EMG1 is essential for mouse pre-implantation embryo development

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Abstract

Background: Essential for mitotic growth 1 (EMG1) is a highly conserved nucleolar protein identified in yeast to have a critical function in ribosome biogenesis. A mutation in the human EMG1 homolog causes Bowen-Conradi syndrome (BCS), a developmental disorder characterized by severe growth failure and psychomotor retardation leading to death in early childhood. To begin to understand the role of EMG1 in mammalian development, and how its deficiency could lead to Bowen-Conradi syndrome, we have used mouse as a model. The expression of Emg1 during mouse development was examined and mice carrying a null mutation for Emg1 were generated and characterized.

Results: Our studies indicated that Emg1 is broadly expressed during early mouse embryonic development. However, in late embryonic stages and during postnatal development, Emg1 exhibited specific expression patterns. To assess a developmental role for EMG1 in vivo, we exploited a mouse gene-targeting approach. Loss of EMG1 function in mice arrested embryonic development prior to the blastocyst stage. The arrested Emg1-/- embryos exhibited defects in early cell lineage specification as well as in nucleologenesis. Further, loss of p53, which has been shown to rescue some phenotypes resulting from defects in ribosome biogenesis, failed to rescue the Emg1-/- pre-implantation lethality.

Conclusion: Our data demonstrate that Emg1 is highly expressed during mouse embryonic development, and essential for mouse pre-implantation development. The absolute requirement for EMG1 in early embryonic development is consistent with its essential role in yeast. Further, our findings also lend support to the previous study that showed Bowen-Conradi syndrome results from a partial EMG1 deficiency. A complete deficiency would not be expected to be compatible with a live birth.
of 60S to 40S ribosomal subunits [5,6]. These findings indicate an important role for EMG1 in the biogenesis of the 40S ribosome.

Deciphering the precise role of EMG1 in 40S ribosome biogenesis has been challenging. A temperature sensitive mutation in yEMG1 could be suppressed by the methyl donor S-adenosyl methionine (SAM) [6] or deletion of the snR57 gene encoding a snoRNA needed for 2′-O-ribose-methylation of G1570 in the 18S rRNA [7]. Furthermore, yEMG1 was found to interact directly with snoRNA [8] and the 18S rRNA [9]. Taken together, these findings suggested that yEMG1 functions to methylate the 18S rRNA, a concept that was later supported by the identification of yEMG1 as a SAM-dependent pseudouridine-N1-specific methyltransferase [10].

The EMG1 protein is highly conserved from archaeobacteria to humans or mice [11]. Expression of the human orthologue of EMG1 in yeast demonstrates that it is capable of suppressing the lethal defect in yEMG1 cells, indicating that EMG1 is both structurally and functionally conserved among these eukaryotes [6]. More recently, a mutation in human EMG1, which significantly reduces EMG1 protein levels, has been found to cause Bowen-Conradi syndrome (BCS), an autosomal recessive disorder characterized by severely impaired prenatal and postnatal growth, profound psychomotor retardation, and death in early childhood [12]. This finding strongly suggests that EMG1, as a key molecule in ribosomal synthesis, could be important for development. To get a better understanding of this, in the present study, we have attempted to generate an EMG1-deficient mouse and characterize the expression of Emg1 during mouse development. Our data demonstrates that EMG1 is essential for mouse pre-implantation development.

Results and Discussion
Expression of Emg1 during mouse embryogenesis
As a first step toward elucidating the role of EMG1 during development, we analyzed the expression of the Emg1 gene in mouse embryos and postnatal tissues. Using RNA in situ hybridization, Emg1 expression was readily detected in E2.5 morula embryos (Figure 1A), with the strongest expression associated with the inside cells in the late stage of morula (Figure 1B). High levels of Emg1 mRNA were also detected in the inner cell mass (ICM) of mouse blastocysts (E3.5), but not in the trophectoderm (Figure 1C). This expression pattern is consistent with Emg1 being expressed highly in the embryo proper, but weakly expressed or not detectable in the trophoblasts of mouse placenta (Figure 2).

Expression of Emg1 in mouse pre-implantation embryos was also analyzed by determining the activity of LacZ in Emg1 knockout heterozygotes in which the expression of the LacZ transgene was regulated by the endogenous Emg1 regulatory elements (see Figure 3). Crosses of wild-type males with Emg1+/− females revealed a positive LacZ signal in zygotes and in 2-cell embryos, which was not present in those from breeding Emg1+/− males with wild-type females (Figure 1D), indicating that these early mouse embryos could contain maternally transmitted EMG1. However, starting at the 8-cell morula stage, there were two distinct LacZ-staining patterns for the embryos collected from the breeding of wild-type males with Emg1+/− females (Figure 1E). Among these embryos, Emg1+/− showed wide-spread LacZ staining, whereas the ones that were genotyped as Emg1+/− exhibited a weak disperse staining which could be from residual maternally transmitted LacZ proteins. This data indicates that the onset of zygotic expression of EMG1 may occur at the 8-cell morula stage. In addition, X-gal staining of E3.5 Emg1+/− blastocysts showed strong LacZ staining in the ICM, but not in the trophectoderm (Figure 1E), which is consistent with Emg1 expression detected by in situ hybridization (Figure 1C).

To determine the expression pattern of Emg1 in post-implantation embryos, mouse embryos at E8.5-E15.5 were analyzed. At E8.5-E9.5, Emg1 was widely and strongly expressed and showed no clear tissue-specific pattern (Figure 2A-B). At later stages (E11.5-E15.5), however, Emg1 was found to be expressed at a low level in most embryonic tissues, but strongly in several regions, including the ventricular zone of the neuroepithelium (Figure 2C), the neural layer of retina (Figure 2D), the follicles of vibrissae (Figure 2E), thymus (Figure 2F), submandibular glands (Figure 2G), brown adipose tissue (Figure 2H), lung (Figure 2I), nephric tubules and renal mesenchyme (Figure 2J) and seminiferous tubules in the testis (not shown).

To examine the expression of Emg1 in extraembryonic tissue, we performed RNA in situ hybridization on E8.5-9.5 mouse placenta. No Emg1 signal was detected in the trophoblast cells, while a control gene, Rtel (Regulator of Telomere length) showed strong expression in this cell lineage (Figure 2K) [13]. In adult mice, similar levels of Emg1 mRNA were detected in multiple tissues by Northern blot hybridization (Figure 2L), suggesting that EMG1 could be widely expressed during postnatal development. However, using RNA in situ hybridization assays, Emg1 cell-specific expression patterns were detected in several tissues (Figure 2M-Q). In the adult testis, Emg1 is highly and specifically expressed in both spermatogonia and early meiotic spermatocytes, but not in late stage spermatocytes (Figure 2M). A strong Emg1 signal was also identified in oocytes and the granulosa cells of the pre-antral follicles in the ovary (Figure 2N). In the adult mouse brain, Emg1 expression was mainly detected in the
Figure 1 Expression of Emg1 in pre-implantation embryos. (A) Whole mount RNA in situ hybridization of mouse pre-implantation embryos. Asterisks indicate the E2.5 morulae, and arrows indicate the blastocysts. Hybridization with Emg1 sense probe is presented as a control. (B) High magnification showing that a stronger Emg1 signal is detected within the inside cells at the morula stage. (C) High magnification demonstrating that the inner cell mass of the mouse blastocyst is strongly stained with Emg1 antisense probe (arrow indicates), whereas the trophectoderm has a very low level of Emg1 (arrowhead). (D) Whole mount X-gal staining of zygotes and 2-cell embryos either from the cross of wild-type males with Emg1+/− females or from Emg1+/− males with wild-type females. Only the embryos from the breeding of wild-type males with Emg1+/− females show LacZ signals. (E) X-gal staining of morulae and blastocysts collected from the breeding of wild-type males with Emg1+/− females, shows wide-spread LacZ signals in the 8-cell morula and in the ICM of Emg1+/−. In Emg1+/− morulae or blastocysts, only a few cells are weakly stained for LacZ, probably reflecting a low level of residual maternally transmitted LacZ protein in these cells. Scale bar in A-D, 50µm, and in E, 100µm.
Figure 2 Expression of Emg1 in post-implantation embryos and adult mice. (A-B) Whole mount RNA *in situ* hybridization of post-implantation mouse embryos (A: E8.5; B: E9.5). (C-J) RNA *in situ* hybridization on the sections of E13.5-E15.5 mouse embryos, demonstrating that Emg1 is expressed at low levels in most tissues, but strongly in the ventricular zone of the neuroepithelium (C, arrow indicates), the neural layer of retina (D), the follicles of vibrissae (E, arrows indicate), thymus (F), submandibular glands (G, arrow indicates), brown adipose tissue (H, arrow indicates), lung (I, arrow indicates). Asterisk indicates liver in which low expression of Emg1 was found, nephric tubules and renal mesenchyme (J, arrow indicates). (K) RNA *in situ* hybridization on the sections of E9.5 placenta, showing that Emg1 mRNA is low or undetectable in the trophoblast cells (arrow indicates). Rtel antisense probe is presented as a positive control. Magnification for images C-K: x10. (L) Northern blot analysis of Emg1 expression in mouse adult tissues. Lane: 1 Brain; 2 Lung; 3 Heart; 4 Liver; 5 Spleen; 6 Kidney; 7 Skeletal muscles. Lower panel shows the total ribosomal RNAs for normalization. (M) RNA *in situ* hybridization on mouse testis, showing that Emg1 is highly and specifically expressed in spermatogonia and meiotic spermatocytes. (N) In ovary, strong Emg1 expression is detected in oocyte (arrow indicates). The granulose cells of the pre-antral follicles are also positive for Emg1 (arrowheads). (O-P) In brain, Emg1 is predominately expressed in the granular neurons of cerebellum (O, arrow indicates) and the neurons located in hippocampus (P, arrows indicate). (Q) In intestine, Emg1 expression was mainly identified in the crypts (arrowhead). Magnification for images O-Q: x10.
granular layer of neurons of the cerebellum (Figure 2O) as well as in the hippocampus (Figure 2P). Specific expression of Emg1 was also found in the crypts of the intestine (Figure 2Q).

Taken together, our gene expression data indicates that EMG1 is broadly distributed in the early developing embryos, but its expression is more restricted in the later stages of development. In adult mice, Emg1 also exhibits cell-specific expression, most notably in the gonads, brain and intestine. This expression pattern suggests that EMG1 may not only be important for early embryonic development, but could also be required for the development of several cell lineages at late developmental stages or during postnatal development.

**Generation of the Emg1 null mouse allele**

To further study the developmental role of EMG1, we have mutated Emg1 in mice by homologous recombination. The mouse Emg1 gene contains 6 exons that encode a protein composed of 244 amino acids. In order to create an Emg1 null allele, a gene-targeting vector with a splice acceptor (SA)-IRES-βgeo-pA cassette was used to replace exons 2-6 and to remove approximately 80% of the Emg1 coding sequence (Figure 3A). Given that Emg1 is highly expressed in ES cells, the inserted SA-IRESβgeo cassette in the first intron of the Emg1 locus will trap exon 1 to turn on the expression of βgeo, a fusion protein of LacZ and neo. This was designed to allow us to significantly increase the targeting frequency, while also allowing us to establish a mouse allele in which a LacZ reporter is regulated by the endogenous Emg1 regulatory elements. Indeed, approximately 25% (11 out of 45) of the ES colonies obtained after G418 selection showed correct homologous recombination by Southern blot analysis using both 5' and 3' probes external to the targeting vector (Figure 3B-C). Furthermore, LacZ transgene expression in Emg1+/− embryos was entirely consistent with the

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**Figure 3 Targeted disruption of EMG1 in mice by homologous recombination.** (A) Structure of the wild-type Emg1 locus, the targeting vector and the mutated locus with exons 2-6 replaced by SA-IRESβgeo cassette. The location of the hybridization probes (5’ probe and 3’ probe) for Southern blot analyses are shown. (B-C) Southern blot analysis of targeted ES cell clones using the 3’ probe (B) and the 5’ probe (C). (D) Whole mount X-gal staining of Emg1+/− embryos (E8.5 and E9).
The expression pattern established by in situ hybridization (Figure 1E and 3D). In this study, we have used two independently targeted ES lines to generate germline-transmitting chimeras that were bred with 129S1 or CD1 females to produce Emg1 null mutants for functional analysis.

The pre-implantation lethality of Emg1 null mutants
Both Emg1+/− males and females were normal and fertile, and transmitted the targeted allele to about 50% of their progeny. However, no homozygous offspring were born from Emg1+/− intercrosses (Table 1, n = 237), indicating that loss of EMG1 function leads to embryonic lethality. To determine at which stage of development the homozygous mutant embryos died, timed heterozygous matings were performed. An analysis of embryos between E8.5-E12.5 revealed that none of the 64 embryos genotyped were Emg1−/−. The expected Mendelian frequency of heterozygote, homozygote and wild-type was only observed in the pre-implantation embryos (Table 1), indicating that mice lacking EMG1 protein stopped developing at the pre- and/or peri-implantation stage embryos.

To address this further, we analyzed more than 100 pre-implantation embryos collected from two independent mouse lines carrying the Emg1 knockout allele. At E2.5, Emg1+/− intercrosses yielded early stage embryos that were indistinguishable from each other, despite the presence of Emg1−/− mutations among these progeny (Figure 4A). In addition, all the Emg1−/− embryos at this developmental stage showed similar BrdU incorporation (Figure 4B), and nearly undetectable levels of apoptosis, suggesting a defect in the specification of early cell lineages from each other, despite the presence of Emg1−/− embryos. The E3.5 embryos from Emg1+/− intercrosses, however, consistently showed a mixture of morula and blastocyst-stage embryos. Genotyping of these embryos demonstrated that whereas the blastocysts were either Emg1+/+ or Emg1−/− heterozygotes, the morula-stage embryos were Emg1−/− mutants (Figure 4C).

| Developmental Stage | No. of mice by genotyping | Total |
|---------------------|---------------------------|-------|
|                     | +/- | +/- | +/- |
| Postnatal           | 107 | 130 | 12 |
| E12.5               | 6   | 10  | 0   |
| E9.5                | 4   | 13  | 0   |
| E8.5                | 11  | 20  | 0   |
| E3.5                | 11  | 22  | 0   |
| E2.5                | 12  | 17  | 11  |

The failure of Emg1−/− embryos to form blastocysts could suggest a defect in the specification of early cell lineages in these mutant embryos. To test this, we analyzed the expression of several early cell lineage markers, including OCT4, NANOG and CDX2, in E3.5 Emg1−/− embryos. These markers have been shown to be widely expressed in the blastomeres of cleavage stage embryos, but become restricted to the different lineages, i.e. OCT4 and NANOG in ICM and CDX2 in trophectoderm, after initiation of blastocyst formation [14-17]. Using these lineage markers, we found that although E3.5 Emg1−/− embryos did not form the OCT-3/4 or OCT4 and NANOG in ICM and CDX2 in trophectoderm, after initiation of blastocyst formation [14-17]. Using these lineage markers, we found that although E3.5 Emg1−/− embryos did not form the OCT-3/4 or NANOG-positive ICM like that of Emg1+/− blastocysts, the blastomeres in the mutant embryos displayed levels of expression of these markers similar to wild type (Figure 5A and Additional file 1). However, the expression of CDX2 was consistently found to be significantly decreased in the mutant embryos as compared to the controls (Figure 5B). These results suggest that loss of EMG1 function more specifically down-regulates the expression of CDX2 in the blastomeres of the cleavage stage embryos, which might then influence the allocation or morphogenesis of cell lineages during early embryogenesis.
Ribosomal biogenesis in *Emg1*<sup>−/−</sup> mutants

EMG1 has been shown to be a highly conserved nucleolar protein required for ribosome biogenesis [5]. *Emg1*-null mutants exhibit arrested development prior to the blastocyst stage, similar to that observed in other mouse models that lack factors involved in ribosomal RNA synthesis or processing, including RBM19 (RNA-binding motif protein 19) [18], pescadillo-1 (PES-1) [19], fibrilлярin [20], RNA polymerase I or II [21], BYSL [22], SURF6 [23] and RPS19 (ribosomal protein S19) [24].
Figure 5 Characterization of the early embryonic lineages in Emg1<sup>−/−</sup> mutant embryos. (A) Immunofluorescence staining with anti-OCT-3/4 antibody, demonstrating that E3.5 Emg1<sup>−/−</sup> embryos do not form the OCT-3/4-positive inner cell mass (ICM) like the wild type blastocyst, but display a similar level of OCT-3/4 expression as wild type controls. (B) Immunofluorescence staining with anti-CDX2 antibody, showing that CDX2 is weakly expressed in the blastomeres of mutant embryos as compared to Emg1<sup>+/+</sup> blastocysts or morulae.
Some of these genetic mutations have been clearly demonstrated to cause severe defects in ribosomal biogenesis [19,21,22]. Thus, loss of EMG1 function in mice could also disrupt this biological pathway, leading to pre-implantation lethality.

To address this question, we first determined whether loss of EMG1 could affect nucleologenesis during pre-implantation development. As demonstrated previously, “de novo” nucleologenesis which begins at the two-cell stage is critical for the resumption of rRNA transcription during early embryogenesis [25-27]. This process involves the morphological transformation of the nucleolus precursor body (NPB) to a mature, tripartite nucleolus as seen in the blastocysts [27,28]. To determine the effect of the Emg1 gene-deletion on nucleologenesis, we performed IF staining for the nucleolar makers B23/nucleophosmin and fibrillarin in E3.5 embryos harvested from Emg1+/− intercrosses. As shown in Figure 6, in E3.5 Emg1+/− or Emg1−/− embryos, the nucleoli are smaller and more irregular than NPBs in E2.5 morula, indicating a striking maturation occurred at this developmental stage. In contrast, the nucleoli in E3.5 Emg1+/− embryos still showed the large ring shape, closely resembling the NPBs observed in E2.5 wild-type morula. Therefore, these data strongly suggest that deletion of the Emg1 gene arrests nucleologenesis during early embryonic development.

To determine if Emg1+/− embryos exhibit defective 40S ribosome biogenesis, similar to yeast depleted in yEMG1, we examined the level of mature 18S rRNA using reverse transcription (RT) followed by PCR. Although the levels of 18S rRNA are significantly reduced in yeast depleted in yEMG1, no detectable decrease in 18S rRNA were detected in Emg1+/− morulae at E2.5 as compared to wild-type embryos at the same developmental stage (Figure 6B). Since E2.5 mouse morulae could contain residual maternal rRNAs, we used the same approach to analyze 18S rRNA in E3.5 Emg1+/− embryos as compared to wild-type embryos with the same developmental stage. Again, no difference was observed (Figure 6C). Given that the levels of 18S rRNA in cells are very high, and small differences would not be detectable using this assay, we also looked for an increase in the precursors to the 18S rRNA, the pre-rRNA, but again no obvious difference between Emg1+/− and wild type or heterozygous embryos was detected (Figure 6C). The unchanged expression of 18S rRNA and 47S rRNA in Emg1+/− embryos was also indicated by RT followed by real-time PCR analysis (data not shown). Although this data differs from that in yeast, it is still possible that there is a delay in ribosomal RNA processing or assembly that was not detected using these assays. More sensitive approaches such as metabolic labeling or pre-rRNA specific probes may be required to show a delay in rRNA processing, as was recently shown in the study of a protein required for the maturation of the 60S ribosomal subunit in human cells [29]. However, due to the early pre-implantation lethality of the Emg1 null allele, we are unable to derive EMG1-deficient cells in which to perform these assays. Future experiments with a conditional knockout of EMG1 will greatly help to address the role of EMG1 in the regulation of ribosomal biogenesis during development.

**PS3 deficiency does not rescue the pre-implantation arrest of Emg1+/− mice**

Previous studies have found that mutations in many proteins involved in ribosome biogenesis lead to an up-regulation of p53 [30-32], a key regulator of the cell cycle and apoptosis. The importance of p53 in the regulation of ribosome biogenesis has been addressed by studies showing that inhibition of p53 can suppress the effects of some defects in ribosome biogenesis. In mice, p53 inhibition was found to suppress the effects of mutation of Tcof (Treacher Collins syndrome-causing gene) [33] and Rps24 (Diamond-Blackfan anemia-causing gene) [34]. These findings suggest that the inhibition of p53 may suppress the detrimental effects of mutations in other disorders of ribosomal biogenesis, such as EMG1 deficiency. To test this, E3.5 embryos were collected from intercrosses of Emg1+/p53 double heterozygotes (Emg1+/−/p53+/−) or Emg1+/−/p53−/−. A total of 5 Emg1+/−/p53−/− E3.5 embryos were identified, and all of them were found to be arrested at morula stage like the Emg1−/− null mutants (Additional file 2). In addition, none of E3.5 Emg1+/−/p53−/− embryos developed to blastocysts during in vitro culture. Taken together, these data demonstrate that p53 inactivation fails to rescue the pre-implantation arrest of the Emg1 null allele.

In summary, we have demonstrated that EMG1 is essential for mouse pre-implantation. We showed that loss of EMG1 function specifically arrests early embryonic development at the morula stage, preventing blastocyst formation. This phenotype is consistent with our expression data showing that Emg1 is highly expressed during this critical developmental stage. However, due to the high expression of Emg1 in mouse oocytes (Figure 2N), which could be maternally transmitted into early developing embryos like other nucleolar components [35] (Figure 1D), we could not exclude the possibility that EMG1 is also required before the morula stage. Future experiments with a conditional knockout of EMG1 specifically in mouse oocytes will allow us to answer this question. Nevertheless, our study highlights a critical role of EMG1 in mouse early embryonic development.
Figure 6 Characterization of nucleologenesis and ribosomal synthesis in $Emg^{+/+}$ embryos. (A) Immunofluorescence staining for the nucleolar markers B23/nucleophosmin and fibrillarin. In E3.5 $Emg^{1/-}$ embryos, the nucleoli are smaller and more irregular than the NPBs in E2.5 morulae. The nucleoli in E3.5 $Emg^{1/-}$ mutants, however, display a large sphere shape, which closely resemble NPBs as seen in E2.5 wild-type embryos. (B) RT-PCR on E2.5 morulae harvested from $Emg^{1/-}$ intercrosses. Arrows indicate the morulae that lack $Emg1$ mRNA but show a similar level of 18S rRNA to that of $Emg1$-expressing morulae. The genotypes for each sample are also presented. M: 50 bp or 100 bp DNA markers. (C) RT-PCR on E3.5 $Emg^{-/-}$ embryos, showing similar levels of 18S rRNA and pre-rRNA in both mutant and wild-type embryos.
The importance of EMG1 in development has also been demonstrated by our recent finding that this gene is mutated in human BCS syndrome, a severe developmental disorder with prenatal and postnatal growth retardation, profound psychomotor deficit, and death in early childhood [36]. Because of this mutation, the EMG1 protein was found to be significantly reduced in fibroblasts of BCS patients [12]. The residual protein that is detected is likely necessary to allow survival, as mice with a complete deficiency of EMG1 exhibit pre-implantation lethality. Therefore, the involvement of EMG1 in development could be dose-dependent. The hypomorphic mutation of EMG1 in BCS could specifically affect late embryonic development or certain cell lineages to cause BCS-associated phenotypes. In line with this, in this study, we found that Emg1 is predominately expressed in distinct cell types at late embryonic developmental stages or in adult (Figure 2). The unique expression of Emg1 in the granular neurons of cerebellum or in hippocampus could underly an important role for EMG1 in the control of psychomotor development, whose dysfunction is characteristic of BCS. Future experiments with mouse alleles to allow knockin of the BCS mutation, or a conditional allele, will allow us to address the pathological role(s) of EMG1 in vivo.

Conclusions
We have provided direct genetic evidence that EMG1 is essential for mouse pre-implantation. Given the requirement of yEMG1 in the biogenesis of the ribosomal 40S subunit, our study also highlights the critical role of ribosomal biogenesis in early development. The absolute requirement for EMG1 in mouse development is consistent with its essential role in yeast. Further, our findings also lend support to the previous study that showed Bowen-Conradi syndrome results from a partial EMG1 deficiency. A complete deficiency would not be expected to be compatible with a live birth.

Methods
Construction of the Emg1 gene-targeting vector
The Emg1 gene-targeting vector was made based on a PCR-based cloning strategy as described previously [37]. Briefly, the mouse Emg1 genomic fragments required for the 5′ and 3′ arms of homology were PCR-amplified from the genomic DNA of R1 ES cells (on 129S1 background) with a high-fidelity polymerase (Clontech). After validation by DNA sequencing, the PCR products were cloned into two individual vectors that contain the SA-IRESpgeo cassette, and a pGKDTA fragment (the negative selection cassette), respectively. Subsequently, using the restriction enzymes and the cloning strategy as described [37], the DNA fragments were isolated, and assembled together to generate the gene-targeting vector.

Generation of Emg1 deficient mice
The Emg1 gene-targeting construct was linearized and electroporated into R1 ES cells, and then selected with G418 (250 μg/ml). The G418 resistant ES clones were screened by Southern blot analysis for the correctly targeted allele using EcoR1 (for the 5′ external probe) and BamH1 (for the 3′ external probe) digestion. Two independently targeted ES cell clones were used to generate chimeric mice that subsequently transmitted the genetic alternation through the germ line. The phenotypes of Emg1−/− mutants derived from both targeted ES cell lines were indistinguishable. Mice were maintained on either 129S1 or on a mixed 129S1 and CD1/ICR background, in which Emg1−/− developed the same phenotype. All mouse experiments were performed in accordance with procedures approved by the University of Manitoba Animal Care and Use Committee.

Genotyping
PCR and Southern blot analysis were applied for genotyping the Emg1 heterozygous mice. PCR was performed on ear-punched DNA. Primers to amplify the targeted allele were the sense primer (P1), located in intron 2 (5′-GTTCCTCAGCATATAGTGCT-3′) and antisense primer (P2) specific for the SA-IRESpgeoA cassette (5′-GGGACAGGATAAGTATGACATCA-3′). To detect the wild-type Emg1 allele, P1 primer and an antisense primer (P3) locating in exon 2 (5′-TGTAACCTGTAGCAAGCCAGC-3′) were used for PCR. Southern blot analysis was undertaken using standard protocols.

To genotype pre-implantation mouse embryos, a nested PCR method was applied. DNA was prepared by incubating E2.5-E3.5 embryos with 10 μl of Proteinase K buffer (10 mM Tris pH 8.3, 50 mM KCl, 2 mM MgCl2, 0.01% gelatin, 0.45% Nonidet P40, 0.45% Tween 20 and 500 μg/ml proteinase K) for 1 h at 55°C followed by incubation at 95°C for 10 min. 5 μl of DNA sample was then directly used for PCR using P1 and P2 (for the Emg1 targeted allele) and P1 and P3 (for the wild-type allele). 2 μl of products from the first round PCR were further PCR-amplified with the internal primers, generating 264 bp (wild-type allele) and 353 bp (targeted allele) products, respectively.

A similar approach was also applied to genotype the p53 null allele in Emg1/p53 double mutant pre-implantation embryos. The first round PCR was done using the primers as described previously [38]. In the second round PCR, the following internal primers were utilized: 5′-TACCTCAC-TACAGGTAGCT-3′ (sense) and 5′-TCTTAGAGA-CAGTTGACTCC-3′ (antisense) (for detecting the p53 wild-type allele), and 5′- TACCTCACTACAGTGACCTG-3′ (sense) and 5′-GTGATATTGCTGAA-GAGCCTTG-3′ (antisense) (for detecting the p53 null allele).
**Early embryo isolation and in vitro culture**

Emg1−/− or Emg1+/−/p53−/− mice were intercrossed, and the females were examined for the presence of a vaginal plug which was set as embryonic day (0.5). Embryos at different stages of development (E2.5 through E3.5) were collected by either dissecting ampullae or flushing oviducts with M2 medium (Millipore). For E8.5-E9.5 oviducts with M2 medium (Millipore), and incubated at 37°C for 24 h. Embryos at different stages (E2.5 through E3.5) were placed in KSOM-1/2AA medium (Millipore), and incubated at 37°C for 24 h.

**Immuno-staining of pre-implantation embryos**

Immuno-staining of pre-implantation embryos was performed based on the protocols provided by Dr. Janet Rossant http://www.sickkids.ca/research/rossant/custom/protocols.asp. The following antibodies were used: monoclonal anti-CDX2 (1:200, CDX2-88, BioGenex, CA, USA), monoclonal mouse anti-OCT3/4 (1:50, C10; Santa Cruz Biotechnology), rabbit anti-NANOG (1:200, Cosmo Bio), Rat anti-E-cadherin (1:100, Sigma), monoclonal mouse anti-B23 (1:50, Invitrogen) and rabbit anti-fibrillarin (1:100, Abcam). Secondary antibodies included Texas Red or Alexa488-conjugated goat anti-mouse, goat antirat and goat anti-rabbit (Molecular Probes). To visualize nuclei, embryos were stained with DAPI (0.5 μg/ml) for 3 min at room temperature. Immuno-stained embryos were mounted onto microscopic slides with ProLong Gold (Invitrogen) and covered with glass cover slips. Images were collected using a Zeiss Axioplan 2 microscope to generate Z-stacks which were deconvolved using iterative algorithms program in Axio Vision 4.6.

For the BrdU labeling assay, E2.5-E3.5 embryos were incubated with KSOM-1/2AA medium containing 10 μM BrdU (Sigma) for 3 h, and were then fixed in 4% paraformaldehyde (PFA) (for 10 min, permeabilized in 0.25% Triton X-100 for 10 min, treated with 2N HCl for 10 min, and detected with anti-BrdU antibody (Sigma). TUNEL staining

E2.5-E3.5 mouse embryos were fixed in 4% PFA in PBS for 1 h at room temperature, and permeabilized for 1 h in PBS-0.5% Triton X-100. The embryos were then washed three times in PBS-0.1% Triton X-100 and incubated at 37°C for 1 h in a staining solution containing biotin-dUTP, terminal deoxynucleotide transferase (TdT), and detected using an ABC staining kit (Vector).

**Whole mount x-gal staining**

Pre-implantation embryos were fixed for 2 min with 1% PFA, 0.2% glutaraldehyde and 0.02% NP40 in PBS. After fixation, embryos were washed three times with PBS containing 0.02% NP40, and stained at 37°C overnight with a staining solution (4 mM K4Fe(CN)6, 4 mM K3Fe(CN)6, 2 mM MgCl2, and 0.2% X-gal in PBS). For E8.5-E9.5 embryos, embryos were fixed for 30 min with 4% PFA. After extensive washing with PBS containing 0.02% NP40 (three times, 20 min each time), embryos were stained as described above.

**RNA in situ hybridization**

RNA in situ analysis of whole mount mouse embryos and frozen sections of mouse tissues were performed according to established protocols [39] with antisense and sense digoxigenin-labeled riboprobes which were in vitro transcribed from the full-length mouse Emg1 coding sequence. Mouse embryos were collected from pregnant outbred ICR female mice at E9.5-E15.5 days of gestation, and the adult tissues were harvested from two-month old ICR mice. All the samples were fixed in DEPC-treated 4% PFA at 4°C overnight.

RNA in situ hybridization on early mouse embryos (E2.5-E3.5) was performed essentially based on a described protocol [40]. To preserve the pre-implantation embryos, the whole procedure was carried out in a transwell-insert (Corning).

**Northern Blot Analysis**

Total RNA from flash-frozen mouse tissues was extracted using TRIzol (Life Technologies, Inc.). 20 μg of total RNA was separated on a 1% agarose-formaldehyde gel and transferred to Hybond nylon membrane (Amersham). Hybridization was carried out in PerfectHyb (Sigma) with 1.5×10⁶ cpm/ml probe which covers the whole coding sequence of Emg1.

**RT-PCR analysis of pre-implantation embryos**

Each blastocyst or morula was lysed with 5 μl ice-cold Cell Lysis II Buffer (Ambion) for 10 min at 75°C. 2 μl of lysisate was used for PCR based genotyping, the rest was digested with DNase1 (0.08 unit/μl, Ambion) at 37°C for 15 min. After inactivation at 75°C for 5 min, 2 μl of DNase1-treated embryonic lysate was used in an RT reaction in a 10 μl volume using the OneStep RT-PCR kit (Qiagen) according to the manufacturer’s instructions. The final concentration of specific primers (see the reverse primers described below) was 0.6 μM each. 2 μl RT mixture were then used for PCR reactions with the Multiplex PCR kit (Qiagen) and the primers described below. The PCR cycles contain an initial denaturation step of 95°C for 15 min and 40 cycles of 94°C for 30 s, 60°C for 90 s, and 72°C for 60 s, and a final 10 min extension step at 72°C. The PCR products were separated on a 12% polyacrylamide gel in 1× Tris-borate-EDTA buffer. The bands on the gel were visualized by the silver staining method as described [41].

The following primers were used for the above RT-PCR analysis: Mouse Emg1: forward (5'-TGAAGTGAACCCC-CAGACTC-3') and reverse (5'-GAAGTGTGGAGACAT-TGGAT-3'). The amplified DNA band is 148 bp. Mouse
pre-rRNA: forward (5'-CTCTTGCTGTGTGTTTGGTA-3') and reverse (5'-TGATAAGGGCCACACAGA-3') in the 5' external transcribed spacer (5'-ETS) region of mouse 47S pre-rRNA [42]. The size of the PCR product is 105 bp. Mouse 18S rRNA: forward (5'-GCAATTATTTCCCATGAACG-3') and reverse (5'-GGCCCTACTAAACCATTCCAA-3'), which gives rise to a DNA band with 123 bp.

Additional material

### Additional file 1: Expression of NANOG in Emg1<sup>+/−</sup> mutant embryos
E3.5 embryos from Emg1<sup>+/−</sup> intercross were co-immunostained with anti-NANOG (red) and anti-B-catenin (green) antibodies. In E3.5 Emg1<sup>+/−</sup> blastocysts, nuclear-localized NANOG is mainly found in the ICM. NANOG is also detected in the blastomeres of E2.5 Emg1<sup>+/−</sup> morulae. At E3.5, Emg1<sup>+/−</sup> embryos arrest at the morula stage, in which the blastomeres express similar levels of NANOG as that in E2.5 Emg1<sup>+/−</sup> morulae.

### Additional file 2: ps3 inactivation fails to rescue the pre-implantation arrest of the Emg1 null allele
E3.5 embryos were collected from intercross of Emg1<sup>−/−</sup>/ps3<sup>−/−</sup> (male) and cross of Emg1<sup>−/−</sup>/ps3<sup>−/−</sup> (female). In both, Emg1<sup>−/−</sup>/ps3<sup>−/−</sup> embryos show the same morula arrest as Emg1<sup>−/−</sup>/ps3<sup>+/+</sup> or Emg1<sup>−/−</sup> embryos. Scale bar, 100 μm.

### Abbreviations

EMG1: Essential for mitotic growth 1; BCS: Bowen-Conradi syndrome; rRNAs: ribosomal RNAs; SAM: S-adenosyl methionine; ICM: inner cell mass; PES-1: pescadillo-1; Rbm19: RNA-binding motif protein 19; Rps19: ribosomal protein S19; IF: immunofluorescence; PFA: paraformaldehyde; TdT: Terminal deoxynucleotidyl transferase; RT: reverse transcription.

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### Authors’ contributions

X.W., B.T. and H.D. conceived and designed the experiments. X.W. and S.S. performed in situ and Northern hybridization. X.W., S.S. and H.D. generated the gene-targeting vector and produced the knockout mice. X.W. and N.P. performed mouse breeding and genotyping, and X.W. did all the analysis of the pre-implantation mouse embryos. B.T. and H.D. wrote the manuscript with subsequent contributions from all authors. All authors read and approved the final manuscript.

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