nanog has different effect on teratoma growth. *Biochem Biophys Res Commun* **517**, 324–329.
42 Buecker C, Srinivasan R, Wu Z, Calo E, Acampora D, Faial T, Simeone A, Tan M, Swigut T and Wysocka J (2014) Reorganization of enhancer patterns in transition from naive to primed pluripotency. *Cell Stem Cell* **14**, 838–853.
43 Aksoy I, Jauch R, Chen J, Dyla M, Divakar U, Bogu GK, Teo R, Leng Ng CK, Herath W, Lili S *et al.* (2013) Oct4 switches partnering from Sox2 to Sox17 to reinterpret the enhancer code and specify endoderm. *EMBO J* **32**, 938–953.
44 Oki S, Ohta T, Shioi G, Hatanaka H, Ogasawara O, Okuda Y, Kawaji H, Nakaki R, Sese J and Meno C (2018) ChIP-Atlas: a data-mining suite powered by full integration of public ChIP-seq data. *EMBO Rep* **19**, e46255.
45 Deluz C, Friman ET, Strebinger D, Benke A, Raccaud M, Callegari A, Leleu M, Manley S and Suter DM (2016) A role for mitotic bookmarking of SOX2 in pluripotency and differentiation. *Genes Dev* **30**, 2538–2550.
46 Strebinger D, Deluz C, Friman ET, Govindan S, Alber AB and Suter DM (2019) Endogenous fluctuations of OCT4 and SOX2 bias pluripotent cell fate decisions. *Mol Syst Biol* **15**, e9002.
47 Tatapudy S, Aloisio F, Barber D and Nystul T (2017) Cell fate decisions: emerging roles for metabolic signals and cell morphology. *EMBO Rep* **18**, 2105–2118.
48 Ryter SW and Choi AMK (2002) Heme oxygenase-1: molecular mechanisms of gene expression in oxygen-related stress. *Antioxidants Redox Signal* **4**, 625–632.
49 Loboda A, Jozkowicz A and Dulak J (2015) HO-1/CO system in tumor growth, angiogenesis and metabolism — targeting HO-1 as an anti-tumor therapy. *Vascul Pharmacol* **74**, 11–22.
50 Was H, Dulak J and Jozkowicz A (2012) Heme oxygenase-1 in tumor biology and therapy. *Curr Drug Targets* **11**, 1551–1570.
51 Jozkowicz A, Was H and Dulak J (2007) Heme oxygenase-1 in tumors: is it a false friend? *Antioxidants Redox Signal* **9**, 2099–2117.
52 Was H, Cichon T, Smolarczyk R, Rudnicka D, Stopa M, Chevalier C, Leger JJ, Lackowska B, Grochot A, Bojkowska K *et al.* (2006) Overexpression of heme oxygenase-1 in murine melanoma. *Am J Pathol* **169**, 2181–2198.
53 Hill M, Pereira V, Chauveau C, Zagani R, Remy S, Tesson L, Mazal D, Ubillos L, Brion R, Ashgar K *et al.* (2005) Heme oxygenase-1 inhibits rat and human breast cancer cell proliferation: mutual cross inhibition with indoleamine 2,3-dioxygenase. *FASEB J* **19**, 1957–1968.
54 Skrzypek K, Tertil M, Golda S, Ciesla M, Weglarczyk K, Collet G, Guichard A, Kozakowska M, Boczkowski J, Was H *et al.* (2013) Interplay between heme oxygenase-1 and miR-378 affects non-small cell lung carcinoma growth, vascularization, and metastasis. *Antioxidants Redox Signal* **19**, 644–660.
55 Gueron G, De Siervi A, Ferrando M, Salierno M, De Luca P, Elguero B, Meiss R, Navone N and Vazquez ES (2009) Critical role of endogenous heme oxygenase 1 as a tuner of the invasive potential of prostate cancer cells. *Mol Cancer Res* **7**, 1745–1755.
56 Ferrando M, Gueron G, Elguero B, Giudice J, Salles A, Leskow FC, Jares-Erijman EA, Colombo L, Meiss R, Navone N *et al.* (2011) Heme oxygenase 1 (HO-1) challenges the angiogenic switch in prostate cancer. *Angiogenesis* **14**, 467–479.
57 Barbagallo I, Tibullo D, Di Rosa M, Giallongo C, Palumbo GA, Raciti G, Campisi A, Vanella A, Green CJ and Motterlini R (2008) A cytoprotective role for the heme oxygenase-1/CO pathway during neural

differentiation of human mesenchymal stem cells. *J Neurosci Res* **86**, 1927–1935.
58 Li Volti G, Ientile R, Abraham NG, Vanella A, Cannavò G, Mazza F, Currò M, Raciti G, Avola R and Campisi A (2004) Immunocytochemical localization and expression of heme oxygenase-1 in primary astroglial cell cultures during differentiation: Effect of glutamate. *Biochem Biophys Res Commun* **315**, 517–524.
59 Cao YA, Wagers AJ, Karsunky H, Zhao H, Reeves R, Wong RJ, Stevenson DK, Weissman IL and Contag CH (2008) Heme oxygenase-1 deficiency leads to disrupted response to acute stress in stem cells and progenitors. *Blood* **112**, 4494–4502.
60 Barbagallo I, Vanella A, Peterson SJ, Kim DH, Tibullo D, Giallongo C, Vanella L, Parrinello N, Palumbo GA, Di RF *et al*. (2010) Overexpression of heme oxygenase-1 increases human osteoblast stem cell differentiation. *J Bone Miner Metab* **28**, 276–288.
61 Kozakowska M, Ciesla M, Stefanska A, Skrzypek K, Was H, Jazwa A, Grochot-Przeczek A, Kotlinowski J, Szymula A, Bartelik A *et al*. (2012) Heme oxygenase-1 inhibits myoblast differentiation by targeting myomirs. *Antioxidants Redox Signal* **16**, 113–127.
62 Toro A, Anselmino N, Solari C, Francia M, Oses C, Sanchis P, Bizzotto J, Vazquez Echegaray C, Petrone MV, Levi V *et al*. (2020) Novel Interplay between p53 and HO-1 in Embryonic Stem Cells. *Cells* **10**, 35.
63 Sun J, Hoshino H, Takaku K, Nakajima O, Muto A, Suzuki H, Tashiro S, Takahashi S, Shibahara S, Alam J *et al*. (2002) Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene. *EMBO J* **21**, 5216–5224.
64 Verneri P, Vazquez Echegaray C, Oses C, Stortz M, Guberman A and Levi V (2020) Dynamical reorganization of the pluripotency transcription factors Oct4 and Sox2 during early differentiation of embryonic stem cells. *Sci Rep* **10**, 1–12.
65 Cao R and Zhang Y (2004) The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. *Curr Opin Genet Dev* **14**, 155–164.
66 Xue Y, Wong J, Moreno GT, Young MK, Côté J and Wang W (1998) NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. *Mol Cell* **2**, 851–861.
67 Pardo M, Lang B, Yu L, Prosser H, Bradley A, Babu MM and Choudhary J (2010) An Expanded Oct4 interaction network: implications for stem cell biology, development, and disease. *Cell Stem Cell* **6**, 382–395.
68 Liang J, Wan M, Zhang Y, Gu P, Xin H, Jung SY, Qin J, Wong J, Cooney AJ, Liu D *et al*. (2008) Nanog and Oct4 associate with unique transcriptional repression complexes in embryonic stem cells. *Nat Cell Biol* **10**, 731–739.
69 Kashyap V, Rezende NC, Scotland KB, Shaffer SM, Persson JL, Gudas LJ and Mongan NP (2009) Regulation of Stem cell pluripotency and differentiation involves a mutual regulatory circuit of the Nanog, OCT4, and SOX2 pluripotency transcription factors with polycomb Repressive Complexes and Stem Cell microRNAs. *Stem Cells Dev* **18**, 1093–1108.
70 Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK *et al*. (2006) Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* **441**, 349–353.
71 Gao Y, Liu X, Tang B, Li C, Kou Z, Li L, Liu W, Wu Y, Kou X, Li J *et al*. (2017) Protein expression landscape of mouse embryos during pre-implantation development. *Cell Rep* **21**, 3957–3969.
72 Matsumoto M, Kondo K, Shiraki T, Brydun A, Funayama R, Nakayama K, Yaegashi N, Katagiri H and Igarashi K (2016) Genomewide approaches for BACH1 target genes in mouse embryonic fibroblasts showed BACH1-Pparg pathway in adipogenesis. *Genes Cells* **21**, 553–567.
73 Niu C, Wang S, Guo J, Wei X, Jia M, Chen Z, Gong W, Qin Y, Wang X, Zhi X *et al*. (2021) BACH1 recruits NANOG and histone H3 lysine 4 methyltransferase MLL/SET1 complexes to regulate enhancer-promoter activity and maintains pluripotency. *Nucleic Acids Res* **49**, 1972–1986.
74 Wei X, Guo J, Li Q, Jia Q, Jing Q, Li Y, Zhou B, Chen J, Gao S, Zhang X *et al*. (2019) Bach1 regulates self-renewal and impedes mesendodermal differentiation of human embryonic stem cells. *Sci Adv* **5**, eaau7887.
75 Lin QS, Weis S, Yang G, Zhuang T, Abate A and Dennery PA (2008) Catalytic inactive heme oxygenase-1 protein regulates its own expression in oxidative stress. *Free Radic Biol Med* **44**, 847–855.
76 Linnenbaum M, Busker M, Kraehling JR and Behrends S (2012) Heme oxygenase isoforms differ in their subcellular trafficking during hypoxia and are differentially modulated by cytochrome p450 reductase. *PLoS ONE* **7**, e35483.
77 Yang G, Biswasa C, Lin QS, La P, Namba F, Zhuang T, Muthu M and Dennery PA (2013) Heme oxygenase-1 regulates postnatal lung repair after hyperoxia: Role of β-catenin/hnRNPK signaling. *Redox Biol* **1**, 234–243.
78 Gandini NA, Fermento ME, Salomón DG, Blasco J, Patel V, Gutkind JS, Molinolo AA, Facchinetti MM and Curino AC (2012) Nuclear localization of heme oxygenase-1 is associated with tumor progression of head and neck squamous cell carcinomas. *Exp Mol Pathol* **93**, 237–245.
79 Krzeptowski W, Chudy P, Sokołowski G, Żukowska M, Kusienicka A, Seretny A, Kalita A, Czmoczek A,

Gubała J, Baran S *et al*. (2021) Proximity ligation assay detection of protein–dna interactions—is there a link between heme oxygenase-1 and g-quadruplexes? *Antioxidants* **10**, 1–22.

80 Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G and Mesirov JP (2011) Integrative genomics viewer. *Nat Biotechnol* **29**, 24–26.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supporting Information includes Supplementary methods, Supplementary methods tables, Supplementary Appendix and Supplementary References