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The Dual Roles Of Geminin During Trophoblast Proliferation And Differentiation

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Abstract

Geminin is a protein involved in both DNA replication and cell fate acquisition. Although it is essential for mammalian preimplantation development, its role remains unclear. In one study, ablation of the geminin gene (*Gmnn*) in mouse preimplantation embryos resulted in apoptosis, suggesting that geminin prevents DNA re-replication, whereas in another study it resulted in differentiation of blastomeres into trophoblast giant cells (TGCs), suggesting that geminin regulates trophoblast specification and differentiation. Other studies concluded that trophoblast differentiation into TGCs is regulated by fibroblast growth factor-4 (FGF4), and that geminin is required to maintain endocycles. Here we show that ablation of *Gmnn* in trophoblast stem cells (TSCs) proliferating in the presence of FGF4 closely mimics the events triggered by FGF4 deprivation: arrest of cell proliferation, formation of giant cells, excessive DNA replication in the absence of DNA damage and apoptosis, and changes in gene expression that include loss of Chk1 with up-regulation of p57 and p21. Moreover, FGF4 deprivation of TSCs reduces geminin to a basal level that is required for maintaining endocycles in TGCs. Thus, geminin acts both like a component of the FGF4 signal transduction pathway that governs trophoblast proliferation and differentiation, and geminin is required to maintain endocycles.

Keywords

geminin; trophoblast stem cells; trophoblast giant cells; differentiation; trophectoderm; endoreplication; endocycles; Chk1; Cdkn1a/p21/Cip1; Cdkn1c/p57/Kip2

Introduction

Despite the numerous targeted mutations generated in mice, only 25 or so zygotically expressed genes have been shown to be essential for the development of a fertilized egg into a blastocyst (Kohn et al., 2011). One of these is geminin, a small protein originally identified in *Xenopus* embryos as both an inhibitor of DNA replication (McGarry and Kirschner, 1998)

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and an inducer of neural plate expansion (Kroll et al., 1998). Geminin plays different roles during the development of multicellular animals. Geminin prevents premature loading of the replicative MCM helicase onto replication origins [(Kisielewska and Blow, 2012; Klotz-Noack et al., 2012) and references therein], and geminin influences cell fate acquisition by associating with transcription factors or chromatin modifying proteins [(Caronna et al., 2013; Lim et al., 2011) and references therein]. Thus, it is not surprising that attempts to identify the role of geminin in preimplantation mammalian development have proven enigmatic.

Preimplantation development begins with fertilization and ends with implantation of the resulting blastocyst. A blastocyst consists of a monolayer of epithelial trophoblast cells (trophectoderm) that envelop the remaining pluripotent blastomeres [inner cell mass (ICM)] within a cavity (blastocoel). Although ablation of the geminin gene (*Gmnn*) in mice arrested development as early as the 4-cell to 8-cell stage in two independent studies, the phenotypes differed significantly. In one study (Hara et al., 2006), *Gmnn-/-* embryos appeared to lack a trophectoderm (although trophoblast biomarkers were not analyzed), and the blastomeres embryos underwent DNA replication in the absence of mitosis, sustained DNA damage, and entered apoptosis. This study was consistent with the established role of geminin in preventing ‘DNA re-replication’, an aberrant event in which cells undergoing mitotic cell divisions initiate a second S-phase before the first S-phase is completed. DNA re-replication results in DNA damage, induction of the DNA damage response, and apoptosis (Ding and MacAlpine, 2010; Kerns et al., 2012; Klotz-Noack et al., 2012; Yanagi et al., 2005; Zhu and Depamphilis, 2009).

In a separate study (Gonzalez et al., 2006), all of the blastomeres in *Gmnn-/-* embryos appeared to differentiate into the trophoblast giant cells (TGCs) that are essential for implantation of the blastocyst and subsequent placentation. These embryos contained a blastocoel cavity but lacked an ICM, expressed genes characteristic of TGCs, and over-replicated their nuclear DNA. Implied, but not proven, was that *Gmnn* ablation induced endoreplication, a hallmark of TGCs. Endoreplication (also termed endoreduplication) refers to the occurrence of a second S-phase in the absence of an intervening mitosis and cytokinesis [reviewed in (Zielke et al., 2013)]. Multiple S-phases in the absence of mitosis and cytokinesis are referred to as ‘endocycles’. Cells that are developmentally programmed for endoreplication, such as TGCs, do not undergo apoptosis, but form viable, nonproliferating, mononuclear, polyploid cells. These results suggested that down-regulation of *Gmnn* expression induces differentiation of pluripotent blastomeres into trophectoderm and eventually into TGCs. Subsequent studies in which *Gmnn* expression was suppressed in P19 embryonal carcinoma cells and embryonic stem cells suggest that high levels of geminin sustain the expression of genes that prevent differentiation of pluripotent cells into trophoblasts (Yang et al., 2011; Yang et al., 2012).

This conclusion, however, is difficult to reconcile with the fact that differentiation of trophoblasts into TGCs first occurs in peri-implantation blastocysts in response to the absence of fibroblast growth factor-4 [FGF4, (Arman et al., 1999; Keramari et al., 2010; Murohashi et al., 2010; Nichols et al., 1998; Xu et al., 1998)] that is produced by the ICM (Roberts and Fisher, 2011). Moreover, trophoblast stem cells (TSCs) differentiate into TGCs

when deprived of FGF4 (Simmons and Cross, 2005; Tanaka et al., 1998), and these TGCs continue to express geminin (Ullah et al., 2008), presumably in order to maintain endocycles in mammals as it does in *Drosophila* (Zielke et al., 2008). If geminin was essential for blastomere viability rather than differentiation, then TGCs in *Gmnn-/-* embryos could have resulted simply from the absence of FGF4 due to the absence of an ICM. Alternatively, if geminin were critical in trophoblast differentiation during preimplantation development, then geminin would be a component of the FGF4 signal transduction pathway that governs TSC proliferation and differentiation.

TSCs are derived from the trophectoderm of the blastocyst and give rise exclusively to all of the trophoblast lineages in the placenta (Oda et al., 2010; Rielland et al., 2009; Tanaka et al., 1998). TSCs proliferate as tightly packed colonies when cultured in medium conditioned by primary embryonic fibroblasts and supplemented with FGF4 (Tanaka et al., 1998). When cultured in the absence of FGF4 and conditioned medium (termed FGF4 deprivation), TSCs differentiate into TGCs. FGF4 deprivation of TSCs *in vitro* mimics the induction of trophoblast differentiation *in vivo* [reviewed in (Hu and Cross, 2010)].

To determine whether or not geminin is essential for either TSC proliferation or differentiation, TSCs were isolated from blastocysts homozygous for a conditional *Gmnn* knockout and containing a tamoxifen sensitive *Cre* recombinase gene. These cells behaved like wild-type TSCs when cultured either in the presence or absence of FGF4 and conditioned medium. However, when the *Gmnn* gene was ablated in proliferating TSCs, and the results compared with TSCs undergoing differentiation in response to FGF4 deprivation, two distinct requirements for geminin were evident. A basal level of geminin was required to maintain endocycles in TGCs, and a high level of geminin was required to prevent TSCs from exiting their mitotic cell cycle and differentiating into TGCs. Moreover, the high level of geminin prevented characteristic changes in gene expression that occur when TSCs are deprived of mitogens (Ullah et al., 2011; Ullah et al., 2008). These results confirm in part the work of Gonzalez et al. (2006) by demonstrating that once trophoblast specification has occurred during preimplantation development, geminin is essential to prevent trophoblast differentiation. In addition, they reveal a dual role for geminin in trophoblast biology; first as a component of the FGF4 signal transduction pathway that governs trophoblast proliferation and differentiation, and second as a regulator of endoreplication.

Materials and Methods

Mice and Cells

[*Gmnn*$^{tm1a(KOMP)Wtsi}$] mice with a LacZ reporter tagged *Gmnn* allele were obtained from the Wellcome Trust Sanger Institute, Cambridge, UK (Skarnes et al., 2011). They were mated with mice expressing the Flp recombinase (Jackson Laboratories; “FLPeR mouse”, Stock#003946) to produce mice containing a *Gmnn* floxed allele [*Gmnn*$^{tm1c(KOMP)Wtsi}$] (Fig. 1A). These mice were then mated with B6.Cg-Tg(CAG-cre/Esr1*)5Amc/J (Stock#004682, Jackson Laboratories) to obtain mice homozygous for the *Gmnn* floxed allele and heterozygous for the Cre recombinase gene (*Gmnn*$^{flox/flox}$*; ErCre/+*). Primary cells isolated from *Gmnn*$^{flox/flox}$*; ErCre/+* mice allows conditional Gmnn ablation by addition of monohydroxytamoxifen (MHT) as described (Kaneko and DePamphilis, 2013).

Due to an out-of-frame stop codon in exon 5, the ablated *Gmnn* alleles produced mRNA that could encode a truncated geminin protein that cannot bind the geminin-associated proteins CDT1, BRG1 or HOX. The *ErCre* allele was confirmed by PCR genotyping using primers recommended by the Jackson Laboratories. Blastocysts were isolated at E3.5 following mating of $Gmnn^{flox/flox}$ mice with $Gmnn^{flox/flox}$; *ErCre/+* mice.

TSCs were isolated from blastocysts and maintained on feeder layers of mitomycin C treated primary mouse embryo fibroblasts (Millipore) to minimize spontaneous differentiation, as described (Himeno et al., 2008; Kohn and DePamphilis, 2013; Ullah et al., 2013). TSC culture medium consisted of conditioned media supplemented with FGF4 and heparin. Experiments were carried out in the absence of a feeder layer using TSCs that had been weaned off feeder layers. TSC differentiation was induced by "FGF4 deprivation" [eliminating conditioned medium, FGF4 and heparin from the culture medium (Ullah et al., 2011; Ullah et al., 2008)].

Reagents

Monohydroxytamoxifen (MHT)/4-Hydroxytamoxifen (Sigma-Aldrich) was prepared as a 10mM solution in absolute ethanol and added to cultured cells at a final concentration of 3μM. Reagents included FGF4 (Sigma-Aldrich), DMEM and RPM1640 (Cellgro or Millipore), ES-qualified fetal bovine serum (Invitrogen) and MG132 (C2211; Sigma). Antibodies included Chk1 (SantaCruz Biotechnologies, sc-8408), Geminin (sc-13015), p21 (sc-397 and sc-6246), p27 (sc-776), p57 (GeneTex, GTX62720), H2A.X (Cell Signaling #2595), phospho-H2A.X (ser139) (Cell Signaling #9718), cleaved caspase-3 (Asp175) (Cell Signaling #9661), cleaved PARP (Cell Signaling #9544), cytokeratin-8 endo-A (Developmental Studies Hybridoma Bank, TROMA-1) and Tubulin (DSHB, Iowa E7). 5-Ethynyl-2′-deoxyuridine (EdU) and its fluorescent azide detection system (Click-iT chemistry) were used as recommended by Invitrogen. The TUNEL assay (Roche, catalog no. 11684795910) was performed as recommended by Roche, except that after fixation, cells were permeabilized for 10 min with phosphate buffered saline containing 0.1% Triton X-100 and 0.02% SDS.

Analytical Methods

Flow cytometry was performed as previously described (Ullah et al., 2008). The DNA content of cells was analyzed by a FACSCalibur flow cytometer (Becton Dickinson) with CellQuestPro (BD Bioscience), and the data were analyzed using FlowJo software (Treestar, Ashland, OR).

Immunofluorescence was performed as described (Ullah et al., 2011), except that nuclei were stained with Hoechst 33342 (Invitrogen) and slides were mounted in Prolong Gold Anti-fade reagent (Invitrogen). Images were acquired on a Leica TCS5 SPII confocal microscope with a 63× objective, using Leica Application Suite Advanced Fluorescence (LAS AF) software. Images were analyzed with ImageJ software. Western immuno-blotting was performed on either total cell lysates (Ullah et al., 2011) or whole cell extracts (2×Laemmli buffer) with similar results.

Polymerase chain reaction (PCR) and Reverse transcription-polymerase chain reaction (RT-PCR) were performed as described (Kaneko et al., 2007; Kaneko et al., 2004) using the PCR primers in Table 1. PCRs used Platinum Taq polymerase (Invitrogen). RT-PCRs were carried out on 500ng of total RNA isolated using RNeasy kit (Qiagen) according to the manufacturer's protocol.

Expression Vector

Mouse *Gmnn* cDNA was cloned into pcDNA-TO/*myc*-HisA (Invitrogen) at BamH1 and Xho1 restrictions sites to produce untagged recombinant Gmnn protein (stop codon included). Constructs were confirmed by sequencing. TGC were transfected with pcDNA-TO-*Gmnn* using Lipofectamine 2000 (Invitrogen).

shRNA Suppression Of *Gmnn*

shRNA oligonucleotides were designed against two different *Gmnn* gene sequences (AAGCCTTTGATCTTATAAGTA and AATTTGATTCTGAAGAAGAAG) and cloned into pLKO.1-TRC [Addgene plasmid 10878, (Moffat et al., 2006)]. Lentivirus particles were produced into Hek-293T cells and used for transduction of TSC, as previously described (Ullah et al., 2011). Both shRNAs targeting *Gmnn* were packaged into a single lentivirus preparation.

Results

Derivation Of Trophoblasts With A Conditional *Gmnn* Knockout

In order to identify the role of geminin in trophoblast proliferation and differentiation, two TSC lines were derived from blastocysts produced by mating $Gmnn^{flox/flox}$; *CAG-cre/*$Esr1^{Tg/+}$ with $Gmnn^{flox/flox}$; *CAG-cre/*$Esr1^{+/+}$mice. $Gmnn^{flox/flox}$; *CAG-cre/*$Esr1^{Tg/+}$ ($Gmnn^{flox/flox}$*; ErCre/*+) TSCs contained two *Gmnn* alleles in which exon 4 was flanked by loxP sites ($Gmnn^{flox/flox}$, Fig. 1A) and one Cre recombinase gene fused to the mutated steroid binding domain of the estrogen receptor (*ErCre/*+). $Gmnn^{flox/flox}$; *CAG-cre/*$Esr1^{+/+}$($Gmnn^{flox/flox}$) TSCs obtained from another blastocyst in the same litter were used as controls. When $Gmnn^{flox/flox}$*; ErCre/*+ TSCs proliferating in the presence of FGF4 were treated with monohydroxytamoxifen (MHT), exon 4 was ablated from the *Gmnn* gene (Fig. 1B), and wild-type *Gmnn* RNA disappeared (Fig. 1C). The truncated *Gmnn* RNA detected in these cells was at least 10-fold less than full length *Gmnn* RNA in control cells (Fig. 1C). Based on its amino acid composition, mouse geminin has a molecular weight of 23.3kDa, but it migrated during gel electrophoresis with an apparent molecular weight ~33 kDa (Fig. 1E). Its identity was confirmed by its disappearance in $Gmnn^{flox/flox}$*; ErCre/*+ TSCs following treatment with MHT, and by the disappearance of the same protein from human cancer cells in response to anti-*Gmnn* siRNA. Furthermore, MHT-mediated depletion of geminin protein was evident as early as 12 hours after addition of MHT, was complete by 24 hours, and remained so for at least five days (Fig. 1D). None of these changes were detected in $Gmnn^{flox/flox}$ TSCs. Thus, MHT selectively and effectively eliminated geminin expression in $Gmnn^{flox/flox}$*; ErCre/*+ TSCs.

Gmnn Ablation Induces Morphological Changes Associated With TSC Differentiation

Differentiation of TSCs into TGCs is characterized by arrest of cell proliferation, by formation of giant cells with a single enlarged nucleus, by changes in the pattern of gene expression, and by induction of endoreplication. FGF4 deprivation quickly arrested proliferation of both $Gmnn^{flox/flox}$*; ErCre/+* and $Gmnn^{flox/flox}$ TSCs (Fig. 2A) and induced their differentiation into giant cells that contained a single enlarged nucleus (Fig. 2B-E, -FGF4). Moreover, TSC colonies contained ~3% mitotic nuclei whereas none were observed in TGC colonies (data not shown).

Gmnn ablation in TSCs mimicked the effects of FGF4 deprivation. Addition of MHT arrested proliferation of $Gmnn^{flox/flox}$*; ErCre/+* TSCs (Fig. 2A). Within 24 to 48 hours of addition of MHT to $Gmnn^{flox/flox}$*; ErCre/+* TSCs, they differentiated into giant cells with a greatly enlarged nucleus (Fig. 2D,E; +FGF4+MHT). This phenomenon was observed with TSCs proliferating on a layer of primary mouse embryonic fibroblasts (data not shown) as well as TSCs cultured in TSC medium after being weaned off feeder layers (Fig. 2). In contrast, addition of MHT to $Gmnn^{flox/flox}$ TSCs neither arrested their proliferation (Fig. 2A) nor produced giant cells (Fig. 2B,C; +FGF4+MHT). Similar results were obtained using shRNAs to suppress *Gmnn* expression in wild-type TSCs. These results revealed that geminin is essential for preventing TSCs from exiting their mitotic cell cycle and differentiating into TGCs when cultured in presence of FGF4.

Gmnn Ablation Induces RNA Changes Associated With TSC Differentiation

Changes in the RNA levels of several genes whose expression patterns were characteristic of TSC differentiation (Hu and Cross, 2010; Simmons et al., 2007) were induced either by FGF4 deprivation or by *Gmnn* ablation. The RNA levels of *Chk1*, *Cdx2* and *ErrB*, each of which is expressed in TSCs but not TGCs, were reduced upon either FGF4 deprivation or *Gmnn* ablation (Fig. 3). Conversely, either FGF4 deprivation or *Gmnn* ablation increased the RNA levels of *Pl1* and *Pl2*, genes that are expressed in TGCs but not in TSCs. Unexpectedly, *Gmnn* ablation down-regulated expression of *Mash2/Ascl2*, a gene encoding a transcription factor required for differentiation of trophectoderm into spongiotrophoblasts (Tanaka et al., 1997), and did not up-regulate expression of *Tpbpa*, a gene characteristic of spongiotrophoblasts (Simmons et al., 2007). FGF4 deprivation had the opposite effects on these two genes. Subsequent studies suggested that this was an artifact of abnormal endocycles (see Discussion). Despite this finding, the changes observed in RNA (Fig. 3) and protein levels (discussed below) are consistent with a role for geminin in the FGF4-dependent signal transduction pathway that governs trophoblast differentiation.

Two members of the Cip/Kip family of CDK-specific inhibitors, Cdkn1C/p57/Kip2 (p57) and Cdkn1a/p21/Cip1 (p21), are genes whose expression is dramatically up-regulated during TSC differentiation as a result of post-translational modifications (Ullah et al., 2011; Ullah et al., 2008). Accordingly, their RNA levels should remain unchanged, regardless of whether TSC differentiation was induced by FGF4 deprivation or by *Gmnn* ablation. To test this hypothesis, $Gmnn^{flox/flox}$*; ErCre/+* TSCs and $Gmnn^{flox/flox}$ TSCs in the presence of FGF4 were treated with MHT. As expected, *Gmnn* RNA was eliminated in the ErCre+ cells, but not in the ErCre- cells (Fig. 3). RNA levels for both p57 and p21 remained unchanged under

both conditions, as well as during FGF4 deprivation of these cells (Fig. 3). In addition, the level of *Gmnn* RNA remained unchanged during FGF4 deprivation of these TSC lines, suggesting that geminin protein, like p57 and p21, is also regulated post-translationally during TSC proliferation and differentiation (see below).

Gmnn Ablation Induces Protein Changes Associated With TSC Differentiation

Previous studies have shown that Chk1 prevents expression of p57 and p21 proteins in TSCs by phosphorylating them at specific sites, which results in their ubiquitin-dependent degradation by the 26S proteasome (Ullah et al., 2011). When Chk1 protein is suppressed by FGF4 deprivation, then p57 and p21 proteins appear concomitant with inhibition of Cdk1 activity and differentiation of TSCs into TGCs (Ullah et al., 2008). In contrast, the protein level of the third member of the Cip/Kip family, Cdkn1B/p27/Kip1 (p27), remains unchanged during TSC differentiation. Therefore, if geminin is part of the FGF4-dependent signal transduction pathway previously described for TSCs, *Gmnn* ablation should result in down-regulation of Chk1 with concomitant up-regulation of p57 and p21 without a change in the level of p27. This hypothesis was confirmed by the fact that treatment of $Gmnn^{flox/flox}$*; ErCre/+* TSCs with MHT in the presence of FGF4 suppressed expression of Chk1 protein, while inducing expression of p57 and p21, but not p27 (Fig. 4A, +FGF4, MHT). In contrast, treatment of $Gmnn^{flox/flox}$ TSCs with MHT in the presence of FGF4 had no effect. Neither did treatment with the ethanol vehicle. Similar results were obtained using shRNAs to suppress Gmnn in wild-type TSCs (data not shown). These results suggested that geminin prevents the down-regulation of Chk1 protein in TSCs.

FGF4 Deprivation Of TSCs Down-regulates *Gmnn* Expression

If geminin is required to prevent TSC differentiation, then FGF4 deprivation of $Gmnn^{flox/flox}$ TSCs should result in down-regulation of geminin with concomitant down-regulation of Chk1 and up-regulation of p57 and p21 proteins. Again, the level of p27 should remain the same. This hypothesis was confirmed for $Gmnn^{flox/flox}$*; ErCre/+* TSCs (Fig. 4A,B; -FGF4), $Gmnn^{flox/flox}$ TSCs (Fig. 4A), and the wild-type TSC line described by Ullah et al. (2008) (Fig. 4C). The results revealed that FGF4 deprivation induced down-regulation of geminin protein, and that this transition was accompanied by down-regulation of Chk1 protein with concomitant up-regulation of p57.

The average decrease in geminin protein during FGF4 deprivation was about 3-fold (Fig. 4D), whereas treatment of $Gmnn^{flox/flox}$*; ErCre/+* TSCs with MHT in the presence of FGF4 essentially eliminated geminin within the first 24 hours (Figs. 1D,E). The same treatment of $Gmnn^{flox/flox}$ TSCs had no effect on geminin levels, confirming that the action of MHT depended on the activation of *ER-Cre*. Finally, cytokeratin-8 endo-A (antigen of Troma-1 antibody) is expressed in the cytoplasm of all trophoblasts, but the amount of this protein in the 3% to 5% of TGCs that arise spontaneously in populations of TSCs was visibly greater in the TGCs than in the neighboring TSCs (Fig. 2E, TS cell panel). Comparisons of the levels and subcellular locations of cytokeratin-8 in TGCs produced either by FGF4 deprivation or by *Gmnn* ablation were indistinguishable (Fig. 2E, TG cell panels). These results suggested that high levels of geminin in TSCs prevent the loss of Chk1 protein that

previous studies showed results in post-translational expression of p57 and inhibition of CDK activity (Ullah et al., 2011; Ullah et al., 2008).

Gmnn Expression In TGCs Does Not Restore Chk1 Expression

Given that the level of Chk1 protein in TSCs was suppressed in response to *Gmnn* ablation, and that both geminin and Chk1 proteins were down-regulated in response to FGF4 deprivation (Fig. 4), geminin expression in TSCs is somehow linked to Chk1 expression. However, although geminin expression was partially restored in $Gmnn^{flox/flox}$;*ErCre/+* TGCs that had been deprived of FGF4 for up to 10 days (Figs. 5A; 7F), this event was not accompanied by renewed expression of Chk1 (Fig. 5A). To determine whether or not high levels of geminin comparable to those in TSCs would restore Chk1 expression, recombinant geminin was ectopically expressed in TGCs (Fig. 5B). Again the level of endogenous Chk1 expression remained unaffected by increased levels of geminin. Previous studies have shown that p57 expression in both TSCs and TGCs is regulated through ubiquitin-dependent degradation (Ullah et al., 2011). To determine whether or not the same is true for Chk1 expression, TGCs were treated with MG132, a specific inhibitor of the 26S proteasome. The level of Chk1 protein was not restored, regardless of the presence or absence of a *Gmnn* gene or geminin protein (Fig. 5C). Thus, Chk1 levels in TGCs could not be restored either by increasing geminin expression or by blocking ubiquitin-dependent degradation of proteins.

Geminin Is Required To Maintain Endocycles

FGF4 deprivation of wild-type TSCs induces endoreplication which results in mononuclear cells containing as many as 16 to 32 times the diploid amount of nuclear DNA, as defined by fluorescence activated cell sorting (FACS) [Figs. 6 and 7A, -FGF4]. Each round of endoreplication produces an integral multiple of 4N DNA. The number of these ‘endocycles’ varies with the cell line and the length of time that cells are subjected to FGF4 deprivation (MacAuley et al., 1998; Parisi et al., 2003; Ullah et al., 2011; Ullah et al., 2008). FGF4 deprivation of either $Gmnn^{flox/flox}$; *ER-Cre/+* or $Gmnn^{flox/flox}$ TSCs resulted in as many as four endocycles in 10 days to produce nuclei with up to 64N DNA content. When $Gmnn^{flox/flox}$ TSCs were treated with MHT (Fig. 6A +FGF4+MHT), they proliferated as normal TSCs with a FACS profile indistinguishable from cells treated with the ethanol vehicle (Fig. 6A +FGF4). In contrast, addition of MHT to $Gmnn^{flox/flox}$; *ER-Cre/+* TSCs proliferating in the presence of FGF4 induced giant cells with enlarged nuclei that contained the same amounts of excess DNA as TGCs produced via FGF4 deprivation; however, distinct endocycles were not detected (Fig. 6B, +FGF4+MHT & -FGF4). Similar results were obtained using shRNAs to suppress *Gmnn* expression in wild-type TSCs (data not shown). Thus, geminin ablation resulted in the same amount of DNA replication but without the distinctive appearance of endocycles.

To determine whether or not *Gmnn* was required to maintain endocycles in TGCs, MHT was added to the FGF4 deprivation medium at various times after FGF4 deprivation had begun (Fig. 7). When added to $Gmnn^{flox/flox}$; *ErCre/+* TSCs at the beginning of FGF4 deprivation (day 0, Fig. 7A), similar amounts of excess DNA were produced, but distinct endocycles were absent after five or even 10 days of FGF4 deprivation (Fig. 7B). Gmnn ablation after

two (Fig. 7C) or three (Fig. 7D) days of FGF4 deprivation also perturbed the pattern of endocycles observed after ten days. Similarly, MHT addition after five days of FGF4 deprivation resulted in cells that lacked later endocycle peaks (i.e. 32N and 64N). Western immuno-blotting confirmed the absence of geminin protein following addition of MHT (Fig. 7F). Thus, elimination of geminin following induction of TGC differentiation by FGF4 deprivation affected subsequent rounds of endoreplication, revealing that geminin is required to generate and maintain endocycles in TGCs.

Geminin Helps Prevent DNA Re-Replication During Endocycles

The fact that the amount of excess DNA replication observed in the absence of geminin was equivalent to that observed in the presence of geminin, despite the fact that distinct endocycles were absent, suggested that geminin prevents DNA re-replication from occurring during endocycles just as it does during mitotic cell cycles (Abbas et al., 2013). During mitotic cell cycles, geminin is one of several mechanisms that prevent assembly and activation of new prereplication complexes on chromatin before S-phase has been completed. Endocycles and DNA re-replication both result in an enlarged nucleus, but endocycles produce both G and S-phase nuclei, whereas DNA re-replication produces only S-phase nuclei. Therefore, to distinguish between these two modes of DNA replication, the fraction of nuclei in S-phase was determined by their ability to incorporate 5-ethynyl-2′-deoxyuridine (EdU, a modified DNA polymerase substrate), and the fraction of nuclei in G-phase was determined by the absence of EdU and the presence of p57 protein (Ullah et al., 2008).

Three populations of cells were compared: *Gmnn*$^{flox/flox}$*; ErCre/+* TSCs proliferating in TSC medium (+FGF4), or in TSC medium subjected to *Gmnn* ablation (+FGF4 +MHT) and the same cells undergoing differentiation in response to FGF4 deprivation (-FGF4). Beginning two days after addition of MHT or the ethanol vehicle or FGF4-deprivation, the cells were cultured for 20 minutes in the presence of EdU. Since *Gmnn* ablation was complete within 24 hours of MHT addition (Fig. 1D), the EdU-labeled nuclei in MHT treated cells represented endoreplication in the absence of geminin. Since CDK activity is required for the initiation and maintenance of endocycle S-phases, expression of p57 occurs exclusively in G-phase nuclei (Hattori et al., 2000; Ullah et al., 2008). Cells in transition from one phase to the next exhibited neither p57 expression nor EdU incorporation during a brief pulse.

Immuno-fluorescence revealed that about 60% of the cells in proliferating cultures of TSCs were undergoing DNA replication, and only about 3% expressed p57 [Fig. 8, TSCs (+FGF4)]. As with wild-type TSCs (Ullah et al., 2008), p57 was expressed only in TGCs that arose spontaneously in TSC cultures (Fig. 2E, +FGF4). During FGF4 deprivation, the fraction of p57 positive nuclei increased rapidly to about 60% with a corresponding decrease in the fraction of nuclei undergoing DNA replication [Fig. 8, TGCs (-FGF4)], consistent with endoreplication occurring with decreasing frequency. However, *Gmnn* ablation resulted in similar numbers of TGCs containing p57 or EdU positive nuclei throughout the duration of the experiment [Fig. 8, TGCs (+FGF4 +MHT)]. The morphology and staining patterns of these TGCs were indistinguishable from those produced by FGF4 deprivation. These results

were consistent with unregulated endocycles in which the length of individual G and S phases varied from cell to cell. Alternatively, some *Gmnn-/-* TGCs might undergo DNA re-replication while others arrest in G-phase.

To further distinguish endocycles from DNA re-replication, cells were cultured with EdU for increasing periods of time and then fixed and stained for p57 in order to determine if the cells that synthesized EdU-labeled nuclear DNA (S-phase) subsequently expressed the CDK inhibitor p57 (G-phase). If cells were undergoing endocycles, the fraction of nuclei labeled with both EdU and p57 would increase over time, whereas if cells were undergoing DNA re-replication, then double-labeled nuclei would be absent, because they remain in S-phase. As expected, the fraction of EdU-labeled nuclei increased as the period allowed for EdU incorporation into nascent DNA was increased (Fig. 9). Similarly, the fraction of nuclei that contained both EdU-labeled DNA and p57 protein also increased from 0% with a 20 minutes pulse of EdU to 7% with a 12 hour pulse of EdU. These were cells that had completed S-phase and entered G-phase. Since the results for MHT treated cells were indistinguishable from those with FGF4 deprived cells, the extent of endoreplication in the absence of geminin was equivalent to the extent in the presence of geminin. However, after 24 hours of EdU incorporation, the fraction of double-labeled nuclei in FGF4 deprived cells increased to 22%, whereas the fraction in *Gmnn* ablated cells was only 8%. Thus, in the absence of geminin, the ability of cells to complete S-phase and transit into G-phase ceased by 36 hours after geminin depletion (three days after MHT addition). However, since 40% of TGCs still actively synthesized nuclear DNA four days after geminin was depleted [Fig. 8, TGCs (+FGF4+MHT)], whereas only 10% of FGF4 deprived TGCs still actively endoreplicated their DNA [Fig. 8, TGCs (-FGF4)], TGCs eventually switched from endoreplication to DNA re-replication when deprived of geminin.

TSC Differentiation Does Not Induce Either DNA Damage Or Apoptosis

The results described above revealed that endoreplication was initiated in the absence of geminin, but that subsequent S-phases eventually underwent DNA re-replication. However, the extent of DNA re-replication was difficult to estimate based solely on changes in DNA replication patterns. Therefore, we considered changes in DNA damage and apoptosis. TGCs produced *in vivo* duplicate their genome uniformly and completely up to six times (Sher et al., 2013), and TGCs produced from TSCs *in vitro* are comparatively insensitive to DNA damage (Ullah et al., 2013; Ullah et al., 2008). In contrast, DNA re-replication induced by suppression of geminin expression in cancer cells results in demonstrable DNA damage and apoptosis (Zhu and Depamphilis, 2009). Therefore, if the extent of DNA re-replication produced in *Gmnn-/-* TGCs was significant, the amount of DNA damage and apoptosis in *Gmnn-/-* TGCs should be greater than in $Gmnn^{flox/flox}$ *TGCs*.

$Gmnn^{flox/flox}$*; ErCre/+* TSCs were assayed for DNA fragmentation using the TUNEL assay (Fig. 10A), and double-stranded DNA breaks were detected by phosphorylation of histone H2AX (γ-H2AX; Fig. 10B). The results revealed that TGCs produced either by FGF4 deprivation (-FGF4) or by *Gmnn* ablation in the presence of FGF4 (+FGF4 +MHT) did not contain any more DNA damage than occurred during proliferation of their TSC counterpart (+FGF4 +EtOH). Furthermore, neither the cleaved poly (ADP-ribose) polymerase (PARP)

fragment nor the cleaved caspase-3 fragments that are normally produced in cells undergoing apoptosis were detected (Fig. 10C). Thus, neither FGF4 deprivation nor geminin ablation of TSCs induced either DNA damage or apoptosis, consistent with the conclusion that the extent of DNA re-replication in *Gmnn*-/- TGCs was insignificant.

Discussion

The results presented here confirm the conclusion of Gonzalez et al. (2006) that *Gmnn* ablation can induce TGC formation, at least in those blastomeres that have already differentiated into trophoblasts. Moreover, the effects of *Gmnn* ablation in TSCs are consistent with a novel role for geminin in the FGF4 signal transduction pathway that governs trophoblast proliferation and differentiation. In this model (outlined in Fig. 11), geminin serves two roles; it governs the transition from cell proliferation to cell differentiation, and it maintains endocycles in terminally differentiated cells. Whether geminin also regulates differentiation of pluripotent blastomeres into trophoblasts, as suggested by Gonzalez and coworkers (Gonzalez et al., 2006; Yang et al., 2011), or is simply required for the propagation and viability of pluripotent blastomeres, as suggested by Hara et al. (2007), remains to be determined.

Geminin Is Essential For Trophoblast Proliferation and Differentiation

The events that followed *Gmnn* ablation in TSCs in the presence of FGF4 and conditioned medium closely mimicked those observed by FGF4 deprivation of TSCs. Both protocols arrested TSC proliferation with their concomitant transformation of into giant cells (Fig. 2). The giant cells contained a single enlarged nucleus that had over-replicated its DNA to the same extent, without inducing either DNA damage or apoptosis, regardless of whether differentiation was triggered by *Gmnn* ablation or FGF4 deprivation (Figs. 6, 10). Both protocols initiated changes in gene expression characteristic of TSC differentiation into TGCs *in vivo* as well as *in vitro* (Fig. 3), albeit with one caveat (spongiotrophoblasts, see below). Finally, both protocols triggered suppression of Chk1 protein with concomitant up-regulation of p57 and p21 proteins (Fig. 4). The p57 gene is among the most strongly expressed genes in TGCs produced *in vivo* (Sher et al., 2013). Consistent with the *Gmnn* ablation experiments, FGF4 deprivation down-regulated expression of geminin protein, thereby revealing that a reduction in *Gmnn* expression was an early event in the FGF4 signal transduction pathway outlined in figure 11.

Previous studies have shown that expression of p57 and p21 in trophoblasts is regulated post-translationally by the Chk1 kinase (Ullah et al., 2011). The results described here reveal that geminin is essential in proliferating trophoblasts to prevent suppression of Chk1 protein levels. This role for geminin might well be specific for trophoblasts as well as other cells that respond to a mitogen dependent G2 checkpoint (Ullah et al., 2011), because suppression of geminin expression in cells derived from human cancers induces DNA re-replication, which results in DNA damage, induction of the Chk1-dependent DNA damage response, and apoptosis. Suppression of geminin in cells derived from normal human tissues has little, if any, affect on cell proliferation or viability (Zhu and Depamphilis, 2009). The ability of geminin to prevent Chk1 suppression in TSCs requires high levels of geminin, because

FGF4 deprivation reduced, but did not eliminate, geminin expression in TSCs. However, once Chk1 was suppressed during TSC differentiation, subsequent expression of geminin, even at levels greater than those observed in TSCs, did not restore Chk1 expression. Selective inhibition of the 26S proteasome also failed to induce Chk1 expression, suggesting that suppression of Chk1 expression occurs at the translational level or earlier.

Expression of many S-phase genes in trophoblast cells, including *Gmnn* (Markey et al., 2004), is likely regulated by E2f. Not only is TSC differentiation accompanied by an overall decrease in E2F DNA-binding complexes and a decrease in E2F-dependent gene expression (Soloveva and Linzer, 2004), but ablation of E2f activators promotes excess nuclear DNA replication (presumably endoreplication) during mouse development in both trophoblasts and hepatocytes (Chen et al., 2012). For example, *Drosophila* E2f regulates expression of cyclin E, which regulates the transition from G to S-phase during endoreplication by regulating cyclin E•Cdk2 activity (Zielke et al., 2011). Although a change in *Gmnn* RNA levels was not detected during TSC differentiation (Fig. 3, and data not shown), regulation of geminin activity can occur by mechanisms that are not dependent on cell cycle specific degradation of geminin protein (Kisielewska and Blow, 2012; Yang et al., 2011).

The fact that either *Gmnn* ablation or FGF4 deprivation induced equivalent amounts of excess DNA replication revealed that Chk1 is down-regulated during TSC differentiation after the TSC S-phase was completed. Previous studies revealed that selective inhibition of Chk1 kinase activity by chemical inhibitors or shRNAs induced TSC differentiation into giant cells expressing genes characteristic of TGCs, but without enlarged nuclei or excess DNA (Ullah et al., 2011). Endoreplication does not occur under these conditions, because Chk1 is present throughout the mitotic cell cycle. When Chk1 activity is inhibited throughout the cell cycle in TSCs, p57 is expressed throughout the cell cycle, thereby preventing any form of CDK-dependent DNA replication (Depamphilis et al., 2012). In contrast to these results, *Gmnn* ablation, like FGF4 deprivation, induced p57 expression in less than half of the TGCs; thereby allowing DNA replication to occur in the remaining TGCs [Figs. 6; 7; (Ullah et al., 2011)]. In order for this to occur, *Gmnn* ablation must suppress Chk1 selectively during G2 phase of the mitotic cell cycle, the only phase wherein selective inhibition of Cdk1 activity induces endoreplication (Hochegger et al., 2007; Ma et al., 2009; Ullah et al., 2008). Consistent with this hypothesis, the role of geminin in preventing DNA re-replication occurs during G2 phase (Klotz-Noack et al., 2012).

Although the mechanism by which *Gmnn* ablation triggers TSC differentiation remains to be elucidated, it cannot result from the induction of DNA re-replication. Suppression of Chk1 occurs before p57 protein appears, and p57 is required to trigger endoreplication. Since these events are confined to G2-phase, DNA re-replication cannot occur until after TSC differentiation has begun. The simplest mechanism by which *Gmnn* ablation triggers TSC differentiation would be if high levels of geminin interfered with the two ubiquitin ligases that target Chk1 for degradation in order to promote mitosis either during unperturbed cell cycles or to suppress the DNA damage response (Huh and Piwnica-Worms, 2013; Zhang et al., 2009). High levels of geminin might prevent Chk1 destruction in TSCs until the anaphase-promoting complex (APC) has triggered the metaphase to anaphase transition, and Cdk1 activity is no longer required. The APC is the ubiquitin ligase that targets geminin for

destruction during the metaphase to anaphase transition (APC•Cdc20) and during G1-phase (APC•Cdh1). In that case, geminin and Chk1 would be targeted concurrently for ubiquitin-dependent degradation, albeit by different ubiquitin ligases. Once TGCs form, Chk1 protein is no longer regulated by ubiquitin-dependent degradation, because Chk1 protein was not recovered upon treatment of TGCs with MG132 (Fig. 5). Therefore, oscillating changes in the levels of geminin protein that occur during endocycles would not affect Chk1 expression.

One difference between *Gmnn* ablation and FGF4 deprivation was that *Gmnn* ablation did not induce genes characteristic of spongiotrophoblasts, whereas FGF4 deprivation did. Spongiotrophoblasts occupy the middle layer of the rodent placenta where they support the underlying villi, secrete several polypeptide hormones, and generate at least two subclasses of TGCs (Hu and Cross, 2010). This difference, however, appears to be linked to the requirement for geminin in maintaining endocycles, because TGCs nullizygous for either *Gmnn* (Fig. 3) or cyclin E (Parisi et al., 2003) exhibited both defective endocycles and the absence of spongiotrophoblast biomarkers *Tpbpa*, *Mash2/Ascl2* and *Flt1*.

Geminin Maintains Endocycles In Trophoblast Giant Cells

The ability of blastomeres in *Gmnn-/-* mouse embryos to differentiate into TGCs with a nuclear DNA content at least three times greater than undifferentiated blastomeres suggested that geminin was essential for endoreplication (Gonzalez et al., 2006). On the other hand, ablation of *Gmnn* in TSCs triggered their differentiation into TGCs with a heterogeneous nuclear DNA content between 4N and 64N, the same as with FGF4 deprived TGCs, but with one striking difference. The distinct endocycles observed during FGF4 deprivation were absent in TGCs produced by Gmnn ablation. FGF4 deprivation reduced, but did not eliminate, geminin, suggesting that a basal level of geminin was required to generate uniform cycles of endoreplication. If this basal level was reduced by ablation of *Gmnn* at various times after FGF4 deprivation, the endocycles were obscured. However, once a round of endoreplication was completed, it was stable even in the absence of geminin. Therefore, geminin was required to maintain distinct endocycles.

The role of geminin in maintaining endocycles appears to be the same as its role in mitotic cell cycles. Geminin helps to establish the periodicity of cell cycle events by preventing the initiation of a new S-phase before the current S-phase is completed (termed DNA re-replication). However, in non-cancer cells as well as in some cancer cells, geminin is only one of several mechanisms that prevent DNA re-replication (Abbas et al., 2013; Zhu and Depamphilis, 2009). Therefore, TGCs can undergo one or more rounds of endoreplication before the absence of geminin produces a phenotype.

If DNA re-replication occurred in the absence of geminin, then G-phase nuclei should transit into S-phase nuclei and remain in S-phase for an extended period of time. In the case of cancer cells, DNA re-replication also results in DNA damage and apoptosis, which limits the extent of excess DNA replication to between 4N and 8N DNA. In the experiments presented here, either *Gmnn* ablation or FGF4 deprivation of TSCs resulted in nuclei with a DNA content of 32N to 64N, without inducing DNA damage or apoptosis. Moreover, both G and S-phases were present, and up until two days after geminin was depleted, TGCs in S-phase

transited into G-phase at the same frequency in *Gmnn-/-* cells as in FGF4 deprived cells, consistent with one or more cycles of endoreplication. However, with continued culture, *Gmnn+/+* TGCs continued to transit from S-phase into G-phase, whereas *Gmnn-/-* TGCs in S-phase remained in S-phase, presumably due to repeated licensing of replication origins.

Variation in the periodicity of G and S-phases during endocycles would be expected in *Gmnn-/-* TGCs, because the level of geminin protein, like cyclin E, oscillates during endocycles, thereby regulating the transitions between G and S phases. This has been demonstrated in *Drosophila* where geminin is targeted for degradation by the anaphase promoting complex during the S to G transition (Narbonne-Reveau et al., 2008; Zielke et al., 2008), and the counter-play of E2F1 and its antagonist CRL4•Cdt2 ensures that CycE activity peaks in late G phase (Zielke et al., 2011). Since the same set of proteins are required for endocycles in TGCs (Garcia-Higuera et al., 2008; Geng et al., 2003; Parisi et al., 2003; Yang et al., 2012), the same mechanisms for maintaining endocycles likely exist in mammals as in flies (Ullah et al., 2009).

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Highlights

- ablation of geminin gene induces trophoblast stem cell (TSC) differentiation into giant cell
- high levels of geminin prevent TSC differentiation into giant cell
- basal levels of geminin maintain endocycles in giant cell
- loss of geminin mimics FGF4 deprivation of TSCs
- geminin acts like a downstream effector of FGF signaling in TSCs

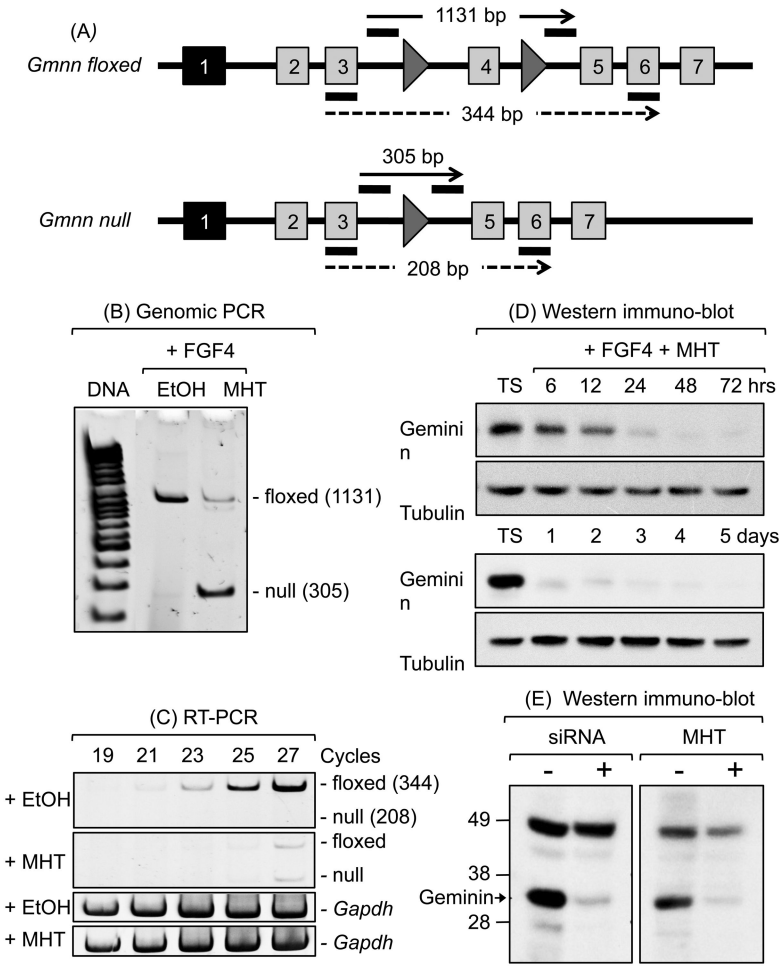

Figure 1. Conditional knockout of *Gmnn* exon 4 in TSCs depletes geminin mRNA and protein

(A) Schematic representation of *Gmnn* floxed allele and null allele after recombination by Cre-recombinase. Black and grey boxes represent non-coding and coding exons, respectively. Exon 4 is flanked by two LoxP sites (triangles) and is deleted after recombination by Cre recombinase. Solid boxes above and below the gene represent PCR primers used for genomic PCR and RT-PCR, respectively, with the expected fragment sizes for genomic PCR indicated by solid arrowed lines and for RT-PCR indicated by dashed arrowed lines. (B) $Gmnn^{flox/flox}$, *ER-Cre/+* TSCs were treated for 3 days with either 3 μM MHT or the equivalent amount of the ethanol vehicle. On day 5, DNA was extracted for genomic PCR. The floxed allele amplicon is 1131bp. The null allele amplicon is 305bp. (C) $Gmnn^{flox/flox}$, *ER-Cre/+* TSCs were treated as in panel B, but total RNA was extracted for RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) provided a loading control. The floxed allele amplicon is 344bp. The null allele amplicon is 208bp. (D) $Gmnn^{flox/flox}$, *ER-Cre/+* TSCs were treated with 3μM MHT for the indicated times. Whole-cell extracts were subjected to Western immuno-blotting to detect geminin levels. Tubulin provided a loading control. (E) Geminin protein was detected by Western immuno-blotting based on its molecular size, its sensitivity to anti-*Gmnn* siRNA in HCT116 cells as described in (Zhu and Depamphilis, 2009), and its sensitivity to ablation of the geminin gene in $Gmnn^{flox/flox}$, *ER-Cre/+* TSCs.

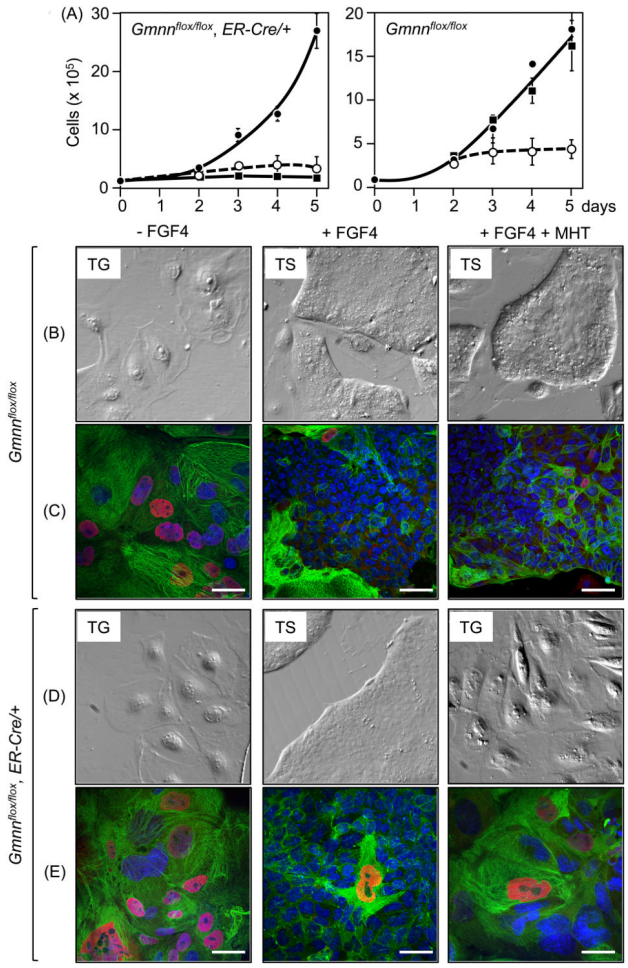

Figure 2. *Gmnn* ablation arrested TSC proliferation and induced changes in morphology characteristic of TGCs

(A) *Gmnn*$^{flox/flox}$, *ER-Cre/+* and *Gmnn*$^{flox/flox}$ TSCs were cultivated for three days in presence of TSC medium (+FGF4) supplemented with the EtOH vehicle (●), TSC medium supplemented with MHT (■), or FGF4 deprivation medium (○ dashed line). After three days, the culture medium was replenished, except that the EtOH and MHT supplement were omitted and the experiment was continued for two more days. The number of cells attached to the plate was scored at the indicated times. Error bars indicate standard deviation of two independent experiments. (B) and (D) Nomarski images (10×) of the indicated cells after 5 days showing either tightly packed monolayers of small TSCs (TS), or TGCs with a single enlarged nucleus (TG). (C) and (E) The same cells were stained with antibodies to visualize p57 protein (RED) and cytokeratin endo-A (GREEN), as well as with Hoechst to visualize nuclear DNA (BLUE). Scale bars represent 50μm.

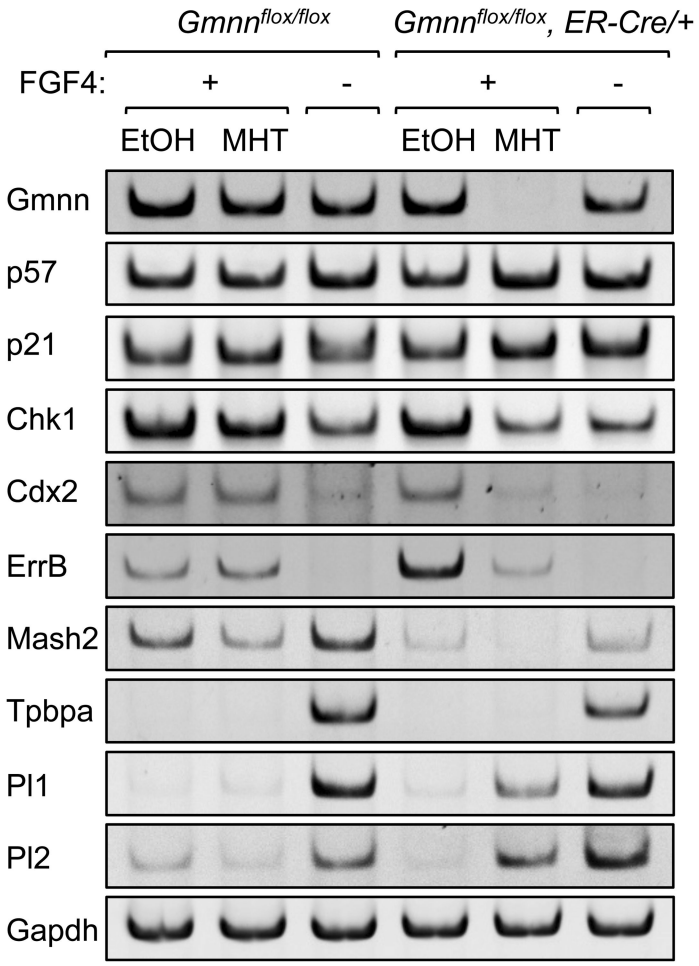

Figure 3. *Gmnn* ablation induced changes in gene expression characteristic of TSC differentiation into TGCs

Gmnn$^{flox/flox}$, *ER-Cre/+* and *Gmnn*$^{flox/flox}$ TSCs were cultured for three days either in FGF4 deprivation medium (-FGF4), or in TSC medium (+FGF4) supplemented either with MHT or with the equivalent amount of EtOH vehicle. Culture medium was replenished after three days, except that the EtOH and MHT supplements were omitted. After 5 days, expression of the indicated RNA was determined by RT-PCR on total cellular RNA.

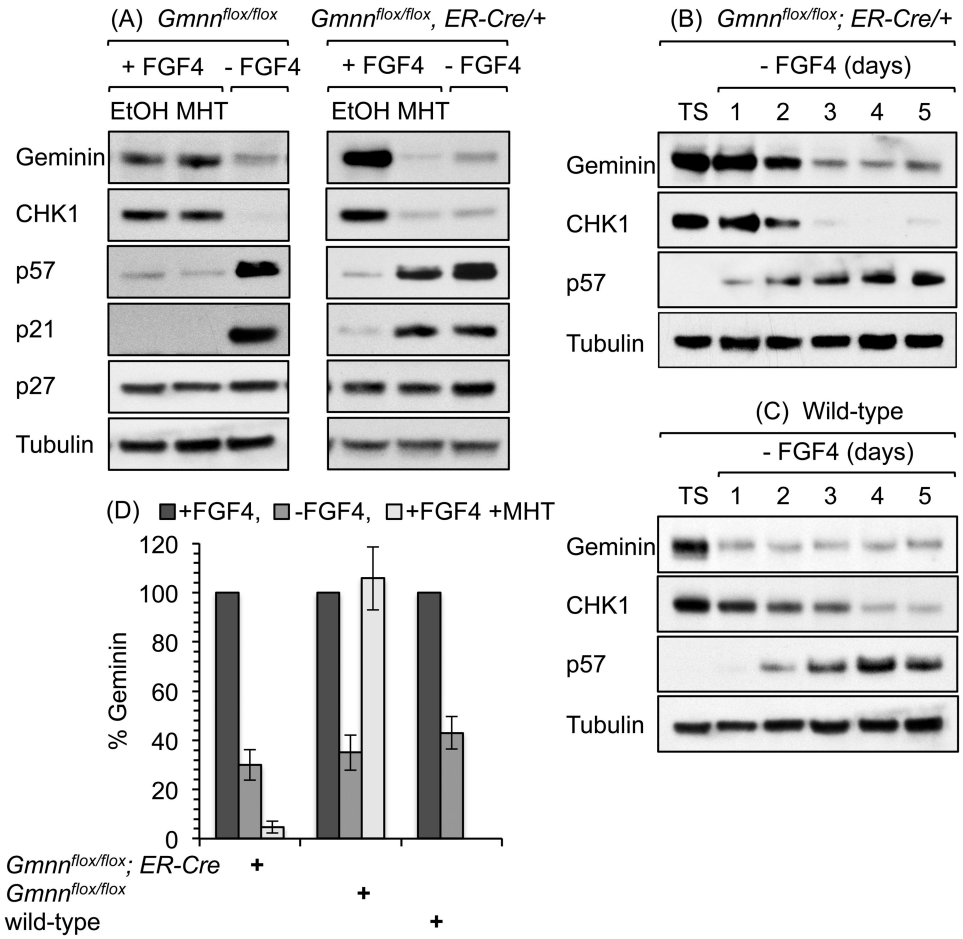

Figure 4. Geminin expression was consistent with a role in the FGF4 signal transduction pathway that governs the transition from proliferation to differentiation

(A) *Gmnn*$^{flox/flox}$, *ER-Cre/+* and *Gmnn*$^{flox/flox}$ TSCs were cultured as in figure 3. After 5 days, expression of the indicated proteins was determined in total cell extracts by Western immuno-blotting. Tubulin provided a loading control. Either *Gmnn*$^{flox/flox}$, *ER-Cre/+* TSCs (B) or wild-type TSCs (Ullah et al., 2008) (C) were subjected to FGF4 deprivation for the indicated times. The indicated proteins were then identified in total cell extracts by Western immuno-blotting. Tubulin provided a loading control. (D) The relative amounts of geminin protein were determined by densitometry of gels exposed for different lengths of time. Results shown are averages of six independent experiments with *Gmnn*$^{flox/flox}$, *ER-Cre/+* TSCs, five with wild-type TSCs, and four with *Gmnn*$^{flox/flox}$ TSCs. Error bars indicate SEM. The amount of geminin present after five days of either FGF4 deprivation (-FGF4, lighter bars) or MHT treatment of TSCs in the presence of FGF4 (+FGF4+MHT, lightest bars) was calculated as a fraction of the geminin in the untreated TSC parent population (+FGF4, black bars).

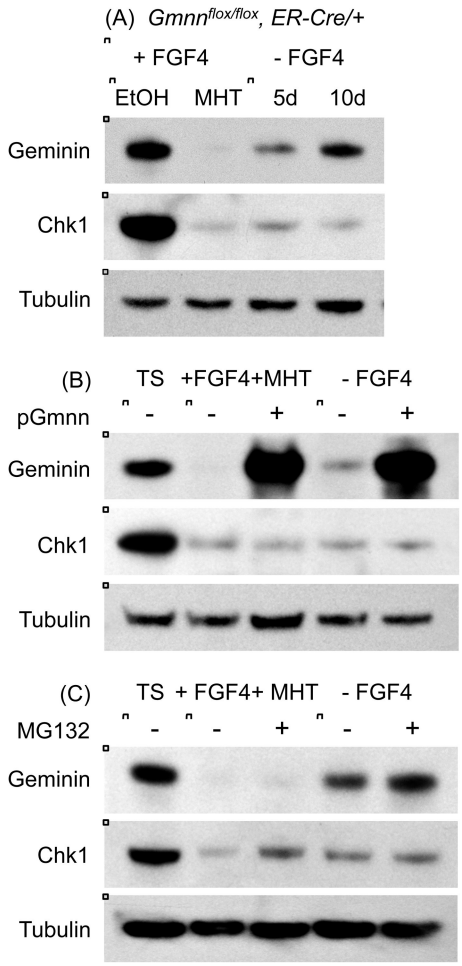

Figure 5. Geminin did not rescue Chk1 expression in TGCs

(A) *Gmnn*$^{flox/flox}$, *ER-Cre/+* TSCs were cultured in presence of FGF4 (+FGF4) and either EtOH or MHT as in figure 2. Alternatively, the same cells were cultured in the absence of FGF4 (-FGF4) for up to 10 days. (B) *Gmnn*$^{flox/flox}$, *ER-Cre/+* TSCs were cultured as in (A), except that the TGCs produced by treatment with +MHT or −FGF4 were transfected with pcDNA-TO-Gmnn plasmid DNA (pGmnn) and collected 24 hours post-transfection. (C) *Gmnn*$^{flox/flox}$, *ER-Cre/+* TSCs were cultured as in (A). After 3 days, 2μM MG132 (+) was added to the culture medium. Cells were harvested six hours later. The indicated proteins in panels A, B and C were assayed by Western immuno-blotting of whole-cell extracts.

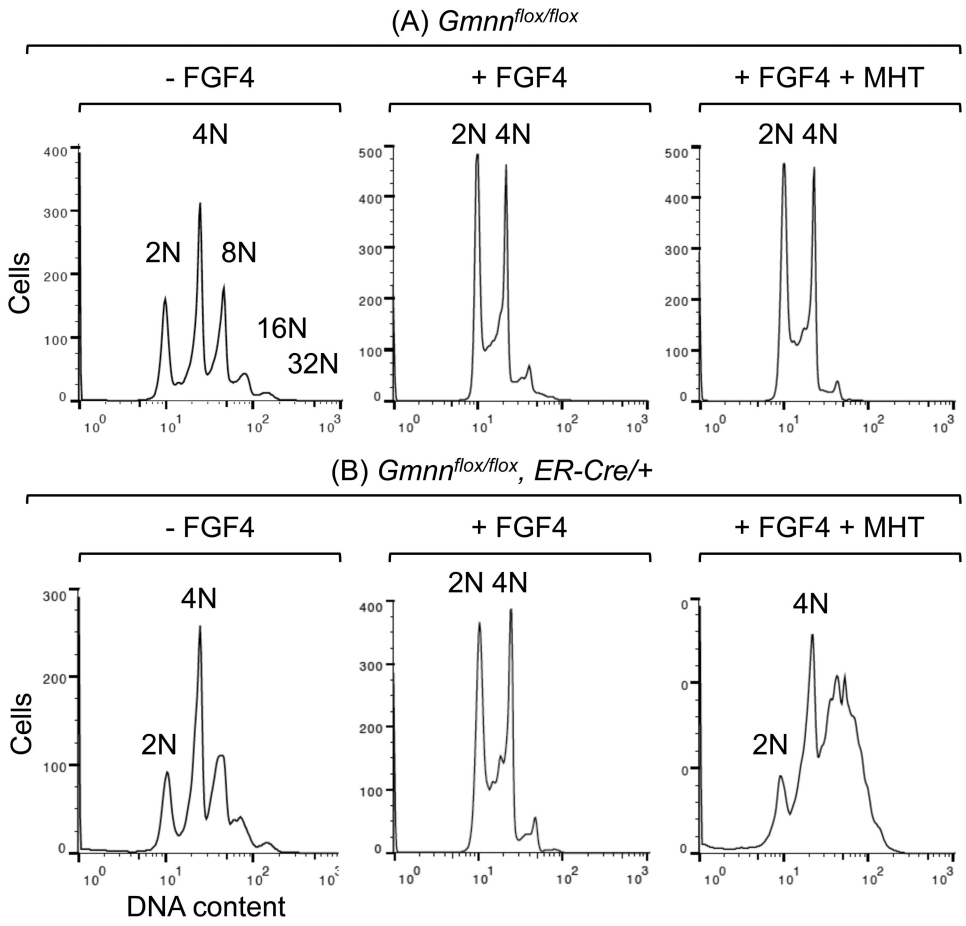

Figure 6. *Gmnn* ablation induced excess DNA replication in TGCs, but not distinct endocycles

(A) *Gmnn*$^{flox/flox}$ TSCs were cultured in FGF4 deprivation medium (-FGF4), or in TSC medium supplemented either with MHT (+FGF4+MHT) or with the equivalent amount of EtOH vehicle (+FGF4). Culture medium was replenished after three days, except that the EtOH and MHT supplements were omitted. After five days, the cellular DNA content was assessed by fluorescence-activated cell sorting (FACS) (B) *Gmnn*$^{flox/flox}$, *ER-Cre/+* TSCs were treated as in (A).

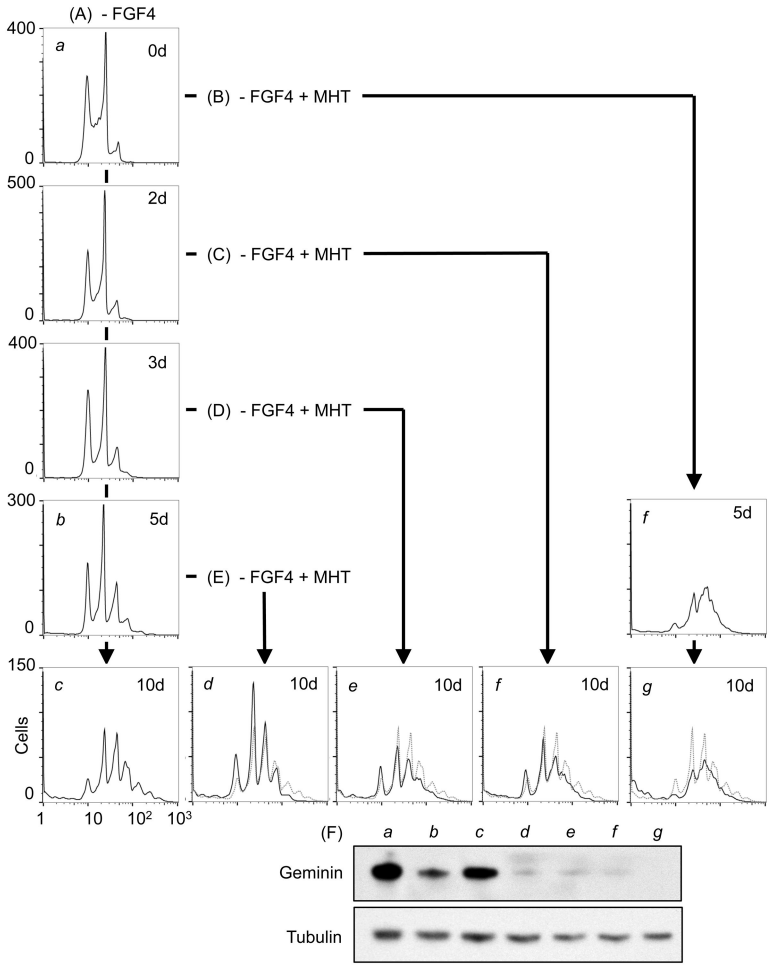

Figure 7. Geminin was required to produce distinct endocycles in TGCs

(A) FGF4 deprivation of *Gmnn*$^{flox/flox}$, *ER-Cre/+* TSCs was carried out for up to 10 days. *Gmnn* ablation was induced by addition of MHT to the culture medium at the indicated times: (B) at the beginning of FGF4 deprivation (zero days), (C) after two days of FGF4 deprivation, (D) after three days, (E) or after five days. Cells were collected at the indicated times. DNA content was analyzed by FACS (solid line) and compared with the FACS profile of TGCs after 10 days of FGF4 deprivation (dotted line). (F) Geminin levels were assayed by Western immuno-blotting on total cell extracts from the indicated time points (a-h). Tubulin provided a loading control.

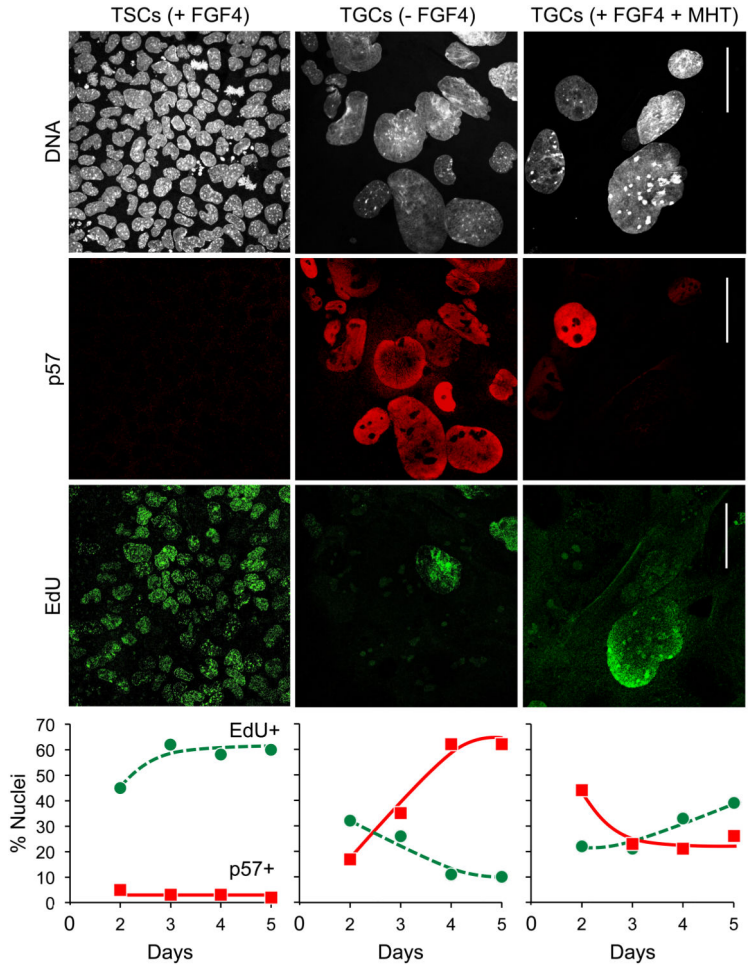

Figure 8. *Gmnn* ablation altered the timing of G and S-phases in TGCs

Gmnn$^{flox/flox}$, *ER-Cre/+* TSCs were cultured in TSC medium [TSCs (+FGF4)], or in FGF4 deprivation medium [TGCs (-FGF4)], or in TSC medium supplemented with MHT [TGCs (+FGF4 +MHT)]. At the indicated times, cells were incubated for 20 minutes with EdU to visualize S-phase nuclei (green). After fixation, cells were stained with antibodies to visualize p57 in G-phase nuclei (red) and with Hoechst to visualize total nuclear DNA (white). Images shown were taken at five days. Scale bars represent 50μm. The fraction of EdU positive nuclei (●, dashed line) and p57 positive nuclei (■, solid line) were quantified at the indicated times. At least 300 cells were scored for each time point.

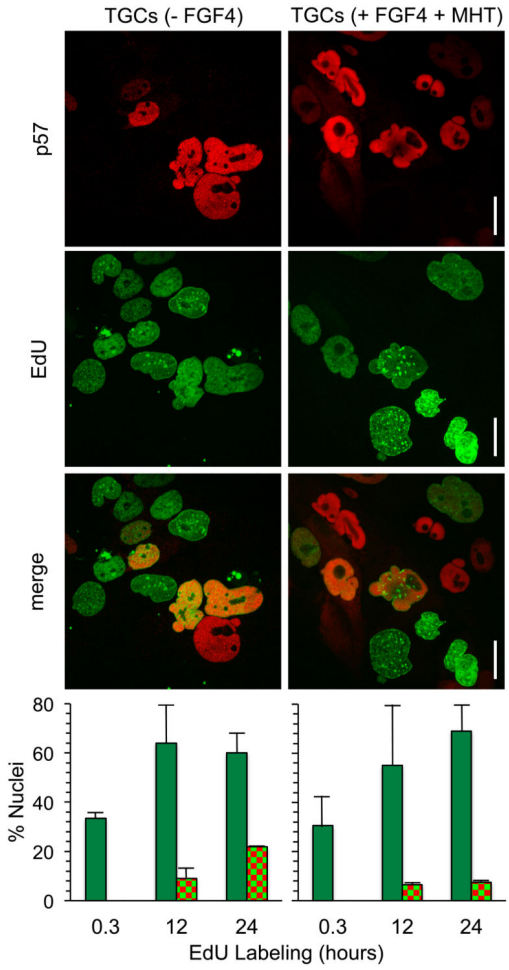

Figure 9. Geminin was required to regulate the S-phase of the endocycles

Gmnn$^{flox/flox}$, *ER-Cre/+* TSC were cultured either in absence of FGF4 [TGCs (-FGF4)] or in presence of FGF4 and MHT [TGCs (+FGF4+MHT)]. After 2 days 10μM of EdU was added to the medium for 12 or 24 hours to visualize cells that transited into S-phase (green). After fixation, cells were stained with antibody recognizing p57 (red). Images shown were taken after 24hours of EdU incorporation. Scale bars represent 25μm. The fraction of nuclei that were in S-phase during the pulse (EdU, green bar) and the fraction of cells that transitioned from S-phase to G-phase (EdU+p57, red and green bars) were calculated. At least 200 nuclei were scored for each time point.

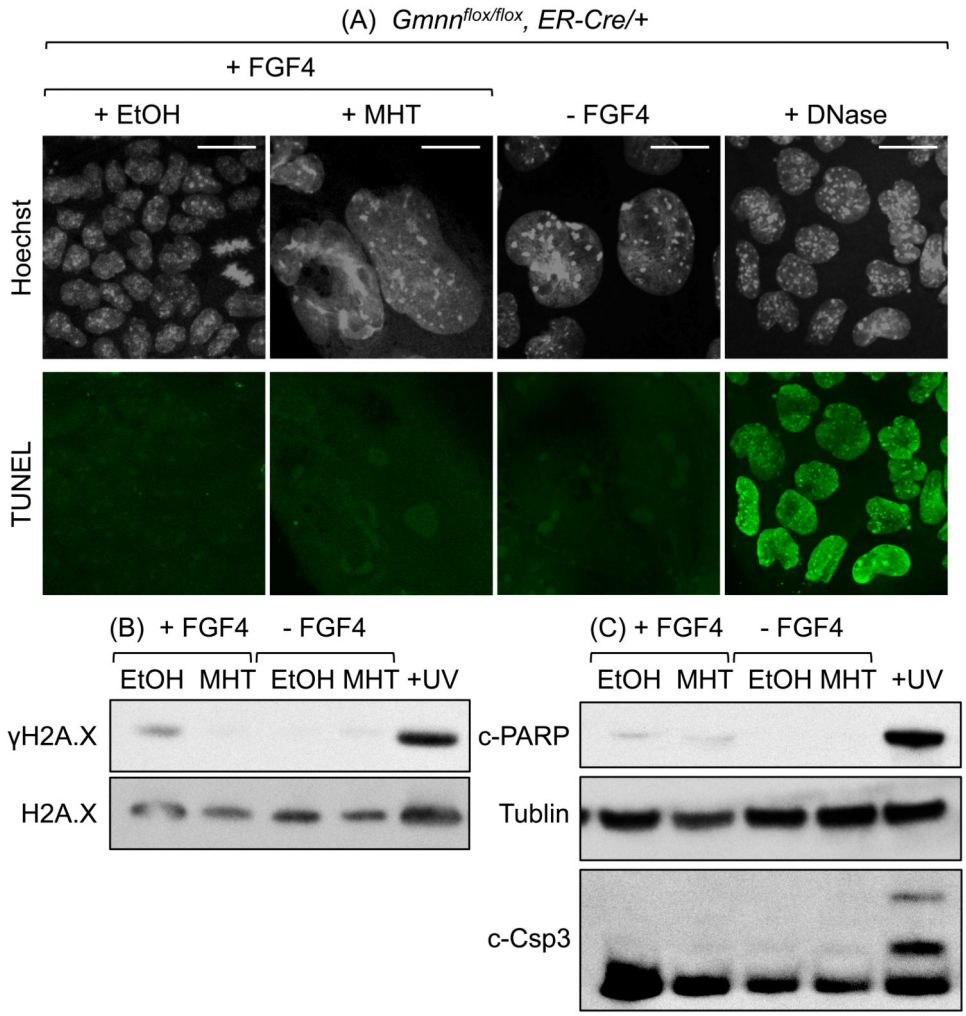

Figure 10. *Gmnn* ablation did not induce either DNA damage or apoptosis

Gmnn$^{flox/flox}$, *ER-Cre/+* TSCs were cultured as in figure 2. (A) After 5 days, cells were fixed and subjected to TUNEL assays to detect nuclear DNA fragmentation. Fixed TSCs treated with DNase I provided a positive control. Scale bars represent 25μm. Total cell extracts of the cells in (A) also were analyzed by Western immuno-blotting for the indicated proteins. (B) H2A.X is a unique core histone that is phosphorylated on S139 (γH2A.X) in response to double strand breaks in DNA. (C) c-PARP is the cleaved form of poly(ADP ribose) polymerase, and c-Csp3 is the cleaved form of caspase-3, two proteins that appear during apoptosis. Antibody specific for cleaved-caspase 3 recognized the large (17/21kDa) fragment of cleaved caspase-3 and also reacted with a lower unspecific band that was used as a loading control. To provide a positive control, TSCs were irradiated by UV (1000J) and harvested 12 hours later (+UV).

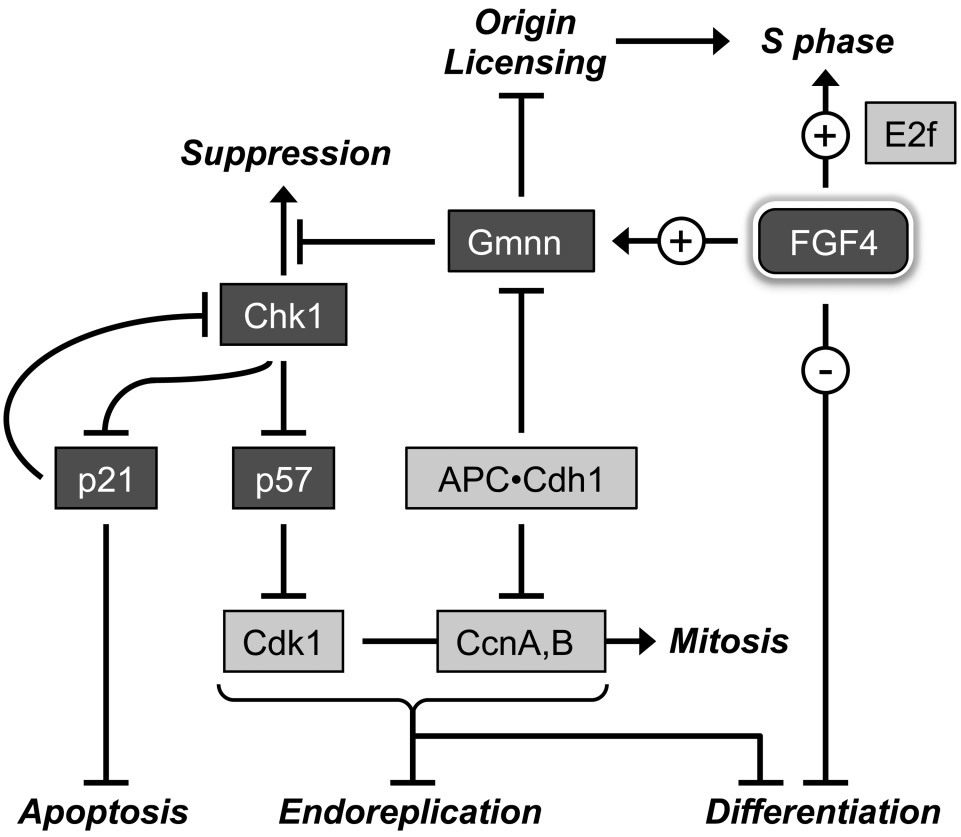

Figure 11. The FGF4 signal transduction pathway governs trophoblast proliferation and differentiation via geminin

FGF4 (and probably other mitogenic proteins as well) is essential for trophoblast proliferation. This mitogenic activity is likely mediated by E2F-dependent gene expression (Chen et al., 2012), and possibly directed at regulating the activity of the anaphase promoting complex (APC) (Yang et al., 2012). FGF4 deprivation results in down-regulation of geminin activity to a level that maintains endocycles, but that does not prevent down-regulation of Chk1 protein. The loss of Chk1 kinase activity results in expression of two CDK-specific inhibitors, p57 and p21 (Ullah et al., 2011). The p57 protein prevents the onset of mitosis by selectively inhibiting Cdk1 activity, thereby triggering the first round of endoreplication (Hattori et al., 2000; Ullah et al., 2008). This event activates the G1-phase APC•Cdh1 ubiquitin ligase, which targets geminin, cyclin B and cyclin A for degradation, thereby allowing licensing of replication origins and the onset of S-phase without passing through mitosis (Ullah et al., 2009). Inhibition of Cdk1 triggers both endoreplication and TSC differentiation. In the absence of p57, FGF4 deprivation produces multinucleated TGCs, revealing the existence of alternative mechanisms to trigger TSC differentiation (Ullah et al., 2008). Endocycles also require p57, which is expressed during G-phase and then suppressed during S-phase to allow sequential assembly and activation of pre-replication complexes (Ullah et al., 2008). Geminin helps to maintain endocycles by preventing DNA re-replication. The p21 protein helps to prevent DNA damage induced apoptosis in TGCs (de Renty et al., 2013; Ullah et al., 2008). It might also maintain suppression of Chk1 by reducing *Chk1* RNA levels (Gottifredi et al., 2001), as observed during FGF4 deprivation (Fig. 3).

Table 1

PCR and RT-PCR primers

PCR Genomic *Gmnn* sense: CTGAAGAGGACCTGAGTTCAGTTC PCR Genomic *Gmnn* antisense: CAACCCCTTTCTCCAGTGATGTTC
Gmnn sense: AGCCTTCTGCAGATGGATCTC *Gmnn* antisense: CTCAGCTACTTCTGCCAAGTC
Mash2 sense: CGCCAACAAGAAGCTGAGTAAG *Mash2* antisense: AGGTTTCTTGGGCTAGAAGCAG
Tpbpa sense: CTGAACTGCAAGAGCAGAAG *Tpbpa* antisense: TTCGCTCGTTGCCTAACTTC
Pl1 sense: CCCTGTGTCATACTGCTTCCATC *Pl1* antisense: AACTCGGCACCTCAAGACTTTG
Pl2 sense: TACCCAGGAGCTGTTGC *Pl2* antisense: AGCAGTTGTTGTTATGAAC
p57 sense: AGAATGGGGAGCCGGTCG *p57* antisense: GAGCCACGTTTGGAGAG)
p21 sense: CAGTACTTCCTCTGCCCT *p21* antisense: GCAGAAGACCAATCTG
Chk1 sense: CTGGAGTACTGTAGTG *Chk1* antisense: CACATCTTGTTCAGTAAG
Cdx2 (Yagi et al., 2007)
ErrB (Cowden Dahl et al., 2005)
Gapdh (Kaneko et al., 2004)