THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 45, Issue of November 8, pp. 43216–43223, 2002

Printed in U.S.A.

Targeted Deletion of the *Tsg101* Gene Results in Cell Cycle Arrest at G_1/S and p53-independent Cell Death*

Received for publication, July 30, 2002
Published, JBC Papers in Press, August 29, 2002, DOI 10.1074/jbc.M207662200

Andrea Krempler‡, MaLinda D. Henry, Aleata A. Triplett, and Kay-Uwe Wagner§

From the Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Nebraska Medical Center, Omaha, Nebraska 68198-6805

The tumor susceptibility gene 101 (*Tsg101*) was originally discovered in a screen for potential tumor suppressors using insertional mutagenesis in immortalized fibroblasts. To investigate essential functions of this gene in cell growth and neoplastic transformation, we derived primary mouse embryonic fibroblasts from *Tsg101* conditional knockout mice. Expression of Cre recombinase from a retroviral vector efficiently downregulated Tsg101. The deletion of *Tsg101* caused growth arrest and cell death but did not result in increased proliferation and cellular transformation. Inactivation of p53 had no influence on the deleterious phenotype, but *Tsg101*$^{-/-}$ cells were rescued through expression of exogenous Tsg101. Fluorescence-activated cell sorting, proliferation assays, and Western blot analysis of crucial regulators of the cell cycle revealed that Tsg101 deficiency resulted in growth arrest at the G_1/S transition through inactivation of cyclin-dependent kinase 2. As a consequence, DNA replication was not initiated in Tsg101-deficient cells. Our results clearly demonstrate that Tsg101 is not a primary tumor suppressor in mouse embryonic fibroblasts. However, the protein is crucial for cell proliferation and cell survival.

Tumor susceptibility gene 101 (Tsg101) was originally identified as a tumor suppressor gene that causes transformation of NIH3T3 cells when this gene is inactivated by a random antisense strategy (1). Cloning and sequencing of the human *TSG101* cDNA revealed that the mouse and human genes encode proteins with 94% similarity (2). The similarity is even higher because both the mouse and human cDNAs encode 10 additional amino acids at the N terminus that are entirely conserved. Implications for a significant role of human TSG101 in tumor formation came from mapping of the gene locus to chromosome 11 p15.1-p15.2. This genomic region is known to be associated with a loss of heterozygosity in several human tumor types (3, 4). However, rearrangements and somatic mutations within the *TSG101* gene locus could not be identified (5). Aberrant splice variants are found frequently in different tumor types, suggesting a role for TSG101 in cellular transformation (5–9). Cloning and sequencing of the human and the mouse *Tsg101* gene structure later showed that many of the previously described aberrant transcripts were in fact alternative splice forms generated solely by exon skipping (10). The significance of these Tsg101 splice forms in cell function and tumorgenesis remains elusive.

Several reports show that Tsg101 may influence cell cycle control. Tsg101 is mainly localized in the cytoplasm, but upon cell cycle progression it can be found in the nucleus and the mitotic spindle. Depletion of Tsg101 by specific antibodies results in cell cycle arrest (11). Interestingly, strong overexpression of Tsg101 *in vitro* also leads to the inhibition of cell division and cell death, suggesting that the amount of Tsg101 within a cell is critical for its function (11, 12). Tsg101 has several conserved protein domains that can exhibit cell cycle regulatory functions. The C-terminal coiled-coil domain of Tg101 was predicted to interact with the cell growth regulating protein stathmin (13), and this region of Tsg101 is also involved in a potential co-repressor activity (14, 15). Furthermore, a proline-rich domain in Tsg101 was found to act as an activation domain in transcriptional regulation (1). The N-terminal region of Tsg101 is similar to a domain found in inactive forms of ubiquitin-conjugating enzymes, proteins that can serve as dominant negative regulators of cell cycle control (16, 17). This domain is suggested to interact directly with the key cell cycle regulator Mdm2, thereby stabilizing the protein (18). The cyclin-dependent kinase inhibitor p21$^{waf1/cip1}$ was also identified as a potential binding partner for Tsg101 (19) although the exact interacting region between Tsg101 and p21$^{waf1/cip1}$ has not been determined.

Analysis of the murine *Tsg101* gene promoter revealed features of a housekeeping gene (10). In mice, mRNA transcripts were identified in all embryonic and adult tissues examined, even as early as embryonic 1-cell and 2-cell stages. Based on these findings we hypothesized that deletion of Tsg101 using a conventional knockout approach would result in embryonic lethality (10). This assumption was confirmed recently in a mouse model that carries a deletion of exons 8 and 9 (21). Therefore, we have chosen the Cre-*loxP* strategy to study the loss-of-function of Tsg101 during proliferation and differentiation *in vivo* and *in vitro*. In this report, we show that Tsg101 is an essential factor for cell growth, cell cycle regulation, and cell survival. Tsg101-deficient cells arrest and die during G_1/S transition. The cell cycle arrest is caused by inactivation of cyclin-dependent kinases (cdk) 2. We also demonstrate that p53 is not a mediator of cell death in Tsg101-deficient cells. Furthermore, the *Tsg101* null mutation does not result in neoplastic transformation suggesting that Tsg101 is not a primary tumor suppressor gene.

MATERIALS AND METHODS

Construction of Retroviral Expression Vectors—The *Tsg101* cDNA (a kind gift of Dr. Cohen, Stanford University) was amplified by PCR,

* This work was supported by National Institutes of Health NCI Grant RO1CA93797 (to K. U. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Deutsche Forschungsgemeinschaft stipend KR 2107/1-1.

§ To whom correspondence should be addressed: Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Rm. 8009, Omaha, NE 68198-6805. Tel.: 402-559-3288; Fax: 402-559-4651; E-mail: kuwagner@unmc.edu.

cloned into the pJ3H vector in-frame with the hemagglutinin (HA)[1] tag, and sequenced. This vector was cut with *Sal*I, blunted, and cut again with *Eco*RI to release the tagged *Tsg101* cDNA. This fragment was cloned directionally into the blunted *Bam*HI and sticky *Eco*RI sites of the retroviral vector pBabe-puro. A pBabe-GFP retroviral vector was cloned by releasing the puromycin coding sequence with *Hin*dIII and *Cla*I from pBabe-puro and replacing it with the enhanced green fluorescent protein cDNA, which was amplified by PCR from pEGFP-N1 (Clontech, Inc.). To generate a retroviral vector expressing the HA-tagged Tsg101 protein and the GFP marker from the same construct, we exchanged the entire SV40-puro cassette (*Sal*I-*Nhe*I fragment) of the pBabe-HA-Tsg101-puro vector with the SV40-EGFP cassette from the pBabe-GFP plasmid. The *Cre* coding sequence from vector pBS185 (a kind gift of Dr. Sauer, NIDDK, to Dr. Hennighausen, NIDDK) was cloned as an *Xho*I-*Mlu*I(blunt) fragment into the *Eco*RV-*Xho*I sites of pZero (Invitrogen). The pBabe-Cre-puro retroviral vector was constructed by subcloning the *Cre recombinase* cDNA as an *Xho*I(blunt)-*Eco*RI fragment into the *Bam*HI(blunt) and *Eco*RI sites of pBabe-puro. We have used the helper-free 293Φ packaging cell line (a kind gift of Dr. Ouellette, University of Nebraska Medical Center) to generate replication deficient viral particles of our pBabe derivatives. Human papilloma E6 retrovirus was a kind gift of Dr. Sgagias, University of Nebraska Medical Center.

Cell Culture—The construction of the $Tsg101^{fl/fl}$ targeting construct and the production of transgenic animals will be described elsewhere.[2] Mouse embryonic fibroblasts (MEFs) from 14.5-day-old $Tsg101^{fl/fl}$, $Tsg101^{+/+}$, or $Tsg101^{fl/fl}p53^{-/-}$ embryos were explanted and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 10 μg/ml gentamycin, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). For retroviral infection, cells at passage 2–4 were plated at a density of $3–4 \times 10^5$ cells per 10-cm culture dish. Infection with retroviral vectors was performed in the presence of 10 μg/ml Polybrene (Sigma). Forty-eight hours later, cells were selected in complete medium containing 7 μg/ml puromycin (Sigma). Immortalized $Tsg101^{fl/fl}$ cells were obtained from primary MEFs through application of a 3T3 protocol.

Fluorescence-activated Cell Sorting Analysis and Cell Proliferation Assays—For flow cytometry analysis, $1–2 \times 10^6$ cells were harvested at various time points after puromycin selection, pelleted, and washed with 1× phosphate-buffered saline. Cells were fixed in ice-cold 70% ethanol for 30 min, washed again in phosphate-buffered saline, and stained with propidium iodide as described previously (22). Stained cells were analyzed with FACScalibur (BD Biosciences). The software packages CELLquest (BD Biosciences) and Modfit LT (Verity) were used for data acquisition and data analysis, respectively. To determine the proliferative capacity, cells were incubated for 1 h with 5-bromo-2-deoxyuridine (BrdUrd) labeling reagent, fixed in ethanol/acetic acid, and incorporated BrdUrd was visualized by immunohistochemistry according to the manufacturer's protocol (Amersham Biosciences). The percentage of BrdUrd-positive cells was estimated by counting 1000 cells of two slides of each genotype. Staining for chromatin-tethered proliferating cellular nuclear antigen (PCNA) was performed as described previously (23). Fluorescein isothiocyanate-labeled PCNA antibody (PC-10, Santa Cruz Biotechnology) was used at a 1:700 dilution. Cells were counterstained with 4,6-diamidino-2-phenylindole, and 1000 cells per coverslip were counted to estimate the percentage of cells that contain DNA bound PCNA protein. MTT growth assay was performed as described earlier (24). MTT was obtained from Sigma. Of each cell type 2×10^4 cells were seeded in triplicate in 96-well microtiter plates. Absorbance was measured at 570 nm with an Elx 808 (Bio-Tek Instruments) enzyme-linked immunosorbent assay reader.

Southern Hybridization and Northern Blot Analysis—MEFs from 15-cm culture dishes infected with pBabe-Cre or pBabe were pelleted and digested at 56 °C in 100 μl of cell lysis buffer (50 mM Tris, pH 8.0, 50 mM EDTA, 100 mM NaCl, 1% SDS, 500 μg/ml proteinase K). The genomic DNA was phenol/chloroform extracted, and 15 μg was digested with *Xba*I at 37 °C overnight. The DNA was separated on a 0.5% agarose gel, blotted onto a nylon membrane (GeneScreen Plus, PerkinElmer Life Sciences), and probed with the ^{32}P-labeled 3′ internal probe. Membranes were washed and exposed for 6 h to a Kodak X-Omat AR film.

Twenty micrograms of total RNA, which was isolated from cell pellets of two 15-cm culture dishes as described previously (10), was separated on a 1.5% formaldehyde gel and transferred to a GeneScreen Plus membrane. *Tsg101* transcripts were detected by probing the membranes with ^{32}P-labeled full-length mouse *Tsg101* cDNA.

Reverse Transcriptase-PCR of p53—Total RNA was isolated from cell pellets of two T75 culture flasks as described previously (10). Reverse transcription was performed with the Superscript II RT system (Invitrogen) using poly(dT) (18) as a primer. Primers p53fwd 5′-ATGACTGCCATGGAGGAGTCAC-3′ and p53rev 5′-GCAGAGGCAGTCAGTCTGAGTC-3′ were then used to amplify the 1166-bp region of the p53 cDNA. PCR products were gel-purified and sequenced in both directions on a Beckman-Spinco CEQ2000XL automated sequencer.

Western Blot Analysis and cdk2 Kinase Assay—Cell pellets were lysed on ice for 30 min in 1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 0.4 units/ml aprotinin, 1 mM NaF, 0.1 mM sodium orthovandate. Protein was quantified using a Bradford assay (Pierce). Twenty to fifty micrograms of protein per lane was resolved by SDS-PAGE and blotted onto polyvinylidene fluoride membranes (Invitrogen). The membranes were blocked for 1 h in 1× TBS, 0.05% Tween 20, and 5% dry milk. Subsequently, membranes were incubated with primary antibodies in blocking buffer at 4 °C overnight, washed three times for 15 min in washing buffer (1× TBS, 0.05% Tween 20), and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies in blocking buffer. Membranes were washed again three times in washing buffer and once for 15 min in 1× TBS without Tween 20. Protein bands were detected using the ECL chemiluminescence kit for Western blot analysis (Amersham Biosciences) according to the manufacturer's instructions. Membranes were stripped using 0.2 M NaOH for consecutive detection of various proteins. The following antibodies were used: α-ActB (I-19), α-Tsg101 (C-2), α-cyclin E (M-20), α-cyclin B1 (M-20), α-cyclin A (C-19), and α-p27 (F-8) from Santa Cruz Biotechnology as well as α-p21 (SX118) from Pharmingen and α-p19^{ARF} (Ab-1) from Oncogene at a 1:1000 dilution. The α-cyclin D1 antibody (1:3000 final dilution) was a kind gift from Dr. Diehl (University of Pennsylvania). Horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology and used at a 1:1000 dilution. Cdk2 kinase assay was performed using standard procedures and cdk2 (M-2) antibody from Santa Cruz Biotechnology. Histone H1 (1 μg/μl) and protein A were purchased from Sigma.

RESULTS

Cre Recombinase Expressed from a Retroviral Vector Efficiently Deletes the Tsg101 Locus—We generated conditional knockout mice to delete the first coding exon and part of the *Tsg101* promoter through Cre-mediated recombination (Fig. 1*A*). A detailed description of the construction and the phenotypes of the knockout mice will be published elsewhere.[2] Initially, we tested the efficiency of Cre-mediated recombination at the floxed *Tsg101* locus. We hypothesized that the deletion of exon 1 and the promoter region would efficiently inhibit transcription and translation of *Tsg101*. For this purpose, we established primary embryonic fibroblast cultures (MEFs) from the conditional knockout mice, and infected them with a retroviral vector expressing Cre recombinase (pBabe-Cre). The unmodified retroviral vector (pBabe) served as a negative control. Infected cells were selected by adding puromycin to the growth medium. Cells were harvested 3 to 4 days after infection to isolate DNA, RNA, and total protein. Cre-infected cells showed a single 2.1-kb *Xba*I fragment in Southern blots, indicating complete recombination of the targeted locus (Fig. 1*B*). This leads to undetectable levels of *Tsg101* mRNA and protein in these cells (Fig. 1, *C* and *D*). In contrast, high levels of Tsg101 were observed in controls. Interestingly, the originally described NIH3T3 *Tsg101* knockout cell line (SL6) (1), which was intended to serve as a negative control, showed a significant amount of Tsg101 protein, suggesting that the conventional

[1] The abbreviations used are: HA, hemagglutinin; GFP, green fluorescent protein; MEFs, mouse embryonic fibroblasts; BrdUrd, 5-bromo-2-deoxyuridine; PCNA, proliferating cellular nuclear antigen; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FACS, fluorescence-activated cell sorting; CKI, cyclin-dependent kinase inhibitor; cdk, cyclin-dependent kinases; MMTV, mouse mammary tumor virus.

[2] K.-U. Wagner, A. Krempler, Y. Qi, K. Park, M. D. Henry, A. A. Triplett, G. Riedlinger, E. B. Rucker III, and L. Henninghausen, submitted for publication.

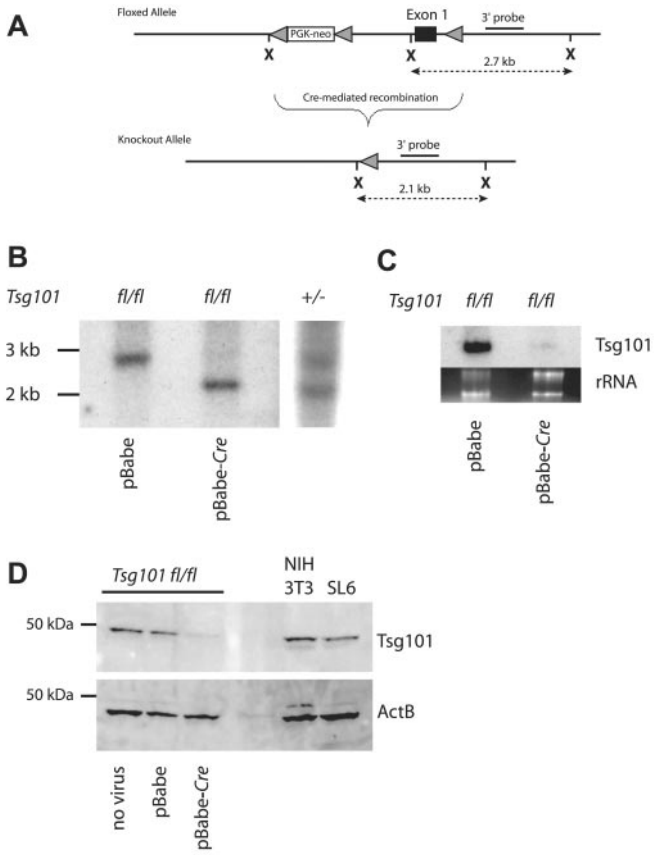


FIG. 1. **Cre-mediated excision and transcriptional down-regulation of the *Tsg101* gene in MEFs, which were derived from *Tsg101*$^{fl/fl}$ embryos.** *A*, diagram of the Cre-mediated deletion of the *Tsg101* gene. *X*, *Xba*I. *B*, verification of the recombination event by *Xba*I Southern analysis in *Tsg101*$^{fl/fl}$ cells (pBabe or pBabe-Cre) as illustrated in *A*. DNA from a tail biopsy of a *Tsg101* heterozygous knockout animal served as a positive control (+/−). The null allele was generated previously by transmitting the floxed allele through the female germline of *MMTV-Cre* (*line A*) transgenic mice (Footnote 2). The 2.1-kb *Xba*I fragment represents the recombined *Tsg101* null allele. Both the wild type and unrecombined floxed allele are represented by a 2.7-kb *Xba*I restriction fragment. *C*, Northern blot analysis. *Tsg101* mRNA was almost undetectable in *Tsg101*$^{fl/fl}$ MEFs expressing Cre recombinase (pBabe-Cre), whereas the infection with a control virus had no effect on *Tsg101* transcription (pBabe). *D*, Western blot analysis. Tsg101 protein was almost undetectable in MEFs expressing Cre recombinase (pBabe-Cre). The infection with a retroviral control vector (pBabe) had no significant effect on Tsg101 translation or its stability compared with untreated control cells (no virus). The SL6 cell line and its parental NIH3T3 cell line (1) were used as negative and positive controls for Tsg101 protein expression. SL6 cells still express significant amounts of the Tsg101 protein. β-Actin (*ActB*) served as a loading control.

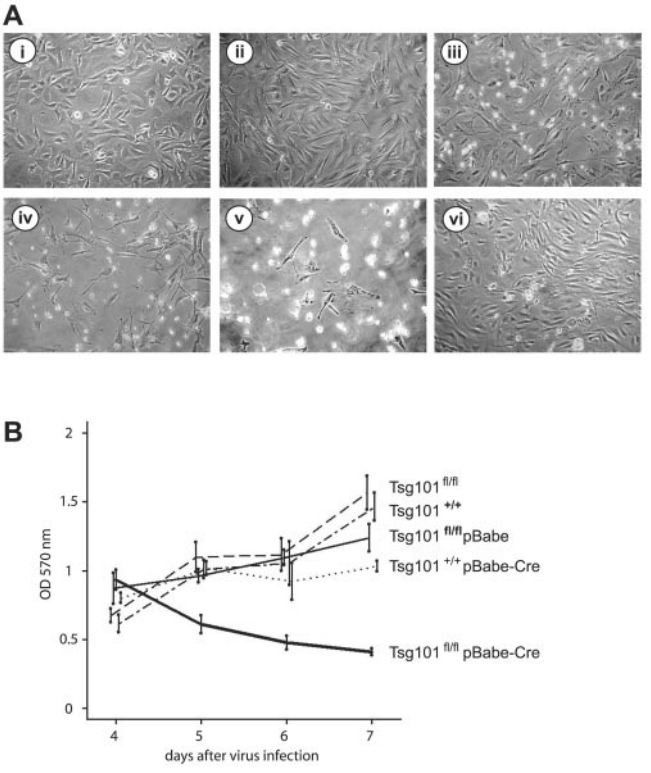


FIG. 2. **Reduced cell growth and cell death associated with Tsg101 deficiency.** *A*: *i*, untreated *Tsg101*$^{fl/fl}$ MEFs in culture. *ii*, 24 h after infection with a retroviral vector expressing Cre recombinase. *iii-v*, 3, 5, and 7 days after infection with a Cre vector and selection with puromycin. *vi*, MEFs derived from wild type embryos (*Tsg101*$^{+/+}$) were infected with the same Cre expressing viral vector and selected for 7 days with puromycin. Low level expression of Cre recombinase and selection with puromycin did not result in significant growth inhibition in wild type control cells. *B*, growth curves of pBabe and pBabe-Cre-infected *Tsg101*$^{fl/fl}$ and *Tsg101*$^{+/+}$ MEFs as determined by MTT color assay. The $A_{570\ nm}$ values corresponding to cell numbers for *Tsg101* knockout cells in the assay decreased, whereas cell numbers in the controls increased within 4 days. *Error bars* correspond to standard deviations.

antisense approach did not entirely inhibit translation of this protein (Fig. 1*D*). The results demonstrate that both floxed alleles in the MEFs were successfully converted into null alleles in the presence of Cre recombinase. Subsequently, this leads to a complete depletion of Tsg101 protein in these cells.

Tsg101 Deficiency Results in Growth Arrest and Cell Death but Not in Neoplastic Transformation—It was reported previously that the functional inhibition of *Tsg101* in immortalized fibroblasts leads to accelerated growth and instant neoplastic transformation (1). Therefore, we hypothesized that the homozygous deletion of *Tsg101* in MEFs from conditional knockout mice should result in transformation and the generation of *Tsg101*-deficient tumor cell lines. *Tsg101*$^{fl/fl}$ MEFs and their wild type controls (*Tsg101*$^{+/+}$) were infected with pBabe-Cre and grown for a prolonged period in the presence of puromycin. In contrast to the previous report, the conditional deletion of the *Tsg101* gene was deleterious (Fig. 2*A*). As determined by MTT growth assay, cell numbers in *Tsg101*$^{fl/fl}$ MEFs infected with Cre recombinase rapidly declined within 7 days after introduction of the virus (Fig. 2*B*). After prolonged incubation (>10 days) very few surviving *Tsg101*$^{fl/fl}$ cells were observed. Clonal expansion of these cells revealed that they had acquired puromycin resistance without expressing Cre recombinase. A recombined *Tsg101* null allele was not detected by PCR in these clones.[3] Wild type (*Tsg101*$^{+/+}$) MEFs infected with the same Cre retroviral vector and selected with puromycin only exhibited slight growth retardation within the first days of selection and resumed normal cell growth thereafter (Fig. 2, *A* and *B*). This suggests that a low-level expression of Cre from the retroviral vector did not inhibit cell growth in our experimental setting. Therefore, toxicity of Cre recombinase can be excluded as a possible factor for the deleterious phenotype caused by *Tsg101* deficiency in the conditional knockout model.

Growth Inhibition and Cell Death Are Caused Solely by Tsg101 Deficiency—Besides cytotoxicity of Cre recombinase, it was feasible to hypothesize that the deleterious phenotype in Tsg101-deficient MEFs was caused by an unintentional inhibition of genes on chromosome 7 in proximity to the *Tsg101* locus. It is known that the insertion of the phosphoglycerine kinase-

[3] A. Krempler, M. D. Henry, A. A. Triplett, and K.-U. Wagner, unpublished observation.

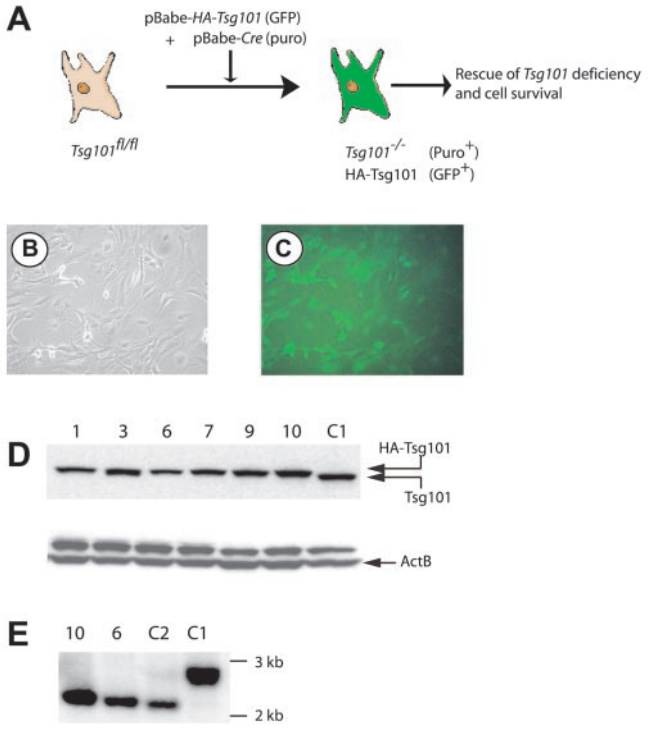
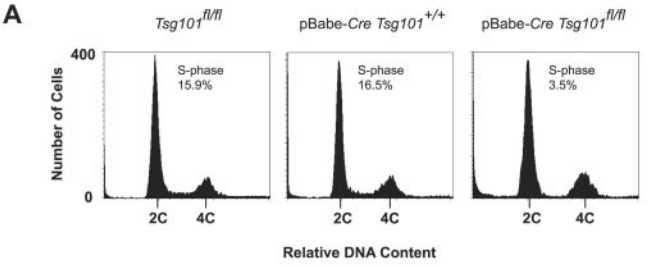


FIG. 3. **Restoration of cell proliferation and survival of cells lacking the endogenous *Tsg101* gene through expression of a HA-tagged Tsg101 protein from a retroviral vector.** *A,* experimental design. Homozygous floxed MEFs were simultaneously infected with retroviral vectors expressing Cre recombinase (puromycin resistance) and an HA-tagged mouse Tsg101 protein (GFP selection marker). *B* and *C,* puromycin-resistant clones expressing the green fluorescent protein were isolated, expanded, and subjected to Western analysis. *D,* Western blot analysis of Tsg101 and β-actin (*ActB*) as loading control on puromycin and GFP positive clones (*numbers 1, 3, 6, 7, 9*, and *10*). *C1*, control cells expressing *Tsg101* from the endogenous locus. The HA-tagged Tsg101 protein is slightly larger than the wild type protein. *E, Xba*I Southern blot analysis to verify the presence of two *Tsg101* knockout alleles on the genomic level. *C1*, control cells with two floxed alleles; *C2*, T*sg101*$^{fl/fl}$ controls infected with pBabe-Cre. Two selected clones numbered 6 and 10 completely lack endogenous *Tsg101* expression.



B

days after infection	Genotype Tsg101	G0/G1	% S	G2/M
4 d	fl/fl pBabe	*68.7*	15.9	*15.4*
4 d	+/+ pBabe-Cre	*66.8*	16.5	*16.7*
4 d	fl/fl pBabe-Cre	*72.2*	**3.5**	*24.3*
5 d	fl/fl pBabe-Cre	70.0	**5.7**	24.3
6 d	fl/fl pBabe-Cre	72.2	**4.6**	23.1
7 d	fl/fl pBabe-Cre	69.5	**5.4**	25.1
	fl/fl starved (24 h)	71.4	**4.7**	23.9

FIG. 4. **Effects of Tsg101 deficiency on cell cycle progression.** *A,* fluorescence-activated cell sorting analysis of the DNA content. The number of Tsg101-deficient cells (*Tsg101*$^{fl/fl}$, pBabe-Cre) in S-phase is significantly reduced compared to untreated cells (*Tsg101*$^{fl/fl}$). Expression of Cre recombinase had no significant effect on cell cycle progression in wild type control MEFs (*Tsg101*$^{+/+}$). *B,* percentages of cells in different stages of the cell cycle over time. pBabe-Cre-infected *Tsg101*$^{fl/fl}$ cells contained only a few cells in S-phase (*bold*). The distribution of cells in *Tsg101* knockout cells resembled the *numbers* observed in serum-starved MEFs.

neomycin cassette can alter the transcriptional regulation of other loci in the vicinity of the insertion site (25). Therefore, we performed a rescue experiment to demonstrate that Tsg101 deficiency alone was the cause for the severe growth retardation and cell death in the conditional knockout model (Fig. 3*A*). *Tsg101*$^{fl/fl}$ MEFs were infected simultaneously with two viral vectors expressing Cre recombinase (pBabe-Cre) or the full-length mouse *Tsg101* cDNA (pBabe-HA-Tsg101-GFP). Individual puromycin-resistant clones were expanded and analyzed for the presence of GFP, which correlated with expression of exogenous Tsg101 (Fig. 3, *B* and *C*). Western blot analysis with an anti-Tsg101 antibody verified the expression of exogenous Tsg101 in all GFP-positive clones. Transgenic Tsg101 that was fused to a HA tag had a slightly different mobility. This feature was useful to distinguish the exogenous HA-tagged protein from endogenous Tsg101. Endogenous Tsg101 was not detected in these individual clones (Fig. 3*D*). Exogenous Tsg101 from a transgenic vector is able to inhibit translation and stability of the endogenous protein (26). To verify that these clones had lost both endogenous *Tsg101* alleles, we performed Southern blot analysis on two selected clones (clone number 6, low expression; and number 10, high expression of HA-Tsg101). Both clones showed a single 2.1-kb band indicating complete recombination of the endogenous *Tsg101* locus (Fig. 3*E*). In summary, the expression of the HA-tagged Tsg101 from a retroviral vector was able to revert the deleterious effects of the loss of both endogenous *Tsg101* alleles. This rescue experiment suggests the deleterious phenotype was caused solely by Tsg101 deficiency. Therefore, inhibition of genes other than *Tsg101* can be excluded as a possible cause for growth inhibition and cell death in the *Tsg101* conditional knockout model.

Loss of Tsg101 Affects Cell Cycle Progression—Tsg101 deficiency in the conditional knockout model resulted in the perturbation of cell growth and in cell death. Because it was suggested earlier that this gene plays a role in cell growth (11, 18, 21), we hypothesized that Tsg101 deficiency leads to cell cycle arrest at one of the major checkpoints before cell death is initiated. We examined *Tsg101*$^{fl/fl}$ MEFs expressing Cre recombinase and their controls (pBabe-Cre *Tsg101*$^{+/+}$ and *Tsg101*$^{fl/fl}$) using fluorescence-activated cell sorting (FACS) analysis of the DNA content (Fig. 4, *A* and *B*). Tsg101-deficient cultures contained significantly fewer cells in S-phase (3.5% in the knockouts *versus* 15.9 and 16.5% in the controls). This reduction in the number of cells in S-phase persisted for at least 4 days (Fig. 4*B*). Moreover, the low number of replicating cells was comparable with the percentage of S-phase cells in serum-starved MEFs, which are normally arrested in G_1/G_0 of the cell cycle. Neither the retroviral transfer nor the expression of Cre had an effect on cell cycle progression in the controls.

To address whether Tsg101-deficient cells enter S-phase and proceed through DNA replication, we examined the incorporation of BrdUrd. Tsg101-deficient cells exhibited a dramatic reduction in BrdUrd incorporation (Fig. 5*A*). Whereas 23 to 26% of the nuclei were labeled in the controls, only 5% of the *Tsg101* knockout cells had incorporated BrdUrd. In addition, the intensity of the BrdUrd staining in most of these *Tsg101*$^{-/-}$ cells was lower than in the controls, indicating incomplete replication of the genome. Successful initiation of DNA replication requires the assembly of several proteins at the DNA replication fork prior to binding of DNA polymerase. One of the key proteins in these complexes is PCNA, which recruits DNA-

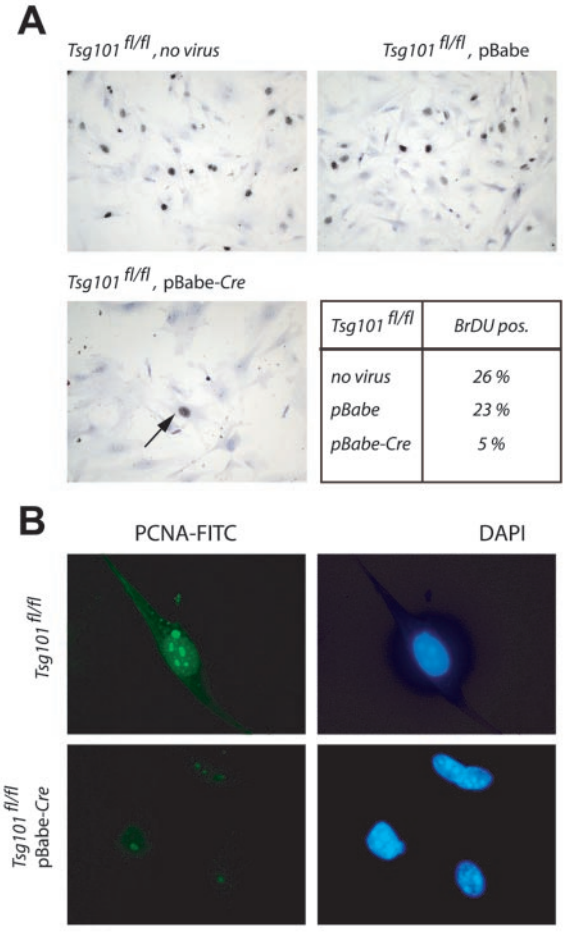


FIG. 5. **Analysis of cell proliferation in Tsg101-deficient MEFs.** *A,* BrdUrd labeling of untreated *Tsg101*$^{fl/fl}$ cells (no virus) as well as *Tsg101*$^{fl/fl}$ cells infected with the pBabe control vector or pBabe-Cre. The *arrow* points to one of the few BrdUrd-labeled cells in the *Tsg101* knockout MEFs. *B,* immunocytochemistry of DNA-bound PCNA protein using a fluorescein isothiocyanate (*FITC*)-labeled antibody against PCNA in *Tsg101*$^{fl/fl}$ controls and *Tsg101*$^{fl/fl}$ MEFs expressing Cre recombinase. DNA bound PCNA was virtually absent from the *Tsg101* knockout MEFs. 4,6-Diamidino-2-phenylindole (DAPI) staining was used to visualize nuclei of Tsg101-deficient cells and their controls.

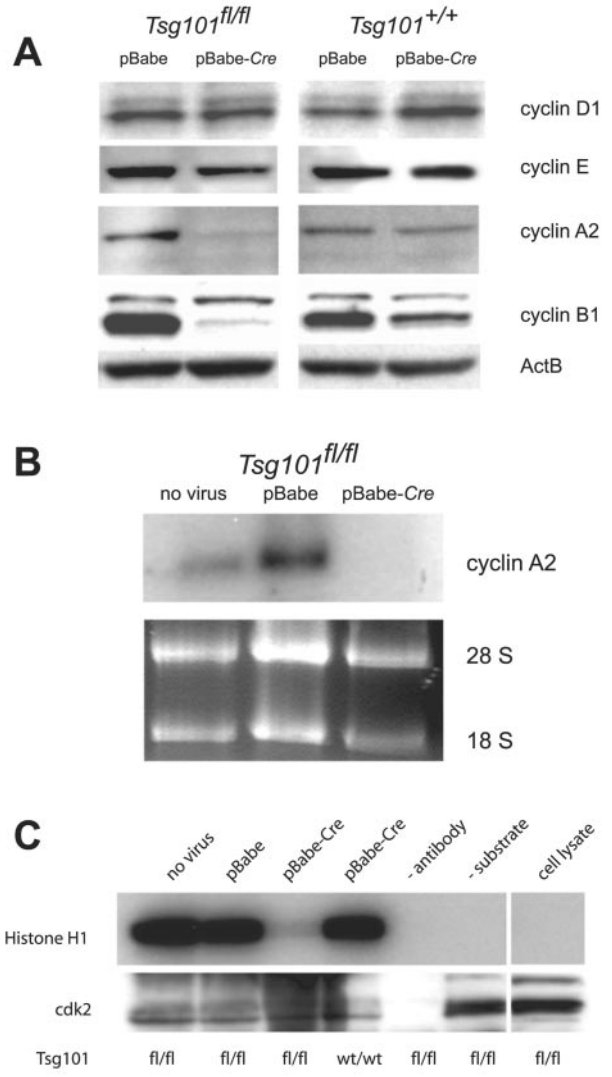


FIG. 6. **Analysis of cyclin expression and cdk2 activity.** *A,* Western blot analysis of G_1, G_1/S, S, and M phase cyclins. The S and M phase cyclins A2 and B1 were markedly down-regulated in Tsg101-deficient cells. *B,* Northern blot showing the expression of cyclin A2. Cyclin A2 mRNA was not detectable in Tsg101-deficient cells. Equal loading of RNA from *Tsg101*$^{fl/fl}$ and Cre-expressing *Tsg101*$^{fl/fl}$ cells was demonstrated by visualization of 28 S and 18 S RNA in the agarose gel used for blotting. *C,* cdk2 kinase assay using histone H1 as a substrate. *Tsg101*$^{fl/fl}$ MEFs infected with pBabe-Cre vector showed reduced cdk2 activity. Equal protein loading was confirmed by reprobing the blot with the cdk2 antibody used for immunoprecipitation.

polymerase δ (27). Tethering of PCNA to DNA is therefore an excellent indicator for initiation of DNA replication. To test whether the DNA replication complex was assembled at the replication fork, we stained Tsg101-deficient and control cells with fluorescein isothiocyanate-conjugated anti-PCNA antibody. Unbound PCNA was removed from the cells by an extraction technique (23). DNA bound PCNA was significantly reduced in *Tsg101*$^{-/-}$ cells. Less than 2% of all conditional knockout cells contained DNA bound PCNA, whereas 14% of the cells were labeled in the controls (Fig. 5*B*). These numbers were consistent with the BrdUrd staining results and with the FACS analysis shown in Fig. 4. Therefore, loss of Tsg101 in primary fibroblasts results in cell cycle arrest late in G_1 or at the transition from G_1 to S and subsequently prevents initiation of DNA replication.

Tsg101 Knockout Cells Exhibit Reduced cdk2 Activity—Cell cycle progression is dependent on the sequential expression of stage-specific cyclins and on the activity of their respective cdks. Based on the FACS analysis, *Tsg101* conditional knockout cells seemed to arrest at the G_1 checkpoint or during G_1/S transition. Therefore, we predicted that the expression of major cyclins, which are responsible for S-phase and G_2/M transition, were deregulated in the Tsg101-deficient cells. In accordance with the observed cell cycle arrest, the G_1 and G_1/S phase cyclins D1 and E did not show any differences between pBabe-Cre infected *Tsg101*$^{fl/fl}$ MEFs and their controls. In contrast, S and M phase cyclins (cyclins A and B) were markedly down-regulated in the knockout cells (Fig. 6*A*). As verified by Northern blot, the down-regulation of cyclin A occurred on the transcriptional level and not through increased degradation of the proteins (Fig. 6*B*).

As cyclin E expression was not altered in the *Tsg101* knockout cells, it was feasible to hypothesize that an inactive cyclin E-associated kinase could be the cause for the profound cell cycle arrest in *Tsg101*$^{-/-}$ cells. Cdk2 associates with cyclin E and is activated shortly before entry into S phase (28, 29). To study the activity of cdk2 in Tsg101 knockout cells, we performed a histone H1 kinase assay with immunoprecipitated cdk2. The ability of cdk2 to phosphorylate histone H1 was greatly impaired in pBabe-Cre-infected *Tsg101*$^{fl/fl}$ MEFs,

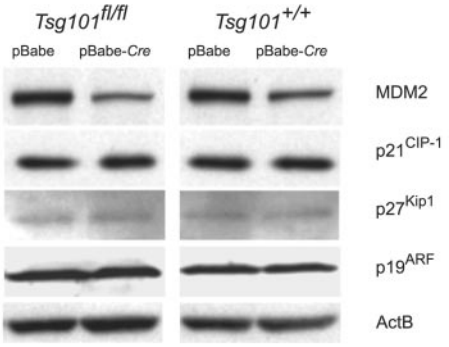


FIG. 7. **Analysis of cell cycle regulator expression.** *A,* Western blot analysis of Mdm2, $p19^{ARF}$, and the main cdk inhibitory proteins $p21^{waf1/cip1}$ and $p27^{Kip1}$. Blots were reprobed with β-actin (*ActB*) to confirm protein loading. Expression of cdk inhibitory proteins was largely unaffected in *Tsg101* knockout cells.

whereas cdk2 from control lysates could strongly phosphorylate histone H1 (Fig. 6*C*). Therefore, the cell cycle arrest imposed by loss of Tsg101 cells is a consequence of reduced cdk2 kinase activity in our primary cells.

Functional Inhibition of p53 Does Not Rescue the Severe Phenotype Caused by Tsg101 Deficiency—The activity of the cyclin E-cdk2 complex is regulated by various mechanisms. One of the negative regulators of this complex is $p21^{waf1/cip1}$ (30). The expression of this cell cycle inhibitor is regulated by p53 (31, 32). Therefore, we hypothesized that p53 and its downstream mediators are involved in cell cycle arrest and cell death caused by Tsg101 deficiency. This preliminary assumption was supported by recent findings that suggest a possible role of Tsg101 in the stabilization Mdm2, which is a negative regulator of p53 (18). In addition, lack of Tsg101 was reported to arrest cells through up-regulation of both the p53 protein and $p21^{waf1/cip1}$ mRNA (21). To support our hypothesis and to verify initial observations by Ruland and coworkers (21), we determined the protein levels of Mdm2, $p21^{waf1/cip1}$, $p27^{Kip1}$, and $p19^{ARF}$ in *Tsg101* conditional knockout cells (Fig. 7). Mdm2 protein levels were decreased in the knockout and the wild type controls. Therefore, the expression of Cre recombinase itself had a slightly negative effect on Mdm2 expression. However, $p21^{waf1/cip1}$ and $p27^{Kip1}$ protein levels remained unaffected in both the knockouts and controls. In addition, we did not observe a change in $p19^{ARF}$ protein levels in Tsg101-deficient cells. $P19^{ARF}$ sequesters Mdm2 and it is therefore an important upstream regulator of the p53 pathway (33). More importantly, it was shown that wild type p53 negatively regulates $p19^{ARF}$ expression through a negative feedback loop (34, 35). A significant up-regulation of p53 in response to Tsg101 deficiency should have resulted in an up-regulation of $p21^{waf1/cip1}$ (downstream of p53) and simultaneously a down-regulation of $p19^{ARF}$ (upstream of p53) to release more Mdm2 that subsequently is able to inhibit p53. Because neither the expression of upstream nor downstream effectors of p53 signaling were altered in the *Tsg101* conditional knockouts, our findings did not support a dominant role for the Mdm2/p53 feedback loop in growth inhibition and cell death as a consequence of Tsg101 deficiency.

To examine whether p53 was involved in the death of *Tsg101* knockout cells, we studied the loss of Tsg101 in three different cell lines with impaired p53 function (Fig. 8*A*). As mentioned earlier, immortalization of primary cells is usually caused by mutations and inactivation of key cell cycle regulators such as p53. We immortalized $Tsg101^{fl/fl}$ MEFs following a 3T3 protocol. After the cells had overcome crisis we sequenced p53 reverse transcriptase-PCR products. Sequence analysis revealed an A765C mutation that caused a E255D change in the highly

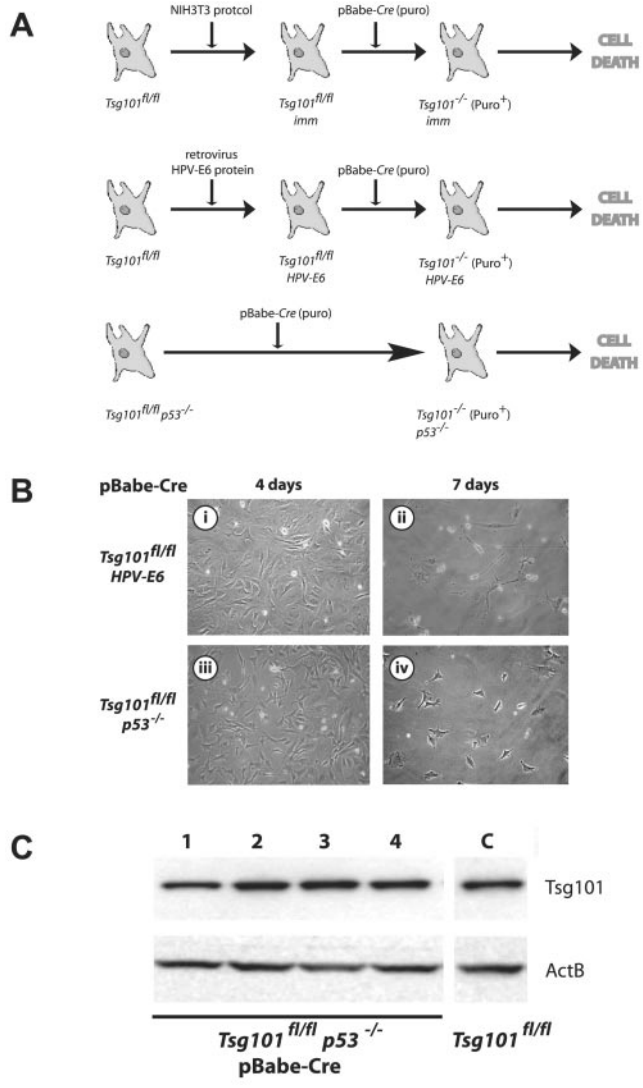


FIG. 8. **Cell death in Tsg101-deficient cells with impaired p53 function.** *A,* different strategies used to obtain p53-deficient $Tsg101^{fl/fl}$ cells. In all three approaches (mutation of p53 through immortalization of $Tsg101^{fl/fl}$ MEFs; infection of $Tsg101^{fl/fl}$ MEFs with human papilloma virus E6 virus; $Tsg101^{fl/fl}p53^{-/-}$ MEFs) deletion of *Tsg101* by Cre-mediated recombination resulted in cell death. *B,* lack of proliferation and cell death in $Tsg101^{-/-}$ p53-deficient cells. *i-iv*, 4 and 7 days after pBabe-Cre infection and puromycin selection of $Tsg101^{fl/fl}$/human papilloma virus E6 and $Tsg101^{fl/fl}p53^{-/-}$ MEFs. Both cell types underwent rapid cell death after deletion of *Tsg101. C,* Western blot analysis of four surviving colonies in $Tsg101^{fl/fl}p53^{-/-}$ cell cultures that were infected with pBabe-Cre and selected with puromycin. The four samples showed normal expression of Tsg101 when compared with a noninfected $Tsg101^{fl/fl}$ cell lysate (*C*).

conserved DNA binding domain of p53 leading to functional inactivation of the protein.[3] As a consequence, cells expressed high levels of p53 but no $p21^{waf1/cip1}$. Infection of this cell line ($Tsg101^{fl/fl}$-imm) with pBabe-Cre resulted in cell death within a few days. Infection of these cells with pBabe had no effect on cell growth. In a second experiment, primary $Tsg101^{fl/fl}$ MEFs were transformed by a retroviral vector harboring the human papilloma virus 16 protein E6, which is best known for mediating the rapid degradation of p53 (for review see Ref. 36). After expansion, the MEF $Tsg101^{fl/fl}$/human papilloma virus-E6 cell line was infected with pBabe-Cre. Again, cells underwent cell death within a few days (Fig. 8*B*), whereas cells infected with pBabe continued proliferation. In a final experiment, $Tsg101^{fl/fl}$ mice were bred into a $p53^{-/-}$ background. MEFs obtained from $Tsg101^{fl/fl}p53^{-/-}$ embryos were infected

with either pBabe-Cre or the control virus. Seven days after infection only very few cells were left in the culture dish containing pBabe-Cre-infected MEFs (Fig. 8*B*). Continued incubation of these cultures resulted in clonal expansion of some remaining cells. However, Western blot analysis on surviving clones revealed that the cells had acquired puromycin resistance without deleting the floxed *Tsg101* locus (Fig. 8*C*). In conclusion, our results demonstrated that p53 did not control cellular pathways leading to cell death in the conditional *Tsg101* knockout model.

DISCUSSION

We developed a conditional knockout model to investigate the tumor suppressive function of the *Tsg101* gene and its involvement in cell cycle regulation. In this report, we used Tsg101-deficient mouse embryonic fibroblasts to study the role of this gene during cell growth and proliferation. We also wanted to verify whether neoplastic transformation occurs as a consequence of Tsg101 deficiency. In contrast to the report by Li and Cohen (1), we were not able to establish tumorigenic cell lines through deletion of *Tsg101*. Instead, we observed cell cycle arrest and rapid cell death. These results suggested that *Tsg101* was not a primary tumor suppressor gene. Our observations *in vitro* were also verified recently in knockout mice that lack *Tsg101* completely or only in selected cell types.[2] Neither haploinsufficiency of Tsg101 ($Tsg101^{+/-}$ mice) nor the Cre-mediated deletion of both alleles in mammary epithelial cells (WAP-Cre $Tsg101^{fl/fl}$) resulted in tumorigenesis by 24 months of age. Our findings were consistent with other reports that suggest an essential role for Tsg101 in proliferation and normal cell function. First, the microinjection of antibodies against Tsg101 led to cell cycle arrest (11). Also, $Tsg101^{\Delta exon\ 8/9-/-}$ mutant mice exhibit a distinct proliferation defect in embryonic tissues as determined by BrdUrd incorporation (21). Recently, small interfering RNAs (38) were used to down-regulate *Tsg101* expression to nearly undetectable levels in 293T (39) and HeLa cells (40). Over a 72-h time course, Garrus and coworkers (39) observed a growth reduction in Tsg101-depleted cells. Moreover, introduction of *Tsg101* small interfering RNAs into human breast cancer cells (MCF-7) was highly toxic when these cells were treated repeatedly for a longer period. Whereas Tsg101-deficient MCF-7 cells die, transfection of other small interfering RNA control vectors had little or no effect on the growth of these cells.[4] In summary, these observations suggest that Tsg101 is important for the growth and survival of primary cells (*e.g.* MEFs and other embryonic cell types) as well as immortalized and transformed cell lines (*e.g.* 293T and MCF-7 cells). Our further studies on transformed $Tsg101^{fl/fl}$ MEFs, which were able to form tumors in nude mice, seemed to support this assumption. The Cre-mediated deletion of *Tsg101* in explanted tumor cells resulted in instant cell death.[3]

Using the conditional knockout model, we were able to demonstrate that the loss of Tsg101 arrests the cell cycle specifically at the G_1/S transition and that the cause for the G_1/S cell cycle arrest in $Tsg101^{-/-}$ cells is an inactive cdk2. The cyclin-dependent kinase 2 interacts directly with cyclin E and cyclin A. The assembly of both complexes is essential for entry into and progression through S phase (28, 29). *Tsg101* knockout cells contain normal levels of cyclin E but lack cyclin A on the transcriptional and protein level. Cyclin A is normally up-regulated during G_1/S transition (41). The cell cycle-controlling element in the cyclin A promoter associates with a multiprotein complex that includes cyclin E/cdk2. Binding of cyclin E alone to the cyclin A promoter is not sufficient for its activation. The complex is dependent on an active kinase (42). Cyclin E and cdk2 were present at normal levels in the Tsg101 conditional knockout cells. Therefore, the cyclin E-cdk2 complex might have assembled at the cyclin A promoter, but they were probably not able to initiate transcription because of an inactive cdk2. The mechanisms that were responsible for the inactivation of cdk2 in the *Tsg101* conditional knockout cells need to be identified. Recently, Oh and coworkers (19) reported that overexpression of Tsg101 from an adenoviral vector causes reduced proliferation of primary keratinocytes through inactivation of cdk2. The authors proposed a mechanism in which Tsg101 is able to bind to and stabilize the major cdk2 inhibitors, $p21^{waf1/cip1}$ and $p27^{Kip1}$, in terminally differentiating keratinocytes. However, this proposed mechanism is not straightforward because Tsg101 had no effect on the stability of $p21^{waf1/cip1}$ in proliferating cells (19). It is possible that the mechanisms, which mediate growth arrest in $Tsg101^{-/-}$ MEFs and the Tsg101 overexpression model, are the same. However, we did not observe a significant change in $p27^{Kip1}$ and $p21^{waf1/cip1}$ protein levels in the conditional knockouts. Several kinases and phosphatases determine the activity of cdk2 (43). Whether any of these activating or inhibiting phosphorylations are involved in the Tsg101-deficient phenotype is currently being investigated.

Whereas Oh and coworkers (19) suggested a direct interaction of Tsg101 with $p21^{waf1/cip1}$, others report an indirect effect of Tsg101 on $p21^{waf1/cip1}$ transcription via the Mdm2-p53 feedback loop (18, 21). Tsg101 possesses an inactive ubiquitin-conjugated enzyme domain near the N terminus (17), encoded by exons 1–5 (10). According to the proposed mechanism by Li and coworkers (18), Tsg101 might act as a dominant negative ubiquitin-conjugated enzyme that inhibits the degradation of Mdm2 (18). The same group of investigators was able to verify their findings in a Tsg101-deficient mouse model that carries a deletion of exons 8 and 9 (21). This mutation, which apparently results in down-regulation of the entire *Tsg101* locus, causes embryonic lethality around E5.5 to E6.5. This deleterious phenotype was mainly the result of a defect in proliferation in a p53-dependent manner. Increased cell death was not reported in this model. The p53 protein and $p21^{waf1/cip1}$ mRNA were up-regulated in the knockouts as determined by immunohistochemistry and PCR. In addition, Ruland and coworkers (21) were able to partially rescue the conventional knockout through introduction of a p53 null mutation into the $Tsg101^{\Delta exon\ 8/9-/-}$ background. Double mutants were reported to survive 2 to 3 days longer. Based on the proposed importance of Tsg101 as a key regulator for Mdm2, this rescue seems to be marginal compared with *Mdm2*/p53 double mutant mice where a knockout of p53 completely rescued embryonic lethality caused by Mdm2 deficiency (20, 37). Theoretically, the direct interaction of Tsg101 with negative regulators of the cell cycle such as $p21^{waf1/cip1}$ or $p27^{Kip1}$ (19) would provide a mechanism for tumor suppressive properties of Tsg101. As a positive regulator of Mdm2, the model proposed by Li and coworkers (18) would categorize Tsg101 as an oncogene and not as a tumor suppressor. Using our conditional knockout model, we attempted to verify the involvement of the Mdm2-p53 feedback loop in mediating the cell cycle arrest caused by Tsg101 deficiency. Various upstream ($p19^{ARF}$, $p16^{Ink4a}$, and Mdm2) and downstream ($p21^{waf1/cip1}$ and $p27^{Kip1}$) targets of p53 exhibited no significant change in gene expression on the protein level in the conditional knockout. Furthermore, neither the functional inhibition of p53 nor the deletion of the p53 gene had a noticeable effect on the deleterious phenotype caused by Tsg101 deficiency. Our observations suggested that cell death caused by a functional inhibition of Tsg101 was mediated through p53 independent mechanisms.

[4] X. Lin and W. Nelson, personal communication.

Taken together, we demonstrated that Tsg101 was an important factor controlling cell cycle regulation and acting as a cell survival factor. Without the Tsg101 protein cells arrest at the G_1/S boundary because of inactive cdk2. Subsequently, they undergo massive and rapid cell death independent of functional p53. In contrast to previous reports, *Tsg101* was not a primary tumor suppressor gene. It did not cause instant neoplastic transformation *in vitro* or *in vivo*. Experiments are underway to determine whether Tsg101 is a modifier for tumor progression in mouse models that are predisposed to cancer. If the functional inhibition of *Tsg101* is able to modify neoplastic transformation, it is very likely that this gene exerts its function through p53 independent mechanisms.

Acknowledgments—We thank Dr. Stanley Cohen (Stanford) for providing the *Tsg101* cDNA as well as SL6 cells and the parental NIH3T3 cell line. We are grateful to Dr. Alan Diehl (University of Pennsylvania) for valuable suggestions and providing the pBabe-puro vector, 293T cells, and the cyclin D1 antibody. We also thank Charles Kuszynski and Linda Wilkie of the UNMC Flow Cytometry Facility for the FACS analysis. Thanks to Drs. Parmender Mehta and Peggy Wheelock (UNMC) for helping us take pictures of the fluorescein isothiocyanate-labeled cells and Joerg Rahnenfuehrer (UC Berkeley) for help in the evaluation of the MTT assay.

REFERENCES

1. Li, L., and Cohen, S. N. (1996) *Cell* **85,** 319–329
2. Li, L., Li, X., Francke, U., and Cohen, S. N. (1997) *Cell* **88,** 143–154
3. Ali, I. U., Lidereau, R., Theillet, C., and Callahan, R. (1987) *Science* **238,** 185–188
4. Reeve, A. E., Sih, S. A., Raizis, A. M., and Feinberg, A. P. (1989) *Mol. Cell. Biol.* **9,** 1799–1803
5. Steiner, P., Barnes, D. M., Harris, W. H., and Weinberg, R. A. (1997) *Nat. Genet.* **16,** 332–333
6. Gayther, S. A., Barski, P., Batley, S. J., Li, L., de Foy, K. A., Cohen, S. N., Ponder, B. A., and Caldas, C. (1997) *Oncogene* **15,** 2119–2126
7. Lee, M. P., and Feinberg, A. P. (1997) *Cancer Res.* **57,** 3131–3134
8. Sun, Z., Pan, J., Bubley, G., and Balk, S. P. (1997) *Oncogene* **15,** 3121–3125
9. Wang, Q., Driouch, K., Courtois, S., Champeme, M. H., Bieche, I., Treilleux, I., Briffod, M., Rimokh, R., Magaud, J. P., Curmi, P., Lidereau, R., and Puisieux, A. (1998) *Oncogene* **16,** 677–679
10. Wagner, K. U., Dierisseau, P., Rucker, E. B., Robinson, G. W., and Hennighausen, L. (1998) *Oncogene* **17,** 2761–2770
11. Zhong, Q., Chen, Y., Jones, D., and Lee, W. H. (1998) *Cancer Res.* **58,** 2699–2702
12. Xie, W., Li, L., and Cohen, S. N. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 1595–1600
13. Sobel, A. (1991) *Trends Biochem. Sci.* **16,** 301–305
14. Watanabe, M., Yanagi, Y., Masuhiro, Y., Yano, T., Yoshikawa, H., Yanagisawa, J., and Kato, S. (1998) *Biochem. Biophys. Res. Commun.* **245,** 900–905
15. Hittelman, A. B., Burakov, D., Iniguez-Lluhi, J. A., Freedman, L. P., and Garabedian, M. J. (1999) *EMBO J.* **18,** 5380–5388
16. Koonin, E. V., and Abagyan, R. A. (1997) *Nat. Genet.* **16,** 330–331
17. Ponting, C. P., Cai, Y. D., and Bork, P. (1997) *J. Mol. Med.* **75,** 467–469
18. Li, L., Liao, J., Ruland, J., Mak, T. W., and Cohen, S. N. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 1619–1624
19. Oh, H., Mammucari, C., Nenci, A., Cabodi, S., Cohen, S. N., and Dotto, G. P. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 5430–5435
20. Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. (1995) *Nature* **378,** 206–208
21. Ruland, J., Sirard, C., Elia, A., MacPherson, D., Wakeham, A., Li, L., Luis, D. L. P., Cohen, S. N., and Mak, T. W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 1859–1864
22. Telford, W. G., King, L. E., and Fraker, P. J. (1991) *Cell Prolif.* **24,** 447–459
23. Sever-Chroneos, Z., Angus, S. P., Fribourg, A. F., Wan, H., Todorov, I., Knudsen, K. E., and Knudsen, E. S. (2001) *Mol. Cell. Biol.* **21,** 4032–4045
24. van de Loosdrecht, A. A., Beelen, R. H., Ossenkoppele, G. J., Broekhoven, M. G., and Langenhuijsen, M. M. (1994) *J. Immunol. Methods* **174,** 311–320
25. Pham, C. T., MacIvor, D. M., Hug, B. A., Heusel, J. W., and Ley, T. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13090–13095
26. Feng, G. H., Lih, C. J., and Cohen, S. N. (2000) *Cancer Res.* **60,** 1736–1741
27. Waga, S., and Stillman, B. (1998) *Annu. Rev. Biochem.* **67,** 721–751
28. Nasmyth, K. (1996) *Science* **274,** 1643–1645
29. Sherr, C. J. (1996) *Science* **274,** 1672–1677
30. Ohtsubo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M., and Pagano, M. (1995) *Mol. Cell. Biol.* **15,** 2612–2624
31. el Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) *Cell* **75,** 817–825
32. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993) *Nature* **366,** 701–704
33. Sherr, C. J. (1998) *Genes Dev.* **12,** 2984–2991
34. Stott, F. J., Bates, S., James, M. C., McConnell, B. B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K. H., and Peters, G. (1998) *EMBO J.* **17,** 5001–5014
35. Robertson, K. D., and Jones, P. A. (1998) *Mol. Cell. Biol.* **18,** 6457–6473
36. Tommasino, M., and Crawford, L. (1995) *Bioessays* **17,** 509–518
37. Montes de Oca, L. R., Wagner, D. S., and Lozano, G. (1995) *Nature* **378,** 203–206
38. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) *Nature* **411,** 494–498
39. Garrus, J. E., von Schwedler, U. K., Pornillos, O. W., Morham, S. G., Zavitz, K. H., Wang, H. E., Wettstein, D. A., Stray, K. M., Cote, M., Rich, R. L., Myszka, D. G., and Sundquist, W. I. (2001) *Cell* **107,** 55–65
40. Bishop, N., Horman, A., and Woodman, P. (2002) *J. Cell Biol.* **157,** 91–101
41. Henglein, B., Chenivesse, X., Wang, J., Eick, D., and Brechot, C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 5490–5494
42. Zerfass-Thome, K., Schulze, A., Zwerschke, W., Vogt, B., Helin, K., Bartek, J., Henglein, B., and Jansen-Durr, P. (1997) *Mol. Cell. Biol.* **17,** 407–415
43. Wu, C. L., Kirley, S. D., Xiao, H., Chuang, Y., Chung, D. C., and Zukerberg, L. R. (2001) *Cancer Res.* **61,** 7325–7332