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Maternal SIN3A Regulates Reprogramming of Gene Expression During Mouse Preimplantation Development¹

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ABSTRACT

The oocyte-to-embryo transition entails genome activation and a dramatic reprogramming of gene expression that is required for continued development. Superimposed on genome activation and reprogramming is development of a transcriptionally repressive state at the level of chromatin structure. Inducing global histone hyperacetylation relieves this repression and histone deacetylases 1 and 2 (HDAC1 and HDAC2) are involved in establishing the repressive state. Because SIN3A is an HDAC1/2-containing complex, we investigated whether it is involved in reprogramming gene expression during the course of genome activation. We find that *Sin3a* mRNA is recruited during maturation and that inhibiting its recruitment not only inhibits development beyond the 2-cell stage but also compromises the fidelity of reprogramming gene expression. The SIN3A that is synthesized during oocyte maturation reaches a maximum level in the mid-1-cell embryo and is essentially absent by the mid-2-cell stage. Overexpressing SIN3A in 1-cell embryos has no obvious effect on pre- and postimplantation development. These results provide a mechanism by which reprogramming can occur using a maternally inherited transcription machinery, namely, recruitment of mRNAs encoding transcription factors and chromatin remodelers, such as SIN3A.

gene expression, histone modifications, preimplantation embryo, reprogramming gene expression, zygotic gene activation

INTRODUCTION

Full-grown mouse oocytes are transcriptionally quiescent. Following fertilization, a low level of transcription initiates in 1-cell embryos with genomewide transcription of intra- and

intergenic regions [1]. This minor wave of genome activation has minimal promoter requirements for transcription, and the transcripts are nonfunctional because they are inefficiently spliced and polyadenylated. The major wave of genome activation occurs during the 2-cell stage and entails a major change in the transcriptome [2–5] that likely is critical for further development and conversion of a highly differentiated oocyte into totipotent blastomeres.

Superimposed on genome activation is development of a chromatin-mediated transcriptionally repressive state. Evidence for this transition comes from work with plasmid-borne reporter genes as well as endogenous genes. For example, 1-cell embryos do not require an enhancer for efficient expression, whereas an enhancer is required for efficient expression in 2-cell embryos [6, 7]. The requirement for an enhancer reflects formation of a transcriptionally repressive state in 2-cell embryos. Consistent with a role of enhancers to relieve chromatin-mediated repression of transcription is the observation that increasing histone acetylation relieves this requirement. Although these conclusions were drawn using viral promoters, similar conclusions were reached using expression the endogenous *Eif1a* gene [8, 9].

Histone deacetylase 1 (HDAC1) is the major HDAC involved in the formation of the transcriptional repressive state that develops during the 2-cell stage because knockdown of HDAC1, but not HDAC2, in preimplantation embryos results in hyperacetylation of histone H4 and prevents the normal decrease of endogenous genes, for example, *Eif1a* [10]. Class I HDACs such as HDAC1 and 2 typically reside in complexes, for example, NuRD, NODE, COREST, and SIN3A [11–13]. We focused our attention on SIN3A, which is a scaffolding protein that interacts with HDAC1/2, because *Sin3a* is essential for mouse development; *Sin3a* embryonic null embryos perish around Embryonic Day 6.5 (E6.5) [14, 15]. Because *Sin3a* null embryos were generated from crossing *Sin3a* heterozygous mice, the role of maternal SIN3A in early preimplantation development, and in particular, the role in development of the chromatin-mediated transcriptionally repressive state was not known.

We report here that SIN3A is encoded by a dormant maternal mRNA that is recruited during oocyte maturation and the newly synthesized protein is essentially degraded by the early 2-cell stage in a proteasome-dependent manner. Inhibiting the maturation-associated increase in SIN3A protein using a combined small interfering RNA (siRNA)/morpholino approach markedly inhibits development beyond the 2-cell stage following in vitro fertilization (IVF). Microarray analysis reveals that a subset of normally expressed genes is expressed at higher levels in SIN3A-knockdown embryos and that another subset of genes not expressed in 2-

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cell embryos become expressed. Interestingly, no significant increase in global histone acetylation is observed in 1-cell embryos. Last, overexpressing SIN3A in 2-cell embryos does not inhibit the incidence of development to the blastocyst or implantation.

MATERIALS AND METHODS

Oocyte and Embryo Collection, and Embryo Culture and Transfer

Germinal vesicle (GV)-intact oocytes were collected from 6-wk-old CF-1 mice primed with eCG for 44 h before isolation as previously described [16]; 10 μ M milrinone was present in the collection medium to prevent resumption of meiosis [17]. Metaphase I (MI) oocytes were collected 7 h after transferring full-grown oocytes to milrinone-free Chatot Ziomek Brinster medium [18]. Metaphase II (MII) eggs were collected from eCG-primed 6-wk-old CF-1 female mice 13–16 h following hCG administration as previously described [19]. MII eggs were also obtained following *in vitro* maturation (IVM) for 16–18 h after transferring full-grown oocytes to milrinone-free Chatot Ziomek Brinster medium. Mid-1-cell, late 1-cell, early 2-cell, mid-2-cell, 8-cell, and blastocyst stage embryos were collected from eCG-primed 6-wk-old CF-1 female mice mated to B6D2F1/J males (Jackson Laboratory) by flushing either the oviduct or uterus 20–21, 30–32, 36, 44, 68, and 94–96 h post-hCG as previously described [20].

Synchronization of 1- and 2-cell embryos was performed as follows. One-cell embryos generated from IVF were cultured in KSOM medium and examined for the appearance of pronuclei at 1 h intervals or for the first cleavage at 1 h intervals starting at 19 h post-IVF. Embryos that formed pronuclei or underwent the first cleavage within the previous hour were collected and cultured separately. One-cell embryos used for global histone modification analysis were fixed 6 h after pronucleus formation. Two-cell embryos used for microarray analysis were collected and frozen 12 h postcleavage. For global transcriptional analysis, 2-cell embryos were added to 2 mM 5-ethynyl uridine (EU) in KSOM medium 12 h postcleavage.

In some experiments, mid-1-cell embryos were cultured in KSOM medium [21, 22] containing either 20 μ M N-(benzyloxycarbonyl)leucinyllucinyllucinal (MG132) (Sigma) or dimethyl sulfoxide (DMSO) (Sigma) at 37°C under an atmosphere of 5% O₂, 5% CO₂, and 90% N₂. After 5 and 10 h of culture, 40 1-cell embryos were collected from the MG132 group and the DMSO control group at each of the two time points for immunoblot analysis.

To assess the effect of overexpressing SIN3A on development, embryo transfer experiments were conducted as follows. *Gfp*^{-/-} virgin CF-1 females were mated to *Gfp*^{+/-} males. The resulting *Gfp*^{+/-} embryos were collected 18 h after fertilization, and embryos with two distinct pronuclei were microinjected at 19–21 h after fertilization with *Sin3a* cRNA; controls were injected with buffer. The embryos were cultured for 96 h (to E4.5) in KSOM medium as described above at which time the number of blastocysts was scored and GFP expression assessed. To determine the incidence of implantation of *Sin3a* cRNA-injected embryos compared with control embryos, blastocyst stage embryos were transferred to pseudopregnant female mice on Postcoital Day 3.5 using the Non-Surgical Embryo Transfer Device (Paratechs) according to the manufacturer's protocol. Each female received 8–10 embryos, half of which were injected with *Sin3a* (*Gfp*^{+/-} or *Gfp*^{-/-}) and the other half injected with (buffer) control (*Gfp*^{+/-} or *Gfp*^{-/-}). Thus, each female received four to five GFP-positive (or negative) *Sin3a* cRNA-injected embryos and four to five GFP-negative (or positive) control embryos. The females were killed 7 days after embryo transfer (E10.5), and the presence of GFP expression in the implanted embryos was assessed. All the animal experiments were approved by the University of Pennsylvania Institutional Animal Use and Care Committee and were consistent with the National Institutes of Health guidelines.

Microinjection

Full-grown GV-intact oocytes isolated from cumulus cell-enclosed oocytes (CEOs) were microinjected with 5 μ l of a solution containing 5 μ M siRNA and 1 mM morpholino that target *Sin3a* mRNA; oocytes were injected while being cultured in bicarbonate-free minimum essential medium (MEM) containing 10 mM HEPES, pH 7.2, 10 μ M milrinone, and 20% fetal bovine serum [23]. The cRNA for *Sin3a*-T7 and GFP were injected at 580 ng/ μ l. The *Sin3a* (s73784; Ambion) and control *Luciferase* siRNA (D-001100-01-05; Dharmacon) were both injected at a concentration of 5 μ M. The concentration of the *Sin3a* (5'-CCTGGTCATCAAACGTCGCTTCAT-3'; GeneTools) and standard control morpholinos (GeneTools) was 1 mM.

IVM and IVF

For IVM and IVF, CEOs from 24-day-old B6SJL/F1/J females primed with eCG 44 h before isolation were collected in MEM supplemented with 10 μ M milrinone and 20% fetal bovine serum as previously described [24]. The cumulus cells were gently stripped from the oocytes using a mouth-operated pipette. The medium used for oocyte maturation was MEM supplemented with 0.23 mM pyruvate and 20% fetal bovine serum. The denuded oocytes were cultured in this medium under drops of mineral oil at 37°C in 5% O₂, 5% CO₂, and 90% N₂ for 13–14 h in the presence of 10–15 CEOs as previously described [25].

Fertilization of eggs *in vitro* was performed as previously described [25]. The fertilization medium was Tyrode medium supplemented with 4 mg/ml of bovine serum albumin [26]. The MII eggs were washed in 4 drops of fertilization medium and placed into 50 μ l drops overlaid with oil. The eggs were then inseminated using sperm (5×10^5 sperm/ml) capacitated for 3 h at 37°C in 5% O₂, 5% CO₂, and 90% N₂. Sperm adhering to the eggs were removed using a fine-bore pipette, and the inseminated eggs were then transferred to and cultured in KSOM medium under oil at 37°C in 5% O₂, 5% CO₂, and 90% N₂. MII eggs were activated by SrCl₂ treatment as previously described [27].

Immunocytochemistry

Oocyte, MI, egg, or embryo samples were collected and fixed in 2.5% paraformaldehyde for 20 min at room temperature within 2 days. The samples were permeabilized for 15 min in PBS containing 0.1% Triton X-100, washed, and blocked for 30 min with PBS containing 0.1% bovine serum albumin, 0.01% Tween-20. The samples were next incubated with a rabbit anti-SIN3A antibody (MBL International) at 1:100 in blocking buffer overnight at 4°C, followed by three 15-min washes in blocking solution (PBS containing 0.1% bovine serum albumin and 0.01% Tween-20). After the washes, the samples were incubated for 1 h with the anti-rabbit cy5-conjugated secondary antibody (Jackson ImmunoResearch) diluted 1:100 in blocking solution. In some cases 1-cell embryos were first permeabilized for 15 min in PBS containing 0.1% Triton X-100, washed, and then fixed in 2.5% paraformaldehyde for 20 min at room temperature. The samples were then processed as described above.

Polyclonal antibodies against histone H3 acetylated on K18 (1:100, 39756; Active Motif), histone H4 acetylated on K5 (1:50, 06-759; Millipore), histone H4 acetylated on K8 (1:100, 06-760; Millipore), histone H4 acetylated on K12 (1:100, 06-761; Millipore), histone H4 acetylated on K16 (1:100, 06-762; Millipore) were used to assess histone modifications. Trichostatin A (TSA) (Sigma), a histone deacetylase inhibitor, was used to induce histone hyperacetylation of early 1-cell embryos by treating the cells with 50 nM TSA for 5 h. After three 15-min washes with blocking solution, the samples were incubated with 1 μ M SYTOX Green (Molecular Probes) to stain DNA. The cells were then mounted under a coverslip in VECTASHIELD medium (Vector Laboratories). A Leica TCS SP laser-scanning confocal microscope captured the images and detected the fluorescence intensity of the samples. For each experiment, all the samples were processed in parallel. For SIN3A, the laser power was adjusted so that the signal intensity was below saturation for the developmental stage that showed the highest signal intensity, and all the images were then scanned at that laser power. The images were processed, and the fluorescence intensity was quantified using ImageJ software (National Institutes of Health).

Immunoblot Analysis

Protein extracts from 40 oocytes, eggs, or embryos were solubilized in Laemmli sample buffer [28], resolved by SDS-PAGE (7.5% gel), and transferred to a polyvinylidene fluoride membrane. The membrane was blocked in 2% Amersham ECL prime blocking reagent (GE Healthcare Life Sciences) for 1 h and incubated at 4°C overnight with the primary antibody in blocking solution. The membrane was then washed four times with PBS with 0.1% Tween-20, incubated with a secondary antibody conjugated with horseradish peroxidase for 1 h in blocking solution, and washed four times with PBS containing 0.1% Tween-20. The signal was detected with the Amersham ECL Select Western blot detection reagent (GE Healthcare Life Sciences) according to the manufacturer's instructions. The SIN3A primary antibody (BMP004; MBL International) was diluted 1:2000 in blocking solution. The TUBA primary antibody (T6074; Sigma) was diluted 1:5000 in blocking solution. The Amersham ECL secondary antibody (NA934V and NA931V; GE Healthcare Life Sciences) was diluted 1:75 000 in blocking solution.

RNA Extraction, RT-PCR, and Real-Time PCR

Total RNA from 20 embryos was extracted using the Arcturus PicoPure RNA Isolation Kit (Life Technologies) according to the manufacturer's instructions

using added *Gfp* RNA as an external standard. The reverse transcription reaction using random hexamers was performed as previously described [10]. The cDNA was then quantified by quantitative real-time PCR (qRT-PCR) using the ABI Taqman Assay-on-demand probe/primer sets for *Sin3a* and *GFP* as previously described [29]. For each qRT-PCR, one embryo equivalent of cDNA was used with a minimum of three replicates as well as a minus reverse transcription and minus template control. Quantification was normalized to *GFP*.

DNA Replication Assay

Inseminated MII eggs were cultured in KSOM medium containing 10 μ M bromodeoxyuridine (BrdU) 3 h postinsemination or soon after first cleavage. One-cell embryos were fixed 19 h postinsemination, and the 2-cell embryos were fixed 34 h postinsemination. The cells were then processed using the aforementioned immunofluorescence protocol with the addition of a denaturing step and neutralizing step after permeabilization. The samples were denatured for 30 min in 2 N HCl and neutralized for 20 min in 100 mM Tris-HCl, pH 8.5, at room temperature. The samples were incubated overnight at 4°C with the mouse monoclonal anti-BrdUTP antibody (11170376001; Roche) at 1:50 in blocking solution. The samples were incubated with the secondary fluorescein isothiocyanate anti-mouse IgG1 antibody (1144-02; Southern Biotech) at 1:100 in blocking solution for 1 h and then mounted in VECTASHIELD medium containing 2 μ M TO-PRO-3 (Life Technologies).

Global Transcriptional Assay

Click-iT RNA Imaging kit (Invitrogen) was used to assay global transcription following the manufacturer's instructions. Briefly, early 2-cell embryos were cultured with 2 mM EU in KSOM medium for 1 h before fixation in 2.5% for 20 min at room temperature. To assay transcription in 1-cell embryos, the cells were incubated in KSOM medium containing 2 mM EU from the time the first pronucleus formed until 17 h postinsemination.

After washing and membrane permeabilization, incorporated EU was detected using the Click-iT detection molecule. The samples were mounted in VECTASHIELD medium containing 2 μ M TO-PRO-3 to visualize the DNA. DNA and EU were visualized using a Leica TCS SP laser-scanning confocal microscopy. The intensity of the fluorescence was quantified using ImageJ software as previously described [30].

Plasmid DNA Constructs

Firefly luciferase reporter constructs under the control of the *Sin3a* 3'-untranslated region (3'-UTR) were generated as previously described [31]. The entire 1-kb *Sin3a* 3'-UTR with a poly (A) site was amplified using the forward primer 5'-GATATCTAGACTGCAGAGCCAGAGCAGGTAGC-3' and reverse primer 5'-GCGCCGAATTCACCTATTTCCTTAAGAATCAAGCT-3'. Amplified *Sin3a* 3'-UTR were digested by *Xba*I and *Eco*RI and subcloned downstream of the coding sequence of the pIVT-*Luc* vector.

To generate a T7 C-terminal-tagged *Sin3a* cRNA, mouse *Sin3a* coding sequence was amplified from mouse cDNA clone 6837144 (Thermo Scientific Open Biosystems) by PCR using the forward primer 5'-gaggGCATGCcATGAAGaGgaGacTGGAcGACCA-3' and the reverse primer 5'-gcgGTCGACCCGGCCACGCGTAGGGGCTTTGAATACTGTGCCGTA-3'. After enzymatic digestion by *Sph*I and *Sal*I-HF, the amplified *Sin3a* coding sequence was subcloned into the pIVT-T7 vector to make pIVT-*Sin3a*-T7.

In Vitro Transcription

The DNA sequence-verified pIVT-*Sin3a*-T7 construct was linearized by *Sfo*I digestion. Capped cRNAs were made using in vitro transcription with T7 mMESSAGE mMachiner (Ambion) according to the manufacturer's instructions. Following in vitro transcription, template plasmid DNAs were digested by adding RNase-free DNase, and the synthesized cRNA were purified by MEGAclear Kit (Ambion), precipitated, and redissolved in RNase-free water.

The DNA sequence-verified pIVT-firefly *Luc*/*Sin3a* 3'-UTR construct was linearized by *Eco*RI digestion. The product was then processed as described above. For both constructs, a single band of the correct size was observed following electrophoresis in an agarose gel containing 1% formaldehyde. Synthesized cRNA was aliquoted and stored at -80°C. For microinjection controls, polyadenylated *Renilla luciferase* cRNA was generated as previously described [31].

Luciferase Assay

Luciferase assays following injection of cRNA encoding the different reporters were performed as previously described [31].

Microarrays Analysis

Total RNA was extracted from 20 oocytes/embryos as described above and amplified with an Ovation Pico WTA system V2 (NuGEN). The material was then fragmented and labeled with the Encore Biotin Module V2 (NuGEN). Four independent biological replicates were hybridized to GeneChip Mouse 2.0 ST microarrays (Affymetrix).

The microarray datasets from the MoGene-2_ (GPL16570) platform were processed using the Oligo package [32] from the Bioconductor framework [33]. Raw data was background corrected, normalized, and summarized using the robust multiarray average procedure.

Differential expression analysis was done using a robust linear model with empirical Bayes from the limma package [34]. We compared the following conditions: α -amanitin versus control 2-cell embryo; *Sin3a* KD (knockdown) 2-cell embryo versus control 2-cell embryo; control 2-cell embryo versus GV oocyte; and *Sin3a* KD 2-cell embryo versus GV oocyte. The *P*-values were calculated using a moderate *t*-statistic. The calculated *P*-values were adjusted for multiple testing using the false discovery rate procedure. Genes were marked as differentially expressed if they had a minimal absolute fold-change >1.5 and the calculated *P*-value was <0.05. A list of all differentially expressed genes for which a gene was differentially expressed for a least one comparison group is found in Supplemental Table S1 (Supplemental Data are available online at www.biolreprod.org).

Platform annotations were downloaded from the Gene Expression Omnibus repository, and all genes that mapped to differentially expressed Affymetrix probesets (defined as false discovery rate <0.05 and absolute logarithmic fold-change >0.58, in any of the comparisons), were kept for further analysis. The 29 680 Affymetrix probesets, which could be annotated, were used for the differential expression analysis, and functional analysis of differentially expressed gene sets was done using the pantherdb database [35].

Statistical Analysis

One-way ANOVA were used to evaluate the differences between groups using GraphPad Prism 6. A level of *P* < 0.05 was considered to be significant.

RESULTS

Sin3a Is a Dormant Maternal mRNA That Is Recruited for Translation During Oocyte Maturation and Following Fertilization

Oocyte maturation triggers degradation of many maternal mRNAs [36], and not surprisingly, the relative abundance of *Sin3a* mRNA decreased during maturation reaching a low point at the 8-cell stage before increasing, likely due to zygotic expression (Fig. 1A). Analysis of the relative amount of SIN3A protein by immunoblotting, however, revealed only a small amount of SIN3A protein in GV oocytes with a dramatic increase between MI and MII and a further increase following fertilization (Fig. 1B). Striking is a dramatic loss of SIN3A protein by the 2-cell stage. Consistent with *Sin3a* transcript abundance is the observation that the amount of SIN3A protein appeared lowest in 8-cell embryos and then increased by the blastocyst stage. Immunocytochemical detection of SIN3A protein, which was nuclear, was consistent with the immunoblotting results (Fig. 1C). The failure to detect SIN3A in MI and MII eggs is attributed to the >25-fold dilution into the cytoplasm following GV breakdown and the pronounced staining in the pronuclei due to accumulation of the newly synthesized protein. In addition, SIN3A localization in male and female pronuclei was not altered when the 1-cell embryos were permeabilized prior to fixation (Supplemental Fig. S1), suggesting that SIN3A is chromatin-associated in both pronuclei.

Further support that *Sin3a* mRNA is recruited during maturation is the observation that there was a pronounced increase in luciferase activity following maturation of GV oocytes injected with a cRNA encoding luciferase fused to the *Sin3a* 3'-UTR (Fig. 2A). In addition, injection of the cRNA

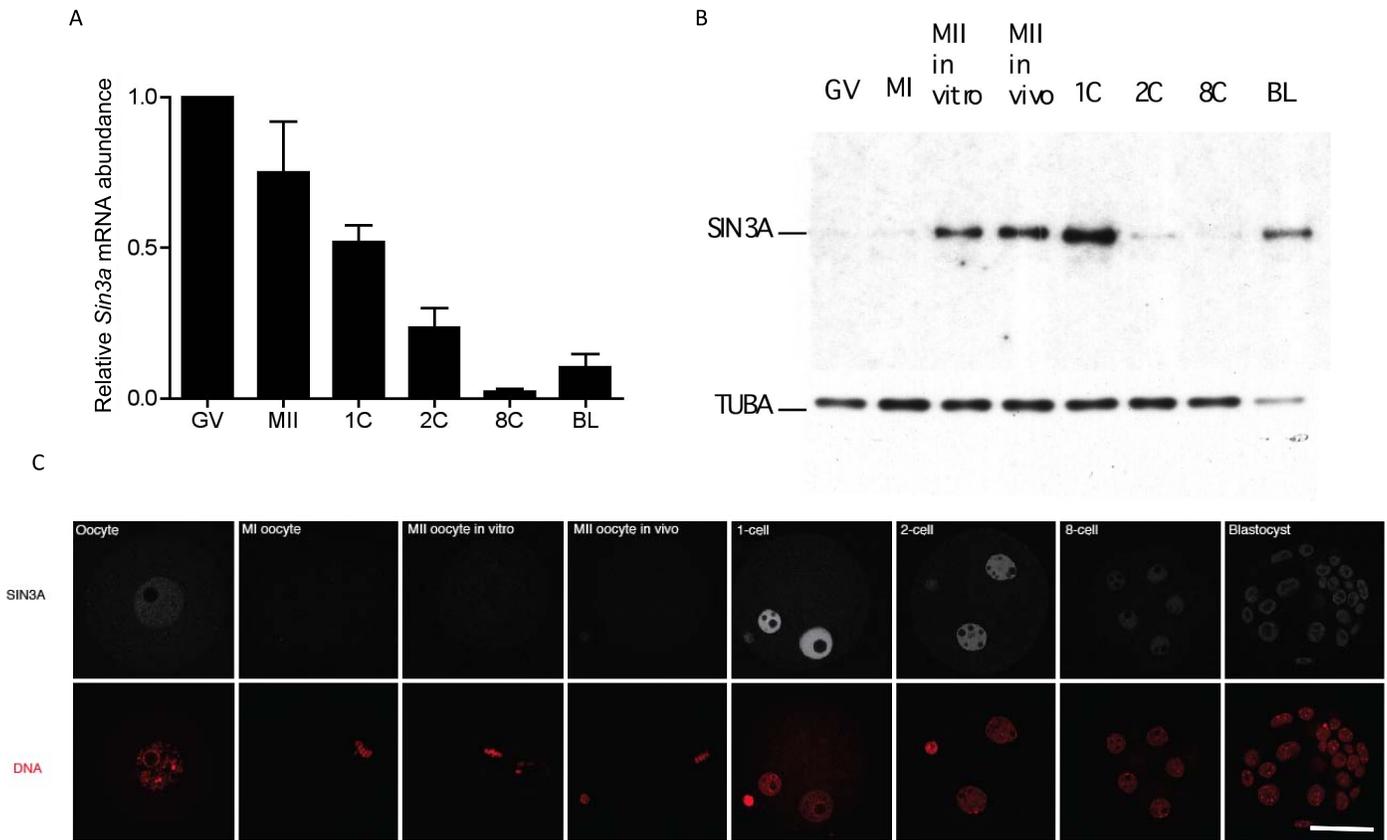


FIG. 1. Developmental expression profile of *Sin3a*/SIN3A. **A**) *Sin3a* mRNA levels were measured by qRT-PCR at the indicated stages. Data were normalized against the detected levels of exogenously added *GFP* and expressed relative to the value obtained for mid-1-cell embryos. The experiment was conducted three times, and the data are expressed as mean \pm SEM. GV, full-grown GV-intact oocytes; MII, metaphase II-arrested egg; 1C, 2C, and 8C refer to 1-cell, 2-cell, and 8-cell stages, respectively; BL, blastocyst. **B**) The relative amount of SIN3A was measured by immunoblot analysis. MII in vitro, oocytes were matured in vitro to MII; MII in vivo, MII eggs were collected following maturation in vivo. The TUBA signal was used to normalize total protein loading. The experiment was performed three times, and similar results were obtained in each case; shown is a representative example. **C**) Immunocytochemical analysis of SIN3A expression during preimplantation development. The experiment was conducted twice, and at least 15 oocytes/embryos were analyzed for each experiment. Bar = 50 μ m.

into MII eggs that were then activated revealed an increase in luciferase activity (Fig. 2B). This increase in luciferase activity is consistent with the additional increase in the amount of SIN3A protein detected by immunoblotting between the MII egg and 1-cell stage (Fig. 1B).

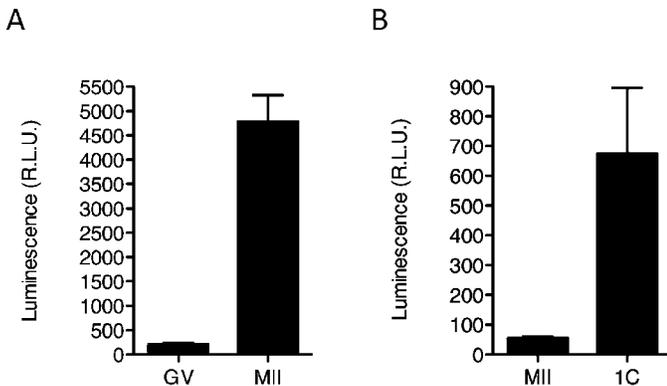


FIG. 2. *Sin3a* 3'-UTR contains elements that drive translational recruitment during oocyte maturation and following activation. Full-grown oocytes (**A**) or MII eggs (**B**) were microinjected with the luciferase reporter cRNAs. Firefly luciferase reporter activities were normalized to the coinjected Renilla luciferase. The data are expressed as mean \pm SEM, and at least 10 oocytes/embryos were analyzed for each group.

To define better the time course for degradation of newly synthesized SIN3A protein, an immunoblot analysis was conducted using 1-cell and 2-cell embryos (Fig. 3). The results of these experiments indicated that \sim 65% of SIN3A is degraded by the late 1-cell stage, \sim 85% by the early 2-cell stage, and it being virtually absent by the mid-2-cell stage. SIN3A degradation was presumably mediated by the proteasome because MG132, a proteasome inhibitor, substantially inhibited SIN3A degradation (Fig. 4).

Inhibiting the Maturation-Associated Increase in SIN3A Perturbs Histone Acetylation in 1-Cell Embryos

The results described above suggest that the function of newly synthesized SIN3A is restricted mainly to the 1-cell stage. Given this restricted window, we focused our efforts on ascertaining the effect of inhibiting the maturation-associated increase in SIN3A on histone acetylation in 1-cell embryos. We used a combined siRNA/morpholino approach that effectively inhibited the maturation-associated increase in SIN3A (Fig. 5). We analyzed 1-cell embryos produced following maturation and insemination in vitro rather than activating the in-vitro matured, microinjected oocytes because of the known differences in histone modifications in the male and female pronuclei [37].

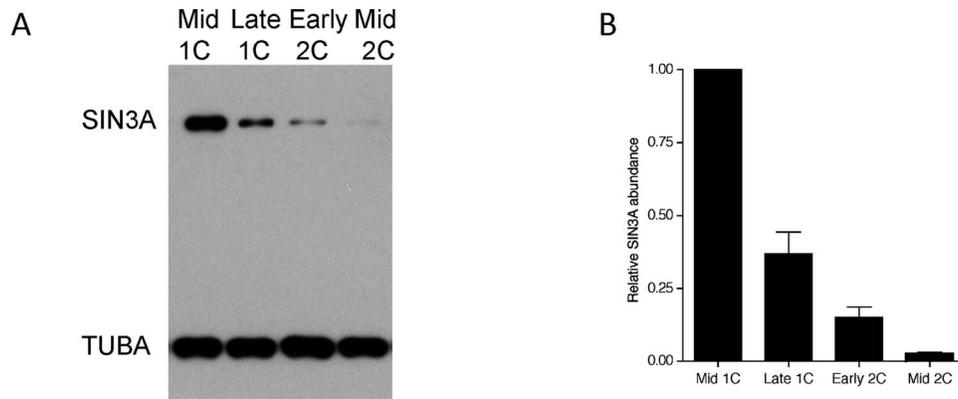


FIG. 3. Time course for SIN3A degradation. **A)** Immunoblot analysis for SIN3A was conducted at the indicated developmental stages. The TUBA signal was used to normalize total protein loading. The experiment was performed three times, and similar results were obtained in each case; shown is a representative example. **B)** Quantification of the relative amount of SIN3A shown in **A**. The data are expressed as mean \pm SEM.

Because SIN3A is part of the SIN3A-HDAC complex, we assessed whether inhibiting the increase in SIN3A that occurs during maturation affects histone acetylation in 1-cell embryos as assessed by immunocytochemistry (Fig. 6). We analyzed H3K18ac, H4K5ac, H4K8ac, H4K12ac, and H4K16ac because these marks are affected when HDAC function is lost (e.g., [10]) and because these acetylated histones are located within three distinct regions of genes; H3K18ac is mainly located in the region surrounding the transcriptional start site, whereas the others are enriched in the promoter and transcribed regions of active genes [38]. Although TSA, an HDAC inhibitor, results in a modest hyperacetylation of all of these marks, surprisingly no hyperacetylation was observed when the maturation-associated increase in SIN3A was inhibited, and if anything, hypoacetylation was observed, for example, H3K18ac, H4K8ac, and H4K12ac.

Inhibiting the Maturation-Associated Increase in SIN3A Inhibits Development Beyond the 2-Cell Stage

We next assessed the effect of inhibiting the maturation-associated increase in SIN3A on preimplantation development

and found that whereas control-injected embryos developed beyond the 2-cell stage, development arrested at the 2-cell stage for the experimental group (Fig. 7). The low incidence of development in the control group likely reflects the deleterious effects on development when denuded oocytes, which have been microinjected, are then matured and inseminated in vitro. It is unlikely that arrest at the 1-cell and 2-cell stages was due to failure to replicate DNA because a similar fraction of both experimental and control embryos displayed BrdU incorporation with comparable signal intensity in both groups (Supplemental Fig. S2).

Inhibiting zygotic genome activation with α -amanitin leads to developmental arrest at the 2-cell stage in mice [39]. Transcription, as assessed by EU incorporation, was reduced in 2-cell embryos derived from eggs in which the maturation-associated increase in SIN3A was inhibited (Fig. 8) relative to controls. The \sim 50% decrease, however, was not sufficient to cause arrest at the 2-cell stage because transcription must be reduced by at least 70% to result in such an arrest [40]. Inhibiting the maturation-associated increase in SIN3A had no effect on transcription in either the male or female pronucleus of 1-cell embryos (Supplemental

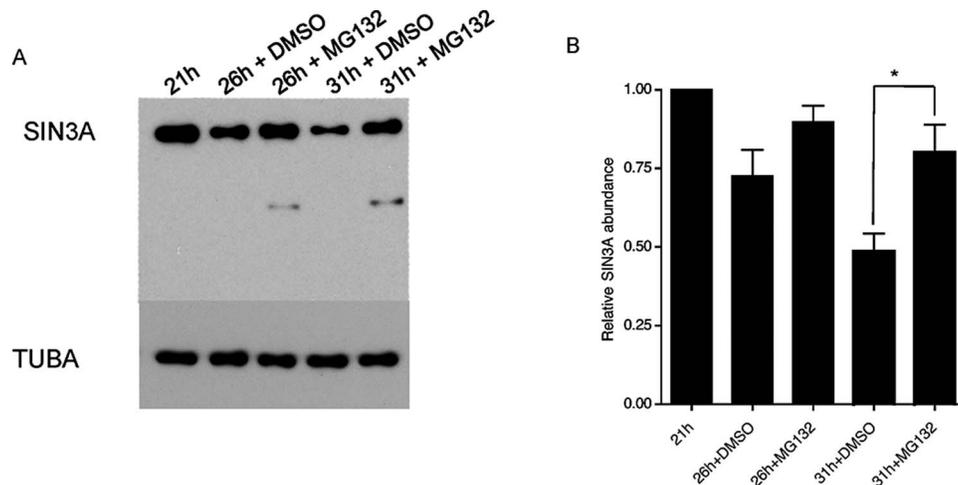


FIG. 4. SIN3A degradation is proteasome-dependent. **A)** Mid-1-cell embryos were isolated and cultured in vitro in the presence of the proteasome inhibitor MG132 or DMSO, the vehicle. SIN3A abundance was measured by immunoblot analysis at the indicated times. The TUBA signal was used to normalize total protein loading. The experiment was performed three times, and a representative example is shown. **B)** Quantification of the data shown in **A**. The data are expressed as the mean \pm SEM, and the SIN3A signal is relative to the mid-1-cell embryo. The times refer to the number of hours post-hCG injection. * $P < 0.05$.

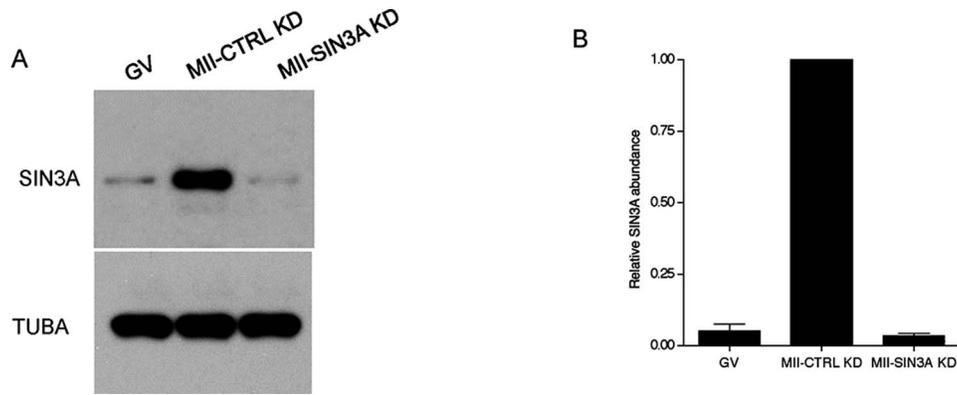


FIG. 5. Combined *Sin3a* siRNA and morpholino inhibit maturation-associated increase in the amount of SIN3A. **A**) Full-grown oocytes microinjected with control or *Sin3a* morpholino (MO) and siRNA were cultured for 1 h in medium containing 2.5 μ M milrinone and then cultured in inhibitor-free medium for maturation. MII eggs were collected 16 h after maturation and used for immunoblot analysis to detect SIN3A protein levels. TUBA was used to normalize total protein loading. The experiment was performed three times, and a representative example is shown. **B**) Quantification of the relative amount of SIN3A shown in **A**. The data are expressed as mean \pm SEM.

Fig. S3). Although transcription in 1-cell embryos is not required for cleavage to the 2-cell stage, recent results have now shown that it is required for development beyond the 2-cell stage [1].

Reprogramming of Gene Expression in SIN3A-Depleted Embryos

A major reprogramming of gene expression occurs during the mid- to late 2-cell stage in mouse [2–5], and inappropriate

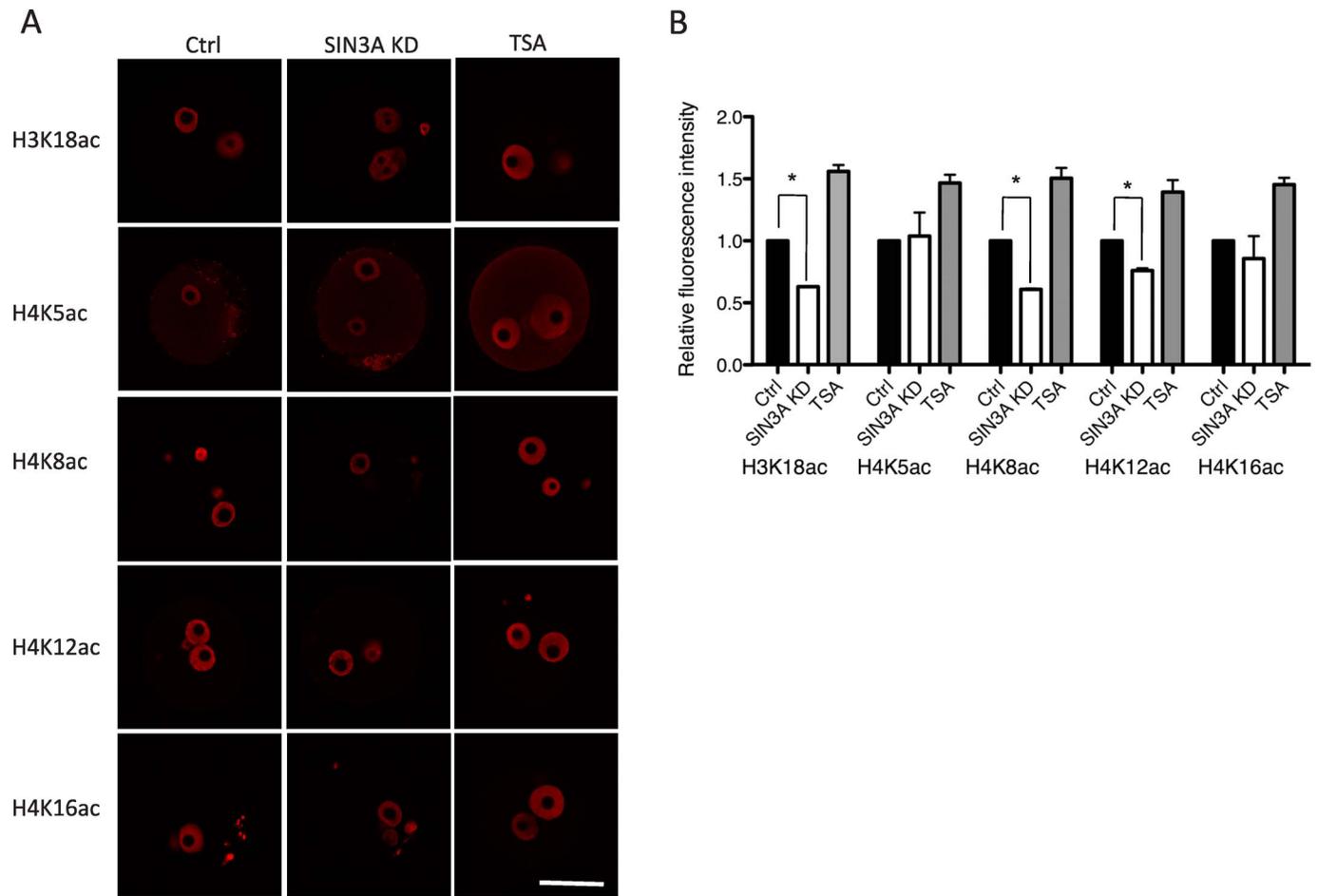


FIG. 6. Effect of inhibiting the maturation-associated increase in SIN3A on histone acetylation in 1-cell embryos. **A**) The indicated histone were assayed by immunocytochemistry for their acetylation status using maternal SIN3A-depleted, control 1-cell embryos (Ctrl), and 1-cell embryos that were incubated with TSA (an HDAC inhibitor) to generate the maximum increase in histone acetylation. The experiment was performed twice, and at least a total of 10 embryos were analyzed for each sample. Bar = 50 μ m. **B**) Quantification of the data shown in **A**. The data are expressed relative to the control 1-cell embryos and are expressed as mean \pm SEM. * $P < 0.05$.

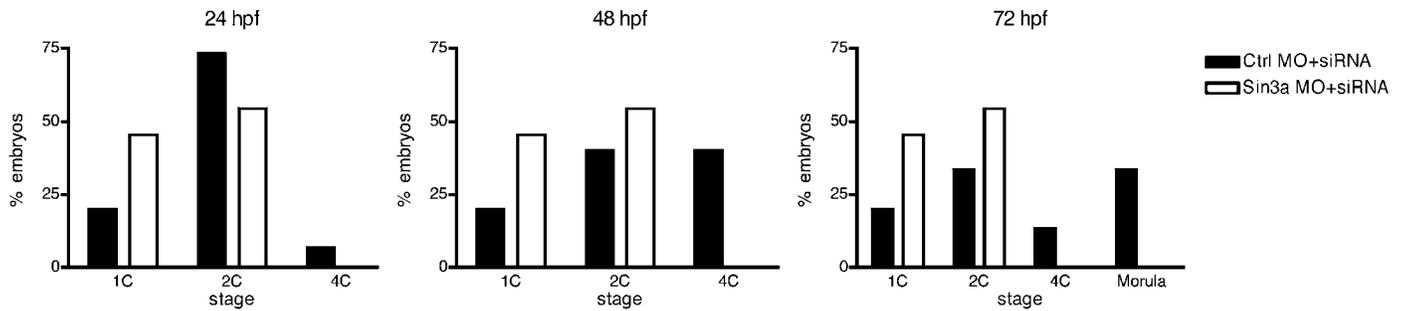


FIG. 7. Inhibiting the maturation-associated increase in SIN3A leads to arrest at the 2-cell stage. After inhibiting the maturation-associated increase in SIN3A, the maternal SIN3A-depleted and control MII eggs were in vitro fertilized (IVF) and embryo development was assessed at 24, 48, and 72 h after fertilization; hpf, hours postfertilization. The experiment was performed three times and the data pooled. A total of 56 experimental and 43 control embryos were analyzed.

reprogramming is associated with 2-cell arrest (e.g., [41]). The observed 2-cell arrest when the maturation-associated increase in SIN3A was inhibited suggested that reprogramming of gene expression was compromised. Accordingly, we conducted microarray analyses with the expectation that a subset of zygotically expressed genes, that is, genes whose expression is inhibited by α -amanitin, would be perturbed.

Hierarchical cluster analysis revealed that as anticipated the transcriptome of GV oocytes and α -amanitin-treated 2-cell embryos was similar and differed from the transcriptome of control and experimental 2-cell embryos (Fig. 9). Furthermore, finding that the transcriptomes of the control and α -amanitin-treated 2-cell embryos was consistent with perturbed expression of a subset of zygotically expressed genes.

A chromatin-based transcriptionally repressive state develops during the 2-cell stage, and this repression is relieved by histone hyperacetylation. Accordingly, we first filtered the dataset for α -amanitin-sensitive genes whose expression was higher in SIN3A-depleted embryos (Supplemental Table S2); the criterion used was that expression in control 2-cell embryos was at least 1.5-fold higher than in GV oocytes. Expression of 145 zygotically expressed genes was higher in 2-cell embryos when the maturation-associated increase in SIN3A was inhibited. These genes included not only protein-coding genes but also ribosomal genes, small nuclear RNAs, and noncoding RNAs.

We also asked whether a subset of genes that are not zygotically expressed in 2-cell embryo were now expressed in

SIN3A-depleted embryos (Supplemental Table S2). We identified 98 genes that displayed no difference in expression between α -amanitin-treated and control 2-cell embryos but whose expression was increased in SIN3A-depleted embryos when compared to control 2-cell embryos. Similar to the situation for zygotically expressed genes, these genes included protein coding genes, ribosomal genes, small nuclear RNAs, and noncoding RNAs but also small nucleolar RNAs, and microRNAs. We also selected four genes from each class to confirm by qRT-PCR that their expression was increased in the SIN3A-depleted 2-cell embryos and found this to be the case for seven of them (Supplemental Table S3).

Mapping the genes whose expression was increased when the maturation-associated increase in SIN3A was inhibited to chromosomes revealed that although there are observable clusters, these clusters fall within gene clusters on the chromosomes, that is, gene-dense regions (Fig. 10). Thus, their positional enrichment is not unexpected. Nevertheless, some chromosomes were enriched for these genes (e.g., chromosome 12), whereas others were not (e.g., chromosome 1) (Supplemental Table S4).

Exogenously Expressing SIN3A Beyond the 1-Cell Stage Does Not Impair Preimplantation Development

The highly restricted window for the presence of SIN3A—it accumulates during maturation and then is essentially absent by the early 2-cell stage—contrasts to other proteins encoded by

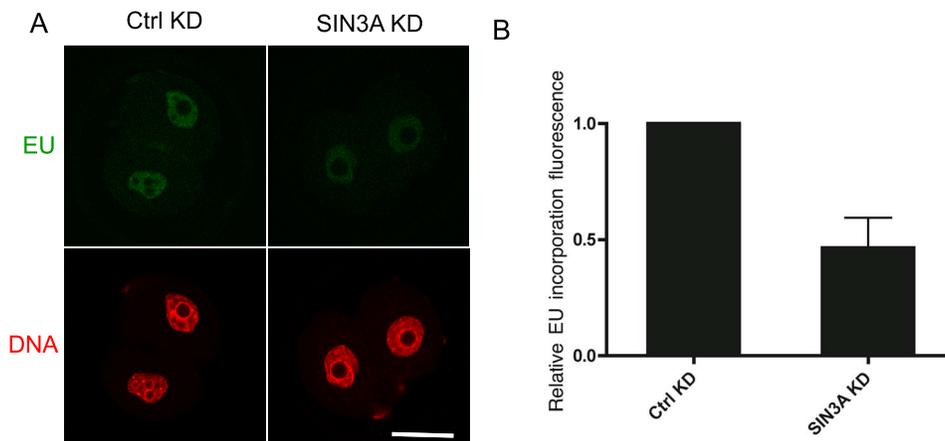


FIG. 8. Transcription is reduced in 2-cell embryo when maternal SIN3A is depleted. **A**) The experiment was performed four times and shown are representative images. At least 10 embryos were analyzed for each treatment group. Bar = 25 μ m. **B**) Quantification of the images shown in **A**. The data are expressed as mean \pm SEM. * P < 0.05.

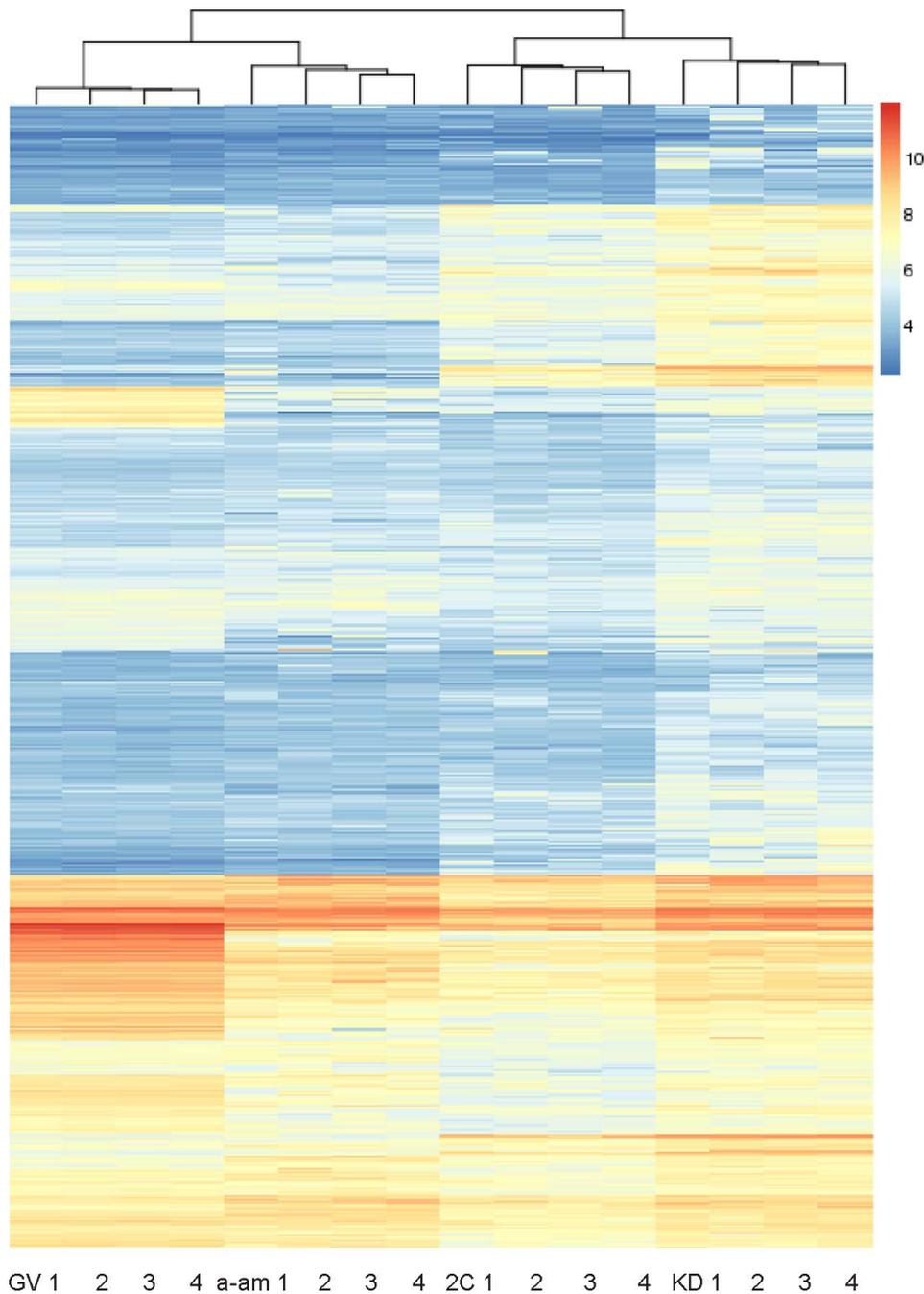


FIG. 9. Heat map of all samples from different treatment groups constructed using hierarchical clustering. Replicate sample numbers are indicated at the bottom of the figure. GV, full-grown GV-intact oocytes; a-am, 1-cell embryos incubated in α -amanitin to the 2-cell stage; 2C, 2-cell embryos derived from oocytes injected with control siRNA and morpholino matured and fertilized in vitro; KD, 2-cell embryos derived from oocytes injected with *Sin3a* siRNA and morpholino matured and fertilized in vitro. Colors correspond to relative RNA abundance (on the log₂ scale) for the detected genes each of which is represented by one horizontal bar. The numbers correspond to each replicate within each group.

dormant maternal mRNAs that remain present for much longer periods of time during preimplantation development, (see, e.g., [31]). This transient pattern of expression suggests that SIN3A function could be restricted to this short window and that the decrease in the amount of SIN3A is required for development beyond the 2-cell stage. To test whether such is the case, we identified conditions in which we increased the amount of SIN3A in 2-cell embryos to an amount comparable to that observed following oocyte maturation by injecting 1-cell embryos with a cRNA encoding *Sin3a* (Fig. 11A).

The control group for these experiments was buffer-injected 1-cell embryos. The rationale for using buffer-injected as a control rather than injecting a cRNA that encodes for some protein, which is the typical control, is that the encoded protein is typically stable, whereas SIN3A is not. In addition, we noted that development to the blastocyst stage depended on what control cRNA was injected (Supplemental Fig. S4). The results of these experiments demonstrated that maintaining elevated levels of SIN3A for several hours beyond that time when it has normally decreased to undetectable levels had no effect on the incidence of development to the blastocyst stage and total cell

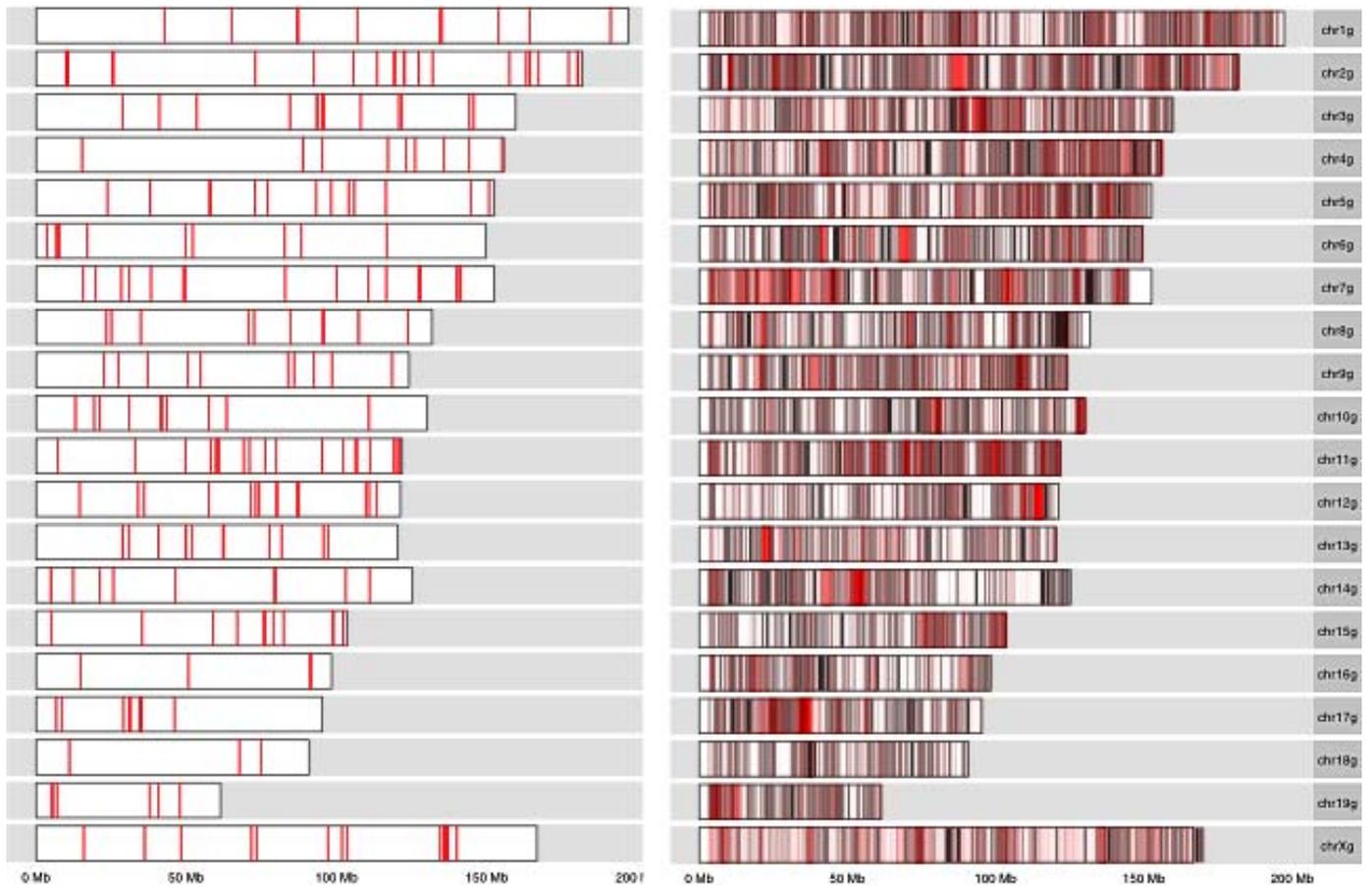


FIG. 10. Karyogram of genes up-regulated in 2-cell embryos depleted of maternal SIN3A (left panel) and density of genes (right panel). The comparison showed that most of the SIN3A sensitive genes emanated from gene dense regions.

numbers in blastocysts and their types (trophectoderm, inner cell mass, epiblast) (Fig. 11B–D). In addition, embryo transfer experiments did not detect any differences in the incidence of implantation and resorption (Fig. 11E). Thus, maintaining elevated amounts of SIN3A, albeit for only a limited duration (Supplemental Fig. S5), has little, if any, effect on development.

DISCUSSION

The results described here suggest that inhibiting recruitment of maternal *Sin3a* mRNA during oocyte maturation relieves repression of a subset of genes during formation of the global transcriptionally repressive state that develops during the course of genome activation. This finding is consistent with the observation that SIN3A is one of several HDAC-containing complexes [11–13] and inducing genomewide hyperacetylation relieves repression of the chromatin-based transcriptionally repressive state that starts to develop during the 2-cell stage [8, 42, 43]. From this perspective, recruitment of maternal *Sin3a* mRNA during oocyte maturation contributes to the first major wave of reprogramming of gene expression during preimplantation development in mouse. The molecular basis for how reprogramming occurs using a maternally inherited transcription machinery remains poorly understood. Although post-translational modifications of existing components of the transcription machinery present in oocytes could contribute to reprogramming, recruitment of dormant maternal mRNAs encoding chromatin remodelers, such as SIN3A, and transcription factors provides an elegant and effective solution.

Examination of microarray data and the nucleotide sequence of *Sin3a* mRNA is consistent with *Sin3a* being a dormant maternal mRNA that is recruited during maturation and following fertilization. Microarray data [5] reveal an increase in the relative abundance of *Sin3a* mRNA between the GV oocyte and 1-cell stages that likely reflects elongation of the poly (A) tail during maturation that leads to more efficient oligo (dT) priming when generating the cDNA libraries. Examination of the 3'-UTR of *Sin3a* mRNA reveals a well-defined cytoplasmic polyadenylation element (U₅AU) [44] within ~200 nucleotides of the polyadenylation signal sequence. In addition, a poly (U₁₃) tract directly preceding the polyadenylation signal sequence could also contribute to the increase in SIN3A observed following fertilization [45]. Microarray data also indicate that *Sin3b* transcript is present in oocytes. Given that this transcript does not exhibit properties that suggest it is a dormant maternal mRNA that is recruited during maturation, for example, no apparent cytoplasmic polyadenylation element, there is every expectation that SIN3B protein is present in oocytes. Nevertheless, such maternal SIN3B is not sufficient to compensate for loss of the maturation-associated increase in SIN3A that is essential for preimplantation development.

A striking observation is that the increase in SIN3A protein is very transient, essentially restricting its function to the 1-cell stage. The presence of a significant amount of *Sin3a* mRNA in 2-cell embryos (when compared to oocytes) makes it unlikely that the decrease in the amount of *Sin3a* mRNA that occurs between the MII and 2-cells is responsible for the virtual

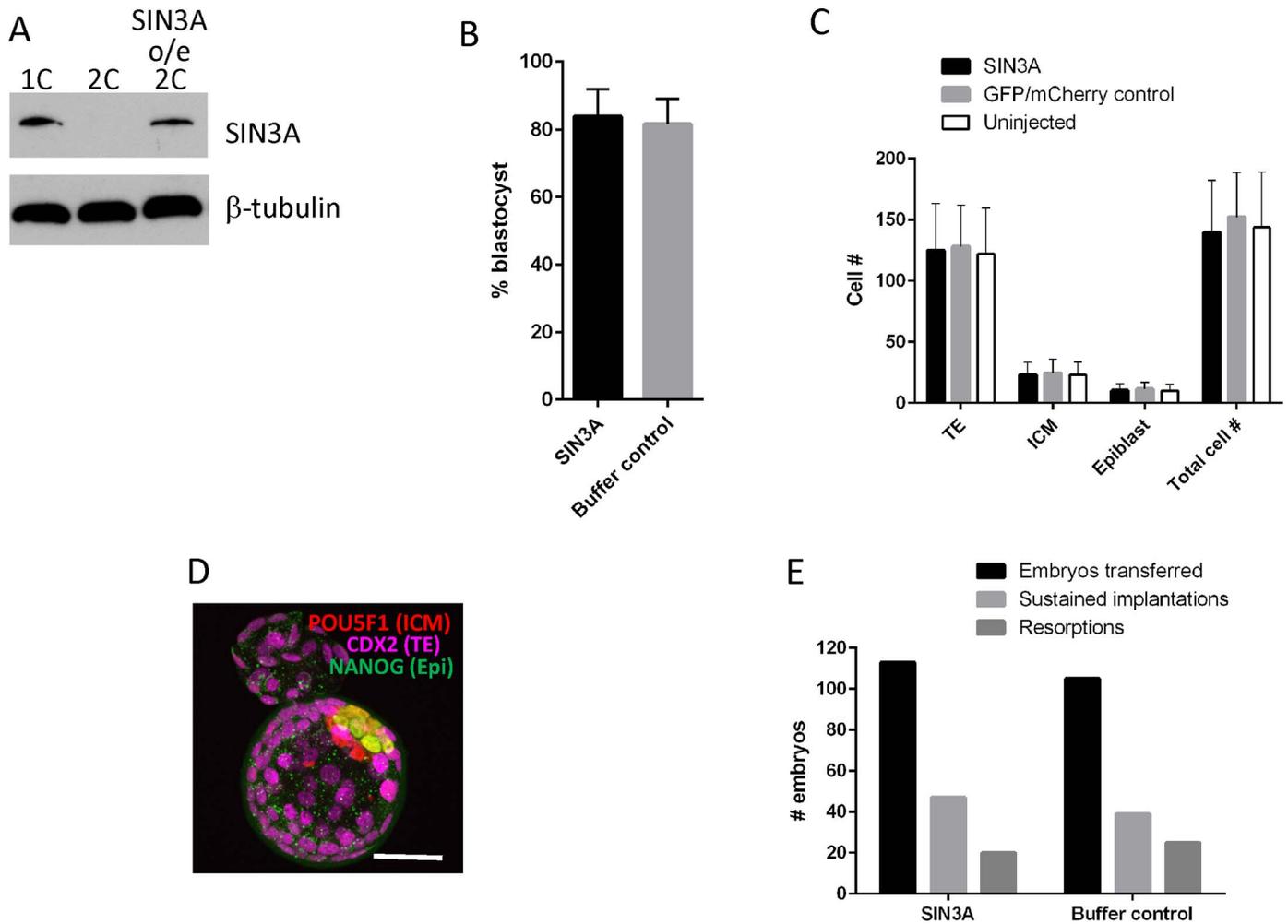


FIG. 11. Overexpressing SIN3A does not affect pre- and postimplantation development. **A**) Immunoblot analysis of SIN3A demonstrating that SIN3A is expressed at a similar level in 2-cell embryos (SIN3A o/e 2C) when compared to 1-cell embryos (1C). As shown in Figure 1, SIN3A is essentially absent in control 2-cell embryos (2C). **B**) Effect of overexpressing SIN3A on development to the blastocyst stage. Control embryos were injected with buffer. The experiment was conducted seven times, and at least 261 embryos were analyzed in each group. The data are expressed as mean \pm SEM. **C**) Effect of overexpressing SIN3A on cell numbers in blastocysts. The experiment was conducted four times, and at least 32 embryos were analyzed in each group. The data are expressed as mean \pm SEM. **D**) Immunocytochemical detection of lineage markers in blastocysts derived from embryos overexpressing SIN3A. Bar = 50 μ m. **E**) Incidence of postimplantation development following blastocyst transfer of control and SIN3A-overexpressing embryos. The experiment was conducted six times.

absence of SIN3A protein in 2-cell embryos. Rather, the newly synthesized SIN3A appears to be intrinsically unstable and subject to proteasome-dependent degradation.

The transient nature of SIN3A expression led us to focus our efforts on the 1-cell stage. We used MII eggs fertilized in vitro, which is technically more demanding than using parthenogenetically activated MII eggs, because of the known differences in modified histone composition between the male and female pronuclei [37] and differences in nuclear concentration of specific chromatin-associated proteins, for example, PG7/STELLA [46]. We did not observe any difference in the pronuclear concentration of SIN3A, regardless of whether the 1-cell embryos were permeabilized before fixation or not. In addition, we did not observe any pronuclear differences in the extent of acetylation of the modified histones that were examined.

Inhibiting the maturation-associated increase in SIN3A led to histone hypoacetylation, not hyperacetylation. It should be noted that nuclear localization of HDAC1 and HDAC2 is not altered in *Sin3a*^{-/-} oocytes (Ma and Schultz, unpublished results). A possible explanation for this observation is that

whereas deletion of HDAC1/2 leads to histone hyperacetylation [10, 47], HDAC1/2 would still be present and could function in an unregulated fashion no longer targeting specific nucleosomes. Alternatively, the HDACs would be available to associate with other HDAC1/2-containing complexes, and should the activity of these complexes be greater than that of SIN3A, this difference in activity could lead to the observed histone hypoacetylation. Although transcription in 1-cell embryos is not affected when the maturation-associated increase in SIN3A is inhibited, the observed hypoacetylation in 1-cell embryos could contribute to the decrease in global transcription in 2-cell embryos. For example, H4K8ac is linked with transcriptional activation and enriched in the promoter and transcribed regions of active genes [38], and H3K18ac may be involved in POL2R recruitment and transcription initiation [48].

Inhibiting the maturation-associated increase in SIN3A results in few embryos developing beyond the 2-cell stage. Such an arrest is the hallmark of failure to activate the genome, in particular, a failure to reprogram correctly. For example, deleting maternal *Brg1* results in arrest at the 2-cell stage and a

failure to reprogram faithfully, and expressing BRG1 in *Brg1*^{-/-} zygotes overcomes the 2-cell arrest [41]. The combination of misexpression of a subset of genes normally activated during the course of genome activation and reprogramming and genes that are not normally expressed in 2-cell embryos is a likely source for the observed developmental arrest. Consistent with SIN3A being one of several HDAC-containing complexes is the observation that only a subset of genes normally activated during the course of genome activation are affected.

Analysis of the misexpressed genes was essentially uninformative, but one interesting correlation emerged when using the molecular signatures database (MSigDB.v5) [49], namely, about 40 of the genes up-regulated when the maturation-associated increase in SIN3A was inhibited 9% to ~20% are genes marked by H3K27me3, EED, or SUZ12 in embryonic stem cells. Further examination of these genes indicates they are marked with a bivalent promoter, that is, promoters enriched both in H3K27me3 and H3K4me3 [50–52]. Any mechanistic linkage between reduced amounts of SIN3A and expression of these genes remains unknown, and the small amounts of biological material essentially preclude chromatin immunoprecipitation experiments to determine whether the promoters of these genes bear these histone marks.

We were unable to maintain elevated amounts of SIN3A following injecting a *Sin3a* cRNA into 1-cell embryos and therefore not able to assess whether the rapid turnover of SIN3A is essential for continued development. Identifying amino acid sequences responsible for rapid SIN3A degradation may permit such an analysis. These overexpression experiments, however, highlight the need to be careful about what constitutes the control in the experiment because certain cRNAs led to decreased development (e.g., *mCherry* cRNA) whereas others do not (e.g., *Luc* cRNA).

In summary, the results presented here document a critical role of recruitment of maternal *Sin3a* mRNA during maturation in reprogramming gene expression during the course of genome activation. Although the precise mechanism as to why expression of certain genes is affected whereas others are unaffected is not known, this strategy of recruiting dormant maternal mRNAs that encode proteins central to a process that should be minimal in the oocyte but then required by the 1-cell stage likely provided a selective pressure for this sophisticated mechanism of regulation of gene expression to evolve in oocytes.

ACKNOWLEDGMENT

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