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Retinoic acid induces Snai1 in stem cells of the preimplantation blastocyst to initiate differentiation.

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Key words: Snai1, Snail, Retinoic acid, Nanog, embryonic stem cells, differentiation, self-renewal.
Abstract (100?)

Although, in general, much is known about the pluripotency self-renewal circuitry, the molecular events that lead ESCs to exit from pluripotency and begin differentiation are largely unknown. We found that the zinc finger transcription factor Snai1, involved in gastrulation and epithelial-mesenchymal transition (EMT) is already expressed in the inner cell mass of the preimplantation blastocysts, where it contributes to ESC exit from plurypotency. In ESCs Snai1 does not respond to TGFβ or BMP4 but is induced by retinoic acid (RA) treatment, which induces the binding, on the Snai1 promoter, of the retinoid receptors RARγ and RXRα, the dissociation of the Polycomb repressor complex 2 (PRC2), and the increase of histone H3 methylation at lysine K4. We found that Snai1 in ESCs mediates the repression of pluripotency genes by binding directly to the promoters of Nanog, Nr5a2, Tcf1, c-Kit, and Tcfcp2l1. Time course analysis shows that the transient activation of Snai1 in Embryoid bodies induces at later time points the expression of the markers of all three germ layers. These results suggest that Snai1 is a key factor that triggers inner cell mass cell of the preimplantation blastocysts to exit from the plurypotency state and initiate their differentiation processes.

Significance

Embryonic stem cells, derived from the inner cell mass, can be maintained indefinitely in their self-replication mode by growing them under defined conditions that sustain the expression of genes involved in their self-renewing. Less is known about the molecular mechanisms that induce stem cells of the inner cell mass to enter into their differentiation processes. We found that retinoic acid, which has been previously shown to be expressed in the trophoectoderm of the preimplantation blastocyst, is a potent inducer of the transcription factors Snai1, which in turn represses the expression of the genes for self-renewal. Thus Snai1, induced by retinoic acid triggers the embryonic stem cells toward their differentiation.
**Introduction.**

Embryonic stem cells (ESCs) derived from the inner cell mass (ICM) are able to self renew or initiate their differentiation by the establishment of multiple regulatory pathways. ESC self renew is maintained by a complex network of transcription factors, which is self-propagated by numerous autoregulatory loops (1). This network is centered around the three leading actors Oct3/4, Sox2, and Nanog, but includes several other transcription factors such as Esrrb, Klf2, Klf4, and Tbx3 (2-5). Although much is known about the regulatory circuitry that maintain ESC identity less is known about the extrinsic stimuli and intrinsic factors that trigger the exit of ICM cells toward their next differentiation steps.

The Snail family of transcription factors that includes the Snai1 (Snail homolog 1), Snai2 (Slug), and Snai3 (Smug) encode zinc finger transcription factors, which function mainly as transcriptional repressors. They are involved in physiological and cancer-associated epithelial-mesenchymal transition (EMT) by directly repressing the E-cadherin promoter and other cell-cell adhesion molecules (6-10). Different signaling pathways, including TGFβ, BMP, FGF, and WNT have been implicated in the induction of Snail family members during the process of EMT (7, 8). In postimplantation embryo Snai1 is expressed in the mesoderm during gastrulation, in neural crest, and in the primitive streak (11, 12). Interestingly, Snail1-deficient embryos are smaller than control littermate embryos already at E7.5 and, besides Brachyury, the expression of the visceral endoderm marker Cerl was severely reduced (13), suggesting a role of Snail before gastrulation and in other tissues than mesoderm.

Retinoic acid (RA), an active metabolite of the vitamin A (retinol), is a potent signaling molecule that exerts pleiotropic roles in patterning, morphogenesis, and organogenesis during vertebrate embryonic development (14). All-trans retinoic acid (atRA) acts through binding to nuclear retinoic acid receptors (RARs). RARs are ligand-inducible nuclear transcription factors, which heterodimerize with the retinoid X receptors (RXRs; nuclear receptors that bind the 9-cis-RA
stereoisomer) and bind to cis-acting retinoic acid response elements (RAREs) in regulatory regions of target genes.

*In vitro* prolonged retinoic acid exposure promotes ESC differentiation into primitive endoderm and cells of the three primary germ layers, depending on culture conditions (15-17). Despite these observations and the well-known effects of retinoids in cellular differentiation and embryonic development, the role of these compounds in preimplantation embryo and early ESC lineage specification has not yet been clarified. It has been previously demonstrated that trophoblast cells of preimplantation blastocysts express retinoids and RA activity has been observed already at the 3.5-day mouse blastocyst in both ICM and trophectoderm (18, 19). However, its function at this early stage of embryo development has not been clarified.

Here, we investigate the mechanisms by which RA promotes early differentiation of ESCs. We demonstrate that the transcription factor Snai1 is expressed in the ICM of preimplantation mouse blastocyst and it is a primary target of RA in ESCs. We further show that Snai1 is able to promote the ESC exit from the pluripotency by direct repression of self-renewal genes *Nanog, Nr5a2, Tcl1, c-Kit, and Tcfcp2l1*. 
Results

*Snail* is induced by RA in undifferentiated ESCs. During embryo development *Snail* has been shown to be required for gastrulation, mesoderm and neural crest formation (7-9, 20). To verify whether *Snail* plays a role in early stages of cell differentiation we analyzed by immunofluorescence its expression in preimplantation mouse blastocysts. At embryonic (E) day 3.5 *Snail* was expressed in Oct3/4 positive nuclei of ICM cells (Fig. 1A and Supplementary Fig. S1). In epithelial-mesenchymal transition (EMT) the transcription of *Snail* is activated by the cytokines of the TGF-β family through the Smad phosphorylation (7). In ESCs, TGF-β1 and BMP4 failed to promote *Snail* expression (Fig. 1B and C) even if BMP4 induced strong Smad2/3 phosphorylation (Fig. S2A). Time course analysis by RT-qPCR and Western blot revealed that *Snail*, but not the related transcription factors Snai2, Snai3, Twist, Zeb1, and Zeb2, is transiently expressed after atRA or its 9-cis isomer treatment achieving the maximum expression level at 2 hours (Fig. 1B, Fig. S2B and data not shown) while the inducers of ESC differentiation bFGF and Activin did not upregulated *Snail* in ESCs (Fig 1C). The regulation of *Snail* by RA is a general feature of ESCs because *Snail* undergoes upregulation upon atRA treatment also in R1 and WW6 mouse ESC lines and even in human BG01V ESCs (Fig. 1D and Fig. S2C).

On the *Snail* promoter we found a canonical conserved retinoic acid response element (RARE-DR5) centered at position -543 with respect to the transcriptional start site (Fig. S3). Interestingly, it has been previously shown that trophoectoderm cells are the major source of retinoids in domestic pigs (18) and RA activity has been measured already at 3.5-day of mouse blastocyst both in the trophoectoderm and in ICM (19). Chromatin immunoprecipitation (ChIP) assays demonstrated that RARγ and RXRα bind the *Snail* promoter in ESCs and their binding increases following atRA treatment (Fig. 1E). *Snail* promoter is bivalent in ESCs as it is marked by both H3K4me3 and H3K27me3. Cell treatment with atRA induced Suz12 dissociation from the promoter which was
accompanied by H3K27me3 demethylation and increase of H3 acetylation and methylation at K4 (Fig. 1F). Cyp26a1 and Oct3/4 were used as positive and negative control, respectively (Fig. S4).

Snail1 is a transcriptional repressor of genes involved in pluripotency and self-renewal. To investigate the role of the early Snail1 expression in ESC differentiation, we generated a stable ESC line expressing the Snail1 whose activity is under the control of 4-hydroxytamoxifen (OHT), (Snail1-ER) (21). Following induction with 1 µM OHT, the chimeric protein Snail1-ER is rapidly localized in the nucleus, where it is stable for about 8 hours (Fig. 2A) and ESCs acquired a more differentiated phenotype (Fig. 2B).

To identify Snail1 regulated genes, we performed a microarray analysis using mRNA from Snail1-ER ESCs induced at different time periods with OHT. We found 131 genes down-regulated more than 2.5-fold at 12 hours post-treatment (p<0.01)(Table S1). Interestingly, 17 of them were genes involved in ESC self-renewal and pluripotency, with a high percentage of transcriptional regulators (Fig. 2C, D). Repression by Snail1 was validated by RT-qPCR (Fig. 2E).

As a control, we treated wild type ESCs with OHT or vehicle alone (EtOH) and did not observe any significant difference in the mRNA expression for all genes tested.

To evaluate the direct binding of Snail1 to the identified target genes we selected out those carrying conserved Snail1 binding sites within their promoter regions (Table S2). These genomic regions were analyzed by ChIP using a specific antibody for Snail1 (Fig. 3A). The promoters of Nanog, Nr5a2, Tcfcp2l1, c-Kit, and Tcl1 showed binding of Snail1-ER that was significantly increased following OHT treatment. Because Nr5a2, Tcfcp2l1, c-Kit, and Tcl1 were described to be targets of Nanog (22), we analysed the repression by Snail1 of these genes in the presence of ectopic Nanog, to investigate whether their repression was due to direct Snail1 binding or Nanog down-regulation. Following OHT treatment, the expression of these genes was still repressed also in the presence of Nanog, while the expression of Esrrb, which was not found to be direct Snail1 target, was no more
repressed in presence of exogenous Nanog (Fig. 3B and C). Thus demonstrating that Nanog, Nr5a2, Tcfcp2l1 c-Kit, and Tcll are all direct Snai1 targets.

In agreement with these observations Snai1 knockdown cells showed more regular ESC colony morphology with an increased and uniform ALP staining and an augmented expression of the key pluripotency markers Nanog, Esrrb, Klf4, and Tcfcp2l1 (Fig. 3D-F).

Snai1 expression induces ESC exit from pluripotency. To verify whether Snai1 expression could promote ESC differentiation we analyzed the effect of transient activation of Snai1-ER in embryoid bodies (EBs). EBs were formed in phenol-red free medium containing Charcoal/Dextran absorbed serum to deplete the basal levels of retinoids and estrogens. After 2 days, EBs were treated with 1 μM 4-OHT, or vehicle alone (EtOH), for 2 hours and then disaggregated and re-aggregated (Fig. 4A) to reduce the Snai1-ER activation to a single pulse and avoid the mesoderm determination due to a prolonged expression of active Snai1 (9). As shown in Figure 4B, the Snai1-dependent differentiation potential was tested by analysis of the mRNA levels of pluripotency and differentiation markers by RT-qPCR. The early pulse of Snai1 activity in ESCs by OHT treatment induced a persistent reduction of Rex1 expression within 24 hours and a transient strong reduction of Nanog expression, while Oct3/4 mRNA level did not change markedly during differentiation.

Analysis of expression of differentiation markers showed a strong increase in the expression of the primitive endoderm (PrE) marker Dab2 (23), the mesendodermal marker Gsc, the primitive streak marker T (Brachyury-Bry) and Flk1, Sox1 and Foxa2, markers of the mesoderm (24), neuroectoderm, and definitive endoderm layers, respectively. It is interesting to note that expression of endogenous Snai1, necessary for the completion of the mesoderm development, was stimulated by the transient activation of Snai1-ER in ESCs.

Collectively these data suggest that Snai1, induced by ER in preimplantation blastocysts, promotes ESCs to exit from pluripotency and initiate their differentiation program.
Discussion

It has been previously demonstrated that preimplantation blastocysts express RA and ICM respond to its signaling (18, 19). Here we found that the transcription factor Snai1 is already expressed in ICM of the preimplantation blastocysts and it is induced in response to RA. We demonstrate that its function in ESCs is to contribute to their exit from the naïve state by direct repression of pluripotency genes.

Interestingly, the induction of Snail by RA appears to be ESC-specific, because it was induced by RA in different ESC lines but was not upregulated by RA in several cell lines, including A549, MCF7, HT29, PANC-1, and PC3, that do express Snail and response to TGF-β (not shown).

It has been previously shown the essential role of RARγ and RXRα in RA-dependent gene regulation in ESCs (25, 26). We found that RARγ and RXRα are constitutively bound to Snail promoter in the absence of ligand and their binding is increased following atRA treatment. This regulation is in agreement with the described model for retinoid receptor activation in which the receptors bound on the DNA repress transcription through the recruitment of the corepressors and the ligand-induced conformational changes increase DNA affinity (27, 28) and favor the switch between corepressors and coactivators (29).
We observed that upon atRA treatment the reduction of PRC2 binding, H3K27me3 mark together with the increase of H3 acetylation and H3K4methylation on the Snai1 promoter. This result is in agreement with the previous observation that in F9 embryonal carcinoma cells the heterodimer RARγ/RXRα was bound on the RAREs elements of the Cyp26A1 and the Hoxa1 promoters in association with the polycomb group (PcG) protein Suz12, promoting methylation on H3K27 and transcriptional repression. This association was attenuated by atRA treatment with consequent transcriptional activation of the target genes (30).

Differently from the prolonged exogenous expression of Snai1 that promotes differentiation of epiblast cells to early mesoderm repressing the microRNA-200 (9), we observed in ESCs the Snai1-dependent repression of genes associated with pluripotency with the consequent increased expression of markers for all three germ layers. This effect was due exclusively to the defined activation window, since we also observed that prolonged expression of Snai1 promoted mesoderm differentiation (not shown). We found that in ESCs Snai1 represes pluripotency and self-renewal genes. Notably, 7 of the identified Snai1 target genes *Esrrb*, *Klf4*, *Klf2*, *Nanog*, *Tbx3*, *Rex1* and *Nr5a2* are key regulators of ESC identity, which have been described to be downregulated during conversion of ESCs into EpiSCs (31).

Snai1 has been previously shown to be involved in post-implant development of the embryo as it is involved in gastrulation and the formation of a primitive streak and several different signalling pathways have been associated with the induction of *Snail1* (7).

Our results now show that Snai1 acts at two independent differentiation steps: it promotes early stages of ESC differentiation by direct repression of pluripotency and self-renewal genes when expressed in ESC and mesoderm formation when expressed in epiblast cells. This result was also confirmed by Snai1 expression before EBs aggregation which recapitulates the first ICM/ESCs lineage segregation between epiblasts and primitive endoderm with the downregulation of the pluripotency markers *Rex1* and *Nanog* while Oct3/4 was not down modulated followed by the spontaneous activation of endogenous Snai1 transcription when Flk1 expression marks mesoderm
differentiation.

In fact it was previously shown that upon ESC differentiation *Rex1* is down-regulated in primitive endoderm and epiblast lineages, *Nanog* is repressed in primitive endoderm and transiently down-modulated in epiblast and pluripotency marker *Oct3/4* is expressed in the ICM cells and later in epiblast and in the primitive endoderm cells at the early stage of blastocyst development (31-36).

In summary, this study has revealed that Snai1, besides inducing mesoderm formation also controls the exit of ESC from pluripotency and self-renewal.
Materials and Methods

**Plasmids DNA constructs.** cDNA of human *Snail* coding region without stop codon, in frame with cDNA of mouse *ERα* ligand-binding region was obtained by PCR from *pBabePuro-hSnai1.ER.NoTag* (Addgene plasmid 19292) (21) using following oligonucleotides: 5’-GAGAGGATCCSCCATGCCGCGCTCTTTCCTC-3’ and 5’-GAGAGTCGACTCAGATCGTGTTGGGGAA-3’, and cloned in the lentiviral vector pCCLsin.hPGK.GFP.pre. The plasmid was confirmed by sequencing. Silencing experiments were performed using lentiviral vectors pLKO.1 from the TRC-Mm1.0 shRNA library (Open Biosystems, Huntsville, AL) expressing specific shRNA for mouse Snai1 (clone TRCN0000096619) and for eGFP as negative control (#RHS4459). Mouse Nanog cDNA was obtained from total ESCs mRNA using the following oligonucleotides: 5’-GAGAGGATCCACCATGAGTGGGTCTTCCTGG-3’ and 5’-GAGAGTCGACCCCATGATGTGGTGGTCCTCTGCTG-3’, and cloned in pCCL lentiviral vector.

**Lentiviral particles production and cells transduction.** Lentiviral vector were co-transfected with *pMD2.VSVG, pMDLg/pRRE, pRSV-Rev* in Lenti-X™ 293 cell line (Clonetech) to produce lentivirus particles as previously described (37). To determine viral titre cells were infected at 2x10⁵ clles/ml in 6 well plates with serial dilution of viral preparation and 8 μg/ml Hexadimetrine-bromide (Sigma) and analyzed 24 hours later for GFP protein expression levels through fluorescence microscope. 2x10⁵ E14 ESCs were plated in 35mm dish and infected with 15 μl of pre-titred viral preparation and 8μg/ml Hexadimetrine-bromide (Sigma). 24 hours after infection medium has been changed and cells were treated with Puromycin 6 μg/ml for 6 days.
**ES cell maintenance.** Mouse ESCs were cultured on gelatin coated dishes in feeders free culture system in serum containing medium consisting of DMEM 4.5 g/l glucose, 15% Embryomax FBS (Millipore), NEAA 100X (Invitrogen), Na-Pyruvate 100X (Invitrogen), LIF ESGRO 1000U/ml (Millipore) and 2-Mercaptoethanol 50 µM (38). Mouse GFP-Bry ES cell line was kindly provided by Dr. Gordon Keller, and cultured in serum free medium on gelatin coated dishes in feeders free culture system as previously described (39).

**ES cell differentiation.** For embryoid-bodies (EBs) formation we used the protocol described previously (40) with the following modification: E14 ESCs expressing Snail1-ER were plated at 2.5x10^5 cells/ml in ultra low attachment plates (Costar) in MEM ALPHA Medium w/o Phenol-red (Invitrogen) supplemented with 10% Charcoal/Dextran Treated FBS (HyClone) and 2-Mercaptoethanol 50 µM. After 46 hours EBs were treated for additional 2 hours with 1 µM 4-Hidroxy-tamoxifen (4-OHT) (#H7904, Sigma-Aldrich). Subsequently, EBs were dissociated with trypsin and cells were reaggregated at 1.5x10^5 cells/ml in the same medium and new EBs were harvested 1,2 and 3 days later. GFP-Bry ESCs were cultured in serum-free medium consisting of 75% IMDM (Invitrogen), 25% Ham’s F12 (Invitrogen) supplemented with 0.5x N2B27 (w/o retinoic acid) supplement (Invitrogen), 0.05% BSA, 2 mM glutamine (Invitrogen), 0.5 mM ascorbic acid (Sigma), 0.45 mM 1-thioglycerol (Sigma). After 24 hours GFP-Bry ESCs were treated for additional 24 hours with 0.1 µM atRA. Subsequently, EBs were dissociated with trypsin and cells were reaggregated at 1.5x10^5 cells/ml in the same medium and new EBs were harvested 3 days later for flow cytometry analysis.

**RT-qPCR.** Total RNA was prepared Total RNA was prepared with TRIreagent (Invitrogen) according to the manufacturer’s protocol. RT-qPCR was performed with SuperScript III One-Step RT-PCR System and SYTO9 Green-Fluorescent Nucleic Acid Stain (Invitrogen) on Rotor Gene 6000 (Corbett Research)(41). The oligonucleotides used for RT-qPCR are listed in Table S3).
**Flow cytometry analysis.** EBs generated from GFP-Bry cell differentiation were dissociated with Dissociation Buffer PBS-based (Invitrogen) for 1-2 min and arranged at 1x10^6 cells/ml in PBS and 1 µl of reconstituted fluorescence reactive dye LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) was added and cell were incubated for 30 min protecting from light and analyzed by FACS CantoII (Becton Dickenson). Data analysis was performed by FlowJo software (Tree Star Inc.)

**Cell Fractionation.** Cell were collected in PBS, centrifuged 10 min at 1850 xg, and then resuspended in 5 volumes (cellular pellet volumes) of Hypotonic Buffer (20 mM HEPES pH 7.9, 1 mM MgCl₂, 10 mM KCl, 0.1% Triton X-100, 20% glycerol plus fresh 0.5 mM DTT, 10mM Na-Butirrate); centrifuged 5 min at 1850 xg at 4°C, and then resuspended in 3 volumes of Hypotonic Buffer. After 10 min of ice incubation cell were centrifuged at 3300 xg for 15 min supernatant was collected as the cytoplasmatic fraction and pellet as the integral nuclear fraction. Nuclei were resuspended in 2 volumes of Hypotonic Buffer plus 3 volumes of 2X-F Buffer (20 mM TRIS HCl pH 7.0, 100 mM NaCl, 60 mM Na₄O₇P₂, 50 mM NaF, 10 µM ZnCl₂, 2% TRITON X-100), mixed by gently rotation for 30 min, and then centrifuged for 30 min at 25000 xg at 4°C. Supernatant is collected as Nuclear fraction.

**Protein extracts, immunoblotting, and immunofluorescence.** Cells were lysed in RIPA buffer and IP performed as previously described (42, 43). Immunofluorescence assays were carried out as previously described (44).

**ChIP assay.** Each Chromatin Immunoprecipitation (ChIP) experiment was performed in at least three independent biological samples as previously described (45). Briefly, 1x10^6 cells were cross-linked with 1% formaldehyde for 10 min at R.T. quenched with 0.125 M Glycin for 5 min at R.T.,
washed twice with PBS and resuspended in 0.2 ml of SDS lysis-buffer, stored on ice for 10 min, sonicated for 15 min 0.5X using Bioruptor (Bio-Rad) and then centrifuged at 20000 xg for 10 min at 4°C. Supernatants were diluted 10 folds with ChIP dilution Buffer (1% kept as input) and incubated with 1-2 µg of primary antibody in gentle rotation at 4°C O/N. Then 10 µl of pre-blocked protein A beads were added and incubated for 1 hour in the same conditions. Then extract and beads complexes were washed for three times respectively with: low-salt buffer, high-salt buffer, LiCl buffer and twice with TE buffer. Elution was performed by adding 0.25 ml of elution buffer for 15 min in gentle rotation at R.T. After RNase and proteinase K treatment, DNA was purified with phenol-chloroform extraction followed by ethanol precipitation. DNA was analyzed by real time quantitative PCR (RT-qPCR) using the SYBR GreenER™ qPCR SuperMix and SYTO9 Green-Fluorescent Nucleic Acid Stain. (Invitrogen). For the amplification of immunoprecipitated DNA, we used the oligonucleotides listed in Table S4.

**Microarray analysis.** RNA was extracted and purified as previously described (46). Microarray was performed on Illumina Platform and analyzed using BeadStudio Gene Expression Module (GX). Data were background adjusted and quantile normalized using default parameters in the BeadStudio Software. Probes with Log[|FC|]>1+and p-value <0.05 were selected for downstream analysis. Heat-map plots were performed through the Bioconductor package in R. Differential expression analysis of the up- or down-regulated genes were performed by plotting genes on their Log₂ expression value using Excel (Microsoft™).

**Antibodies and Reagents.** All-trans Retinoic acid (atRA) (R2625) and 9-cis Retinoic acid (R4643), Retinol (R72632) were obtained from Sigma-Aldrich (Missouri, USA). TGF-β1 (240-B-010), mBMP4 (5020-BP-010) and Activin A (338-AC-010) were obtained from R&D System. bFGF (AA-10-155) was obtained from Life Technology.
For Western Blot primary antibodies: anti SP1 (sc-59), anti ERα (sc-542), anti Tubulin β (sc-9104) Santa-Cruz Biotechnology, anti Lamin B1 (#33-2000) Zymed Research, anti Snai1 (#3879), anti human Snai1 (#4719), anti Slug (#9585), anti Smad2/3 (#3102), anti Smad1 (#9743) and anti pSmad1/5 (#9516) Cell Signalling, anti pSmad2/3 (#AB3849) Millipore and anti-Nanog (#ab14959) Abcam. For ChIP assay: Suz12 (#D39F6, Cell Signalling), RARγ (#abin123009, Antibodies-online), RARα (sc-553-X, Santa-Cruz Biotechnology), rabbit IgG (sc-2027, Santa-Cruz Biotechnology), Histone H3-trimethyl-K4 (#07-473, Millipore), Histone-trimethyl-K27 (#07-449, Millipore), Histone H3-diacetyl (#06-599, Millipore) and anti Snai1 (#3879, Santa-Cruz Biotechnology).

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Author Contribution
F.G., C.L, and F.N, designed experiments, performed the research and analyzed the data; C.DeC, M.O, F.A, M.G., D.D., and S. B performed research. F.G. and S.O. designed the research, analyzed the data and wrote the paper.

The authors declare no conflict of interest.
References


Figure legends.

Fig. 1. Snai1 is expressed in preplantation blastocysts and induced in ESC by retinoic acid. (A) Snail is localized to the nucleus of ICM-Oct3/4 positive cells of the pre-implant mouse blastocyst at E3.5. (B) Snail expression is transiently induced by 0.1 µM atRA, but not by TGF-β1 (3ng/ml) or Bmp4 (10ng/ml). The results were normalized to β-Actin expression. Mean values from three independent experiments are shown with standard deviations. (C) Western blot analysis of Snai1 expression in ESCs. Activin and bFgf were used at the concentration of 10 ng/ml. Proteins levels confirmed the mRNA expression data. The ubiquitous transcription factor Sp1 was used to verify equal loading. (D) The expression levels of Snai1 in atRA treated human ESCs were analyzed by Western blot. (E) Snail promoter is bound by retinoic acid receptors in ESCs. ChIP analysis of RARγ, RXRα, and Suz12 on the promoter region (-600 with respect to the transcriptional start site). atRA 0.1 µM was added at the indicated time points (min). IgG purified from non-immune serum were used as negative control. Data are expressed as percentage of DNA inputs. (F) ChIP analysis of histone modifications on the Snail promoter in control and atRA induced ESCs. Data represent average ±SD of three independent experiments.

Fig. 2. Snail directly represses key self-renewal regulators. (A) Time course analysis of Snail-ER localization showing the rapid and transient Snail-ER nuclear localization and stabilization following 1 µM OHT treatment. Laminin and Tubulin were used as loading and fractionation controls of nucleus and cytoplasm, respectively. (B) Bright field of ESC colonies in control and treated with OHT for 48 hours. (C) Venn diagram of microarray analysis showing genes more than 2.5-fold down-regulated after
12 hours of 1 µM 4-OHT treatment (D) Heat map of microarray analysis showing the down-regulated self-renewal markers at the indicated time points after 4-OHT treatment. (FC: Fold Change). (E) Validation of microarray analysis by reverse transcription real-time polymerase quantitative chain reaction (RT-qPCR) analysis in wild type and Snai1-ER expressing ESCs treated for 12 hours with OHT or EtOH. Values are normalized to Actin β expression and represented as fold change, comparing OHT to EtOH treated cells (expressed in log10 scale). Oct3/4 expression analysis was performed as negative control. Data represent average values ±SD of three independent experiments. P value was calculated with the Student's t test by comparing mRNA fold change of wt cells to fold change of Snai1-ER expressing cells.

**Fig. 3.** Snai1 binds to the promoter regions of pluripotency genes. (A) ChIP of Snai1-ER expressing ESC treated with 1 µM OHT or EtOH for 2 hours and immunoprecipitated with Snai1 antibody. Enrichment relative to the Actin β promoter subtracted of IgG background is measured by RT-qPCR using the primers specific for the indicated genes. Data represent average ±SD of three independent experiments. P value was calculated with the Student's t test by comparing OHT to EtOH treated cells. (B) Western blot analysis of Nanog expression in ESCs Snai1-ER transduced with GFP (negative control) or Nanog (Nanog). (C) RT-qPCR expression analysis of the indicated genes. Values are normalized to Actin β expression and represented as fold change, comparing OHT to EtOH treated cells. Data represent average values ±SD of three independent experiments. P value was calculated with the Student's t test by comparing normalized mRNA values of treated cells to untreated cells. (D) Western blot analysis of Snai1 in control or silenced cells as indicated. (E) Morphology and Alcaline phosphatase (ALP) staining of control or Snai1 silenced ESCs. Snai1 silenced cells present a more regular ESCs colony morphology and an increased and uniform ALP staining. (F) RT-qPCR expression analysis of key pluripotency genes in control or Snai1 silenced ESCs. Data represent average values ±SD of three independent experiments. P value was calculated
with the Student's t test.

**Fig. 4.** (A) Schematic representation of the experimental procedure used to test the ESCs differentiation potential after a single pulse treatment with OHT. (B) Time course analysis of the indicated genes by RT-qPCR. Values are normalized to Actin β expression and represented as fold change relative to value of day 2. Gray line indicates the single pulse OHT treatment. Data represent average values ±SD of three independent experiments. P value was calculated with the Student's t test by comparing mRNA fold change of EtOH treated cells to fold change of OHT treated cells.