Inability to enter S phase and defective RNA polymerase II CTD phosphorylation in mice lacking *Mat1*

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The trimeric Cdk7–cyclin H–Mat1 complex comprises the kinase subunit of basal transcription factor TFIIH and has been shown to function as a cyclin-dependent kinase (Cdk)-activating kinase. Herein we report that disruption of the murine *Mat1* gene leads to peri-implantation lethality coincident with depletion of maternal Mat1 protein. In culture, *Mat1*^{−/−} blastocysts gave rise to viable post-mitotic trophoblast giant cells while mitotic lineages failed to proliferate and survive. In contrast to wild-type trophoblast giant cells, *Mat1*^{−/−} cells exhibited a rapid arrest in endoreduplication, which was characterized by an inability to enter S phase. Additionally, *Mat1*^{−/−} cells exhibited defects in phosphorylation of the C-terminal domain (CTD) of RNA polymerase II on both Ser5 and Ser2 of the heptapeptide repeat. Despite this, *Mat1*^{−/−} cells demonstrated apparent transcriptional and translational integrity. These data indicate an essential role for Mat1 in progression through the endocycle and suggest that while Mat1 modulates CTD phosphorylation, it does not appear to be essential for RNA polymerase II-mediated transcription.

Keywords: Cak/CTD phosphorylation/endoreduplication/Mat1/RNA polymerase II

Introduction

Progression through the cell cycle is mediated by the sequential activation of cyclin-dependent kinases (Cdks), which phosphorylate substrates critical for advancing the cell cycle. Cdk activity is regulated by cyclin binding, Cdk inhibitors, proteolysis, localization and phosphorylation (reviewed in Morgan, 1997). Several cell cycle Cdks require phosphorylation of a conserved threonine residue within the T-loop for full activity (reviewed in Kaldis, 1999). The enzymes that catalyze this activation are known as Cdk-activating kinases or Caks. Biochemical purification of an activity from mammalian and *Xenopus* lysates capable of activating cdc2 *in vitro* (Solomon *et al.*, 1992) led to the identification of a trimeric complex of the MO15/Cdk7 kinase together with cyclin H and a third subunit termed Mat1 (ménage-à-trois; reviewed in Nigg, 1996). *In vitro*, the mammalian Cdk7–cyclin H–Mat1 trimer can phosphorylate and activate Cdc2/Cdk1, Cdk2, Cdk3, Cdk4 and Cdk6 in complex with their cognate cyclin partners (reviewed in Kaldis, 1999).

Shortly after their identification in Cak fractions, Cdk7–cyclin H–Mat1, along with the homologous complex Kin28–Ccl1–Tfb3 in budding yeast *Saccharomyces cerevisiae*, were found to be components of the nine-subunit basal transcription factor TFIIH (Feaver *et al.*, 1994; Mäkelä *et al.*, 1995; Serizawa *et al.*, 1995; Shiekhattar *et al.*, 1995). TFIIH provides several catalytic functions in mediating both basal transcription by RNA polymerase II (pol II) and transcription-coupled nucleotide excision repair (see Tirode *et al.*, 1999, and references therein). These include the helicase activity afforded by the XPB and XPD subunits, which are involved in promoter melting prior to transcription initiation, in addition to the kinase activity provided by Cdk7–cyclin H–Mat1. TFIIH kinase activity is believed to be directed primarily at Ser5 of the repeated heptapeptide consensus sequence YSPTSPS (Gebara *et al.*, 1997; Hengartner *et al.*, 1998; Sun *et al.*, 1998; Trigon *et al.*, 1998), which comprises the C-terminal domain (CTD) of the large subunit of pol II. Phosphorylation of the CTD during pol II-mediated transcription has been shown to be critical in the recruitment of proteins necessary for proper processing of the nascent transcript (McCracken *et al.*, 1997; Hirose and Manley, 1998; Hirose *et al.*, 1999; Misteli and Spector, 1999; Komarnitsky *et al.*, 2000; Rodriguez *et al.*, 2000).

The functions of Mat1 in the trimeric Cdk7–cyclin H–Mat1 kinase and as a subunit of TFIIH are not well understood. Mat1 enhances complex formation of cyclin H and Cdk7 in the absence of Cdk7 T-loop phosphorylation (Devault *et al.*, 1995; Fisher *et al.*, 1995; Tassan *et al.*, 1995; Martinez *et al.*, 1997). Several studies have suggested a role for Mat1 in the control of substrate specificity whereby Mat1 shifts the kinase activity of dimeric Cdk7–cyclin H from Cdk substrates towards CTD substrates *in vitro* (Inamoto *et al.*, 1997; Rossignol *et al.*, 1997; Yankulov and Bentley, 1997). Cdk7–cyclin H–Mat1 has been shown to have a wide range of additional *in vitro* substrates, and phosphorylation of several of these appears to be Mat1 dependent and is likely to occur within the context of TFIIH. These include p53 (Ko *et al.*, 1997), pRb (Wu *et al.*, 2001), the POU domains of the Oct factors (Inamoto *et al.*, 1997), retinoic acid receptor-α

(Rochette-Egly *et al.*, 1997) and estrogen receptor-α (Chen *et al.*, 2000).

Primary sequence analysis indicates that Mat1 contains two conserved structural domains: a canonical RING finger domain and a coiled-coil domain. Structure–function mapping of Mat1 has suggested that the N-terminally located RING finger domain is associated with TFIIH-mediated transcriptional activation (Busso *et al.*, 2000). The central coiled-coil domain is involved in establishing contacts with other TFIIH subunits, primarily with the XPD helicase (Busso *et al.*, 2000). C-terminal sequences appear to be sufficient to mediate the assembly of Cdk7–cyclin H–Mat1 trimers (Tassan *et al.*, 1995; Busso *et al.*, 2000).

The two principal pathways in which Cdk7–cyclin H–Mat1 are suggested to act, namely Cdk activation and pol II-mediated transcription, are both essential for the viability of the cell. Studies have shown that homologous molecules in non-mammalian species are essential genes, and thus a clear dissection of the functions of Cdk7–cyclin H–Mat1 in a physiological setting has proven difficult (Valay *et al.*, 1995; Faye *et al.*, 1997; Larochelle *et al.*, 1998). Compounding this problem is the fact that it has become apparent that differences in the actual *in vivo* capacities of homologous kinases from different species exist. Notably, the Kin28–Ccl1–Tfb3 complex of *S.cerevisiae* has been shown to function only in TFIIH-mediated transcription (Cismowski *et al.*, 1995; Valay *et al.*, 1995; Holstege *et al.*, 1998) while Cdk activation is provided by a separate monomeric kinase named Cak1/Civ1 (Espinoza *et al.*, 1996; Kaldis *et al.*, 1996; Thuret *et al.*, 1996; Kimmelman *et al.*, 1999). In *Schizosaccharomyces pombe*, on the other hand, both the Cdk7-related Mcs6 kinase complex and a single-subunit kinase Csk1 activate Cdks *in vivo* (Hermand *et al.*, 1998; Lee *et al.*, 1999; Hermand *et al.*, 2001).

To date, the best *in vivo* evidence that Cdk7–cyclin H–Mat1 functions as a Cak in metazoan species comes from experiments in *Drosophila* that utilized a temperature-sensitive allele of Cdk7 (DmCdk7) to show that activation of mitotic Cdk–cyclins was impeded at the restrictive temperature (Larochelle *et al.*, 1998). However, a separate study utilizing a dominant-negative allele of DmCdk7 was unable to detect defects in Cak activity and instead described transcriptional defects (Leclerc *et al.*, 2000). Although genetic studies in mammalian systems have not yet been reported, the recent biochemical characterization of a potential budding yeast Cak1/Civ1 homolog from mammalian cells (Nagahara *et al.*, 1999; Kaldis and Solomon, 2000) has suggested that mammalian Cdk activation *in vivo* could be mediated by a single-subunit Cak in addition to, or perhaps in place of, Cdk7–cyclin H–Mat1. These putative kinases are unlikely to represent close sequence homologs of Cak1 based on the lack of evident Cak1 homologs in currently available mammalian databases.

Thus the actual *in vivo* functions of the mammalian Cdk7–cyclin H–Mat1 kinase remain elusive. In this report, we have attempted to add some solvency to this issue by generating a loss-of-function allele of *Mat1* in mouse to address whether the Cdk7–cyclin H–Mat1 kinase functions in pol II-mediated transcription, cell cycle progression or both.

Results

Targeted disruption of the murine Mat1 gene

Mat1 genomic sequences were isolated from a 129-Sv library using a full-length human *MAT1* cDNA as a probe. Restriction analysis and sequencing of several overlapping clones revealed that a single exon of *Mat1* spanning 153 nucleotides of the cDNA (nucleotides 242–394 in DDBJ/EMBL/GenBank accession No. U35249) had been isolated. This exon encodes 51 N-terminally located amino acids encompassing most of the RING finger domain. A replacement-type targeting vector (Figure 1A) was constructed and electroporated into embryonic stem (ES) cells. Three clones were isolated (of 800 screened) that were confirmed to be targeted correctly by Southern blotting with both 5′ and 3′ external probes (Figure 1B). Targeted cells were then injected into BL/6 blastocysts and several of the resulting chimeras were found to transmit the targeted allele through the germline (Figure 1C).

Splicing around targeted exons has been demonstrated with other loci and in this case could lead potentially to the production of an in-frame *Mat1* transcript. To investigate whether a truncated protein might be synthesized by the targeted allele, we performed western blot analysis on total lysates obtained from ES cells heterozygous for the targeted allele using an antibody against Mat1 (Figure 1D). No truncated Mat1 proteins were observed in the ES cell lysates, suggesting that the targeted allele represents a null allele of *Mat1*.

Disruption of Mat1 leads to early embryonic lethality

Mat1 heterozygous (*Mat1*^{+/–}) intercrosses were set to ascertain the viability of *Mat1* null homozygotes (*Mat1*^{–/–}). PCR genotyping analysis of 104 adult offspring indicated that while both *Mat1*^{+/+} and *Mat1*^{+/–} animals were observed at the expected frequencies, no homozygote null animals were obtained (Table I). This demonstrated that disruption of *Mat1* leads to embryonic lethality. Dissection and genotyping of embryos at embryonic day E10.5, E9.0 and E7.5 of development also failed to identify viable homozygotes. In contrast, blastocysts at E4.0 showed close to Mendelian ratios for all genotypes, indicating that lethality probably occurred after implantation but before gastrulation (Table I). Indeed, the observation of resorbing embryos and empty decidua in roughly Mendelian numbers at later times of gestation suggests that *Mat1*^{–/–} embryos die shortly after implantation.

As *Mat1* is an essential gene in *S.cerevisiae*, deletion of mammalian *Mat1* might also be expected to compromise cell viability (Faye *et al.*, 1997). Thus it was surprising that early embryogenesis would proceed in a genetically *Mat1* null background. We reasoned that survival of *Mat1*^{–/–} embryos to the implantation stage of development might be due to maternally provided Mat1. In order to test this hypothesis, embryos obtained from *Mat1*^{+/–} intercrosses were isolated at eight-cell, 16-cell and blastocyst stages and immunostained with an anti-Mat1 antibody. Immunofluorescence analysis demonstrated decreasing Mat1 signal in approximately one-quarter of the embryos as development proceeded such that by the blastocyst stage, Mat1 immunoreactivity was barely observable
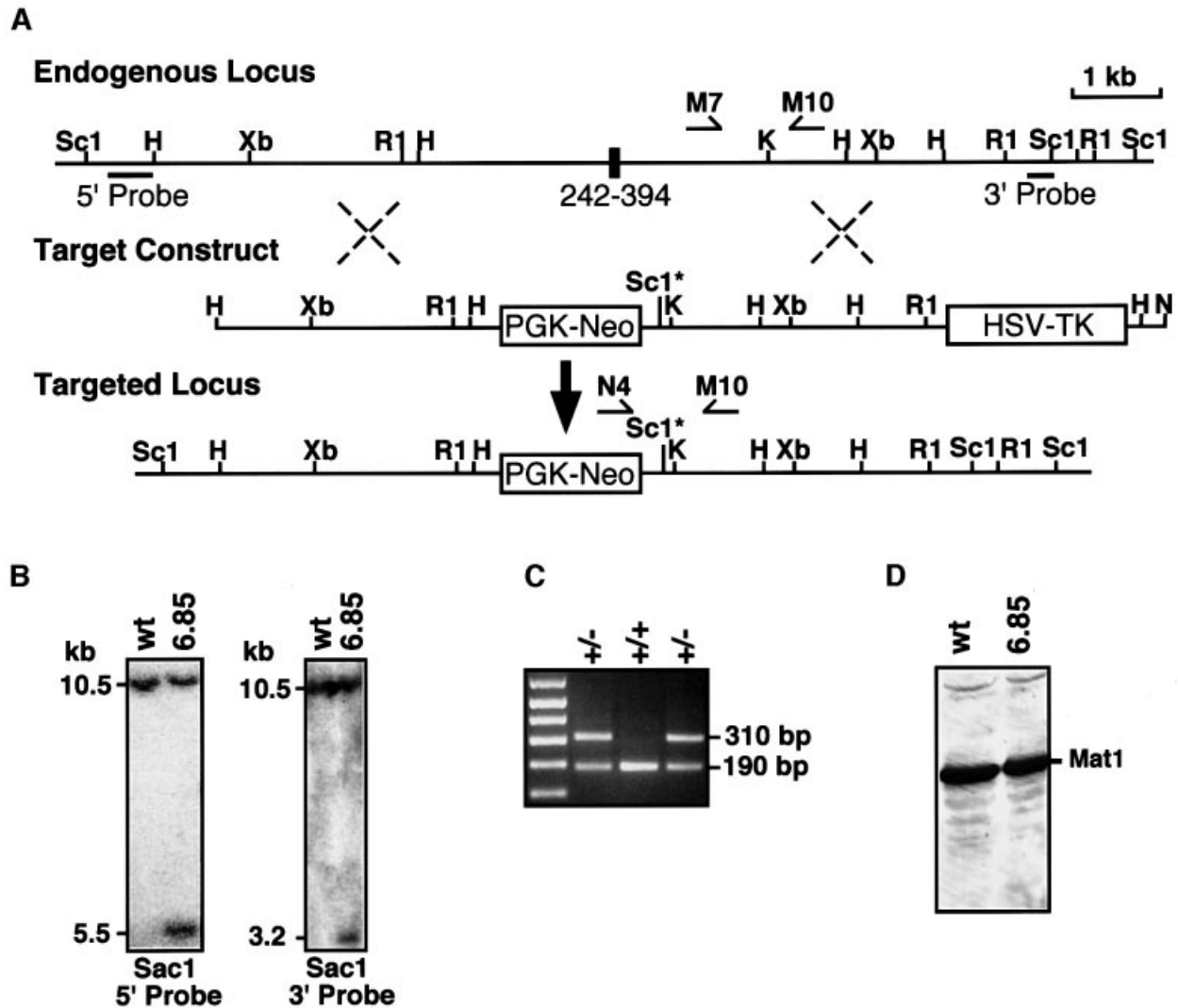
A

B

C

D

Fig. 1. Generation of a *Mat1* null allele. (**A**) Partial genomic structure of the murine *Mat1* gene encompassing the targeted exon (nucleotides 242–394, amino acids 31–81) and target vector used to disrupt the locus. Upon homologous recombination into the murine genome, the PGK-neomycin resistance cassette of the target vector replaces 3 kb of genomic sequences between the *Hin*dIII (H) and *Kpn*I (K) restriction sites flanking the targeted exon. The HSV-TK cassette was used as a negative selection marker. An introduced *Sac*I restriction site (Sc1*) was used in screening for targeted events with both 5′ and 3′ probes. (**B**) Southern blotting analysis of targeted ES cell lines. *Sac*I-digested genomic DNA from wild-type (wt) and targeted ES cell line 6.85 yielding predicted 5.5 kb (5′ probe) and 3.2 kb (3′ probe) bands in addition to a 10.5 kb wild-type band. (**C**) PCR genotyping of DNA isolated from tail cuts of F₁ animals resulting from crossing a chimeric male (ES cell line 6.85) to wild-type females. A 190 bp wild-type band and a 310 mutant band are amplified with primer pairs M7–M10 and N4–M10 shown in (A). (**D**) Western blot of targeted ES cell lines. Total cell lysates from a wild-type ES cell (wt) and targeted ES cell line 6.85 western blotted with α-Mat1.

(Figure 2). Subsequent PCR analysis revealed that embryos with diminishing signal genotyped as *Mat1⁻/⁻* (Figure 2 and data not shown). The timing of the lethality in *Mat1⁻/⁻* embryos therefore probably reflects the depletion of maternal Mat1 protein below threshold levels required to sustain an essential function.

Interestingly, *Mat1⁻/⁻* blastocysts were phenotypically indistinguishable from wild-type controls, demonstrating that *Mat1⁻/⁻* cells have the developmental capacity to differentiate into both inner cell mass (ICM) and trophectodermal lineages. TUNEL analysis of blastocyst stage embryos derived from *Mat1⁺/⁻* intercrosses showed no differences in either the ICM or trophectoderm cells of *Mat1⁻/⁻* and wild-type controls (data not shown). This indicated that pre-implantation embryos severely depleted

of Mat1 protein stores were not committed rapidly to an apoptotic cell fate at this stage.

Mat1 is required for the survival of mitotic but not post-mitotic lineages

To examine more directly the role of *Mat1* in cell proliferation and differentiation, we followed the development of pre-implantation embryos derived from *Mat1⁺/⁻* intercrosses in culture. Regardless of genotype, most embryos hatched from the zona pelucida and formed blastocyst outgrowths, which were maintained in culture for 7 days. Both *Mat1⁺/⁻* and *Mat1⁺/⁺* outgrowths were characterized by the establishment of a rapidly proliferating cluster of ICM-derived cells growing on a base of trophectoderm-derived trophoblast giant cells that had

attached to the culture dish (Figure 3A and B). After 4–5 days in culture, a subpopulation of cells, presumably representing endodermal lineages by morphology, was often observed to differentiate from the ICM and migrate away from the wild-type outgrowths.

Table I. Genotypes resulting from $Mat^{+/-}$ intercrosses

	+/+	+/–	–/–	Resorbed	Total
3 weeks	31	73	0	–	104
E7.5–E10.5	13	30	0	19	62
E3.5	14	31	15	–	61

In contrast, the $Mat1^{-/-}$ embryos gave rise to outgrowths with trophectodermal cells yet failed to develop proliferative ICM cells and, as a consequence, endodermal derivatives (Figure 3A and B). Cells derived from the ICM of the blastocyst would often be observed associated with the remnants of the zona pelucida in $Mat1^{-/-}$ outgrowths, but these cells were never noted to proliferate. Hoechst staining of these cells revealed condensed, fragmented nuclei, suggesting that the $Mat1^{-/-}$ ICM cells underwent apoptosis during the first few days in culture (data not shown). The trophoblast cells established in the $Mat1^{-/-}$ outgrowths, on the other hand, were indistinguishable from wild-type counterparts by light microscopy over the
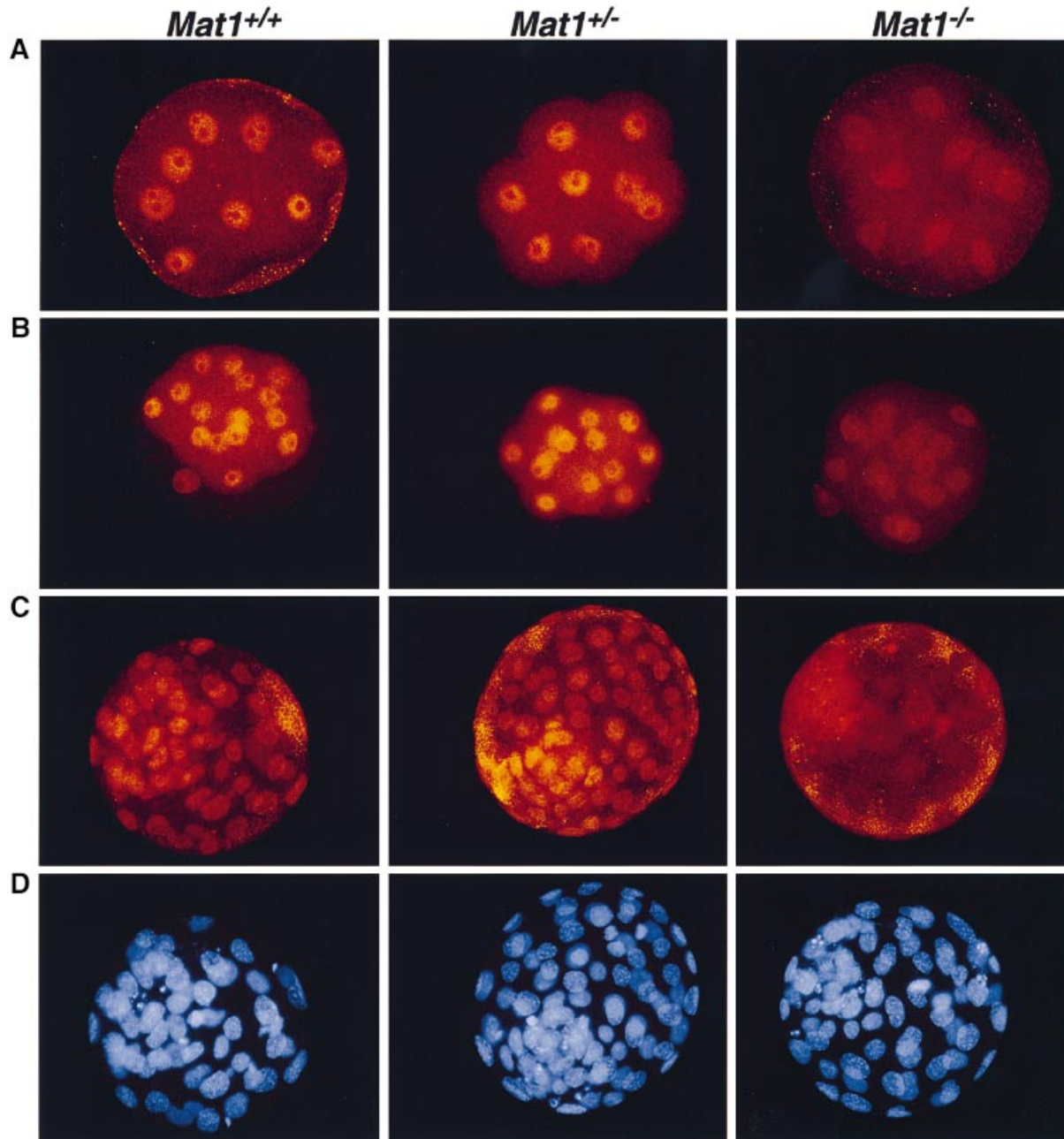

Fig. 2. Depletion of maternal Mat1 protein in $Mat1^{-/-}$ pre-implantation embryos. α-Mat1 immunofluorescence analysis of embryos derived from $Mat1^{+/-}$ intercrosses isolated at the eight-cell (**A**), 16-cell morula (**B**) or blastocyst (**C**) stage of development. The genotypes indicated on top were determined by PCR genotyping. Nuclei of blastocysts depicted in (**C**) were visualized by staining DNA with Hoechst 33342 (**D**).

first few days in culture (Figure 3A). However, as the cells were maintained further in culture, the *Mat1*^{–/–} trophoblast cells were noted to be smaller than the controls. We determined that this was not a secondary phenotype resulting from the absence of ICM lineages in the *Mat1*^{–/–} outgrowths, as control outgrowths dissected of their ICM

early in culture developed giant cells comparable to those with an intact ICM.

In order to address the function of *Mat1* beyond embryonic lethality, we took advantage of the blastocyst outgrowth system as a means of generating a population of trophoblast giant cells on coverslips (20–40 cells per
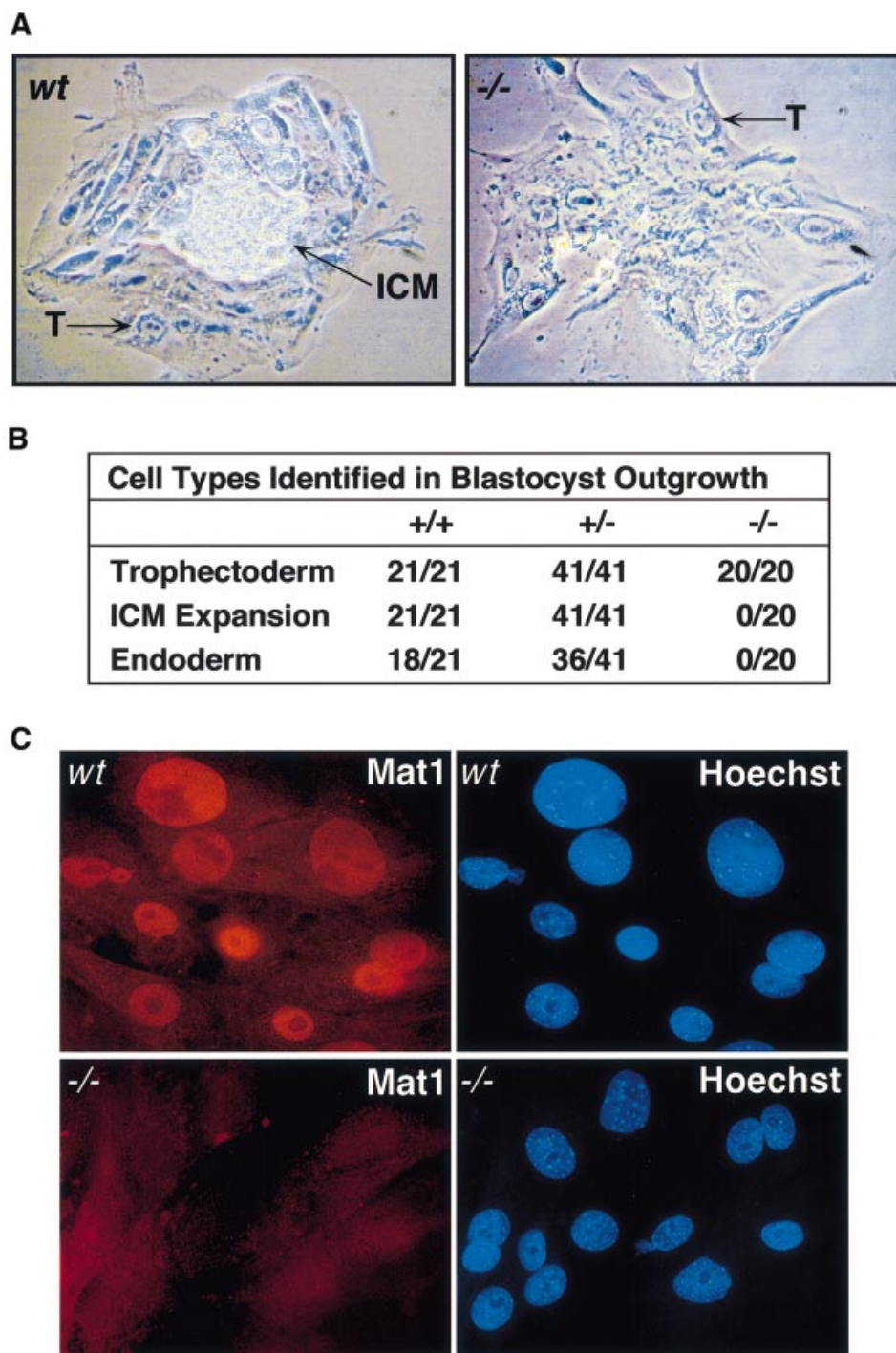

Fig. 3. Cultured *Mat1*^{–/–} embryos give rise to post-mitotic but not mitotic lineages. (**A**) Phase contrast micrographs of *Mat1*^{+/+} (left panel) or *Mat1*^{–/–} (right panel) blastocyst outgrowths at day 6 in culture (10.5 days post-coitum). Trophoblast giant cells (T) and inner cell mass (ICM) cells (absent in the *Mat1*^{–/–} outgrowth) are indicated. Endodermal lineages that differentiate from wild-type ICM have migrated outside the field of this micrograph. (**B**) Summary of cell types identified in blastocyst outgrowth. Trophectoderm, ICM and endodermal cell types were scored from outgrowths derived from *Mat1*^{+/–} intercrosses after 7 days in culture. PCR genotyping as described in the text was used to determine the indicated genotype. (**C**) Establishing immunofluorescence as a genotyping tool. PCR-genotyped *Mat1*^{+/+} (wt) or *Mat1*^{–/–} (–/–) outgrowths were analyzed following Mat1 antibody immunofluorescence and Hoechst 33342 staining (200× magnification).

outgrowth). Genotyping of outgrown cells was accomplished by staining with Mat1 antibodies (Figure 3C), which in all cases analyzed was in agreement with PCR genotyping of ICM cells dissected away from wild-type outgrowths. It should be noted that genotyping by immunofluorescence does not distinguish between $Mat1^{+/+}$ and $Mat1^{+/-}$, and so outgrowths genotyped in this manner are referred to collectively as wild-type ($Mat1^{wt}$).

Mat1 specifically regulates the steady-state levels of Cdk7 and cyclin H

Mat1 has been suggested to act as an assembly factor for the Cdk7–cyclin H–Mat1 trimer (Devault et al., 1995; Fisher et al., 1995; Tassan et al., 1995). We therefore wanted to address whether or not the expression of Cdk7 and cyclin H was affected in $Mat1^{-/-}$ cells. In order to do this, outgrown trophoblast cells were subjected to immunofluorescence with antibodies specific for Cdk7 and cyclin H (Figure 4A). Analysis of wild-type cells revealed strong nuclear staining of both Cdk7 and cyclin H (Figure 4A). $Mat1^{-/-}$ cells, however, consistently revealed that the signals for both Cdk7 and cyclin H were severely diminished (Figure 4A). In order to be sure that this was not due to epitope masking in the $Mat1^{-/-}$ cells, immunofluorescence with additional antibodies directed at Cdk7 and cyclin H was performed and found to confirm our initial observations (data not shown).

In S.cerevisiae, strains harboring temperature-sensitive alleles of the Mat1 homolog (TFB3) exhibit severely compromised transcription upon shifting to the non-permissive temperature (Faye et al., 1997). We therefore wanted to determine whether the diminished levels of Cdk7 and cyclin H were specific to Mat1 loss or instead a manifestation of a more general transcriptional deregulation. Steady-state transcription can be assayed indirectly by monitoring protein expression, particularly when assaying the levels of proteins with a rapid turnover. We therefore analyzed cells derived from blastocyst outgrowths for expression of several proteins with variable turnover rates by immunofluorescence. These included proliferating cell nuclear antigen (PCNA), p53, Cdk2, cyclin E, cyclin D1, Cdk6 and the large subunit of pol II (Figures 4B and 5A). We found that the expression of all of these proteins was comparable in both $Mat1^{-/-}$ and $Mat1^{wt}$ cells. These observations suggest that the loss of mammalian Mat1 does not deregulate transcription or translation globally and that Mat1 specifically regulates the steady-state levels of Cdk7 and cyclin H in vivo.

Mat1 modulates pol II CTD phosphorylation

Many studies have implicated the CTD of the large subunit of RNA polymerase II as a substrate of TFIIH kinase activity. We therefore wanted to determine whether the phosphorylation status of the CTD was affected in $Mat1^{-/-}$ cells. This was accomplished by immunofluorescence analysis utilizing monoclonal antibodies recognizing specific phospho-epitopes of pol II (Figure 5).

Using an antibody (8WG16) that is specific for non-phosphorylated Ser2 of the CTD but that also recognizes partially phosphorylated pol II (Thompson et al., 1989), we were able to visualize the majority of cellular pol II. We found that both $Mat1^{wt}$ and $Mat1^{-/-}$ nuclei immuno-

stained comparably throughout the nucleus, excluding nucleolar compartments (Figure 5A). This observation indicated that expression of pol II was unaffected by the loss of Mat1.

Phosphorylation of Ser5 of the heptapeptide repeat was visualized using an antibody (H14) specific for this phospho-epitope (Patturajan et al., 1998). Immuno-staining of Mat1 wild-type cells with H14 revealed fine punctate immunoreactivity throughout the nucleus (Figure 5B and C). Individual cells of an outgrowth often showed varying degrees of intensity of H14 signal, with some cells exhibiting very intense staining (Figure 5B and C). In contrast, $Mat1^{-/-}$ cells revealed a considerable decrease in H14 immunoreactivity (Figure 5B and C). Although some degree of variability in H14 staining was also noted in $Mat1^{-/-}$ cells, we never observed any cells exhibiting the high levels of Ser5 immunoreactivity that had been noted in wild-type cells.

We then looked at the phosphorylation status of Ser2 using an antibody (H5) specific for this phospho-epitope (Patturajan et al., 1998). As had been observed with H14 staining, $Mat1^{wt}$ cells exhibited a finely particulate staining throughout the nucleoplasm that, unlike the H14 staining, did not vary significantly from one cell to another (Figure 5D and E). Immunostaining of $Mat1^{-/-}$ cells with H5 revealed a dramatic decrease in signal when compared with $Mat1^{wt}$ control cells (Figure 5D and E). Additionally, in ~30% of the $Mat1^{-/-}$ cells (Figure 5D and E; 67 of 231 cells scored), we observed intense H5 immunoreactivity localizing to 20–30 discreet nuclear domains. A similar staining pattern in $Mat1^{wt}$ control cells was only noted in a single nucleus of several hundred scored.

Functional de novo transcription and translation in $Mat1^{-/-}$ cells

The apparent discrepancy between our observation that multiple proteins were detected at wild-type levels and the observed reduction in pol II CTD phosphorylation prompted us to assay more directly the ability of $Mat1^{-/-}$ cells to engage in de novo transcription and translation. To this end, a cytomegalovirus (CMV) promoter-driven green fluorescent protein (GFP) expression plasmid was micro-injected into outgrown cells at day 6.0 in culture (equivalent to 10.5 days post-coitum). Injected cells were returned to an incubator and assayed for GFP expression by fluorescence microscopy the following day. Regardless of genotype, all microinjected cells exhibited strong expression of the GFP protein (Figure 6). This result showed that $Mat1^{-/-}$ cells were comparable with control cells in their ability to engage in de novo transcription and translation of the CMV-GFP expression plasmid.

Mat1 is required for S phase entry in trophoblast giant cells

DNA synthesis in trophoblast giant cells occurs by endoreduplication whereby successive rounds of G and S phases proceed without intervening mitosis (Barlow et al., 1972; Gardner, 1983). As a result, endocycling giant cells acquire vast quantities of DNA in their nuclei, thereby becoming polyploid (Varmuza et al., 1988). Throughout our analysis of outgrown cells, it was noted that $Mat1^{-/-}$ nuclei were consistently smaller in size and stained less intensely with the DNA stain Hoechst 33342

A

B
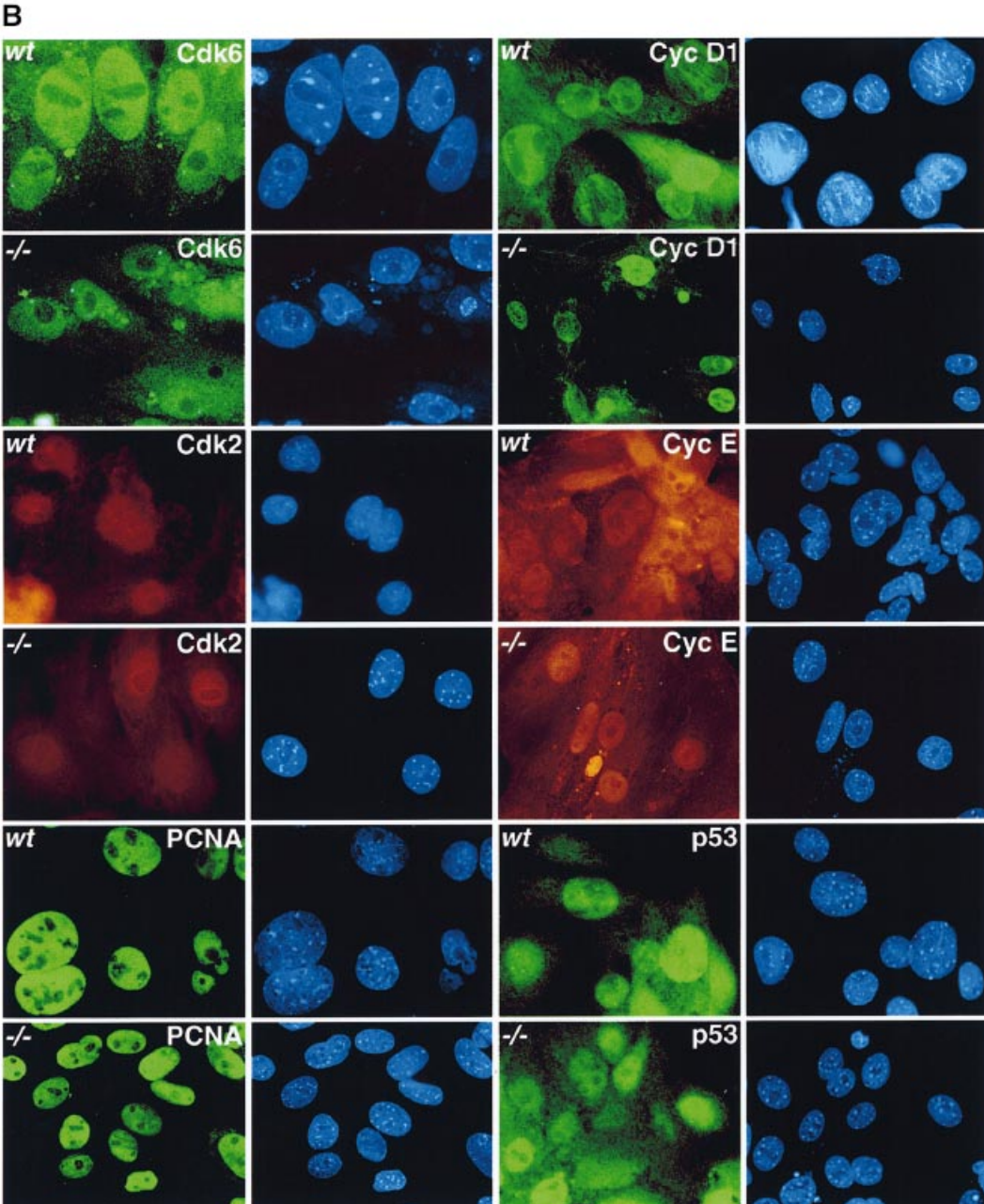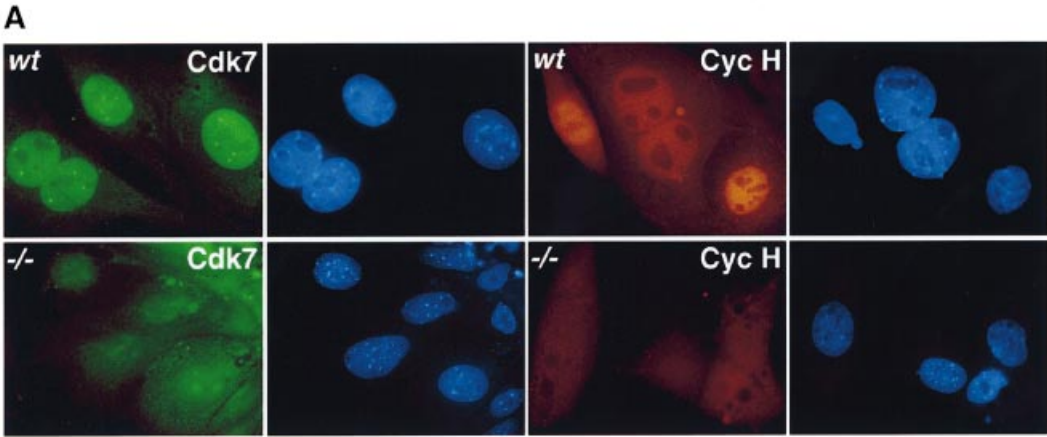
than control nuclei (compare $Mat1^{-/-}$ and $Mat1^{wt}$ nuclei in Figures 3–6 and inset in Figure 7). These observations suggested that the $Mat1^{-/-}$ cells had reduced amounts of DNA in their nuclei.

In order to examine this possibility in detail, the DNA content of outgrown cells and control mouse embryo fibroblasts (MEFs) was quantified by measuring the fluorescence intensity of Hoechst 33342-stained nuclei from normalized digital images [Figure 7; $n(MEF) = 3146$, $n(Mat1^{-/-}) = 171$, $n(Mat1^{wt}) = 362$]. This analysis revealed the DNA content of the $Mat1^{-/-}$ cells to be heterogeneous and was on average greater than the 2C and 4C amounts measured for the MEFs (Figure 7). This indicated that the majority of $Mat1^{-/-}$ cells had engaged in endoreduplication and become polyploid. However, comparison with $Mat1^{wt}$ cells uncovered a dramatic reduction of DNA content in the $Mat1^{-/-}$ cells (Figure 7). This observation indicated that although the $Mat1^{-/-}$ trophoblasts had entered cycles of endoreduplication, they were nonetheless compromised in some aspect of the endocycle.

We reasoned that the lower DNA content in $Mat1^{-/-}$ trophoblasts could reflect either that the endocycle was progressing more slowly than in $Mat1^{wt}$ counterparts or, alternatively, it could reflect an arrest of the endocycle. To differentiate between these possibilities, E3.5 blastocysts and blastocyst outgrowths (day 4.5 in culture equivalent to 9 days post-coitum) were cultured in the presence of bromodeoxyuridine (BrdU; for 15 and 46 h, respectively) and then immunostained with an antibody specific for BrdU to identify cells that had undergone DNA synthesis during the labeling period (Figure 8). Incorporation of BrdU into blastocyst stage nuclei revealed no difference between the $Mat1^{-/-}$ and $Mat1^{wt}$ embryos, both of which showed extensive labeling of ICM and trophectoderm cells (Figure 8A). In contrast, while the vast majority of giant cells readily incorporated BrdU in control outgrowths, no BrdU-positive cells were detected in the $Mat1^{-/-}$ outgrowths (Figure 8B). These results demonstrate that the $Mat1^{-/-}$ cells were unable to enter S phase and were arrested in the endocycle.

Discussion

Mat1 is an essential gene

Studies in yeast and *Drosophila* have indicated that Cdk7–cyclin H–Mat1 homologs are essential genes (Valay *et al*., 1995; Faye *et al*., 1997; Larochelle *et al*., 1998). It was therefore surprising that although disruption of the murine *Mat1* gene leads to embryonic lethality, the timing of the lethality was relatively advanced in terms of murine development. We observed that $Mat1^{-/-}$ embryos inherit a reservoir of maternal Mat1 protein (and possibly mRNA) that diminishes with time as development proceeds. Depletion of maternal Mat1 is likely to reflect the half-life of Mat1 protein as well as the fact that the protein is diluted to ever increasing numbers of nuclei as embryonic cells divide. The coincidence of the peri-

implantation lethality of $Mat1^{-/-}$ embryos with the depletion of observable Mat1 therefore suggests that protein levels are reduced to a point no longer capable of sustaining a vital Mat1 function, and lethality ensues.

Mat1 is required for mitotic proliferation and for endoreduplication: implications for Cak activity

Our blastocyst outgrowth experiments indicated that mitotic ICM cells required Mat1 for survival while post-mitotic trophoblast giant cells did not. This suggests that Mat1 is essential for some aspect of the mitotic cell cycle. As *Drosophila* Cdk7 has been demonstrated to be required for activation of mitotic Cdk–cyclins (Larochelle *et al*., 1998), it is tempting to speculate that the inability of murine $Mat1^{-/-}$ ICM cells to proliferate might also reflect a defect in the activating phosphorylation of mammalian mitotic Cdks.

The switch from the mitotic cell cycle to the endocycle consisting of alternating S and G phases is coupled to several realignments of the cell cycle machinery. Most notably, oscillating cyclin E activity, presumably in complex with Cdk2, has emerged as the critical regulator of endocycles. In *Drosophila*, this is achieved by oscillation of cyclin E protein levels (Knoblich *et al*., 1994; Sauer *et al*., 1995; Lilly and Spradling, 1996). Cyclin E activity also appears to be critical for murine trophoblast giant cell endocycles as constitutive levels of cyclin E lead to arrested endoreduplication (Singer *et al*., 1999). Our data demonstrate that Mat1 is required for progression of murine trophoblast cells through the endocycle. As Cdk7–cyclin H–Mat1 activates Cdk2–cyclin E complexes *in vitro*, the endocycle arrest we have described could reflect a defect in the activating phosphorylation of Cdk2–cyclin E. Formal proof of this hypothesis, however, requires further biochemical examination that the paucity of cells in our experimental system unfortunately does not allow.

Mat1 loss leads to destabilization of Cdk7 and cyclin H and disruption of TFIIH kinase

Mat1 has been described variably as an assembly or targeting factor for the Cdk7–cyclin H–Mat1 kinase. Our data provide *in vivo* evidence that the steady-state levels of both Cdk7 and cyclin H are Mat1 dependent. The decrease in Cdk7 and cyclin H levels we have observed is probably due to the disruption of the trimeric complex and subsequent destabilization of Cdk7 and cyclin H proteins. This observation, together with the evidence indicating that Mat1 mediates the interaction of Cdk7 and cyclin H with core TFIIH (Rossignol *et al*., 1997; Busso *et al*., 2000; Schultz *et al*., 2000), suggests that loss of Mat1 would effectively disrupt TFIIH kinase activity. Destabilization of core TFIIH does not appear to accompany loss of Mat1, as suggested by the viability of $Mat1^{-/-}$ cells as well as the apparent transcriptional integrity of these cells.

Fig. 4. Mat1 regulates steady-state expression of Cdk7 and cyclin H. (**A**) Steady-state levels of Cdk7 and cyclin H in $Mat1^{-/-}$ cells. Wild-type (wt) or $Mat1^{-/-}$ (–/–) blastocyst outgrowths were analyzed by immunofluorescence with antibodies against Cdk7 or cyclin H as indicated, and co-stained with Hoechst 33342 (400× magnification). (**B**) Immunofluorescence analysis of wild-type (wt) and $Mat1^{-/-}$ (–/–) blastocyst outgrowths with the indicated antibodies and co-stained with Hoechst 33342.
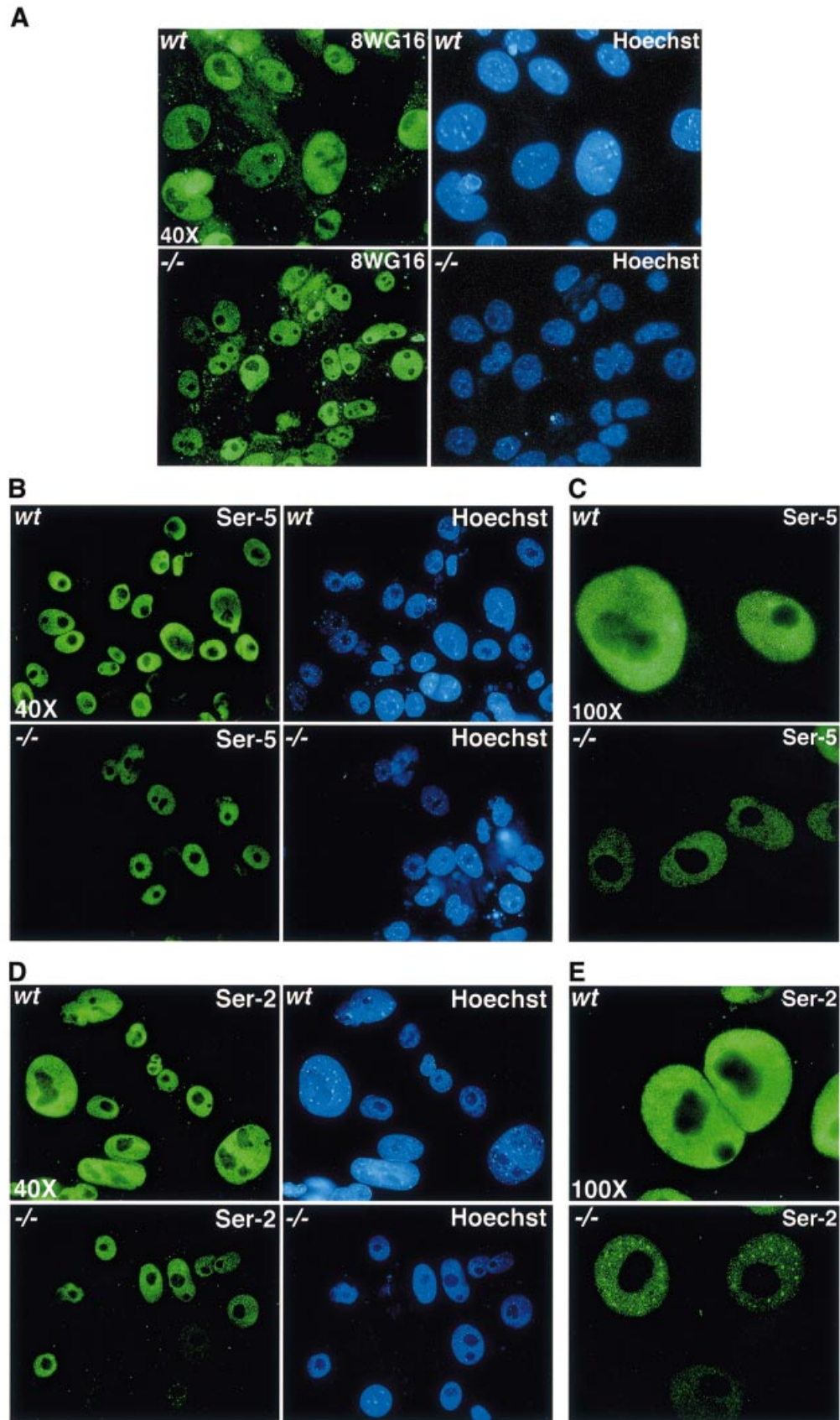
Fig. 5. Mat1 modulates CTD phosphorylation. (**A**) Immunofluorescence analysis of wild-type (wt) and *Mat1⁻/⁻* (–/–) blastocyst outgrowths with RNA polymerase II large subunit antibody 8WG16 co-stained with Hoechst (400× magnification). (**B** and **C**) Immunofluorescence analysis as in (A) using the monoclonal antibody H14 recognizing pol II phosphorylated on Ser5 of the CTD heptapeptide repeat [400× (B) and 1000× (C) magnification]. (**D** and **E**) Immunofluorescence analysis as in (A) using the monoclonal antibody H5 recognizing pol II phosphorylated on Ser2 of the CTD heptapeptide repeat [400× (D) and 1000× (E) magnification]. Note the intense localization of Ser2 to discrete foci in the *Mat1⁻/⁻* micrograph.

Mat1 modulates RNA polymerase II CTD phosphorylation in vivo

The two residues within the heptapeptide repeat sequence of the CTD believed to be the most highly modified *in vivo* are Ser2 and Ser5 (reviewed in Dahmus, 1996). *In vitro*, amino acid substitution of synthetic heptapeptide repeats has been used principally to link TFIIH kinase activity to phosphorylation of Ser5 (Gebara *et al.*, 1997; Hengartner *et al.*, 1998; Sun *et al.*, 1998; Trigon *et al.*, 1998). Our data provide evidence that mammalian Mat1 mediates phosphorylation of pol II on Ser5 to a great extent *in vivo*. The fact that most but not all cellular Ser5 phosphorylation is depleted suggests the existence of additional CTD kinases with *in vivo* affinity for this residue.

We have also observed a decrease in total cellular Ser2 phosphorylation in *Mat1*^{−/−} cells, suggesting that TFIIH kinase might also catalyze the phosphorylation of Ser2 *in vivo*. Interestingly, a recent report utilizing chromatin immunoprecipitation in yeast showed that Ser2 phosphorylation is associated predominantly with the 3′ ends of actively transcribed loci while TFIIH kinase is believed to dissociate from the nascent transcript shortly after promoter clearance (Komarnitsky *et al.*, 2000). Thus the decrease in Ser2 phosphorylation that we observed in the *Mat1*^{−/−} cells could be an indirect consequence of Mat1 loss rather than a direct defect in TFIIH-catalyzed Ser2 phosphorylation. If so, our data are consistent with a model in which TFIIH-mediated kinase activity during promoter clearance would in some way regulate the activity of Ser2 kinase(s) that then act during elongation. Such regulation could involve the direct activation of Ser2 kinases by TFIIH kinase. Alternatively, TFIIH may serve to enhance the substrate affinity of Ser2 kinases by phosphorylating Ser5 during promoter clearance.

Is Mat1-mediated TFIIH kinase function required for pol II transcription?

Surprisingly, disruption of murine *Mat1* was not accompanied by a detectable decrease in transcription or translation in *Mat1*^{−/−} cells despite apparent defects in CTD phosphorylation. This could be inferred indirectly by observing that the steady-state levels of several cellular proteins (including short-lived cyclin D1) were found to be comparable in both mutant and control cells. More directly, *Mat1*^{−/−} cells were capable of *de novo* transcription and translation of a microinjected reporter plasmid. This status of apparent transcriptional integrity was maintained several days after the cells arrested in the
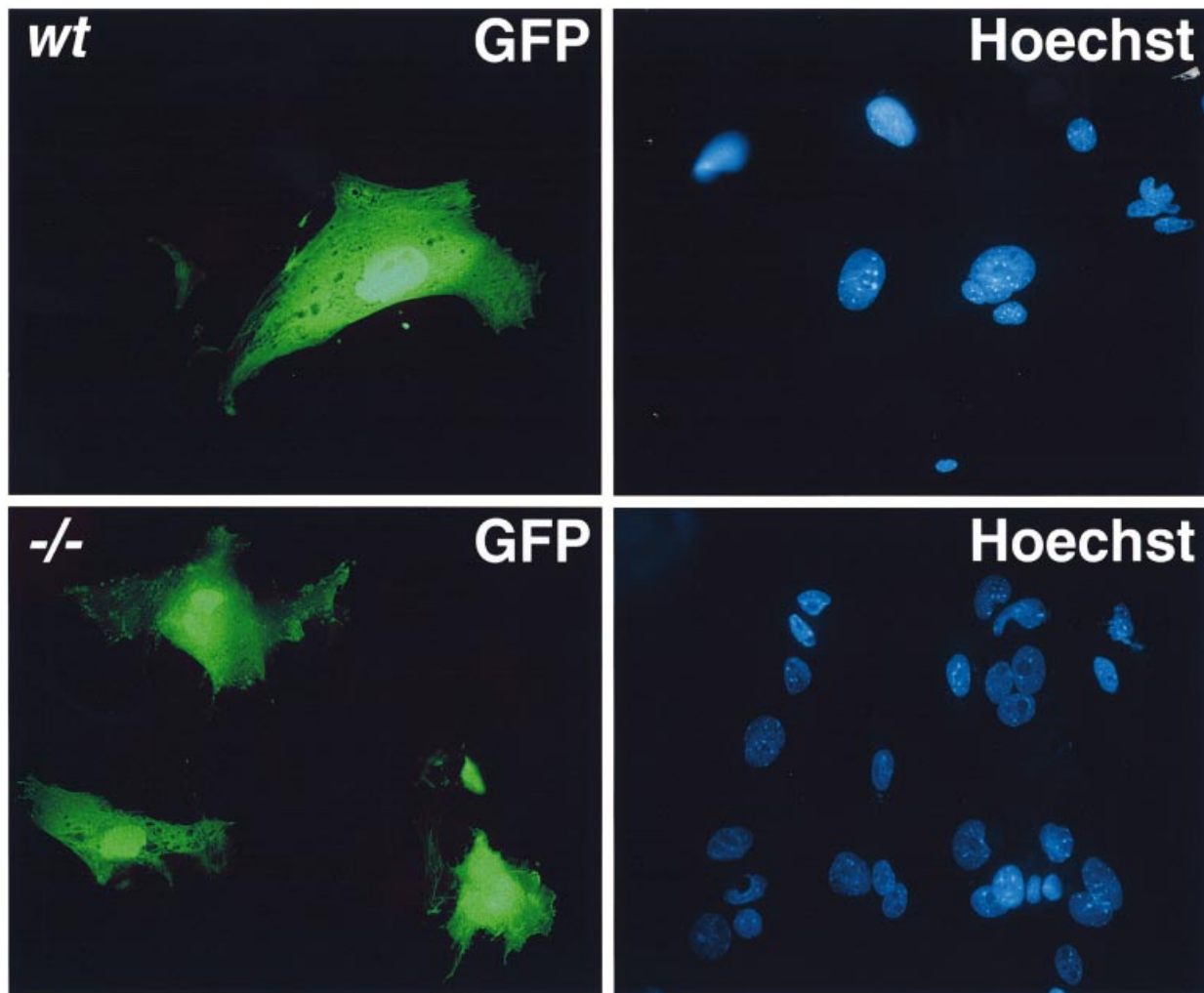

Fig. 6. *De novo* transcription and translation in *Mat1*^{−/−} cells. Fluorescence microscopy analysis of GFP expression in wild-type (wt) and *Mat1*^{−/−} (−/−) cells 1 day after microinjection with pEGFP-N2 plasmid counterstained with Hoechst (200× magnification).
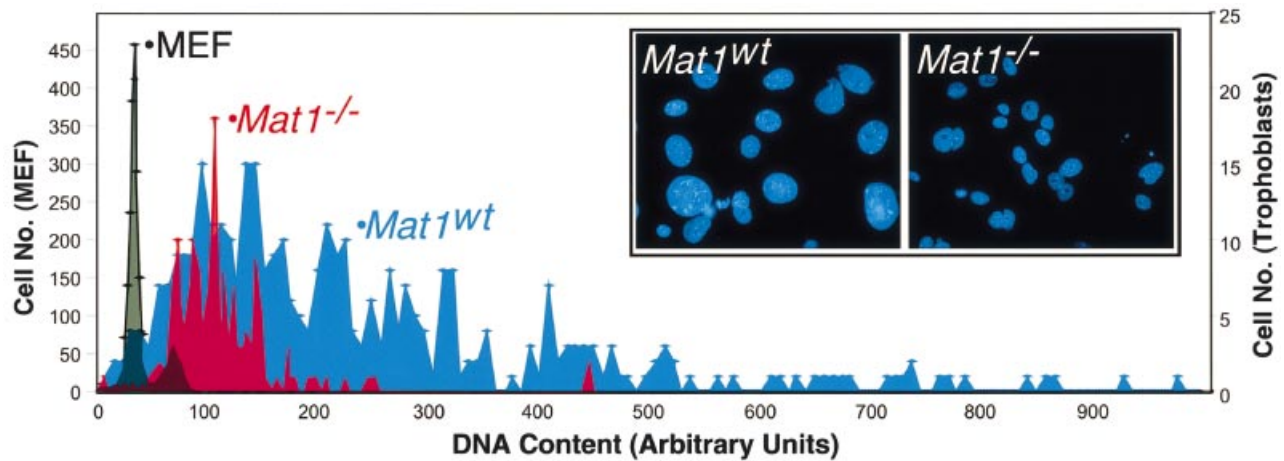
Fig. 7. Defective endoreduplication in *Mat1*−/− trophoblast giant cells. DNA content in mouse embryo fibroblasts (MEFs), wild-type (wt) and *Mat1*−/− (–/–) blastocyst outgrowths. Quantitation was performed from digital images of Hoechst-stained nuclei (such as those shown in the inset). Numbers of cells used for quantitation are indicated on the left for MEFs and on the right for trophoblasts.
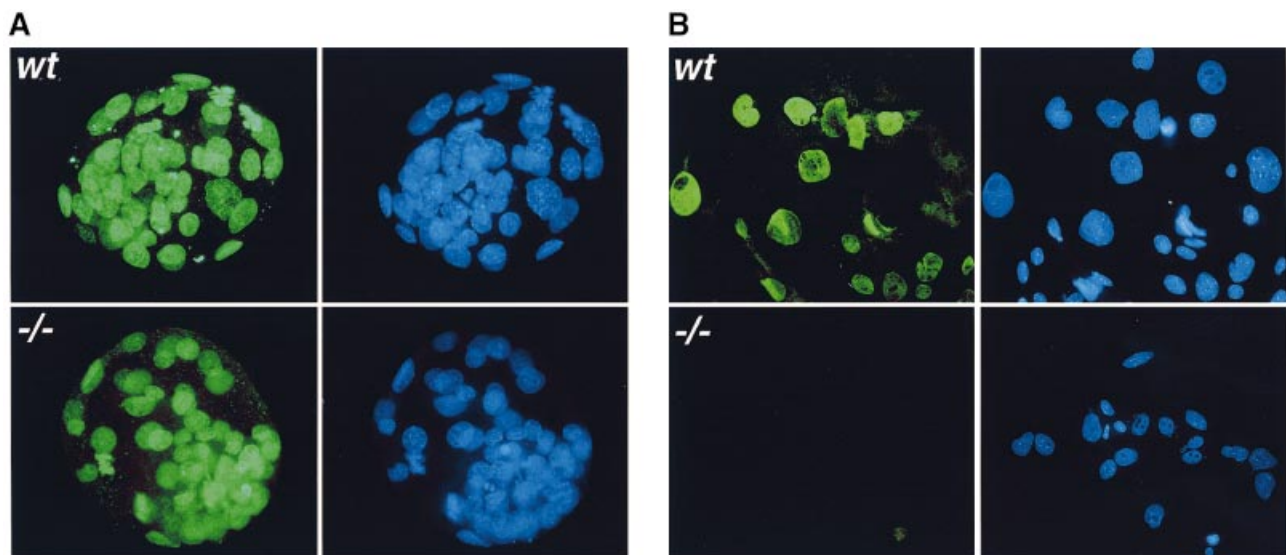

Fig. 8. Endocycle arrest in *Mat1*−/− trophoblast giant cells. (**A**) DNA synthesis in *Mat1*−/− blastocysts. BrdU incorporation for 16 h of wild-type (wt) and *Mat1*−/− (–/–) blastocysts visualized by α-BrdU immunofluorescence and co-stained with Hoechst (400× magnification). (**B**) DNA synthesis in *Mat1*−/− trophoblasts. BrdU incorporation for 46 h of wild-type (wt) and *Mat1*−/− (–/–) trophoblasts visualized as in (A) (200× magnification).

endocycle, which appeared to occur concomitantly with loss of maternally provided Mat1 protein.

Our results differ from those obtained in budding yeast, where loss of TFIIH kinase activity leads to an extremely rapid and near total loss in the transcription of the vast majority of *S.cerevisiae* genes (Holstege *et al.*, 1998). Accordingly, the transcriptional deficit accompanying the disruption of *Mat1* or *Cdk7* homologs in *S.cerevisiae* rapidly leads to loss of viability (Valay *et al.*, 1995; Faye *et al.*, 1997). Our results suggest that the strict dependence of pol II transcription on TFIIH kinase activity in yeast is not maintained in murine trophoblast giant cells, perhaps reflecting unexplored differences in the role of TFIIH kinase in yeast and mammals.

It will be interesting to determine whether the phenotypes associated with Mat1 loss that we describe are specific to early embryonic cell lineages. In this regard, preliminary data utilizing a Cre recombinase-mediated loxP conditional disruption of *Mat1* suggest that the Mat1 functions described herein may represent a more general feature of mammalian cells as adult post-mitotic lineages are viable for many months *in vivo* in the absence of Mat1 (N.Korsisaari, D.J.Rossi, M.Henkemeyer and T.P.Mäkelä, unpublished data).

Materials and methods

Targeting of murine Mat1 and PCR genotyping

A 129-Sv mouse genomic library (Stratagene) was screened with a ^{32}P-labeled probe from a human *MAT1* cDNA corresponding to the open reading frame. Two identified positive clones were subjected to restriction mapping and sequencing, leading to the identification of a single exon corresponding to nucleotides 242–394 of the murine *Mat1*

cDNA (DDBJ/EMBL/GenBank accession No. U35249) with large introns on both sides. A target vector was constructed by inserting 3.0 kb *Hind*III, and 3.2 kb *Kpn*I–*Eco*RI genomic fragments flanking the targeted exon into a conditional replacement vector (pDELBOY-3X; http://www.hi.helsinki.fi/ tm/pub/Del/Delboy3x.jpg) derived from pK-11 (kind gift of Drs E.Meyers and G.Martin). *Xho*I-linearized vector was electroporated into the R1 ES cell line (Nagy *et al.*, 1993), and gancyclovir and G418 double-resistant clones were isolated (Joyner, 1993). Homologous recombination was screened for by Southern blotting with 5′ and 3′ external probes, both of which were diagnostic upon *Sac*I digestion of genomic DNA. Three out of 800 clones confirmed with both probes and cells from one of these lines (clone 6.85) were injected into C57/BL6 blastocysts. Three chimeric males were identified to be transmitting the targeted allele by Southern blotting and by PCR genotyping. Mice were maintained on inbred (129-Sv), mixed (129-Sv X CD1) and outbred (CD1 >90%) backgrounds with no observable difference in phenotype. PCR genotyping was achieved with the following primers: M7, CAACTAAAGATACCTCCAGCTCC; M10, GCCCTATTTCAGGAGCCAGTCC; and N4, GTCAGTTTCATA-GCCTGAAGAACG. M7 and M10 amplify a 190 bp wild-type band, while N4 and M10 amplify a 310 bp mutant band.

Blastocyst outgrowth

Mat1^{+/−} heterozygous animals were naturally mated and fertilized embryos were flushed from the oviducts at E2.5 with M2 medium (Sigma). Embryos were transferred into M16 medium (Sigma) at 37°C, 5% CO_2 under mineral oil (Sigma) for 48 h. Expanded blastocysts were then transferred to 24-well plates seeded with glass coverslips and maintained in ES medium (Joyner, 1993) for up to 7 days.

BrdU labeling

E3.5 blastocysts and outgrowths at day 5 in culture were cultured in ES cell medium supplemented with 10 µM BrdU (Sigma) for 16 and 46 h, respectively. Cells were then fixed in paraformaldehyde (PFA) for 20 min. Following washing in phosphate-buffered saline (PBS), the cells were treated with 0.5 M HCl for 30 min, after which immunofluorescence was performed as described below.

Immunofluorescence and antibodies

For immunofluorescence experiments, we used rabbit polyclonal antibodies against Mat1 (FL-309), Cdk2 (M-2), cyclin E (M-20) and mouse monoclonal antibodies against PCNA (PC10), cyclin D1 (72–13G) and Cdk7 (C-4), all from Santa Cruz. In addition, mouse monoclonal antibodies were used against pol II epitopes H5, 8WG16 (Research Diagnostics) and H14 (Covance), against p53 (Pab 122; Pharmingen), against Cdk6 (Neomarkers), against BrdU (Roche Molecular Biochemicals) and against Mat1 (Transduction Labs). The Cdk7 and cyclin H antibodies were rabbit polyclonal antibodies (Mäkelä *et al.*, 1995). Secondary antibodies included fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM (Chemicon), FITC-conjugated anti-mouse IgG (Roche Molecular Biochemicals) and rhodamine-conjugated anti-rabbit IgG (Roche Molecular Biochemicals) all used at 1:200 dilution in 5% fetal calf serum (FCS) and 0.5% saponin in PBS. Immunofluoresence was performed variably depending upon the antibodies used. In general, outgrowths grown on glass coverslips were fixed for 20 min in 1.75–3.5% PFA followed by a permeabilization with either 0.5% Triton X-100 in PBS for 20 min or with cold methanol for 2 min and blocking for 30 min in 10% FCS in PBS. Primary and secondary antibodies were left on cells for 1 h, after which DNA was stained with Hoechst 33342 (0.5 g/ml) for 5 min. Each step was followed by three washes of 5 min with PBS. Coverslips were mounted onto glass slides for epifluorescence microscopy with a Zeiss Axiophot 2 microscope and images were photographed with a Sensicam 12 bit digital camera (PCO CCD Imaging). DNA content was quantified from the digital images with NIH Image software.

Microinjection and detection of GFP

Cells cultured in ES cell medium buffered with 10 mM HEPES pH 7.4 were injected with 25 ng/µl of pEGFP-N2 (Clontech), 0.1 mg/ml Texas Red dextran (mol. wt 70 000 Da; Molecular Probes) in water. Injection duration was 0.5 s with 120 hPa pressure using an Eppendorf microinjector and transjector (Eppendorf 5246) and a Zeiss Axiovert 135 microscope. After injection, cells were washed twice with ES medium, returned to a 37°C incubator with 5% CO_2 and analyzed 24 h later by fluorescence microscopy.

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