Requirement for Sun1 in the expression of meiotic reproductive genes and piRNA

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The inner nuclear envelope (NE) proteins interact with the nuclear lamina and participate in the architectural compartmentalization of chromosomes. The association of NE proteins with DNA contributes to the spatial rearrangement of chromosomes and their gene expression. Sun1 is an inner nuclear membrane (INM) protein that locates to telomeres and anchors chromosome movement in the prophase of meiosis. Here, we have created Sun1−/− mice and have found that these mice are born and grow normally but are reproductively infertile. Detailed molecular analyses showed that Sun1−/− P14 testes are repressed for the expression of reproductive genes and have no detectable piRNA. These findings raise a heretofore unrecognized role of Sun1 in the selective gene expression of coding and non-coding RNAs needed for gametogenesis.

KEY WORDS: Sun1, Sad1, Unc84, Nuclear envelope, Gametogenesis, piRNA, Mouse

INTRODUCTION

The nuclear envelope (NE) sequesters chromosomes in the nucleus, separating them from the cytoplasm. In addition to providing anchoring sites for chromatin, the nuclear membrane is important for cellular metabolic activities. For example, the nuclear pore complex (NPC) supports the selective exchange of macromolecules between the nucleus and the cytoplasm (Schneider and Grosschedl, 2007), and the nuclear lamina can bind chromatin and regulate DNA replication and transcription (Ellis, 2006). Moreover, the loss of NE function has been linked to a variety of dystrophies, collectively termed ‘nuclear envelopapathies’ (Schirmer et al., 2003). These diseases have phenotypes that include cardiac and skeletal myopathies, lipodystrophy, peripheral neuropathy and premature aging (Burke et al., 2001; Burke, 2001; Burke and Stewart, 2002; Espada et al., 2008; Worman and Courvalin, 2004).

How genes in the nucleus become accessible for processes such as transcription, replication or repair, and what factors are involved in these regulatory machineries are incompletely understood (Trinkle-Mulcahy and Lamond, 2007). One notion is that the NE proteins participate in modulating chromosome organization via direct contact or through indirect epigenetic events (Liu et al., 2007), while the C terminus of the protein connects to cytoplasmic anchorage (Lee et al., 2002). In mammalian cells, the N terminus of Sun1 targets the protein to the inner nuclear membrane (Chi et al., 2007), while the C terminus of the protein connects to cytoplasmic actin through a direct interaction with Nespin (Crisp et al., 2006; Padmakumar et al., 2005). In somatic cells, human SUN1 has been described to be one of the early INM factors that associate with segregated daughter chromosomes in anaphase, participating in post-mitotic chromatin de-condensation by recruiting a membrane-associated histone acetyl transferase, hALP (Chi et al., 2007).

To gain additional physiological insights into the function(s) of Sun1, we created Sun1−/− mice. Sun1−/− mice are born and grow normally; however, they are reproductively sterile. Gametogenesis in these mice was halted at meiotic prophase I. An analysis of the Sun1−/− mice revealed a prevalent loss in the expression of reproductive genes and small non-coding piRNAs. Although it has been suggested that the sterility of Sun1−/− mice arose from the loss of the mechanical function of Sun1 at meiotic telomeres (Ding et al., 2007), our current findings support that failed expression of reproductive genes and piRNAs further explains Sun1−/− sterility.

MATERIALS AND METHODS

Construction of Sun1 knockout mice

The Sun1 knockout vector was constructed by cloning a 0.9 kb fragment containing exon 10, intron 10 and exon 11 of the mouse Sun1 gene into vector pGEM-7 (Promega, Madison, WI, USA). Neo was positioned upstream of exon 10. A 3.0 kb DNA fragment upstream of exon 10 and a 3.4 kb fragment downstream of exon 11 were placed before the 5′ end of Neo and after the 3′ end of exon 11, respectively (Fig. 1A). The HSV-TK gene was placed at the 5′ end for negative selection. In addition, three loxp (locus of X-over of bacteriophage P1) sites were created at 5′ end of Neo, between

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Neo and exon 10, and at 3’ end of exon 11, respectively (Fig. 1A). Sun1 knockout mice were generated by the Mouse Genome Engineering Facility at the University of Nebraska. In brief, the Sun1 target vector was introduced into embryonic stem (ES) cells by electroporation and doubly selected using G418 and ganciclovir. Surviving clones were confirmed by polymerase chain reaction (PCR). Heterozygous ES cells were injected into C57BL/6J blastocysts. Mosaic founder animals were screened for germline transmission of the knockout genotype by breeding to C57BL/6J mice. F1 mice heterozygous for the knockout allele were mated to a Cre-expressing transgenic mouse [strain B6.FVB-Tg(EIIa-cre)C5379Lmgd/J, the Jackson Laboratory] resulting in the deletion of exon 10 and 11 of Sun1 (Fig. 1A) in some offspring. Loss of exon 10-exon 11 was verified by PCR using primers: 5’-GGCAAGTGGATCTCTTGTGAATTCTTGAC-3’ and 5’-GTGACCCACCCCTTTGGGATGTCGTCATC-3’. Cre confirmation was confirmed using primers: 5’-CGGCCTCCTGGCATGAAACTATCT-3’ and 5’-GGTAAACAGCATTGCCTGTCATT-3’.

Preparation of anti-mouse Sun1 antibody
A DNA fragment corresponding to amino acids 701-913 of mouse Sun1 was cloned into the pET47b+ vector (Novagen, Gibbstown, NJ, USA) and expressed in Escherichia coli BL21(DE3) cells. The recombinant His-tagged mouse Sun1 (701-913) protein was purified using Ni-NTA agarose (Qiagen, Valencia, CA, USA), and the purified protein was used for rabbit immunization (Spring Valley Laboratories, Woodbine, MD, USA). Rabbit mouse Sun1 antiserum (zmusSun1-I-C) was further purified using protein A-agarose (Bio-Rad, Hercules, CA, USA).

Immunohistochemistry
Sections from paraffin-embedded fixed tissues were deparaffinized and rehydrated using xylene and ethanol. Antigen retrieval was achieved by placing the slides in boiling citrate buffer, pH 6.4 for 20 minutes. After cooling at room temperature for 20 minutes, slides were rinsed with ddH2O and TBST (Tris-buffered saline with 0.1% Tween-20) successively. Endogenous peroxides were quenched by 3% H2O2 treatment for 10 minutes. To prevent non-specific binding, slides were blocked with 1% BSA in PBS for 30 minutes. After blocking, slides were incubated with the secondary antibody was added followed by incubation with a peroxidase-chamber for 1 hour. After rinsing with TBST, a biotinylated anti-rabbit IgG diluted with TBST was applied and slides were incubated in a humidified background eraser (Biocare Medical, Concord, CA, USA) containing 10% for 1 hour at room temperature. Alexa-488-, Alexa-594- or Alexa-647-conjugated secondary antibodies were used for immunofluorescent detection. Fluorescent-conjugated secondary antibodies was washed three times with 2×SSC, 0.1% SDS solution at 30°C. Membranes were exposed to an IP plate, which was then scanned using a FLA-7000 imager (Fujifilm, Stamford, CT, USA).

RT-PCR of small RNAs
Total RNA from mouse testis was prepared, and small RNA (~200 nucleotides) was enriched using the mirVana miRNA isolation kit (Applied Biosystems, Austin, TX, USA). The extracted total small RNAs were poly-A extended at their 3’ ends using poly(A) polymerase (Applied Biosystems). After annealing with a poly(T) adaptor (5’-GGGAGACGAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTTTTTTTTTTTTTTTT), RNA was reverse transcribed using Superscript II reverse transcriptase. PCR was performed using a universal reverse primer (5’-GGGAGACGAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTTTTTTTTTTTTTTTT) and U6 snRNA (5’-GGCAGG GCCATGCTAATCTTCTCTGTATCG-3’) as an internal control. Primers used for RT-PCR are described in the Results and Discussion section. The RT-PCR products of small RNAs were analyzed by 15% denaturing ura-polyacrylamide gels. Primer sequences used for RT-PCR of Line-1 type A element (Line-1 A-F1, 5’-GAGTTTTTGGACTGTGATTCC-3’; Line-1 A-R1, 5’-CTCTCCTAGTATTTCAGCTGGG-3’) were designed by Kuramochi-Miyagawa et al. (Kuramochi-Miyagawa et al., 2008).

RESULTS
Construction of Sun1−/− mice
Mouse Sun1 is an inner nuclear membrane protein with three nuclear transmembrane domains spanning amino acids 358 to 431 (Padmakumar et al., 2005). The N terminus of Sun1 is required for its inner nuclear membrane localization (Chi et al., 2007; Liu et al., 2007), while the SUN C terminus connects to the cytoskeleton through nesprin (Padmakumar et al., 2005). To investigate the physiological function of mammalian Sun1, we generated a Sun1 knockout mouse by targeted deletion of exons 10 and 11 (amino acids 344 to 408) that correspond to the transmembrane domains. To guard against embryonic lethality of Sun1 homoygous null animals, we created first a conditionally knocked out mouse. As illustrated in Fig. 1A, independent founder mice with three loxp sequences flanking a Neo (neomycin) gene, exon 10, and exon 11 were created; germ line transmission from founder to F1 offspring was confirmed by PCR (Fig. 1B, left panel). Next, to delete exons 10 and 11 from Sun1, we mated F1 animals to a strain of transgenic mouse from the Jackson Laboratory [strain B6.FVB-Tg(EIIa-cre)C5379Lmgd/J] that expresses an adenovirus EIIa promoter-driven Cre-expression line-1 type A element (Line-1 A-F1, 5’-GAGTTTTTGGACTGTGATTCC-3’; Line-1 A-R1, 5’-CTCTCCTAGTATTTCAGCTGGG-3’) were reported.
vector (Fig. 1A). Cre-mediated removal of loxP-flanked exon 10 and exon 11 in F2 mice (Sun1+/–) was confirmed by PCR (Fig. 1B, right panel). We then bred pairs of Sun1+/– mice and successfully generated Sun1–/– offspring. Sun1–/– mice are reproductively sterile. Although Sun1 is expressed ubiquitously in most mouse tissues (see Fig. S1A in the supplementary material) (Crisp et al., 2006), Sun1–/– mice were born grossly normal, and they grew to 7 months of age with body weights that were indistinguishable from those of wild-type siblings (see Fig. S1B in the supplementary material). However, when we paired Sun1–/– mice for mating, we discovered that the mice were infertile. This observation agrees with the results from a previously reported, albeit non-identical, Sun1 knockout (KO) mouse (Ding et al., 2007). The reproductive sterility of our Sun1–/– mice was confirmed by multiple parings of 6-week-old male Sun1+/+ with female Sun1–/– (four pairs) and male Sun1–/– with female Sun1+/+ (four pairs) animals. None of the eight paired animals produced any pregnancy, supporting that both male and female Sun1–/– mice are infertile.

When the mice were sacrificed and necropsies were performed, we observed that adult Sun1–/– male gonads were significantly smaller than their counterpart wild-type organs (Fig. 1C). Large weight differences in the male gonads were seen when the animals were compared at 28 days post birth (Fig. 1C). Moreover, when compared to Sun1+/+ mice, no sperm was seen in the epididymides of adult Sun1–/– mice (Fig. 1C; data not shown). In female Sun1–/– mice, no follicle was detected in the uterine tubes (see Fig. S1C in the supplementary material). Four-week-old Sun1+/+ testes had well-differentiated spermatocytes and spermatids (Fig. 1D); however, in Sun1–/– mice, the seminiferous tubules were blocked at the prophase of meiosis I (Fig. 1D). In the Sun1–/– mice, increased numbers of apoptotic cells were observed by TUNEL assay (see Fig. S1D in the supplementary material). Additionally, 4-week-old Sun1–/– ovaries contained no oocytes (see Fig. S1E in the supplementary material). The phenotype of our Sun1 knockout mice is similar to that reported by Ding et al. (Ding et al., 2007). Unlike female mice whose oogenesis at meiosis I completes before birth, the first wave of spermatogenesis initiates 2 days postnatal. To further characterize
which stage of meiosis was affected by Sun1 depletion, we focused our analysis on postnatal male, rather than prenatal female, mice due to their easier experimental accessibility.

To characterize the gametogenesis defect, we used SCP3 (also called SYCP3) as an indicator for synaptonemal complex (SC) formation, and phosphorylation of histone H2A at Ser139 (i.e. γH2AX) as a marker for synapsis (Viera et al., 2004). SCP3 is a part of the lateral element of SC, a meiosis-specific protein structure that forms between two homologous chromosomes during meiosis and is essential for synapsis of homologous chromosomes (Zickler, 2006). Accumulation of γH2AX is an early response to induced double-strand breaks (DSBs), such as those seen in meiotic recombination. In the budding yeast and in the mouse, the initiation of synapsis is dependent on the occurrence of DSBs during leptotene and zygotene (Viera et al., 2004). During normal meiotic progression, SCP3 and γH2AX first appear diffusely in the leptotene stage of spermocytes (Fig. 2A-C). As meiosis progresses to the zygotene stage, SCP3 forms a line-shaped structure, and γH2AX configures into discrete loci (Fig. 2D-F). In late zygotene and pachytene, γH2AX staining becomes restricted to the sex (XY) body, and prominent synapsis marked by SCP3 was observed [Fig. 2G-L, indicated by arrow] (Hamer et al., 2003; Sciurano et al., 2007). Intriguingly, although wild-type cells showed above expected progression in SCP3 and γH2AX staining (Fig. 2M-O), the distribution of γH2AX in Sun1−/− cells failed at the step of congress to the XY body (Fig. 2P-U). Thus, γH2AX in Sun1−/− cells remained multiply retained on various chromosomes (Fig. 2P-U), indicating that meiotic chromosome synapsis is impaired in Sun1-depleted cells.

**Sun1 localization during spermatogenesis**

To further investigate the role of Sun1 in gonad maturation, we generated an antibody (αmusSun1-C) to mouse Sun1. As shown in Fig. 3A, αmusSun1-C stained the nuclear envelope of mouse embryonic fibroblasts (MEFs), consistent with previous Sun1 localization studies (Chi et al., 2007; Padmakumar et al., 2005). Using αmusSun1-C, we performed immunohistochemical staining of mouse testes (Fig. 3B, part a), using Davidson’s fixative with paraffin-embedding to preserve the organization of the tissue and the morphology of the cells. Employing Methyl Green to counterstain the nuclei, Sun1 was localized in progressively dividing meiotic cells (Fig. 3B, part a) and was found initially at the nuclear periphery in spermatagonia (Fig. 3B, part b). As the cells entered meiotic prophase I, the chromosomes condensed and Sun1 concentrated at the chromosome ends (Fig. 3B, parts c-f). Using a telomere marker, Trf1 (Scherthan et al., 2000), Sun1 stained coincidently, although did not completely overlap, with Trf1 at the telomeres; moreover, Sun1 was also associated with the nuclear membrane, as revealed by lamin B1 staining (Fig. 3C). By the second stage of meiosis when haploid chromatids were produced, Sun1 reappeared at the nuclear periphery (Fig. 3B, part g). As the spermatids elongated, an acrosome-acroplaxome-manchette complex formed to shape the chromatid head for packaging chromatin (Kierszenbaum and Tres, 2004). At this juncture, Sun1 distributed to the lateral element of SC, a meiosis-specific protein structure that forms between two homologous chromosomes during meiosis and is essential for synapsis of homologous chromosomes (Zickler, 2006). Accumulation of γH2AX in Sun1−/− cells failed at the step of congress to the XY body (Fig. 2P-U). Thus, γH2AX in Sun1−/− cells remained multiply retained on various chromosomes (Fig. 2P-U), indicating that meiotic chromosome synapsis is impaired in Sun1-depleted cells.

**Prevalently changed meiotic gene expression in Sun1−/− versus control testes**

The spatial organization of chromosomes in the nucleus can influence gene expression (Kumar et al., 2008; Marshall, 2007; Stewart et al., 2007). Evidence suggests that a Sun1-telomere interaction tethers the chromosomes to the nuclear periphery, compartmentalizing DNA during the various stages of meiosis (Fig. 3). We wondered next how the depletion of Sun1 in Sun1−/− animals might impact meiotic gene expression.
In postnatal mice, the first wave of spermatogenesis transpires stepwise within the testis (Fig. 4A) (Goetz et al., 1984). At defined times, new germ cell types populate the testis, and the more immature cells progress to mature cells until the entire spectrum of germ cells is generated (Fig. 4A). To investigate how Sun1 might affect gene transcription, we compared male Sun1+/+ and Sun1–/– germ cells in the initial stages of mouse spermatogenesis by using cDNA microarrays. Total RNAs were extracted from 9- (P9, before prophase I) and 14- (P14, zygotene/pachytene) day-old mouse testes (Fig. 4A,B) and were analyzed using a 38 k gene chip representing 25,000 known germ cells. Fig. 3. Localization of Sun1 in the wild-type testis. (A) Immunofluorescent staining of Sun1 in mouse embryonic fibroblasts (MEFs) using αmusSun1-C. A clear nuclear envelope localization of Sun1 (green, Alexa-488) was seen. DNA stained by DAPI is in red. (B) Localization of Sun1 in the wild-type adult testis (Davidson’s fixed, paraffin-embedded tissue section), as detected by immunohistochemistry. Sun1 is stained brown, and the nuclei were counterstained with Methyl Green (green). Part a shows an overall view of Sun1 staining in seminiferous tubule. Sun1 was localized to the nuclear periphery in spermatogonia (b) and round spermatids (g), showed punctate staining at chromosome ends in prophase I (c-f), and decorated an acrosome-like structure in elongated spermatids (h). Sun1 staining was not seen in spermatozoa (i). (C) Immunofluorescent staining of a frozen testis section (28-day-old) by αmusSun1-C (green, Alexa-488), anti-Trf1 (red, Alexa-594) and anti-lamin B1 (gray, Alexa-633) antibodies. Nuclei are labeled by DAPI (blue). Insets show higher-magnification images of the framed sections. sc, spermatocyte; sp, spermatozoan; Esd, elongated spermatid.

Fig. 4. Histology of testes from Sun1+/+ and Sun1–/– mice during the first wave of spermatogenesis. (A) A schematic time line for the first wave of mouse spermatogenesis. (B) H&E stainings of testes from 9- and 14-day-old Sun1+/+ and Sun1–/– mice are shown. Insets show higher-magnification images of the tissues. In P9 testes (a,b), no significant morphological difference was observed between Sun1+/+ and Sun1–/– mice. In P14 mice, although the distribution of spermatogonia/sertoli cells and meiotic prophase I spermatocytes are similar (quantified in C) in the wild-type and Sun1–/– testes, wild type mostly showed clear ‘bouquet’ structures (c) while Sun1–/– cells did not (d). Cell stages later than pachytene were not observed in either P9 or P14 testes. (C) Cell compositions in P9 and P14 Sun1+/+ and Sun1–/– testes. Two thousand cells were counted in each set.
mouse genes (NCBI GEO accession number GPL6806). cDNA expression from three pairs of Sun1–/– versus Sun1+/+ animals was evaluated at each of the two time points. We also compared the expression profiles of Sun1–/– versus Sun1+/+ mouse embryonic fibroblasts (MEFs). Not all 25,000 array points were detected in the samples; however, the reproducibly detectable signals (P<0.05, t-test) from three separate arrays were collated. Overall, 10,043 (P9 testes), 9280 (P14 testes) and 6414 (MEFs) data points were captured (Table 1). Comparing the Sun1–/– versus Sun1+/+ samples, the expression of control ‘housekeeping’ genes such as actin or Gapdh was unchanged, and as expected Sun1–/– was consistently low in the Sun1–/– cDNA arrays (see Table S1 in the supplementary material).

The expression profiles of P9 Sun1–/– and Sun1+/+ mouse testes showed that 9448 cDNAs were essentially unchanged, and approximately an equal number of genes were either two fold up- (222 out of 10,043, 2.2%) or down- (373 out of 10,043, 3.7%) regulated [a ratio of 1:1.6 in up- versus down-regulated genes (Table 1)]. This pattern became significantly different at P14 when the first wave of spermatogenesis proceeded to a stage between zygotene and pachytene and when bouquet structures were observed in most of the wild-type (Fig. 4B, part c) but not the Sun1–/– (Fig. 4B, part d) testis cells. At P14, 1.2% (109 out of 9280) of genes were upregulated and 9.0% (834 out of 9280) of genes were downregulated [a ratio of 1:7.5 in up- versus down-regulated genes (Table 1)]. Conversely, in non-germ control cells (i.e. MEFs), we actually noted that more genes were upregulated than were downregulated in the Sun1–/– compared with the Sun1+/+ sample [a ratio of 1:0.5 in up- versus down-regulated genes (compare 643 to 323; Table 1)]. Collectively, these numbers suggest that a loss of Sun1 results in selectively downregulated gene expression in P14 germ cells.

Because Sun1–/– mice are infertile, we considered next whether reproductive genes (ontology analyses were made with the mAdb software developed by CIT, NIH, USA) were specifically repressed in these animals. We detected microarray signals for 119 reproductive genes in P9, and 162 reproductive genes in P14 germ cells. Although Sun1–/– and Sun1+/+ P9 samples expressed essentially the same reproductive genes, P14 cells showed a 1.29 ratio in up- versus down-regulated genes in Sun1+/+ samples (see Table 2). Of note, several genes, such as Gykl1, Acr, Spag6, Odf4 and Piwil1, were repressed more than 10-fold [i.e. \( \log_{2}(\text{Sun1}+/+) < -3.3 \)] in P14 Sun1–/– compared with Sun1+/+ testis (see Table S2 in the supplementary material).

### Reduction in Mili- and Miwi-associated piRNA in Sun1–/– testis

In our cDNA microarrays, reduced expression of Miwi (i.e. Piwi1; see Table S2 in the supplementary material) was seen in the Sun1–/– samples. Miwi and Mili are murine Piwi gene family members whose expression is restricted to mouse germ cells (Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004); they have been implicated in stem cell renewal, RNA silencing and germ cell development (Hartig et al., 2007; Klattenhoff and Theurkauf, 2008; Seto et al., 2007). To confirm the microarray results, we employed a semi-quantitative RT-PCR assay to measure the expression of Miwi and other reproduction-relevant genes in P9, P14 and P28 testes (Fig. 5A). Indeed, a reduction in Miwi, Mili and Mvh1, but not Mhl1, was observed.

Mili expression is crucial for germ cell progression to spermatids (Deng and Lin, 2002). The reduced expression of Mili could create a block in meiosis at a stage prior to pachytene in Sun1–/– cells. Mvh1 and Mili function before the zygotene stage (Kuramochi-Miyagawa et al., 2004; Tanaka et al., 2000), and it is intriguing that these two factors were also repressed in Sun1–/– animals. We noted that not all meiotic factors were affected. For example, the synaptonemal complex protein Scp3, the expression of which is restricted in leptotene to diplotene spermatocytes (Di Carlo et al., 2000; Meuwissen et al., 1992), was unchanged (Fig. 5A). Thus, the collective results suggested that an absence of Sun1 selectively reduced the expression of many, but not all, reproductive genes.

Mili and Miwi function in the biogenesis of a novel class of 24- to 29-nucleotide piRNAs that accumulate during meiosis (Aravin et al., 2002). In addition, Mili forms a complex with Mvh1 in germ cells (Kuramochi-Miyagawa et al., 2004). Above, we documented the reduced expression of Mili and Mvh1 in Sun1–/– germ cells.
cells (Fig. 5A). Because Mili and Miwi are necessary for piRNA production, their reduction could be independently confirmed by verifying the reduced expression of piRNA. To this end, we end-labeled total RNA with [γ-32P]-ATP and assayed the abundance of small RNAs by denaturing gels. Notably, although wild-type mice harbored a surfeit of ~30-nucleotide piRNAs, these small RNA signals were not detected in 28-day-old Sun1−/− mice (Fig. 5B). Northern blotting using piRNA-specific probes (see Materials and methods) confirmed that the expected Mili- and Miwi-associated piRNAs were not seen in 28-day-old Sun1−/− tests (Fig. 5C). Moreover, we characterized the expression of several individual piRNAs using a highly sensitive RT-PCR assay. Thus, while Mili- and Miwi-associated piRNAs were seen in P14 Sun1−/− tests, little to no piRNAs were detected in P14 or P28 Sun1−/− tests by RT-PCR (Fig. 5D).

The silencing of transposable elements via de novo DNA methylation is required during gametogenesis, and the loss of Mili and Miwi2 is associated with the activation of retrotransposons (Kuramochi-Miyagawa et al., 2008). To investigate whether the reduction in Mili and Miwi caused by Sun1 depletion affected the expression of retrotransposons, we checked a representative Line-1 element using semi-quantitative RT-PCR (Kuramochi-Miyagawa et al., 2008). Indeed similar to findings from Mili−/− mice, the expression of type A Line-1 was de-repressed in two-week-old Sun1−/− tests (Fig. 5E).

DISCUSSION

The nuclear membrane and its associated proteins affect many cellular processes, including the spatial architecture of the DNA in the eukaryotic nucleus, which influences gene expression (Schneider and Grosschedl, 2007). We previously reported on the contribution of the inner nuclear membrane protein Sun1 to somatic cell mitosis and cell cycle progression (Chi et al., 2007). Others have presented findings that Sun1 provides a structural bridge that connects the nucleus to cytoplasmic actin and is involved in nuclear anchorage (Crisp et al., 2006; Padmakumar et al., 2005). Here, we have created a Sun1 knockout mouse, and have found, surprisingly, that the mouse is born somatically normal, and that the major in vivo developmental deficit in the Sun1−/− animal is reproductive infertility with failed gametogenesis.

Our experimental results agree with those of Ding et al. (Ding et al., 2007) that Sun1 locates to telomeres during the prophase of meiosis I (Fig. 3B,C). In lower eukaryotes that do not undergo meiotic reproduction, the published literature suggests that Sun1 behaves differently. For instance, the S. pombe Sun1 homolog Sad1 is a component of the spindle pole body (SPB) that serves as a microtubule organization center (Chikashige et al., 2006; Raff, 1999; Tomita and Cooper, 2006), and Matefin/SUN-1 of C. elegans has been reported to mediate the attachment of centrosomes to the nucleus (Penkner et al., 2007). In mammalian germ cells, Sun1 tethers telomeres to the nuclear periphery (Fig. 3D) (Ding et al., 2007; Kierszenbaum and Tres, 2004). Our current study suggests that loss of this DNA compartmentalization reshapes the meiotic gene expression needed for normal gametogenesis (Table 2).

The report from Ding et al. (Ding et al., 2007) suggested that the association of Sun1 with telomeres is a prerequisite for efficient homolog paring and synopsis. At the initial stages of meiosis, paternal and maternal chromosomes converge and pair. Subsequently, homologous recombination could occur, and meiosis would then proceed to the next phase (Meier and Ahmed, 2001). Ding et al. (Ding et al., 2007) hypothesized that Sun1 functions in telomere clustering and bouquet formation, and that the bouquet structure facilitates the probability of encounter between chromosomes for recombination (Chikashige et al., 2006; Tomita and Cooper, 2006). Although this attractive hypothesis provides details on how Sun1 might contribute to bouquet formation and synopsis, it does not explain how these
events mechanistically influence downstream gametogenesis. Our data do not question the influence of Sun1 on bouquet formation, but they do suggest that loss of Sun1 further results in failed expression of selective reproductive genes and piRNAs, which would causally impact gametogenesis.

The cDNA microarray analyses revealed that in Sun1−/− versus Sun1+/+ P9 testis, the ratio of up- versus down-regulated genes (1:1.6) was significantly different to the same ratio (1:7.5) in P14 testes (see Table 1). In Sun1−/− P9 testes, out of 119 reproductive genes with detectable signals, only two were different between Sun1−/− and Sun1+/+ animals, whereas in P14 testes 30 reproductive genes were different, and 29 of these were repressed in Sun1−/− animals. How then does one link a loss of Sun1 to selectively changed meiotic gene expression? The answer is not known; however, recent experiments do show that siRNA-depletion of Sun1 interfered with the organization of the INM (inner nuclear membrane) constituents and the NPC (Chi et al., 2007; Liu et al., 2007). Transcriptionally active genes are frequently NPC associated (Taddei et al., 2006). Perhaps, a Sun1-depletion effect on the NPC could, in part, explain the selectively perturbed transcription in P14 Sun1−/− testes.

That Sun1 contributes to meiotic transcription is consistent with emerging data that some nuclear proteins previously touted only for their structural roles do significantly affect gene expression. For example, the cohesin protein whose function was attributed exclusively to connecting sister chromatids during mitosis and meiosis has recently been shown to serve a role in developmental gene regulation (Hallson et al., 2008; Misulovin et al., 2008). Likewise, the inner nuclear membrane protein Src1 was also revealed to regulate subtelomeric gene expression (Grund et al., 2008). Sun1 is a lamin A-binding protein (Crisp et al., 2006; Haque et al., 2006), and its currently invoked role in selective gene transcription may have implications for the competing models, the ‘mechanical model’ versus the ‘gene expression model’, that explain the pathogenesis of human laminopathies which arise from lamin A mutation (Wagner and Krohn, 2007). In the mechanical model, lamin A mutation weakens the cytoskeletal structure leading to disease pathology. By contrast, the gene expression model posits that lamin A mutation affects either directly or indirectly the expression of disease-associated genes that engender pathology. Considering the gametogenesis defect in Sun1−/− mice, one surmises that this could arise in two ways. First, Sun1 depletion could abrogate proper chromosomal organization, interrupting meiosis and arresting gene expression. Second, Sun1 depletion could selectively interfere with gene expression, whereby the loss of expression interrupts meiosis preventing proper chromosomal organization. Currently, the findings from Sun1−/− mice do not fully differentiate between whether Sun1 depletion impacts first chromosome organization or gene expression, or both simultaneously. Indeed, further dissection of the Sun1 knockout mouse could potentially permit the delineation of which genes are involved in meiotic progression before and after bouquet formation.

Sun1 tethers lamin A through nesprin to cytoplasmic actin (Haque et al., 2006), and loss of Sun1 might be expected to show a similar mechano-structural weakness to that seen with a lamin A mutation. Sun1−/− mice, unlike Lmna−/− mice (Sullivan et al., 1999), however, do not exhibit a laminopathy. We speculate that the Sun1 function in Sun1−/− somatic cells may be redundantly constituted by the expression of other Sun domain proteins. However, based on our finding of selective repression of reproductive genes in Sun1−/− versus Sun1+/+ testes, it may be worthwhile comparing in detail the gene expression patterns of relevant somatic tissues from Lmna−/− versus Lmna+/+ mice for correlation with the development of pathology.

Another unanticipated observation to emerge from our work was the requirement for mouse Sun1 in the expression of Piwi-like proteins, Mulli and Mili. Mulli and Mili are expressed highly in testes and bind 24- to 29-nucleotide piRNAs. Both genes are required for male fertility as mice knocked out for either the Mulli or the Mili gene have degenerative male germ cells (Deng and Lin, 2002; Kuramoto-Miyagawa et al., 2004). In flies, piRNA mutations lead to the activation of retrotransposons. Mobilization of retrotransposition can create germline DNA damage and trigger cellular apoptotic responses (Breenecke et al., 2007). Indeed, piRNAs may serve ubiquitously to control chromatin organization, gene transcription, RNA stability or translation (Klattenhoff and Theurkauf, 2008). Consistent with these data, our results show that Sun1−/− germ cells do have higher retrotransposon expression (Fig. 5E). The detailed linkage between piRNA processing and Sun1 and the absence of piRNAs in Sun1−/− germ line cells needs further investigation, which could reveal a gamete-specific mechanism(s) that accounts for reproductive infertility.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/6/965/DC1

**References**


Table S1. Expression ($Sun1^{-/-}$ versus $Sun1^{+/+}$) of housekeeping genes (Actin and Gapdh) and $Sun1$ by microarray

<table>
<thead>
<tr>
<th>Gene</th>
<th>Entrez Gene ID</th>
<th>Log$_2$ ($Sun1^{-/-}$/wt)</th>
<th>P value</th>
<th>Log$_2$ ($Sun1^{-/-}$/wt)</th>
<th>P value</th>
<th>Log$_2$ ($Sun1^{-/-}$/wt)</th>
<th>P value</th>
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<td>11461</td>
<td>0.32</td>
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<td>0.86</td>
<td>0.0050</td>
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<td>11461</td>
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Data are presented in log$_2$ scale.
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Gene descriptions:
- **Strbp**: spermatid perinuclear RNA binding protein (Strbp), mRNA.
- **Golga3**: golgi autoantigen, golgin subfamily a, 3 (Golga3), mRNA.
- **Psg19**: pregnancy specific glycoprotein 19 (Psg19), mRNA.
- **Ddx1**: DEAD (Asp-Glu-Ala-Asp) box polypeptide 1 (Ddx1), mRNA.
- **Creb1**: cAMP responsive element binding protein 1 (Creb1), transcript variant B, mRNA.
- **Zfp41**: zinc finger protein 41 (Zfp41), transcript variant 2, mRNA.
- **Sbf1**: SET binding factor 1 (Sbf1), mRNA.
- **Rps6kb1**: ribosomal protein S6 kinase, polypeptide 1 (Rps6kb1), mRNA.
- **Kdr**: kinase insert domain protein receptor (Kdr), mRNA.
- **Tcf7l2**: transcription factor 7-like 2, T-cell specific, HMGG-box.
- **Hrb**: HIV-1 Rev binding protein.
- **Tbp1**: TATA box binding protein-like 1 (Tbp1), mRNA.
- **Bmp1r1**: bone morphogenetic protein receptor, type 1A (Bmp1r1), mRNA.
- **Dnaj1**: DnaJ (Hsp40) homolog, subfamily A, member 1 (Dnaj1), mRNA.
- **Cdc25b**: cell division cycle 25 homolog B (S. pombe).
- **Mih1**: mutl homolog 1 (E. coli) (Mih1), mRNA.
- **Diap2**: Diaphanos homolog 2 (Drosophila).
- **Spata6**: spermatogenesis associated 6.
- **Acox1**: acyl-Coenzyme A oxidase 1, palmitoyl (Acox1), mRNA.
- **Nr2c2**: nuclear receptor subfamily 2, group C, member 2.
- **Spata20**: spermatogenesis associated 20 (Spata20), mRNA.
- **Ercc1**: excision repair cross-complementing rodent repair deficiency, complementation group 1 (Ercc1), mRNA.
- **Siah1a**: seven in absence 1A (Siah1a), mRNA.
- **Sox9**: SRY-box containing gene 9 (Sox9), mRNA.
- **Nr0b1**: nuclear receptor subfamily 0, group B, member 1 (Nr0b1), mRNA.
- **Fkbp4**: FK506 binding protein 4 (Fkbp4), mRNA.
- **Tcf7l2**: transcription factor 7-like 2, T-cell specific, HMGG-box.
- **Tgbf1**: transforming growth factor, beta 1 (Tgbf1), mRNA.
- **Tcf7l2**: transcription factor 7-like 2, T-cell specific, HMGG-box (Tcf7l2), mRNA.
- **Cetn2**: centrin 2 (Cetn2), mRNA.
- **Crem**: cAMP responsive element modulator (Crem), mRNA.
- **P42pop**: Myb protein P42POP (P42pop), mRNA.
- **Dhc2r4**: 24-dehydrocholesterol reductase (Dhc2r4), mRNA.
- **Dnd1**: dead end homolog 1 (zebrafish) (Dnd1), mRNA.
- **Citr**: Citron.
- **Tial1**: Tia1 cytoxic granule-associated RNA binding protein-like 1 (Tial1), mRNA.
- **Zfp148**: zinc finger protein 148 (Zfp148), mRNA.
- **Cdc25b**: cell division cycle 25 homolog B (S. pombe).
- **Nkd1**: naked cuticle 1 homolog (Drosophila) (Nkd1), mRNA.
- **Hrb**: HIV-1 Rev binding protein.
- **Dmrt3**: doublesex and mab-3 related transcription factor 3 (Dmrt3), mRNA.
- **Sox9**: SRY-box containing gene 9 (Sox9), mRNA.
- **Brd2**: bromodomain containing 2 (Brd2), transcript variant 1, mRNA.
- **Cgtap**: cartilage associated protein (Cgtap), mRNA.
- **Rhox5**: reproductive homeobox 5 (Rhox5), mRNA.
- **Cugbp1**: CUG triplet repeat, RNA binding protein 1.
- **Rhox5**: reproductive homeobox 5 (Rhox5), mRNA.
- **Gpx4**: glutathione peroxidase 4 (Gpx4), transcript variant 2, mRNA.
- **Hexb**: hexosaminidase B (Hexb), mRNA.
- **Sox3**: SRY-box containing gene 3 (Sox3), mRNA.
- **Gli1**: GLI-Kruppel family member GLI1 (Gli1), mRNA.
- **Nanog**: Nanog homeobox.
- **Figla**: folliculogenesis specific basic helix-loop-helix (Figla), mRNA.
- **Gtgeo22**: gene trap ROSA b-geo 22 (Gtgeo22), mRNA.
- **Dynl1**: dynin light chain LC8-type 1 (Dynl1), mRNA.
- **2010012**: RIKEN cDNA 2010012C16 gene (2010012C16rik), mRNA.
- **C16Rik**: mago-nashi homolog, proliferation-associated (Drosophila) (Magoh), mRNA.
- **Utx2**: upstream transcription factor 2 (Utx2), mRNA.
- **Adamt2s**: A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 2.
- **Btg1**: B-cell translocation gene 1, anti-proliferative (Btg1), mRNA.
- **Phthl**: Parathyroid hormone-like peptide.
- **Nr5a1**: nuclear receptor subfamily 5, group A, member 1 (Nr5a1), mRNA.
- **Pakah1b3**: platelet-activating factor acylhydrolase, isoform 1b, alpha 1 subunit (Pakah1b3), mRNA.
- **Pebp1**: phosphatidylethanolamine binding protein 1 (Pebp1), mRNA.
- **Nhp2l1**: NHP2 non-histone chromosome protein 2-like 1 (S. cerevisiae).
- **Amhr2**: anti-Mullerian hormone type 2 receptor (Amhr2), mRNA.
<table>
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<tr>
<th>Gene</th>
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<th>mRNA</th>
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