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Sin3a is essential for the genome integrity and viability of pluripotent cells

Patrick McDonel, Jeroen Demmers, David W.M. Tan, Fiona Watt, Brian D. Hendrich

Abstract

The Sin3a/HDAC co-repressor complex is a critical regulator of transcription networks that govern cell cycle control and apoptosis throughout development. Previous studies have identified Sin3a as essential for embryonic development around the time of implantation, during which the epiblast cell cycle is uniquely structured to achieve very rapid divisions with little tolerance of DNA damage. This study investigates the specific requirement for Sin3a in the early mouse embryo and shows that embryos lacking Sin3a suffer unresolved DNA damage and acute p53-independent apoptosis specifically in the E3.5–4.5 epiblast. Surprisingly, Myc and E2F targets in Sin3a-null ICMs are downregulated, suggesting a central but non-canonical role for Sin3a in regulating the pluripotent embryonic cell cycle. ES cells deleted for Sin3a mount a DNA damage response indicative of unresolved double-strand breaks, profoundly arrest at G2, and undergo apoptosis. These results indicate that Sin3a protects the genomic integrity of pluripotent embryonic cells and governs their unusual cell cycle.

Keywords:
Sin3a
ICM
Apoptosis
Embryonic stem cells
Cell cycle
DNA damage

Introduction

Robust mechanisms are required to protect the genomic integrity of pluripotent embryonic cells that give rise to the entire organism and future generations through the germ line. Consequently, embryonic stem (ES) cells and, by inference, cells of the inner cell mass (ICM) have considerably lower mutation frequencies than somatic cells (Hong et al., 2007) and are highly sensitive to DNA damage and acute p53-independent apoptosis specifically in the E3.5–4.5 epiblast. Indeed, ES cells deleted for Trp53 readily undergo apoptosis in response to DNA damage with kinetics similar to wild-type cells (Aladjem et al., 1998; Lin et al., 2005; Solozobova et al., 2009). Indeed, ES cells deleted for Trp53 readily undergo apoptosis in response to DNA damage with kinetics similar to wild-type cells (Aladjem et al., 1998; Lin et al., 2005; Solozobova et al., 2009). Despite a failure to induce canonical somatic targets of p53.

As with the response to DNA damage, models of cell cycle control in the epiblast are largely inferred from studies of mouse ES cells (for reviews see (Orford and Scadden, 2008; White and Dalton, 2005)). Indeed, ES cells deleted for Sin3a fail to complete G1 and the replication machinery, thus rapidly driving ES cells from mitosis into S phase regardless of any damage or stress that might have occurred.

A key regulator of the Myc, E2F, and p53 transcription networks that govern cell cycle control and apoptosis throughout development is the Sin3a co-repressor complex (McDonel et al., 2009; Silverstein and Ekwall, 2005). This conserved complex is scaffolded by the Sin3a protein, which binds class I histone deacetylases (HDACs)
Hdac1 and Hdac2 (Laherty et al., 1997) and a diverse array of sequence-specific repressors via its paired amphipathic helices (Le Guezennec et al., 2006; Sahu et al., 2008), thus recruiting HDAC activity to target promoters.

Sin3a function is essential for the growth and viability of mouse embryonic fibroblasts (MEFs). Deletion of Sin3a in MEFs results in a profound growth defect, significant G2/M accumulation, and increased apoptosis in conjunction with de-repression of hundreds of Myc, E2f1, E2f2, and p53 targets that control cell cycle progression, DNA replication and repair, and cell death (Dannenberg et al., 2005). Interestingly, while the induction of p21CIP (MGI: Cdkn1a) in response to Sin3a deletion requires p53, the growth arrest, replicative defects, and apoptosis were not alleviated by either ablation or functional inactivation of p53. Furthermore, genes involved in both non-homologous end-joining (NHEJ) and homologous recombination (HR) repair pathways were aberrantly upregulated in MEFs lacking Sin3a (Dannenberg et al., 2005), suggesting a novel role in balancing the relative activities of these two double-strand break (DSB) repair strategies in addition to its reported chromatin-modifying functions during NHEJ (Jazayeri et al., 2004) and DNA replication (Aparicio et al., 2004) in yeast.

Genetic studies have shown that Sin3a is required for early mouse embryonic development between E3.5 and gastrulation at E6.5 (Cowley et al., 2005; Dannenberg et al., 2005). Similarly, knock-down of Sin3a by siRNAs resulted in severely impaired proliferation in ES cells (Fazzio et al., 2008). However, as outlined above, many of the genes overexpressed in Sin3a−/− MEFs that led to growth defects and apoptosis are already highly expressed in early embryonic cells, and thus their de-repression in the ICM or ES cells is not necessarily expected to have such adverse effects. Therefore, the mechanisms underlying this embryonic requirement for Sin3a are not at all clear.

To better understand the requirement for Sin3a/HDAC complexes in early mammalian embryogenesis, we examined in detail embryos and ES cells deleted for Sin3a. We found that Sin3a is absolutely required for proliferation and survival of cells in the ICM as embryos implant, but cells in the trophectoderm lineage appeared largely normal in Sin3a−/− embryos. We identify a number of proteins interacting with Sin3a in ES cells, further implicating the complex in cell cycle control and the DNA damage response. We propose that Sin3a is essential to maintain both the unusual cell cycle of pluripotent embryonic cells and their genomic integrity.

Materials and methods

Mice and embryos

Sin3a−/− ES cells were a kind gift of Gregory David (New York). ES cells were injected into C57Bl/6 host blastocysts to generate chimeric mice using standard methods (Hogan et al., 1994). Resulting Sin3a−/− mice were then intercrossed to create a homozygous Sin3a−/− mice line or crossed to mice expressing aCre transgene under the control of the Sox2 promoter (Hayashi et al., 2002), kindly provided by Jennifer Nichols (Cambridge). Maternal contribution of the Cre protein to the zygote resulted in recombination between the LoxP sites and deletion of Sin3a. To better understand the requirement for Sin3a/HDAC complexes in early mammalian embryogenesis, we examined in detail embryos and ES cells deleted for Sin3a. We found that Sin3a is absolutely required for proliferation and survival of cells in the ICM as embryos implant, but cells in the trophectoderm lineage appeared largely normal in Sin3a−/− embryos. We identify a number of proteins interacting with Sin3a in ES cells, further implicating the complex in cell cycle control and the DNA damage response. We propose that Sin3a is essential to maintain both the unusual cell cycle of pluripotent embryonic cells and their genomic integrity.

All mice were housed under standard conditions. All procedures were covered by a license granted by the UK Home Office and were approved by institutional ethics committees.

Immunofluorescence

Embryos were fixed in 2.5% paraformaldehyde, permeabilised in 0.25% Triton X-100 in PBS, and blocked in PBS containing either 3% donkey serum, 0.1% BSA, and 0.01% Tween-20 or 10% fetal calf serum and 0.1% Triton X-100. The primary antibodies were applied in blocking solution at 4 °C overnight, and secondary antibodies were applied for an hour at room temperature. Primary antibodies used were the following: anti-Sin3a (1/200, sc-768, Santa Cruz Biotechnology), anti-Oct4 (1/100, sc-5279 and sc-8628, Santa Cruz Biotechnology), anti-Nanog (1/250, ab21603, Abcam and RCAB0002P-F, Cosmo Bio Co), anti-Cdx2 (1/200, Cdx2-88, BioGenIX), anti-Eomes (1/200, ab23345, Abcam), anti-Gata4 (1/200, sc-1237, Santa Cruz Biotechnology), anti-Mi-2β (1/200, 39289, Active Motif), anti-activated Caspase 3 (1/500, AF835, R&D Systems), and anti-phospho-H2AX (S139) (1/500, 05-636, Millipore). For the phosho-H2AX (γH2AX) quantitation, mean staining intensity values obtained from confocal images were measured for DAPI-positive nuclei using Volocity software (Perkin Elmer) for three wild-type embryos, eight heterozygous embryos, and six null embryos. Cells were scored as being ICM or TE based upon Oct4 expression levels. This resulted in 78, 184, and 96 wild-type, heterozygous, or null ICM cells respectively, and 118, 250, and 216 wild-type, heterozygous and null TE cells, respectively. Mean intensity values of anti-H2AX staining were used to build box plots (http://www.physics.csbsju.edu/stats/display.distribution.html). P-values were calculated using a single tailed Mann-Whitney test (http://faculty.vassar.edu/lowry/utest.html).

Single-ICM gene expression analyses

Individual ICMS from Sin3a+/− intercrosses were dissected away from trophectoderm by immunosurgery (Nagy et al., 2006) at 3-5 dpc and placed into either first-strand buffer (1× Superscript III buffer (Invitrogen), 0.5% NP-40 (Pierce), 10 mM DNT mixture (Roche), 3.4 mM Mo4dT30 primer (AAGCGATTGATACAAGGCGATGCGATGCCGCT AC30dT30) (Osawa et al., 2005), 1 mM DTT (Invitrogen), 5 U SuperRNase (Ambion), 7.5 U PrimerRnase inhibitor (Eppendorf®) or miRNA lysis buffer [1 mg/mL BSA (NEB), 0.5% NP-40 (Pierce)], snap-frozen on dry ice, and stored at −80 °C. Trophectoderm from each embryo were lysed in PCR lysis buffer (1× Thermol buffer (NEB), 0.45 mM Igepal CA-630 (Sigma), 0.45% Tween 20 (Sigma), 200 μg/mL Proteinase K (Roche)) at 55 °C for 2 h followed by inactivation for 15 min at 95 °C and genotyped for Sin3a by duplex PCR as described above.

For cDNA synthesis, ICMS frozen in first-strand buffer were lysed at 65 °C for 5 min, diluted 1:10 in 4.5 μL first-strand buffer, and re-heated to 65 °C for 5 min. Primer was allowed to anneal at 45 °C for 2 min before addition of 0.5 μL of Superscript III and incubation at 45 °C for 15 min. The reaction was inactivated at 65 °C for 10 min. Unannealed primer was digested by the addition of 4 U Esonuclease I (Thermo Fisher Scientific) in 1.0 L of 1.6 mM MgCl2 and incubated at 37 °C for 30 min before inactivation at 80 °C for 25 min. Removal of the RNA template and polyadenylation of the cDNA was carried out concurrently by the addition of 5 μL RNaseH (Invitrogen), 2.6 μL of 5× terminal deoxynucleotidyltransferase buffer (Promega) supplemented with 1.5 mM dATP (Roche), and 30 U terminal deoxynucleotidyltransferase (TdT) (Promega) followed by incubation for 15 min at 37 °C before inactivation at 65 °C for 10 min. 4 μL of polyadenylated cDNA was used as template for PCR amplification in 1× ExTaq buffer (TaKaRa), 0.65 mM dNTP (Roche), 8.25 μM Mo4dT30 primer, and 5 U ExTaq (TaKaRa) by incubating at 94 °C for 1 min, 50 °C for 2 min, and 72 °C for 2 min to allow second-strand synthesis. Subsequently, 35 cycles of amplification were performed by incubating at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min. The first round of amplification was
performed in triplicate, after which the amplified cDNA was pooled. A second round of amplification was performed in duplicate as above by using 2 μl of the pooled amplified cDNA as template. The amplified cDNA was again pooled before purification with the QiAquick PCR purification kit (Qiagen). The eluate was diluted 1:50 in water for subsequent qPCR using an Applied Biosystems StepOne Plus PCR system with Roche SYBR Green Master reagents and primers listed in Suppl. Table 1.

Reverse transcription and qPCR analysis of miRNAs were conducted exactly as described (Tang et al., 2006b), using only the gene-specific forward primers listed in Suppl. Table 1 for the cDNA amplification step.

ES cells

Sin3aFlox/– ES cells were derived from mixed C57/129 blastocysts and cultured in ES cell medium supplemented with 10% fetal bovine serum and recombinant human LIF on gelatin-coated flasks, as described (Smith, 1991). Stable integration of a plasmid expressing Cre-ER2;ires-Puro from a CAG promoter was achieved by electroporating 5× 105 Sin3aFlox/– ES cells with 80 μg linearised vector, plating of serial dilutions, and selection with 1 μg/mL puromycin for 6 days until colonies were clearly visible. Clones were picked into individual gelatin-coated wells and subsequently expanded under puromycin selection. In transfected lines, Cre was translocated into the nucleus by the addition of 50 μg/mL hygromycin, resistant colonies expressing either E14tg2a ES cells with 80 μg/mL hygromycin, resistant colonies expressing either esen or discarded. Typical

Results

Sin3a is required for epiblast formation

To investigate the function of Sin3a in pluripotent cells, we first investigated the role of Sin3a in pre- and peri-implantation development in mice. Nuclear Sin3a protein is visible in both wild-type and extracts. Briefly, cells were harvested by trypsinisation and rinsed twice in ice-cold PBS, then once in ice-cold lysis buffer (10 mM HEPES-KOH pH 7.6, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM PMSF, 0.5 mM DTT). Cells were suspended in lysis buffer for 10 min on ice, then gently lysed using a glass dounce homogeniser. Nuclei were pelleted by centrifugation at 3300 g and resuspended in low-salt nuclear extract buffer (25 mM HEPES-KOH pH 7.6, 20 mM KCl, 1.5 mM MgCl2, 10% glycerol, 0.5 mM PMSF, 0.5 mM DTT) and then lysed by adding DTT to 450 mM and Igepal CA-630 to 0.15% and rotating at 4 °C for 1 h. Insoluble material was pelleted by centrifugation for 30 min at 20,000 g, and the supernatant was aliquoted and flash-frozen in liquid nitrogen.

For immunoprecipitations, 13–15 μg nuclear extract was diluted in IP buffer [25 mM HEPES-KOH pH 7.6, 140 mM KCl, 1% glycerol, 0.1% Igepal CA-630, 0.5 mM EDTA, 0.5 mM DTT, 1X Protease Inhibitor Cocktail III (Calbiochem)], pre-cleared against protein G sepharose, and then rocked for 4 hours at 4 °C with 75 μL (packed) anti-FLAG M2 resin (Sigma). Following extensive washes in IP buffer containing 250 mM KCl, Sin3a complexes were eluted 3 times in IP buffer containing 200 μg/mL 3× FLAG peptide for 30 min. Eluates were pooled and precipitated with trichloroacetic acid. The pellets were washed in acetone and solubilised in Laemml buffer for SDS-PAGE on separate 4–12% Bis–Tris Novex (Invitrogen) polyacrylamide gels for each sample.

Mass spectrometric analysis

1D SDS-PAGE gel lanes were cut into 2 mm slices using an automatic gel slicer and subjected to in-gel reduction with DTT alkylation with iodoacetamide, and digestion with trypsin (Promega, sequencing grade), essentially as described (Wilms et al., 1996). Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ-Orbitrap mass spectrometer (Thermo) operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm × 100 μm, packed in-house) at a flow rate of 8 μL/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm × 50 μm, packed in-house) using a linear gradient from 0% to 80% B (A = 0.1% formic acid; B = 80% (v/v) acetonitrile, 0.1% formic acid) over 70 min at a constant flow rate of 200 nL/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode. Peak lists were automatically created from raw data files using the Mascot Distiller software (version 2.3; MatrixScience). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against the SwissProt FASTA protein sequence database (version SwissProt_56.4, taxonomy: Mus musculus). The peptide tolerance was set to 10 ppm and the fragment ion tolerance was set to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidised methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 65. Individual peptide MS/MS spectra with Mascot scores below 40 were checked manually and either interpreted as valid identifications or discarded. Typical contaminants, also present in immunopurifications using beads coated with pre-immune serum or antibodies directed against irrelevant proteins, were omitted from the table.

Results

Sin3a is required for epiblast formation

To investigate the function of Sin3a in pluripotent cells, we first investigated the role of Sin3a in pre- and peri-implantation development in mice. Nuclear Sin3a protein is visible in both wild-type and
Sin3a<sup>−/-</sup> 2-cell embryos, indicating that Sin3a protein is deposited in the egg and that this maternal protein persists through the first few cleavage divisions (Fig. 1A). By the 8-cell stage nuclear Sin3a is less apparent in mutant embryos than in wild-type littermates, and by the 16-cell stage the maternal protein has largely been depleted and null embryos show no nuclear Sin3a protein (Fig. 1B). At the blastocyst stage Sin3a is expressed ubiquitously in wild-type and heterozygous embryos (Fig. 1B). Despite lacking any detectable Sin3a protein, null blastocysts are able to cavitate and, as judged by patterns of antibody staining for the Oct4, Nanog, and Cdx2 proteins, are able to execute the first lineage decision to segregate inner cell mass (ICM) and trophectoderm (Fig. 1C). Indeed, Sin3a blastocysts were recovered from heterozygote intercrosses at near Mendelian ratios (Table 1). As maternal Sin3a is largely depleted by the 8-cell stage, these results indicate that Sin3a does not have an essential role in cellular proliferation or early cell fate specification in late preimplantation stage embryos.

**Fig. 1.** Sin3a expression in preimplantation development. A. Maternal Sin3A protein (green) can be detected as nuclear signal in wild-type and Sin3a<sup>−/-</sup> 2-cell embryos. Mi2β expression (white) and DAPI staining (blue) are also indicated. B. Staining of 8- and 16-cell embryos and early blastocysts with an anti-Sin3a antibody (green) shows nuclear signal in wild-type and heterozygotes, but loss of nuclear staining in Sin3a<sup>−/-</sup> embryos from the 8-cell stage onwards. Staining for Oct4 (red) and DAPI (blue) is also indicated. C. Staining wild-type and Sin3a<sup>−/-</sup> blastocysts for the ICM markers Oct4 (red) and Nanog (green), for the trophectoderm marker Cdx2 (pink), and for DAPI (blue). A panel showing both Nanog and Cdx2 staining is shown to highlight segregation of the ICM and TE lineages. For all embryos the genotypes are shown to the right. Scale bar = 50 μm.
Despite the apparently normal preimplantation development, implanting Sin3a−−/− embryos were recovered at sub-Mendelian ratios, and those recovered were obviously smaller than their wild-type and heterozygous littermates, with the Oct4- and Gata4-expressing cell populations appearing to be particularly affected (Table 1, Fig. 2A, and Fig. 3A right panel). Nevertheless, most embryos were able to implant, indicating that trophectoderm cells do not require Sin3a to form an attachment to the uterus, consistent with a previous report that seemingly empty implantation sites could be identified at 6.5 dpc (Cowley et al., 2005). By 5.5 dpc few mutant embryos were recovered, but those identified appeared to have completely lost their embryonic compartment, as judged by Oct4 and Gata4 staining, and consisted entirely of Eomes-expressing trophectoderm (Table 1, Fig. 2B). Therefore, we conclude that Sin3a is absolutely required for the viability and/or proliferation of epiblast cells in peri-implantation embryos.

We next wished to assess whether the specific loss of epiblast cells in Sin3a mutant embryos was an intrinsic property of the embryo or was induced externally during implantation. To distinguish these possibilities, we first examined the status of wild-type and mutant embryos in which the preimplantation period was extended by inducing diapause (Buehr and Smith, 2003). Strikingly, subjecting Sin3a−−/− blastocysts to diapause for two days resulted in complete loss of the ICM (Fig. 2C), whereas wild-type (not shown) and heterozygous blastocysts maintained their characteristic ICM morphology. To remove blastocysts from the uterine environment completely, blastocysts were flushed early on the fourth day and allowed to outgrow in standard ES cell serum and LIF conditions. Consistent with a previous report (Cowley et al., 2005), we found that Sin3a-null embryos were able to attach to the substrate but completely failed to outgrow (data not shown).

Failure of blastocyst outgrowth could be due either to a general proliferation defect or to a specific failure of the trophectoderm cells. To assess the proliferation potential of pluripotent cell populations lacking Sin3a, embryos were collected at the 8-cell stage and incubated in the presence of the ERK inhibitor PD0325901 until the expanded blastocyst stage, as described (Nichols et al., 2009). This prevented formation of hypoblast in the resulting blastocysts, allowing us to isolate pure epiblasts after immunosurgery. Outgrowth of isolated wild-type or heterozygous epiblasts in 2i media (Nichols et al., 2009) resulted in derivation of embryonic stem cell lines in all cases, but Sin3a−−/− epiblasts failed to outgrow (data not shown). Therefore, we conclude that Sin3a is required for the expansion of pluripotent cell populations both in vivo and ex vivo.

**Apoptosis specifically eliminates the epiblasts from embryos lacking Sin3a**

Our data thus far indicated a specific loss of the Sin3a−null pluripotent cell population during implantation, but little if any effect upon trophectoderm cells. Sin3a activity has been shown to prevent p53-independent apoptosis in murine embryonic fibroblasts (MEFs) (Cowley et al., 2005; Dannenberg et al., 2005). To determine whether ICM cells of Sin3a−/− embryos were dying via apoptosis, pre- and peri-implantation stage embryos were stained for activated Caspase-3, a marker of apoptotic cells (Nicholson, 1999). Apoptosis occurs at a very low rate in the ICM of wild-type blastocysts during normal development (Jang et al., 2005). Consistent with this, we saw that the vast majority of wild-type or Sin3a−/− blastocysts had either zero or one Caspase 3-positive cell (Fig. 3). By contrast, half of the Sin3a−−/− blastocysts had three or more Caspase 3-positive cells, usually located in the ICM (Figs. 3A left panel, B). One day later almost all wild-type or heterozygous embryos had zero or one Caspase 3-positive cell, whereas over 80% of Sin3a−−/− embryos showed three or more Caspase 3-positive cells (Figs. 3A right panel, B). Those embryos showing multiple apoptotic cells also showed little or no Oct4 staining, consistent with death of ICM cells in the absence of Sin3a (Fig. 3B). Therefore, we conclude that Sin3a is specifically required for survival of pluripotent cells in implanting blastocysts, but it is not required for development of the trophectoderm until at least 5.5 dpc.

**Apoptosis in Sin3−−/− ICMs is independent of p53 activity**

We next addressed whether the growth defect and apoptosis observed in Sin3a−−/− ICMs were the result of p53 activation of arrest

![Table 1](image)

| Sin3a genotype | 3.5 d.p.c | 4.5 d.p.c | 5.5 d.p.c | Weaning |
|---------------|----------|-----------|----------|---------|
| +/+           | 28 (28.6%) | 49 (50.0%) | 21 (21.4%) | 98      |
| +/−           | 25 (31.6%) | 40 (50.0%) | 14 (17.7%) | 79      |
| −/−           | 21 (32.8%) | 39 (60.9%) | 4 (6.3%)  | 64      |
| N             | 84        | 134       | 39       | 260     |

![Fig. 2](image)
and cell death pathways. We found that although E3.5 ICM cells deleted for Sin3a were destined for apoptosis, they did not show induction of the p53 targets p21 (cell cycle arrest), Apaf-1 (apoptosis, (Moroni et al., 2001)), or Dram (autophagy and apoptosis, (Crighton et al., 2006)) (Fig. 4B). By contrast, levels of both p21 and Dram transcripts showed a dose-dependent reduction in Sin3a heterozygous and null ICMs, possibly indicating a previously unrecognised relationship between Sin3a and these p53 targets in early embryonic cells. Our observations are consistent with a previous report that MEFs deleted for Sin3a undergo p53-independent apoptosis (Cowley et al., 2005) and suggest that the cell death induced in pluripotent cells lacking Sin3a is exerted independently of p53 activity.

Sin3a−/− ICMs fail to express key cell cycle promoting factors

To investigate the underlying causes of the growth and apoptosis defects observed in Sin3a−/− embryos, we compared the transcript levels of key cell cycle regulatory genes in wild-type, heterozygous, and null ICMs at E3.5, the stage at which these phenotypes initially emerged. We found that as early as E3.5 transcripts for key cell cycle regulatory proteins were either not expressed or expressed at substantially reduced levels in ICMs deleted for Sin3a as compared with their wild-type or heterozygous counterparts (Fig. 4A). Most notably, whereas Sin3a−/− ICMs express normal levels of Oct4 and Nanog, they expressed nearly undetectable levels of E2f1 and one of its key targets, Ccne1 (Cyclin E1), both of which are critical for driving ES cells rapidly through G1 (Fujii-Yamamoto et al., 2005; Stead et al., 2002). Sin3a-null ICM cells also expressed less than half the wild-type levels of the replication licensing factor Mcm2 and the principal mitotic cyclin Ccnb1 (Cyclin B1). The absence of transcripts for these key cell cycle proteins makes it very unlikely that the cells would be able to progress through DNA replication and mitosis.

Sin3a−/− cell cycle misregulation and cell death phenotypes are not caused by mis-expression of ESCC or Myc-regulated miRNAs

One potential explanation for decreased E2f1 levels and p53-independent cell cycle disruption (and possibly the resulting cell death) comes from recent studies on miRNA control of the cell cycle, particularly in ES cells. Artificially high levels of c-Myc expression were shown in human B cells to induce the miR-20 family of miRNAs as part of a feedback loop, resulting in a block in the translation of E2f1 transcripts (Zhang and Pugh, 2011). This effectively mimics the E2f-inhibitory function of Rb and is expected to prevent expression of E2f targets such as the G1 cyclins and replication factors such as Mcm2. Moreover, so-called “ES Cell Cycle Control” (or ESCC) miRNAs in the miR-290 family stimulate ES cell proliferation by indirectly activating c-Myc and N-Myc, and thus by extension Myc targets, while also blocking p21 translation and inhibiting the Myc inhibitor miRNA let-7 (Gilchrist et al., 2008, 2010; Kumar et al., 2007). We therefore measured the levels of miRNAs in Sin3a−/− ICMs as compared with wild-type littermate ICMs (Fig. 4C). Our analysis of miRNAs in Sin3a-null ICMs revealed no induction of miR-20a nor increased expression of miR-290 family miRNAs, thereby demonstrating that the apoptosis and repression of Myc/E2F targets we observe in Sin3a null ICMs are not due to misregulation of these miRNAs.
Sin3a protects against DNA double-strand breaks and cell cycle arrest in pluripotent cells

In an effort to understand why pluripotent cells lacking Sin3a undergo apoptosis, we created an ES cell line in which Sin3a could be deleted conditionally. Multiple independent ES cell lines were derived from mice harbouring a Sin3a allele that could be deleted conditionally (Sin3a<sup>Flox</sup>−/<sup>Flox</sup>) (Dannenberg et al., 2005). Resulting ES cell lines were then stably transfected with a construct allowing expression of CreERT<sup>2</sup>, in which the Cre recombinase is fused to a modified estrogen receptor (Feil et al., 1997). Addition of 4-hydroxytamoxifen to Sin3a<sup>Flox</sup>−/<sup>Flox</sup>:CreERT<sup>2</sup> ES cells rapidly induced deletion of the floxed Sin3a allele (data not shown) and loss of Sin3a protein (Fig. 5D). As was seen in ICM cells, Sin3a−/− ES cells were not viable and began to die by apoptosis. Propidium iodide staining and flow cytometry showed a marked accumulation of Sin3a-deleted cells with a 4 C DNA content, as compared with heterozygous controls (Fig. 5A). Furthermore, the fraction of 4 C cells entering mitosis, as assessed by the mitotic marker phospho-histone H3 (Ser10), was significantly (p<0.001) reduced in Sin3a-deleted cells (Fig. 5B). Floating and attached cells in tamoxifen-treated and untreated Sin3a<sup>Flox</sup>−/<sup>Flox</sup>:CreERT<sup>2</sup> cultures were assayed for apoptosis by probing with fluorescently labelled Annexin V, which binds to phosphatidyl serine on the cell membrane of apoptotic cells. By 36 h after tamoxifen addition, approximately 68% of ES cells deleted for Sin3a undergo apoptosis, as compared with approximately 12% of untreated cells (Fig. 5C). Taken together, these results indicate that cells lacking Sin3a arrest at the G2/M checkpoint and die by apoptosis.

Given the lack of a functional G1/S damage checkpoint in ES cells, and prior gene expression evidence implicating Sin3a in DSB repair pathways in somatic cells, we hypothesised that the observed G2 arrest in ES cells upon loss of Sin3a could arise from failure to resolve DSBs. Indeed, probing whole-cell lysates from untreated and tamoxifen-treated ES cells revealed a rapid increase in ATM/ATR phosphorylation targets γH2AX (S139), phospho-53BP1 (S166), and phospho-SMC1 (S966), coincident with the loss of Sin3a (Fig. 5D). Whereas H2AX and 53BP1 are hyperphosphorylated in response to a variety of DSB types, SMC1 is typically phosphorylated by ATM as part of the S-phase damage checkpoint (Kim et al., 2002; Yazdi et al., 2002). An increase in the abundance of these markers is consistent with DSBs arising during DNA replication in the absence of Sin3a. From this, we conclude that Sin3a is required to prevent or repair DSBs that arise in ES cells, possibly during DNA replication.

To test whether failure to resolve replicative damage could account for the apparent p53-independent apoptosis seen in mutant ICMs, we next quantified levels of γH2AX in Sin3a−/− ICMs by immunofluorescence. Intensity of anti-γH2AX staining was measured in individual ICM and trophectoderm (TE) nuclei and normalised to anti-Oct4 staining intensity in the same cells. Plotting the resulting distributions reveals that both Sin3a−/− ICM and TE cells indeed have significantly increased H2AX staining as compared to wild-type or heterozygous littermates (Fig. 6). It is important to...
Sin3a associates with cell cycle control and DNA damage response proteins in ES cells

Sin3a has been reported to interact with key cell cycle regulatory proteins such as p53 and Rb, but these proteins play minor roles, if any, in self-renewal of pluripotent cells. To gain a better understanding of how Sin3a may interact with the cell cycle and/or DNA repair machinery in pluripotent cells, we took an unbiased approach to identifying its interacting proteins in ES cells. An epitope-tagged Sin3a protein was expressed in embryonic stem cells and used to pull down the Sin3a complex and any interacting proteins (Table 2). Data were obtained from four independent pull down experiments, providing a list of very high confidence interactors. The related protein Sin3b, which has been reported to interact with Sin3a in NIH3T3 cells (Lienert et al., 2011), was not significantly enriched in the Sin3a immunoprecipitate compared to control cells. This indicates that Sin3a and Sin3b function independently in ES cells, consistent with the dramatic difference in the
requirement for Sin3a and Sin3b during embryogenesis [this study and (Cowley et al., 2005; Dannenberg et al., 2005; David et al., 2008)]. A number of known interactors implicated in control of cell cycle and/or proliferation were found co-purifying with Sin3a in ES cells, including Ogt (Hart et al., 2007; Yang et al., 2002), Ing1 (Nikolaev et al., 2004), Mnt (Meroni et al., 2000) and Max (Laherty et al., 1997).

In addition to Sin3a complex components and known interactors, we also identified a number of proteins not previously known to interact with the Sin3a complex. Two of these novel interacting proteins, Bbx and Ppp1cc, have been implicated in cell cycle control (Lee et al., 2010a; Sánchez-Díaz et al., 2001). The interaction with Bbx is particularly robust as it co-purifies with Sin3a with scores and peptide numbers comparable to those seen for bona fide Sin3a complex components. Notably, six of the high confidence (Mascot Scores ≥700) interacting proteins are phosphorylated upon DNA damage by ATM or ATR (Matsuoka et al., 2007). Therefore we conclude that Sin3a is intimately linked to regulators of cell cycle and the DNA damage response in ES cells.

**Discussion**

Gene regulation by a number of chromatin-modifying complexes has been demonstrated to be important for early murine development, but not necessarily for the survival of pluripotent cells (Niwa, 2007). The Sin3a/HDAC complex is a critical co-repressor of transcription networks that govern cell cycle control, DNA replication and repair, and apoptosis in somatic cells (Cowley et al., 2005; Dannenberg et al., 2005). The requirement for genomic integrity differs considerably between somatic cells and pluripotent cells of the ICM, and correspondingly so does the regulation of these vital processes. Here we show that pluripotent cells exhibit a specific requirement for Sin3a function during early mouse development. Whereas the epiblast compartment is completely lost in Sin3a mutant embryos shortly after implantation, the trophectoderm cells remain largely unaffected. Despite lacking key cell cycle regulatory proteins that interact with Sin3a in somatic cells, Sin3a is required for proper cell cycle control in both undifferentiated ES cells and in embryonic cells fully two days prior the point when they convert to canonical cell cycle regulation (i.e. gastrulation at E6.5). Our data are consistent with Sin3a specifically protecting pluripotent cells from DNA damage leading to cell cycle arrest and elimination by apoptosis both in culture and in implantation stage embryos.

The cell death phenotype we observe in the ICM upon Sin3a loss is likely to be independent of p53, as we did not observe induction of the direct p53 targets p21(Cip1), Apaf1, and Dрам upon Sin3a deletion. This is consistent with previous reports that deletion or inactivation of p53 in MEFs fails to abrogate the growth and apoptosis phenotypes caused by Sin3a deletion (Cowley et al., 2005; Dannenberg et al., 2005) and reports that p53 is largely dispensable for apoptosis both induced by double-stranded breaks in ES cells (Aladjem et al., 1998) and as part of normal development prior to gastrulation (Armstrong et al., 1995; Copp, 1978; El-Shershaby and Hinchliffe, 1974; Macleod et al., 1995). However, the relationship between p53 and DNA damage in pluripotent embryonic cells remains controversial (Corbet et al., 1999; de Vries et al., 2002; Solozobova et al., 2009), and more recent evidence suggests p53 maintains genomic stability in the early embryo by suppressing pluripotency and promoting differentiation to eliminate damaged cells (Lee et al., 2010b; Lin et al., 2005). Our data support non-canonical roles for p53 in ES cells and suggest that parallel or novel apoptotic pathways should be investigated more thoroughly in the early embryo.

ICM cells deleted for Sin3a led to a loss of E2F1 and Ccn1e ( Cyclin E ) expression and a dramatic decrease in both Mcm2 and Ccn1b ( Cyclin B ) transcript levels. This increased repression is counter-intuitive, especially when considering that Mcm genes, Cdk/cyclins, and other Myc/E2F targets are de-repressed in somatic cells lacking Sin3a (Dannenberg et al., 2005). While this decrease in transcription might simply be a consequence of cells whose proliferation has been arrested at G2 by genotoxic stress (see below), the situation is likely more complex in the ICM. In this rapidly proliferating cell type, Myc and E2F targets that drive the cell cycle are very highly expressed during all phases of the cell cycle (Fujii-Yamamoto et al., 2005; Stead et al., 2002), so cells arrested at G2 should still express the factors that drive G1. The notable exception is Ccn1b (Cyclin B1), which is most highly expressed during G2 in ES cells (Fujii-Yamamoto et al., 2005; Stead et al., 2002) to activate Cdk1 and promote mitotic entry. However, Ccn1b levels are reduced in ICM cells lacking Sin3a (Fig. 4A), the opposite result expected for cells accumulated in G2.

The very high expression levels of Myc and E2F targets in wild-type pluripotent embryonic cells demonstrate that they are not generally repressed by Sin3a as they are in much of the soma. An alternative explanation of our results, therefore, is that Sin3a, also robustly expressed in ES cells and the ICM (Fig. 1A, Fig. 5A), facilitates the high expression of Myc/E2F targets by indirectly activating the expression of Myc and E2Fs themselves through repression of Myc/E2F repressors.

One category of possible Sin3a targets that repress Myc and E2F expression would be miRNAs. Indeed, Myc is known to be downregulated directly by the let-7 miRNA family (Kumar et al., 2007), whose expression is normally kept very low in ES cells (Marson et al., 2008; Tang et al., 2006a), and miR-294 is thought to promote Myc activity in ES cells indirectly through repression of an unidentified Myc repressor (Melton et al., 2010). Similarly, human E2F1 is repressed by the miR-20 miRNA family (O’Donnell et al., 2005), whose members are either nearly or completely undetectable in ES cells and the ICM (Tang et al., 2006a, this study). While we did not observe derepression of let-7a, miR-20a, or miR-17-5p in ICMs lacking Sin3a, it is possible that other members of these miRNA families, different miRNAs altogether, or traditional protein repressors of Myc or E2F1 are instead misexpressed in these mutant cells, causing loss of Myc/E2F target expression and abortion of the cell cycle.

Our combined observations of severe G2 arrest and phosphorylation of both H2AX and SMC1 upon Sin3a deletion suggest a specific
and role for Sin3a in protecting pluripotent cells against DSBs acquired during replication. However, of the cause of this damage might be indirect, especially considering that S. cerevisiae lacking Sin3p are hypersensitive to DSBs, but not those specific to S phase (Jazayeri et al., 2001). The reduced Mcm2 levels we observe in Sin3a-null ICMs (Jazayeri et al., 2001) provide a possible explanation. Mammalian cells with reduced Mcm levels are unable to activate a sufficient number of origins could lead to culling by the functional G1 checkpoint, ES cells can readily initiate replication without regard for genome integrity or sufficient available materials to complete the task. Without Sin3a, Mcm2 levels in the ICM are considerably reduced, likely from the loss of E2F expression. If these cells nevertheless enter S phase, then the resulting replicative damage from insufficient origins could lead to culling by the functional G2/M damage checkpoint.

It is nevertheless possible that Sin3a plays a more direct role in the response to DNA damage in pluripotent cells. Sin3a directly interacts with proteins implicated in cell cycle control. The highest-scoring Sin3a interactors in ES cells.

| Protein name | Max Mascot score | Unique Entrez ID | Control Totalb | Unique Entrez ID | Control Totalb | Comment |
|--------------|------------------|------------------|----------------|------------------|----------------|---------|
| Sin3a        | 5086             | 64 458 59        | 537 717 58     | 605 0 12         | 20466          | Sin3a complex component |
| Hdac1        | 2118             | 26 194 19        | 260 263 23     | 269 8 4           | 433759         | Sin3a complex component |
| Anid4b       | 1867             | 25 75 18        | 64 107 19      | 76 3 3           | 94246          | Sin3a complex component |
| Sap130       | 1849             | 21 140 23       | 166 216 23     | 185 4 4           | 269003         | Sin3a complex component |
| Anid4a       | 1609             | 0 0 0          | 0 24 109 24    | 85 0 0           | 238247         | Sin3a complex component |
| Rbp7         | 1474             | 15 112 14       | 142 196 16     | 227 1 1           | 264588         | Sin3a complex component |
| Hdac2        | 1341             | 19 141 13       | 116 155 20     | 159 0 0           | 15182          | Sin3a complex component |
| Suid3        | 1309             | 18 124 15       | 119 177 15     | 156 0 0           | 71954          | Sin3a complex component |
| Brms1l       | 1220             | 16 60 13        | 76 66 12       | 12 58 0           | 52592          | Sin3a complex component |
| Rbp4         | 1136             | 13 83 12        | 125 127 11     | 143 5 5           | 19646          | Sin3a complex component |
| Ing2         | 1013             | 11 26 6         | 20 4 38        | 6 26 0           | 69260          | Sin3a complex component |
| Sap30        | 892              | 13 56 3         | 53 60 7        | 46 0 0           | 60406          | Sin3a complex component |
| Brms1        | 855              | 10 35 10        | 47 66 12       | 12 58 0           | 107392         | Sin3a complex component |
| Sap30l       | 629              | 8 16 6         | 13 35 6        | 32 0 0           | 50724          | Sin3a complex component |
| Sap25        | 316              | 5 11 3         | 3 1 2          | 1 2 0           | 751865         | Sin3a complex component |
| Ogt          | 3371             | 43 84 33        | 110 27 123     | 27 80 1          | 108155         | Known interactor: (Yang et al., 2002) |
| Bbx          | 1837             | 26 59 19        | 78 19 120      | 22 92 0          | 70508          | HMG box transcription factor that is necessary for cell cycle progression from G1 to S phase (Sánchez-Díaz et al., 2001) |

| Protein name | Max Mascot score | Unique Entrez ID | Control Totalb | Unique Entrez ID | Control Totalb | Comment |
|--------------|------------------|------------------|----------------|------------------|----------------|---------|
| Tet1         | 1699             | 0 0 0          | 0 24 111 29    | 96 0 0           | 52463          | Known interactor: (Williams et al., 2011) |
| Fox2k        | 1359             | 18 19 12       | 26 16 21       | 10 19 0          | 68837          | Phosphorylated upon DNA damage, probably by ATM or ATR: (Matsuo et al., 2007) |
| Pspc1        | 1055             | 13 23 11       | 23 13 20       | 9 16 2           | 66645          | Phosphorylated upon DNA damage, probably by ATM or ATR: (Matsuo et al., 2007) |
| Fam56oa      | 901              | 11 34 12       | 53 8 49        | 11 52 0          | 56306          | Phosphorylated upon DNA damage, probably by ATM or ATR: (Matsuo et al., 2007) |
| Phf23        | 765              | 6 16 9         | 25 4 21        | 5 21 0           | 78246          | Phosphorylated upon DNA damage, probably by ATM or ATR: (Matsuo et al., 2007) |
| Bahe1c1      | 726              | 7 14 10       | 16 4 5         | 7 11 2           | 268515         | Phosphorylated upon DNA damage, probably by ATM or ATR: (Matsuo et al., 2007) |
| Fox1k        | 702              | 12 9 6         | 14 7 15        | 15 15 0          | 17425          | Phosphorylated upon DNA damage, probably by ATM or ATR: (Matsuo et al., 2007) |
| 2810046L04Rik| 551              | 10 14 3        | 5 4 7          | 4 6 0           | 212127         | Uncharacterised protein KIAA0232 homolog |
| Chx3         | 499              | 2 10 6        | 2 0 0          | 0 1 2           | 12417          | Hexp1gamma |
| Zgpl1        | 453              | 5 6 2         | 2 1 2         | 1 3 0           | 100009600      | GATA-like protein 1 |
| Ing1         | 428              | 8 13 2        | 18 4 38       | 2 23 0          | 26356          | Known interactor: (Nikolaev et al., 2004). |
| Ppp1cc       | 369              | 6 5 4         | 7 0 5         | 5 5 0          | 19047          | Implicated in cell cycle progression during the transition from mitosis to interphase: (Lee et al., 2010a) |
| Dnajb6       | 346              | 5 6 2         | 2 1 2         | 1 2 0           | 23950          | Dnaj homolog subfamily B member 6 |
| Mnt          | 332              | 5 5 1         | 1 0 0         | 3 3 0          | 17428          | Known interactor: (Meroni et al., 2000) |
| Ythdf1       | 284              | 3 4 1         | 1 1 1         | 1 0 0          | 228994         | YTH domain family protein 1 |
| Hbkp1        | 263              | 4 6 3        | 4 0 0         | 4 7 0           | 73389          | Known interactor: (Swanson et al., 2004) |
| Klf13        | 242              | 2 10 6        | 3 2 2         | 1 1 0           | 507347         | Methylcytosine dioxygenase |
| Tet2         | 226              | 5 6 4         | 1 0 0         | 0 0 0          | 214133         | Methylytosine dioxygenase |
| H2afj        | 235              | 10 3 14       | 3 8 0         | 4 12           | 232440         | Histone H2A |
| Hist2h2ac    | 235              | 10 3 14       | 3 8 0         | 4 12           | 8338           | Histone H2A type 2-C |
| Max          | 188              | 2 3 1         | 1 2 2         | 1 1 0          | 17187          | Known interactor: (Laherty et al., 1997) |

a Maximum Mascot score from the four different experiments.
b Number of unique peptides obtained from individual experiments.
c Total number of peptides obtained from individual experiments.
abundance as bona fide Sin3a complex components (Table 2), is a relatively uncharacterised HMG box-containing protein shown to complement a cell cycle defect in S. pombe (Sánchez-Díaz et al., 2001). Its function in mammalian cells remains unknown. Secondly, our analysis identified several Sin3a interacting proteins that become phosphorylated by ATM and/or ATR in response to DNA damage (Table 2), providing a possible direct link between Sin3a and DNA damage response pathways.

In conclusion, we have found that Sin3a is absolutely required for the proliferation and survival of pluripotent embryonic cells around the time of implantation. We propose that Sin3a is crucial for regulating the pluripotent cell cycle by indirectly facilitating Myc and E2F activity in the ICM. Loss of Myc/E2F target expression in regenerating the stringent G2/M checkpoint that results in death of mutant cells. This is in stark contrast to the situation in T cells or cells of the epidermis, where Sin3a is not required for cellular proliferation (Cowley et al., 2005; Nascimento et al., 2011), and provides insight into the molecular control of both the unusual cell cycle of pluripotent embryonic cells and their genomic integrity.

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