

Essential Role of p400/mDomino Chromatin-remodeling ATPase in Bone Marrow Hematopoiesis and Cell-cycle Progression^{*[5]}

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p400/mDomino is an ATP-dependent chromatin-remodeling protein that catalyzes the deposition of histone variant H2A.Z into nucleosomes to regulate gene expression. We previously showed that p400/mDomino is essential for embryonic development and primitive hematopoiesis. Here we generated a conditional knock-out mouse for the p400/mDomino gene and investigated the role of p400/mDomino in adult bone marrow hematopoiesis and in the cell-cycle progression of embryonic fibroblasts. The Mx1-Cre-mediated deletion of p400/mDomino resulted in an acute loss of nucleated cells in the bone marrow, including committed myeloid and erythroid cells as well as hematopoietic progenitor and stem cells. A hematopoietic colony assay revealed a drastic reduction in colony-forming activity after the deletion of p400/mDomino. Moreover, the loss of p400/mDomino in mouse embryonic fibroblasts (MEFs) resulted in strong growth inhibition. Cell-cycle analysis revealed that the mDomino-deficient MEFs exhibited a pleiotropic cell-cycle defect at the S and G₂/M phases, and polyploid and multinucleated cells with micronuclei emerged. DNA microarray analysis revealed that the p400/mDomino deletion from MEFs caused the impaired expression of many cell-cycle-regulatory genes, including G₂/M-specific genes targeted by the transcription factors FoxM1 and c-Myc. These results indicate that p400/mDomino plays a key role in cellular proliferation by controlling the expression of cell-cycle-regulatory genes.

The alteration of chromatin structure and function is a critical process in the transcriptional activation or repression of various cell-type-specific or developmentally regulated genes and is mainly executed by covalent histone modification and ATP-hydrolysis-dependent chromatin remodeling. All of the ATP-dependent chromatin remodelers are multisubunit protein complexes containing a SWI2/SNF2 family ATPase subunit, which plays a

central role in chromatin-remodeling activities (1, 2). p400/mammalian Domino (p400/mDomino,³ Gene symbol: *Ep400*), which was identified as an interaction partner for adenovirus E1A (3) and a myeloid-specific transcription factor, MZF-2A (4), is an SWR1-class chromatin-remodeling ATPase that is homologous to the yeast Swr1p and *Drosophila* Domino proteins (5, 6). The p400/mDomino-containing protein complex consists of more than 10 subunits, including the Tip60 histone acetyltransferase and a PI3K family protein kinase TRRAP (3, 7, 8). The SWR1-class remodelers are responsible for the regulated exchange of selective histone H2A variants, such as H2A.Z, with the canonical H2A in nucleosomes (5, 9–11). This histone-exchanging activity plays a key role in the epigenetic regulation of gene expression as well as in DNA repair (12–16).

p400/mDomino is known to interact physically and/or functionally with growth-regulating transcription factors, such as Myc, p53, E2F, and adenovirus E1A (3, 17–20). In primary human fibroblasts and osteosarcoma-derived U2OS cells, the knockdown of p400/mDomino results in cell-cycle arrest at the G₁ phase with the induction of p21 expression (21, 22). At the p21 promoter, p400/mDomino colocalizes with p53 at a p53-binding site and with *c-myc* at a TATA region to regulate p21 expression (16, 19). Thus, p400/mDomino appears to regulate both the p53-dependent and c-Myc-modulated expression of p21, which blocks cell proliferation and leads to cellular senescence. However, a recent study showed that the siRNA-mediated knockdown of p400/mDomino in mouse cells reduces the proliferation rate of embryonic stem cells without p21 up-regulation, and has only a modest effect on the cell cycle of embryonic fibroblasts (23). This discrepancy in the effect of p400 knockdown on cellular proliferation could be attributable to cell-type-specific roles of p400/mDomino, or different levels of residual p400/mDomino protein might have resulted in the distinct phenotypes in these knockdown experiments.

We previously reported that mice with an N-terminally deleted p400/mDomino mutation die *in utero* with a defect in primitive erythropoiesis, indicating that the mDomino complex is essential for early development (24). In this study, we

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³ The abbreviations used are: mDomino, mammalian Domino; MEF, mouse embryonic fibroblast; pl:pC, poly(I):poly(C); SPB, sodium phosphate buffer; ER, estrogen receptor; OHT, 4-hydroxytamoxifen; CreER, a fusion of protein Cre with the estrogen receptor; BM, bone marrow; TRRAP, transformation/transactivation domain-associated protein.

generated conditional knock-out mice to examine the role of p400/mDomino in adult mice. The induced deletion of mDomino in mice led to lethality within 2 weeks, accompanied by the rapid loss of bone marrow cells, including hematopoietic progenitor/stem cells. Analysis of the cell-cycle profile of the mDomino-deleted fibroblasts showed that mDomino was essential for cell-cycle progression. Gene expression analysis revealed that the mDomino deletion caused the reduced expression of cell-cycle-regulatory genes that are targets of the transcription factors FoxM1 and c-Myc. These results indicated that mDomino plays a key role in cellular proliferation by regulating the expression of genes involved in cell-cycle progression.

EXPERIMENTAL PROCEDURES

Construction of Targeting Vectors and Generation of Conditional Knock-out Mice—FLPe transgenic mice (25) were kindly provided by Dr. S. Itohara (Brain Science Institute, RIKEN, Japan) through the RIKEN BioResource Center (Tsukuba, Japan). *E2a-Cre* (26) and *Mx1-Cre* transgenic mice (27) were from the Jackson Laboratory. A targeting vector was constructed in which exon 15 of the *mDomino* gene was flanked with two *loxP* sites (Fig. 1A). In brief, a *loxP* site was inserted into intron 14, and an *FRT*- and *loxP*-flanked neomycin-resistance gene (*NeoFRT* cassette) was inserted into intron 15 of the *mDomino* gene. The diphtheria toxin A fragment gene (*DTA* cassette) for negative selection was ligated to the 3'-end of the 3'-homologous arm.

mDom^{+/*NeoFRT*} mice were produced as described previously (28). In brief, mouse R1 embryonic stem (ES) cells were transfected with the targeting vector by electroporation, and G418-resistant clones were screened for homologous recombination by PCR. ES clones carrying the single *mDomino*-targeted allele were injected into BDF1 blastocysts, which were then implanted into recipient ICR female mice. Chimeric mice with a high ES contribution were crossed to C57BL/6 females to yield *mDom*^{+/*NeoFRT*} mice. Germ line transmission was identified by coat color and then confirmed by PCR. To remove the *NeoFRT* cassette, *mDom*^{+/*NeoFRT*} heterozygotes were crossed with CAG-FLPe transgenic mice (25), generating *mDom*^{+/*fl*} mice. To delete the *mDom*^{fl} allele *in vivo*, poly(I):poly(C) (pI:pC, BD Biosciences) was given by one intraperitoneal injection (5 mg/kg body weight) every other day, for a total of three injections. All the mice were housed in a specific pathogen-free facility at Kyoto University, and all animal experiments were carried out in accordance with protocols approved by Kyoto University (Kyoto, Japan).

Genomic DNA for PCR was prepared from tail snips. The genotype of the *mDomino* gene was determined by PCR using a mixture of three specific primers: an exon-14 sense primer (5'-ATTGGAAAATCCAACACCAAGGA-3') and two antisense primers for the wild-type (WT) exon 15 (5'-GTCTCGGAGAGCACCATACAACAAAGATGG-3') and its floxed allele (5'-CCCTGGGATGCCTGCAAGCTTATAACTTCG-3').

For Southern hybridization, genomic DNA extracted from parental and *mDom*^{+/*NeoFRT*} ES cells was digested with restriction enzymes, separated by agarose gel electrophoresis, and transferred to a BA85 nitrocellulose filter (Schleicher &

Schuell). Hybridization was carried out using a ³²P-labeled probe (Fig. 1A).

Flow Cytometry and Western Blotting—For flow cytometric analyses, antibodies against the following proteins were purchased from BD Pharmingen: CD3 (145-2C11), CD4 (L3T4), CD8 (53-6.7), c-Kit (2B8), Sca-1 (D7), Mac-1 (M1/70), B220 (RA3-6B2), Gr-1 (RB6-8C5), FcR2/III (2.4G2), Ter119, and CD71 (C2). The flow cytometric characterization of bone marrow hematopoietic cells was performed as described before (29).

To detect endogenous mDomino protein, mouse embryonic fibroblasts (MEFs) were lysed directly in Laemmli sample loading buffer and heated for 30 min at 85 °C and for 5 min at 95 °C. The samples were separated by electrophoresis on a 5% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane filter (Millipore). Immunoblotting analysis was carried out using the enhanced chemiluminescence system (Millipore) with a rabbit anti-Dom-C polyclonal antibody (4) and peroxidase-conjugated goat anti-rabbit immunoglobulin (Dako).

Histology—To prepare bone marrow sections, femurs were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2, SPB) containing 4% sucrose, and then incubated at room temperature for 24 h in Morse's solution (0.4 M sodium citrate and 22.5% formic acid) for decalcification, and embedded in paraffin. The blocks were sectioned at 4 μm, deparaffinized, and subjected to staining with hematoxylin and eosin.

To detect apoptotic cells, tissues were fixed in 0.1 M SPB containing 4% sucrose and 4% paraformaldehyde at 4 °C for 2 h, then transferred to 0.1 M SPB containing 10% sucrose at 4 °C for 4 h, and then to 0.1 M SPB containing 20% sucrose at 4 °C overnight. The fixed tissues were embedded in OCT compound (Sakura), quickly frozen, sectioned at 8 μm, and mounted onto APS-coated glass slides (Matsunami). For TUNEL staining, the fixed sections were stained with an ApopTag fluorescein *in situ* apoptosis detection kit from Chemicon International, and the nuclei were counterstained with DAPI. The sections were mounted with Fluoromount (Calbiochem) and were visualized by fluorescence microscopy (Keyence BIOREVO).

Cell Culture and Establishment of CreER MEFs—Primary *mDom*^{fl/fl} MEFs were prepared from E13.5 embryos and immortalized according to the 3T3 protocol. The MEFs were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (hereafter "culture medium") in a humidified atmosphere. The pMXs-CreER^{T2}-IRES-EGFP-puro^R plasmid (CreER refers to a fusion of protein Cre with the estrogen receptor) was transfected into the packaging cell line Plat-E (30) using FuGENE transfection reagent (Roche Diagnostics), and the transfected cells were cultured for 2 days to produce a helper-free retrovirus encoding CreER^{T2}. The culture supernatant was recovered and used directly as a retrovirus stock. Immortalized MEFs (1 × 10⁵ cells) in 10-cm dishes were infected by adding 10 ml of the culture medium containing 10 μg/ml Polybrene and 1% of the retrovirus stock. To delete the *mDom*^{fl} allele from *mDom*^{fl/fl}:CreER MEFs, the cells were treated with 7.5 nM 4-hydroxytamoxifen (OHT) for 8 h, washed with the culture medium, and further incubated in

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culture medium under a humidified atmosphere. To establish MEFs expressing exogenous *mDomino*, the *mDom^{fl/fl};CreER* MEFs were transfected with the pNEF-DomHA plasmid encoding a neomycin-resistance gene and the C-terminally HA-tagged *mDomino* cDNA (1/2-disrupted Myc-*mDomino*-HA (4)) by electroporation using the Amaxa system (Amaxa), and stable transfectants were selected by G418 resistance.

Cell-cycle Analysis—Cells were trypsinized and fixed in 70% ethanol overnight at -20°C , treated with $100\ \mu\text{g/ml}$ RNase A (Sigma) in phosphate-buffered saline (PBS) for 30 min at room temperature, and then incubated with $40\ \mu\text{g/ml}$ propidium iodide in PBS for 30 min on ice. Data were acquired using a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (TreeStar, Inc.).

Microarray Analysis—For DNA microarray analysis, RNA was extracted from *Dom^{fl/fl};CreER* MEFs that were left untreated or treated with $7.5\ \text{nM}$ OHT for 8 h and then cultured for 2 days. Biotin labeling of complementary RNA was performed using the GeneChip 3' IVT Express kit (Affymetrix). The biotinylated RNA was fragmented and hybridized to Mouse Gene 430 2.0 chips (Affymetrix) as per the manufacturer's protocol. Both raw image (.dat) and intensity (.cel) files were generated using Gene Chip Operating Software (Affymetrix). All the data, including the signal intensity of each gene, were determined with Microarray Analysis Suite Version 5.0 (Affymetrix). The overall signal intensity of each array was normalized so that the average would be 500. The -fold change analysis was done using the average of OHT-untreated MEFs ($n = 3$) relative to the average of OHT-treated MEFs ($n = 3$). *p* values were calculated using Student's *t* test. The data were filtered for a change in expression exceeding 2.0-fold and a *p* value of <0.015 . In addition, in the assessment of down-regulated genes, genes presenting with a negative value in at least one of the three profiles or with an average intensity of <100 were deleted in the profiles of OHT-untreated MEFs.

Real-time PCR for the Quantitative Analysis of mRNA—For the reverse-transcribed (RT)-PCR reaction, cDNA was synthesized from DNase I-treated total RNA ($0.5\ \mu\text{g}$) using an oligo(dT) primer and Superscript III (Invitrogen) in a $10\text{-}\mu\text{l}$ reaction mixture. Quantitative real-time RT-PCR was carried out using the LightCycler 480 SYBR Green system (Roche Diagnostics), as described before (31), under the following conditions: 10 s at 95°C , 10 s at 60°C , and 20 s at 72°C for 40 cycles. The primer sets used were: 5'-AGCGTTAAGCAGGAAGT-GGA-3' and 5'-TCTGCTGTGATTCCAAGTGC-3' for FoxM1, 5'-TCACTTCTGGCTACATCCCC-3' and 5'-ATAGGACT-CCGTGCCATCAC-3' for PLK1, 5'-TGAGGAGAAGCAGT-GAGGAA-3' and 5'-CTGAGAGGTATTCTTAGCCT-3' for CENP-F, 5'-GGGAGAACTTCCAGGTGTC-3' and 5'-AGA-GAGACTCATCGAGCGAG-3' for Skp2, 5'-GTGGGTCAG-CGCCACACCTC-3' and 5'-GGGAGAGGCGCTTGTGC-AGG-3' for p53, 5'-TGGCTGGCGGTAAGGCTGGA-3' and 5'-ACGTCCGTGGCTGGTTGTCC-3' for H2A.Z, 5'-GGAC-TGTATGTGGAGCGGTT-3' and 5'-GAATCGGACGAGG-TACAGGA-3' for c-Myc, and 5'-CCCAGCGCCTGGCC-TATGTG-3' and 5'-TGCAGTCCGGTCTCCCCAG-3' for E2F1. All the PCR data were normalized to standards and

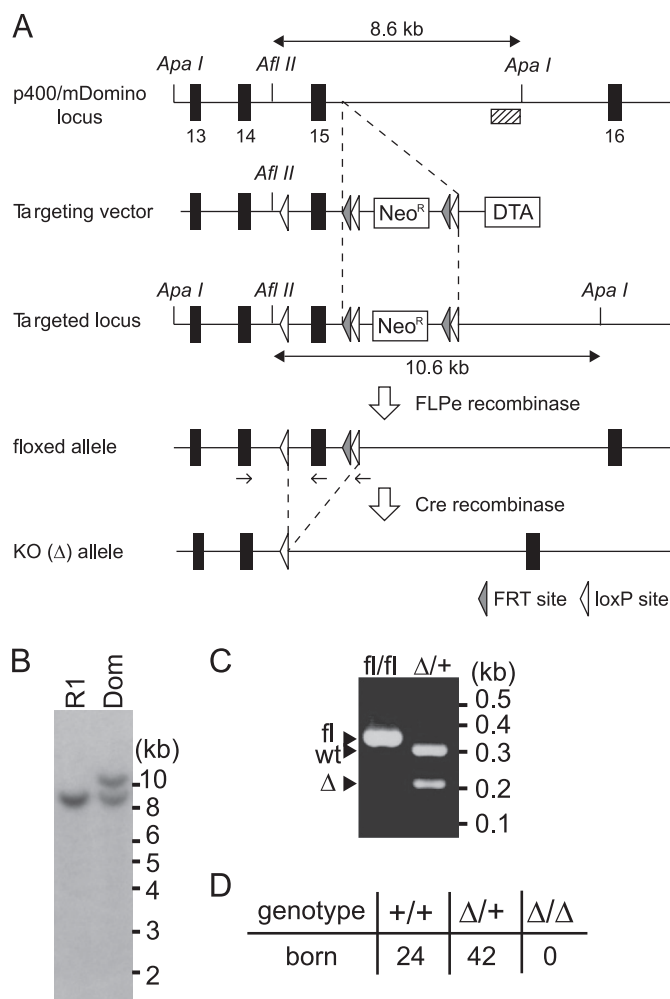


FIGURE 1. p400/mDomino is essential for embryonic development. A, schematic illustration of the exon organization of the p400/mDomino gene and targeting strategy. A targeting vector was designed in which exon 15 was flanked by two loxP sites, and an *FRT/loxP*-flanked Neo cassette (*NeoFRT*) was inserted into intron 15. Removal of the *NeoFRT* cassette by FLPe recombinase generated the *mDom^{fl}* allele. Cre-mediated recombination between the loxP sites generated the exon-15-deleted allele (*mDom^Δ*). The positions of the primers used for genotyping are indicated by short arrows. B, Southern blot analysis of a correctly targeted ES clone. Genomic DNA from parental (R1) or targeted (Dom) ES cells was digested with *ApaI* and *AflII*, and analyzed by Southern blotting using the probe indicated by the hatched box in A. The WT and targeted *mDom* alleles were predicted to result in 8.6- and 10.6-kb bands, respectively, as shown in A. C, PCR genotyping of *mDom^{fl/fl}* and *mDom^{Δ/+}* mice. D, genotype analysis of the progeny from *mDom^{Δ/+}* heterozygous matings.

expressed as copy numbers of target mRNA per nanogram of total RNA.

RESULTS

Generation of p400/mDomino Conditional Knock-out Mice—The targeting construct that was designed to delete exon 15 of the *mDomino* gene by Cre-mediated recombination is shown in Fig. 1A. The deletion of exon 15 from the *mDomino* locus is expected to remove the catalytic center of the ATPase domain and to generate a mutant *mDomino* that lacks a major portion (encoded by exons 15–52) of the protein (4). We used an *FRT*- and *loxP*-flanked Neo cassette (*NeoFRT*) and a DTA cassette as positive and negative selection markers, respectively, for homologous recombination. Mouse ES cell clones containing

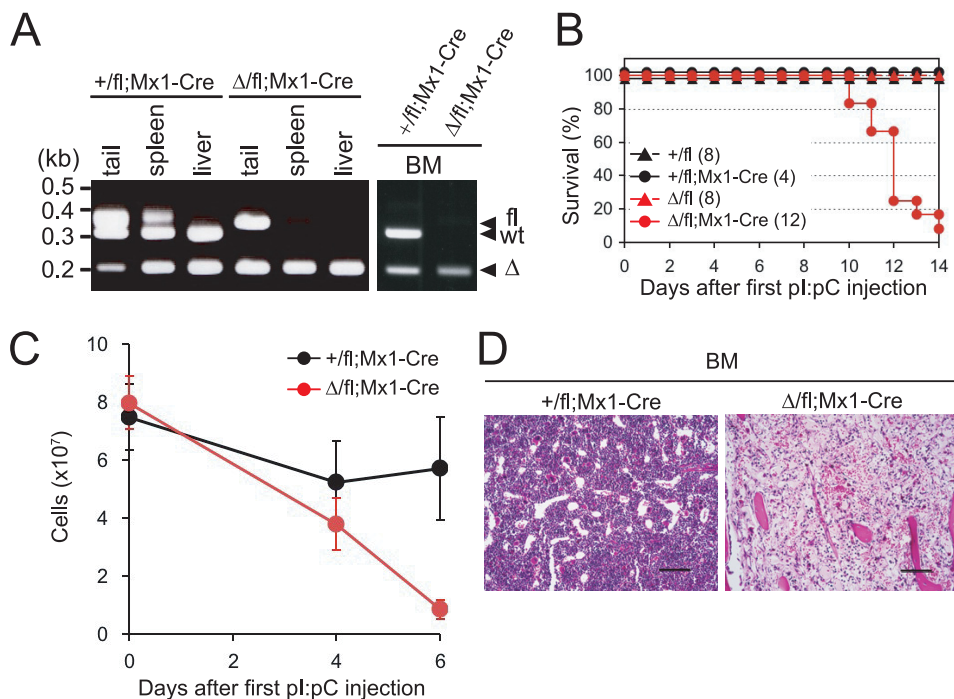


FIGURE 2. Mx1-Cre-mediated conditional deletion of mDomino leads to rapid mortality and a loss of bone marrow cells. Mice were given three intraperitoneal injections of pl:pC, one each on days 0, 2, and 4, and then analyzed. *A*, PCR genotyping of DNA from the tail, spleen, liver, and BM was performed on day 6 for the pl:pC-treated $mDom^{+/fl};Mx1-Cre$ and $mDom^{\Delta/fl};Mx1-Cre$ mice. *B*, survival of the pl:pC-treated $mDom^{\Delta/fl};Mx1-Cre$ mice ($n = 12$) and control mice ($n = 20$ in total) was observed daily. *C*, $mDom^{+/fl};Mx1-Cre$ mice (black, $n = 3$) and $mDom^{\Delta/fl};Mx1-Cre$ mice (red, $n = 3$) were treated with pl:pC, and the total number of nucleated BM cells in all the femurs and tibiae from a single mouse was determined on the indicated days. *D*, hematoxylin and eosin staining of a paraffin-fixed section of the femur 8 days after the first pl:pC injection. Scale bars represent 100 μ m.

the *mDomino*-targeted allele ($mDom^{NeoFRT}$) were identified by PCR (not shown) and Southern blot analyses (Fig. 1*B*) and were used to generate mice carrying the $mDom^{NeoFRT}$ allele in the germ line. Then, the $mDom^{NeoFRT}$ mice were crossed with *CAG-FLPe* transgenic mice (25) to remove the *NeoFRT* cassette. The resulting *mDomino* allele containing two *loxP* sites in introns 14 and 15 (the “floxed” allele) was designated as $mDom^{fl/fl}$ (Fig. 1, *A* and *C*). $mDom^{fl/fl}$ mice were obtained from heterozygous matings at a Mendelian ratio and were phenotypically indistinguishable from their wild-type (WT) or heterozygous littermates, indicating that the $mDom^{fl}$ allele was functional (data not shown). We also crossed $mDom^{NeoFRT}$ mice with *E2a-Cre* transgenic mice (26), to obtain an *mDomino* allele lacking exon 15 in the germ line, which was designated as $mDom^{\Delta}$ (Fig. 1, *A* and *C*). Heterozygous $mDom^{\Delta/+}$ mice were born and developed normally, but no homozygous mutant offspring were born from the intercross of $mDom^{\Delta/+}$ mice, indicating that the homozygous $mDom^{\Delta/\Delta}$ mutation is lethal during embryonic development (Fig. 1*D*). We have not determined whether $mDom^{\Delta/\Delta}$ embryos die in a very early stage of embryogenesis, or can develop at least to embryonic day 8.5 as observed in the homozygous embryos expressing the N-terminally deleted *mDomino* mutant (24).

Acute Loss of Bone Marrow Hematopoietic Cells Caused by the Induced Deletion of *mDomino*—To generate mice in which the *mDomino* gene was inducibly inactivated, $mDom^{fl}$ mice were crossed with *Mx1-Cre* transgenic mice (*Mx1-Cre*), which carry the Cre gene under the control of the *Mx1* promoter. The

Mx1-Cre gene is induced in adult mice by the administration of poly(I):poly(C) (pl:pC) via interferon (IFN) induction (27). To estimate the efficiency of the *mDom* deletion in adult tissues, pl:pC was administered intraperitoneally to *mDom*-floxed mice three times, once each on days 0, 2, and 4. Two days after the last pl:pC injection, genomic DNA from the tail, spleen, liver, and bone marrow (BM) was analyzed by PCR. Although the deletion of the floxed exon 15 of the $mDom^{fl}$ allele was inefficient in the tail, an efficient deletion of the floxed allele (50–70%) was observed in the spleen, and almost complete deletion was achieved in the liver and BM in the $mDom^{\Delta/fl};Mx1-Cre$ and the $mDom^{+/fl};Mx1-Cre$ mice (Fig. 2*A*).

To explore the role of *mDomino* in adult mice, $mDom^{\Delta/fl};Mx1-Cre$ mice were injected with pl:pC, as described above. This treatment resulted in the death of almost all of the $mDom^{\Delta/fl};Mx1-Cre$ mice within 14 days after the first pl:pC injection, whereas no mortality was

observed in any of the pl:pC-injected control mice ($mDom^{+/fl};Mx1-Cre$, $mDom^{\Delta/fl}$, and $mDom^{+/fl}$) (Fig. 2*B*). Our previous study showed that mice with the N-terminally deleted *mDom* mutation die during embryonic development with defects in primitive hematopoiesis (24). Therefore, to investigate the roles of *mDomino* in adult hematopoiesis, we examined the BM phenotypes of the pl:pC-injected $mDom^{\Delta/fl};Mx1-Cre$ mice on days 4 and 6 (*i.e.* 0 and 2 days after the last pl:pC injection, respectively), and found a drastic reduction of nucleated cells (Fig. 2*C*). Hematoxylin-eosin staining revealed the obvious disappearance of BM hematopoietic cells, except for the anucleate mature erythrocytes (Fig. 2*D*). These results indicated that the proliferation and maintenance of hematopoietic cells in the BM are severely impaired by the inactivation of *mDomino*.

To determine which of the BM hematopoietic lineages was affected by the *mDomino* deficiency, we analyzed the BM cells of pl:pC-treated $mDom^{\Delta/fl}$ mice by flow cytometry. The treatment of control mice, such as $mDom^{+/fl};Mx1-Cre$ mice (Fig. 3, *A* and *B*) and $mDom^{\Delta/fl}$ mice (free of *Mx1-Cre*, data not shown), with pl:pC resulted in a significant reduction of bone marrow B220⁺ B cells and Ter119⁺ erythroid cells but not of Mac-1⁺ or Gr-1⁺ myeloid cells, indicating that IFN (and/or pl:pC itself) has some deleterious effect on the lymphoid and erythroid lineages but not on the myeloid lineage. In contrast, the deletion of *mDomino* in BM cells resulted in a significant reduction of the Mac-1⁺Gr-1^{mid-lo} monocyte/macrophage lineage and Mac-1⁺Gr-1^{hi} granulocyte lineage (Fig. 3, *A* and *B*). The CD71⁺Ter119^{mid-hi} erythroid progenitors were also dimin-

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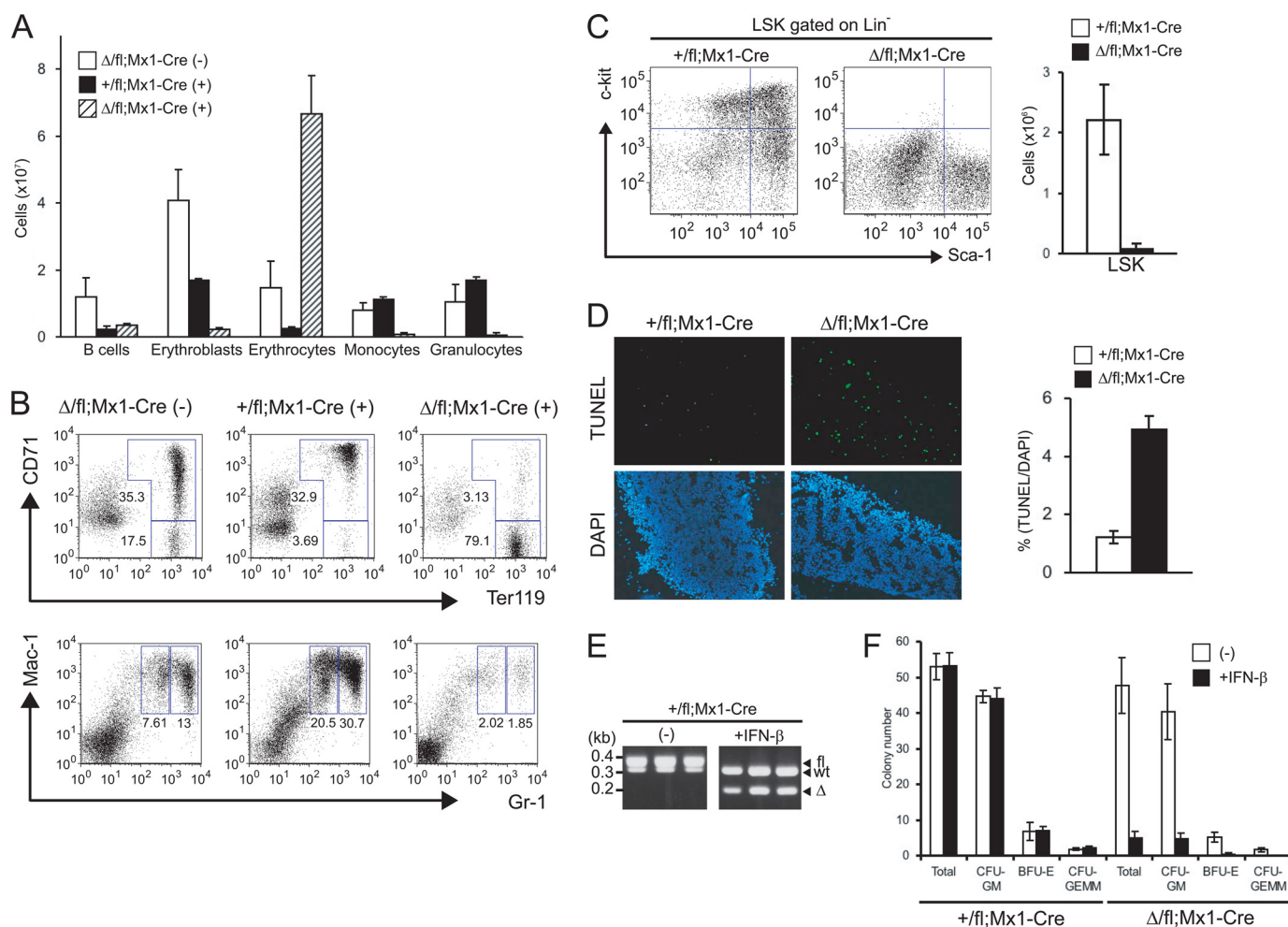


FIGURE 3. Loss of BM hematopoietic cells by mDomino deletion. *A*, total number of BM hematopoietic subsets ($n = 3$, mean \pm S.E.) in pl:pC-treated (+) or untreated (-) mice on day 4. The number of cells in each subset was calculated from the total number of nucleated BM cells and the flow cytometric analysis of hematopoietic subsets shown in *B*. The hematopoietic subsets were defined by lineage markers as follows: B cells (B220⁺), erythroblasts (CD71^{hi}Ter119^{mid} and CD71^{lo-hi}Ter119^{hi}), erythrocytes (CD71⁺Ter119^{hi}), monocytes (Mac-1⁺Gr-1^{mid}), and granulocytes (Mac-1⁺Gr-1^{hi}). *B*, flow cytometric profiles of BM cells from pl:pC-treated (+) or untreated (-) mice on day 4. *C*, flow cytometric analysis of lineage-marker-negative (Lin⁻) hematopoietic stem and progenitor cells (left), and the total number of Lin⁻Sca-1⁺c-Kit⁺ (LSK) stem cells (right) among the BM cells from pl:pC-injected mice. *D*, apoptotic cell death in the mDomino-deleted BM cells. Cryosections of BM were prepared from pl:pC-injected mice on day 3. The BM samples were stained with TUNEL and counterstained with DAPI (left). The TUNEL-positive cells were counted, and the percentage of DAPI-positive cells that were TUNEL-positive was calculated (right). *E* and *F*, *in vitro* hematopoietic colony assay of $mDom^{\Delta/fl};Mx1-Cre$ BM cells under the induced deletion of mDomino by IFN- β . BM cells (2×10^4 nucleated cells) from $mDom^{+/fl};Mx1-Cre$ or $mDom^{\Delta/fl};Mx1-Cre$ mice were cultured using the MethoCult M3434 system (Stem Cell Technologies) in 35-mm culture dishes in the presence or absence of 100 units/ml mouse IFN- β (Merck), in duplicate. *E*, three colonies from each of the IFN-treated or untreated (-) cultures of $mDom^{+/fl};Mx1-Cre$ BM cells were analyzed for mDomino deletion by PCR. *F*, BM cells were cultured in the presence (filled bars) or absence (open bars) of IFN- β , and colonies of the colony-forming unit (CFU)-GEMM (granulocyte/erythrocyte/macrophage/megakaryocyte) and CFU-GM (granulocyte/macrophage), and the burst-forming unit of erythroid (BFU-E) containing more than 30 cells were counted after 12 days of culture.

ished when compared with the pl:pC-injected control mice. The reduction in nucleated hematopoietic cells was accompanied by a dramatic increase in CD71⁺Ter119^{high} mature erythrocytes, which comprised nearly 80% of the non-adherent cells in the BM cavity (Fig. 3, *A* and *B*). Furthermore, lineage-marker-negative (Lin⁻)c-kit⁺Sca-1⁺ hematopoietic stem cells and Lin⁻c-kit⁺Sca-1⁻ progenitor cells were lost in the pl:pC-treated $mDom^{\Delta/fl};Mx1-Cre$ mice (Fig. 3*C*), indicating that mDomino is necessary for the proliferation and/or maintenance of hematopoietic progenitor/stem cells as well as of committed myeloid cells. To address whether the mDomino-deleted hematopoietic cells underwent apoptosis, we analyzed BM cells using the TUNEL assay. One day after the second pl:pC injection, the BM cells from $mDom^{\Delta/fl};Mx1-Cre$ mice displayed more TUNEL-positive

cells than the control (Fig. 3*D*). Similar results were obtained by immunohistochemical staining for active caspase-3 (data not shown), indicating that the rapid extinction of nucleated hematopoietic cells from the mDom-deleted BM is, at least in part, due to an increase in their apoptotic cell death.

Next, we examined the effect of mDomino deletion on the *in vitro* growth and differentiation of erythroid and myeloid progenitors using a multilineage colony assay. The BM cells from $mDom^{\Delta/fl};Mx1-Cre$ mice or control $mDom^{+/fl};Mx1-Cre$ mice were cultured in methylcellulose medium containing multiple cytokines in the presence or absence of IFN- β . The presence of 100 units/ml IFN- β , which effectively deleted the $mDom^{fl}$ allele (Fig. 3*E*), did not have any deleterious effect on the hematopoietic colony formation (CFU-GM, BFU-E, and CFU-GEMM) of control BM cells (Fig. 3*F*, left), but resulted in a drastic reduc-

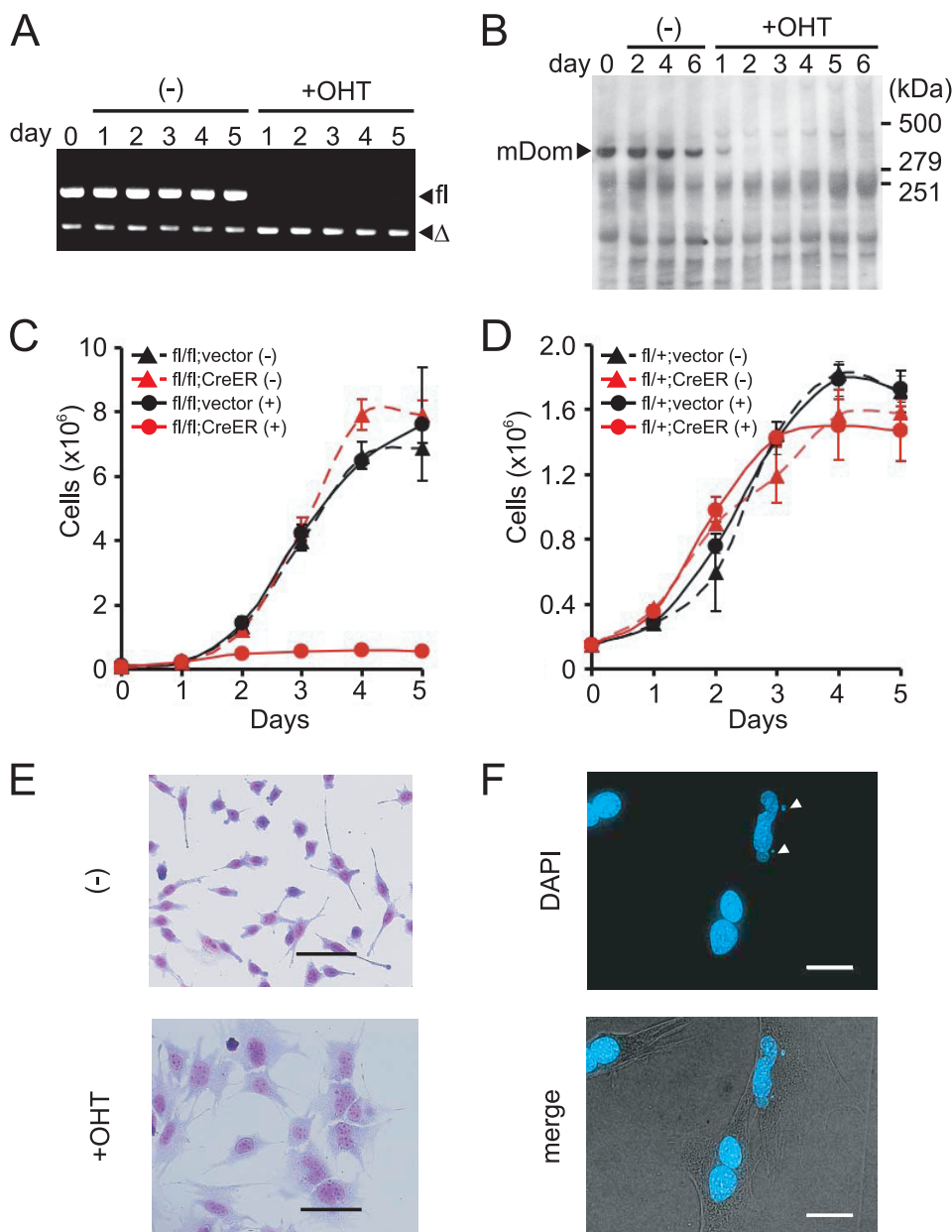


FIGURE 4. Strong growth inhibition of conditional mDomino-deficient embryonic fibroblasts. *A*, PCR analysis of the *mDom*^{fl} deletion by OHT. *mDom*^{fl/fl};CreER MEFs were left untreated (-) or treated with 7.5 nM OHT for 8 h, washed and further cultured, and then analyzed for the status of the *mDom*^{fl} allele on the indicated days. *B*, Western blot analysis of the mDomino protein in the whole cell lysate of *mDom*^{fl/fl};CreER MEFs treated with OHT. *C* and *D*, growth defect of mDomino-deficient MEFs. Four types of retrovirally transduced MEF lines, *mDom*^{fl/fl};CreER (left), *mDom*^{fl/+};CreER (right), and their respective vector controls, were left untreated (-) or treated with OHT (+), and then cultured in the normal medium. At the indicated times, the cells were trypsinized and counted. Data are the means \pm S.D. of three separate experiments. *E* and *F*, nuclear abnormalities observed in mDomino-deficient MEFs. *E*, Wright-Giemsa staining of the OHT-treated or untreated (-) *mDom*^{fl/fl};CreER MEFs after 6 days of culture. Scale bars represent 100 μ m. *F*, DAPI staining of OHT-treated *mDom*^{fl/fl};CreER MEFs (upper). Arrowhead indicates cells with micronuclei. The DAPI image is merged with its bright-field image (lower). Scale bars represent 25 μ m.

tion in the colony formation of the *mDom* ^{Δ /fl};Mx1-Cre cells (Fig. 3*F*, right). PCR genotyping of the colonies that survived in the IFN-treated *mDom* ^{Δ /fl};Mx1-Cre BM culture revealed that the small number of surviving colonies arose from cells that escaped the induced deletion of the floxed mDom gene (data not shown). These results indicated that mDomino-deleted hematopoietic progenitors rapidly, and cell autonomously, lose the ability to proliferate.

mDomino Is Necessary for the Cell-cycle Progression of Embryonic Fibroblasts—The rapid proliferation arrest and/or apoptosis in mDom-deleted BM hematopoietic cells suggested that mDomino could be involved generally in the cell growth regulation. To examine this possibility, we prepared MEFs from *mDom*^{fl/fl} mice and control *mDom*^{fl/+} mice, and immortalized them by the 3T3 passage method. To inducibly inactivate the *mDom*^{fl} allele, the immortalized MEFs were infected with a recombinant retrovirus expressing Cre-ER^{T2} (hereafter simply CreER), which can be activated by treatment with OHT (32). In the CreER-expressing MEFs, the *mDom*^{fl} allele was deleted within a day after OHT treatment (Fig. 4*A*), and the mDomino protein disappeared concomitantly from the cell lysates (Fig. 4*B*). The OHT-induced deletion of mDomino resulted in the rapid and strong growth inhibition of *mDom*^{fl/fl};CreER MEFs but not of the vector control *mDom*^{fl/fl} MEFs (Fig. 4*C*). Another control cell line, *mDom*^{fl/+};CreER MEF, which was established by the CreER-retrovirus infection of heterozygous *mDom*^{fl/+} MEFs, grew normally even after the induced activation of CreER (Fig. 4*D*). These results indicated that the growth of MEFs is not affected by the addition of OHT or by the activation of the CreER protein and that mDomino is essential for their proliferation. In addition, microscopic examination revealed the emergence of a bi- or multi-nucleated population among the mDomino-deleted MEFs that had an enlarged cell body, abnormal nuclear morphology, and micronuclei (Fig. 4, *E* and *F*).

The above results suggested that mDomino plays an important role in cell-cycle regulation. We therefore analyzed the cell-cycle profiles of mDomino-deleted MEFs by flow cytometry. As shown in Fig. 5 (*A* and *B*), the *mDom*^{fl/fl};CreER MEFs treated with OHT showed a significant increase in the population with 4N DNA content and the emergence of polyploid (>4N) cells. To examine the effect of the mDomino deficiency on cell-cycle progression, *mDom*^{fl/fl};CreER MEFs were treated with OHT, driven into quiescence by serum starvation, and then released

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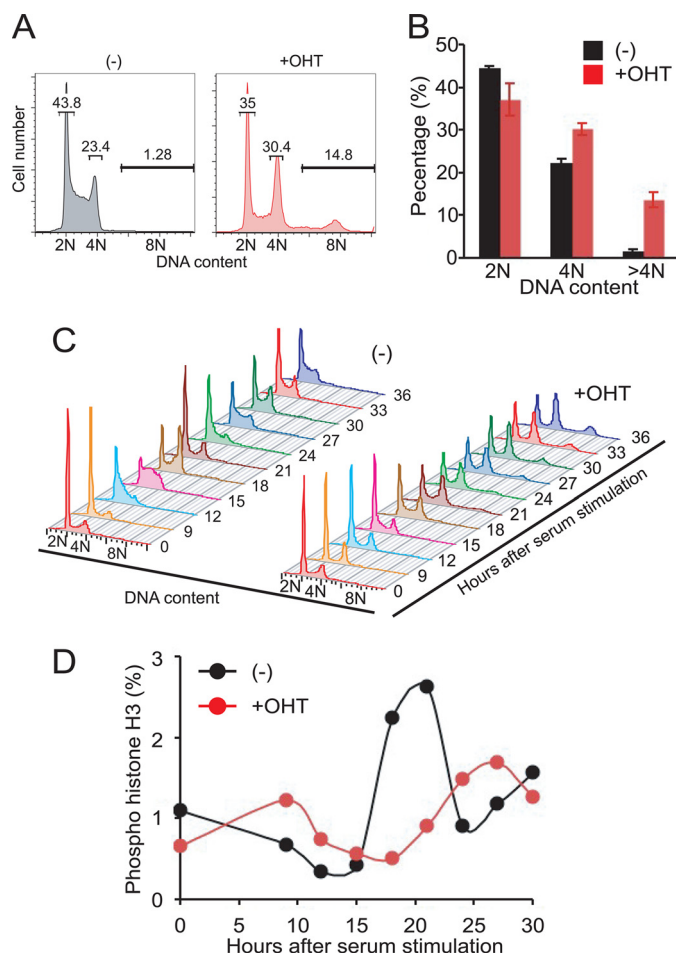


FIGURE 5. mDomino-deficient MEFs exhibit polyploidy and defective cell-cycle progression. A, the DNA content of OHT-treated or untreated (–) *mDom^{fl/fl};CreER* MEFs was determined by flow cytometry after propidium iodide staining. B, the percentage of cells with 2N, 4N, and >4N DNA content in A was determined in three independent experiments. C and D, cell-cycle profile of *mDom^{fl/fl};CreER* MEFs. Cells were treated with (+) or without (–) OHT for 8 h, serum-starved for 24 h, and then released from the starvation by stimulation with 10% fetal bovine serum. The cells were then collected at the indicated times and subjected to the flow cytometric analysis of their DNA content by propidium iodide staining (C), or of the mitotic index by staining with a phospho-Ser-10-specific, anti-histone H3 antibody (D).

back into the cell-cycle by the addition of serum. As shown in Fig. 5C, the mock-treated cells synchronously entered the S phase 12–15 h after serum stimulation, reached the G₂/M phase at about 18 h, and returned to the G₁ phase at 21 h. In contrast, MEFs treated with OHT displayed a small delay in entering the S phase and a slow but continuous increase in a population with 4N DNA content. This 4N population seemed to include not only the G₂/M population but also an aberrant cell population that had initiated a new round of DNA synthesis without cytokinesis (endoreduplication), which resulted in the accumulation of tetraploid cells with ~8N DNA content. To examine whether mDomino-deficient MEFs entered mitosis, MEFs re-stimulated with serum were stained with a phospho-Ser10-specific anti-histone H3 antibody, and quantified by flow cytometry (Fig. 5D). Mock-treated MEFs showed a sharp M-phase peak 18–20 h after stimulation, whereas the mDomino-deficient MEFs showed a delayed entry into mitosis. These results indicated that the depletion of mDomino causes

the malfunction of the cell-cycle regulation at multiple steps, especially in the G₂/M phase, and eventually leads to the aberrant mitosis known as “mitotic catastrophe.”

p400/mDomino was shown to participate in the adenovirus-E1A-induced transformation process through interaction with the N-terminal region of E1A (3). To examine whether expression of E1A affects the cell cycle defect caused by mDomino deletion, *mDom^{fl/fl};CreER* MEFs were infected with retroviral constructs expressing wild-type 12 S E1A or *dl1102*(Δ26–35) mutant that was defective in interaction with mDomino. Resulting MEFs that overexpressed E1A or *dl1102* grew faster than the parental MEFs or vector control cells, but were growth-arrested soon after the OHT treatment (supplemental Fig. 1), suggesting that overexpression of E1A protein cannot compensate for the mDomino deletion.

To confirm that the observed phenotypes of mDomino-deleted MEFs were caused by the absence of mDomino protein and not by other unexpected events, such as genomic recombination caused by CreER, *mDom^{fl/fl};CreER* MEFs were transfected with an expression plasmid for HA-tagged mDomino (pNEF-DomHA), and stable cell lines were analyzed. As shown in Fig. 6, *mDom^{fl/fl};CreER* cells expressing mDomino-HA displayed normal proliferation, cell-cycle distribution, and nuclear morphology irrespective of the OHT-induced deletion of the endogenous mDomino. This showed that the cell-cycle deficiency could be rescued by the exogenous expression of the wild-type mDomino protein and was, therefore, due to the absence of mDomino.

Impairment of Cell-cycle Regulatory Gene Expression in mDomino-deficient Cells—The p400/mDomino chromatin-remodeling complex is known to interact physically or functionally with cell-cycle-regulatory transcription factors, including c-Myc, E1A, p53, and E2F (3, 17–20). To gain insight into the molecular function of mDomino in cell-cycle regulation, we characterized the gene expression profiles of *mDom^{fl/fl};CreER* MEFs treated or untreated with OHT by DNA microarray analysis ($n = 3$ for both untreated and OHT-treated cells), which revealed that 770 genes were differentially (≥ 2.0 -fold) expressed by mDomino depletion, including 191 down-regulated genes (supplemental Table 1) and 579 up-regulated genes (data not shown). A gene annotation analysis revealed that, among the 93 genes whose expression was reduced to <45% by OHT treatment, many (27 genes) are characterized as cell-cycle-related genes, such as *E2F2*, *E2F7*, *E2F8*, *CENP-F* (*cenpf*), *Skp2*, *Nek2*, and *Cyclin A2* (*ccna2*) (Table 1). Moreover, 17 of the 93 genes are known Myc targets (supplemental Table 1) (Myc Cancer Gene, available on-line).

To confirm the microarray data and to characterize the time course of mRNA expression during cell-cycle progression, we carried out quantitative RT-PCR analysis of the genes for *Skp2*, *PLK1*, *CENP-F*, and *FoxM1*. As reported previously, the expressions of these genes were dependent on cell-cycle progression in the control *mDom^{fl/fl};CreER* MEFs (Fig. 7A). In cells treated with OHT, the expression levels of these genes were low, and they were not clearly induced after the serum stimulation. We have also compared expression of *c-Myc*, *E2F1*, *p53*, and *H2A.Z* genes in the OHT-treated or -untreated cells. Expression levels of *p53* and *H2A.Z* in the mDomino-deleted cells were consis-

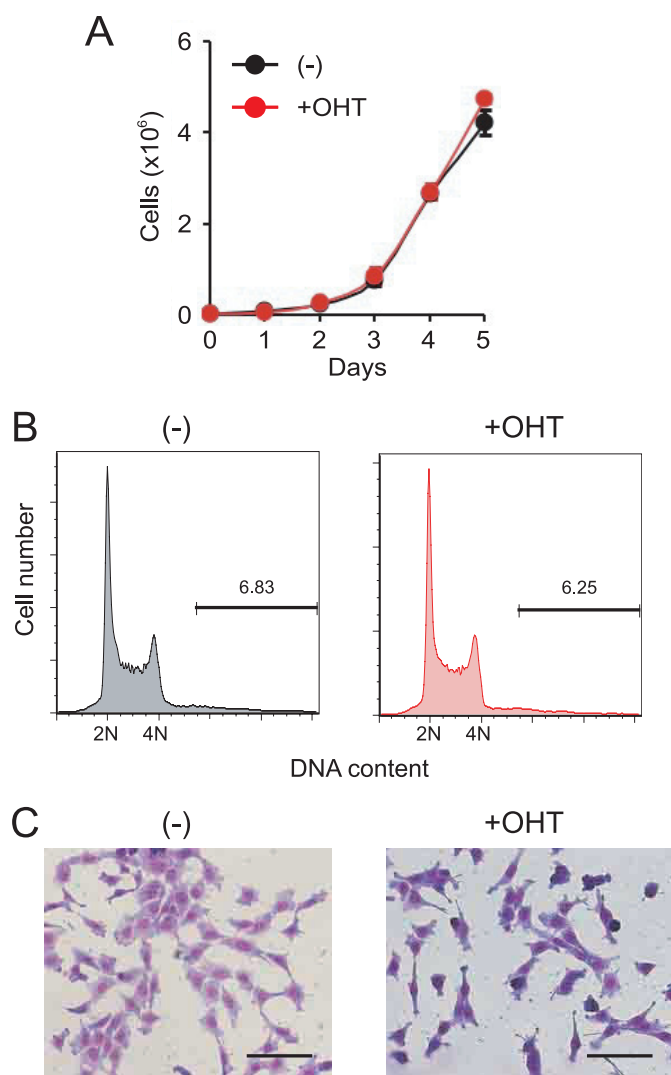


FIGURE 6. Rescue of the cell-cycle phenotype by the exogenous expression of mDomino cDNA. *mDom^{fl/fl};CreER* MEFs were transfected with an mDomino expression plasmid, and stable transformants were cultured with or without OHT treatment. *A*, growth curve of the transformant MEFs treated with (+) or without (-) OHT. Data are the means \pm S.D. of three separate experiments. *B*, cell-cycle profiles of the transformants treated without or with OHT and then cultured for 6 days. *C*, Wright-Giemsa staining of the transformants grown as in *B*. Scale bars represent 100 μ m.

tently lower than those in the untreated cells. Unexpectedly, the expression of *c-Myc* and *E2F1* genes was rather up-regulated in the mDomino-deficient MEFs (Fig. 7*B*), suggesting that mDomino may be involved in repression of these genes by a direct mechanism or by an indirect, feedback pathway. RT-PCR analysis of the mDomino mRNA showed that the expression of mDomino in normal cells was independent of the cell cycle (supplemental Fig. 2). Finally, we examined whether the impaired expression of the cell-cycle-related genes could be rescued by the exogenous expression of mDomino protein. The *mDom^{fl/fl};CreER* MEFs expressing mDomino-HA exhibited a normal expression of the cell-cycle-regulated genes irrespective of OHT treatment (Fig. 7*C*). In contrast, the expression of *c-Myc*, which was up-regulated in the mDom-deficient cells, was reduced to normal by the mDomino expression (Fig. 7*D*). Together, these results indicated that mDomino plays an essen-

TABLE 1

Cell-cycle-related genes affected by mDomino deletion

DNA microarray analysis of OHT-treated (+OHT) or untreated (-) *mDom^{fl/fl};CreER* MEFs were performed as described under "Experimental Procedures." Among the 93 genes whose expression was reduced to <45% by OHT treatment (supplemental Table 1), genes that are characterized as "cell cycle" are listed.

Gene symbol	-fold change	+OHT	(-)	<i>p</i> value	Accession number
<i>E2f2</i>	3.5	30	107	7.33E-05	BB543028
<i>Mybl2</i>	3.2	208	666	2.42E-04	NM_008652
<i>Cenpf</i>	3.2	97	310	1.02E-02	BB667318
<i>Psrc1</i>	3.0	288	878	4.66E-04	NM_019976
<i>Fbxo5</i>	3.0	618	1851	7.31E-05	AK011820
<i>E2f7</i>	2.7	557	1517	3.98E-03	BG069355
<i>Cdc6</i>	2.7	564	1531	4.25E-05	NM_011799
<i>Ccna2</i>	2.6	2615	6868	2.04E-03	X75483
<i>Ccnf</i>	2.6	144	378	3.59E-03	BB089717
<i>Rbl1</i>	2.6	178	465	2.64E-04	U27178
<i>E2f8</i>	2.6	336	876	2.80E-05	BM247465
<i>Rcc1</i>	2.6	470	1213	5.99E-06	NM_133878
<i>2810433K01Rik</i>	2.5	581	1461	2.32E-04	NM_025581
<i>Mapk12</i>	2.5	134	336	6.35E-04	BC021640
<i>Cdca3</i>	2.5	1732	4277	1.52E-03	B1081061
<i>Uhrf1</i>	2.4	192	470	6.75E-03	BB702754
<i>Suv39h2</i>	2.4	353	854	7.45E-04	NM_022724
<i>Plk1</i>	2.4	742	1787	3.42E-05	NM_011121
<i>Rassf1</i>	2.4	83	198	2.07E-03	BB385028
<i>Cdc25a</i>	2.4	590	1405	1.46E-02	C76119
<i>Nek2</i>	2.4	339	802	3.80E-04	NM_010892
<i>Skp2</i>	2.3	221	518	1.43E-02	AV259620
<i>Sgol1</i>	2.3	314	732	3.92E-03	BB410537
<i>Incenp</i>	2.3	87	201	2.06E-04	AV301185
<i>Mcm3</i>	2.3	234	531	1.02E-03	B1658327
<i>Cdc20</i>	2.2	2367	5311	3.80E-03	NM_023223
<i>Ncapg2</i>	2.2	1610	3593	1.26E-03	NM_133762

tial role in the expression of various genes that are involved in cell-cycle regulation and suggested that the impaired expression of those genes causes the aberrant cell-cycle progression, growth arrest, and mitotic catastrophe of the mDomino-deficient cells.

DISCUSSION

In this report, we generated conditional knock-out mice of the p400/mDomino gene. These mice died within 2 weeks of pI:pC-induced, Mx1-Cre-mediated mDomino depletion. We showed that the mDomino deletion in BM cells resulted in the rapid loss of committed myeloid and erythroid cells as well as hematopoietic progenitor/stem cells. The extinction of hematopoietic cells from the BM was probably due to both a decrease in the proliferation and an increase in the apoptotic cell death of the mDomino-deleted cells, followed by their clearance and replacement by mature erythrocytes. A hematopoietic colony formation assay demonstrated that the IFN-induced deletion of mDomino strongly inhibited the growth of all the colony-forming progenitors, including the myeloid, erythroid, and megakaryocytic lineages, suggesting that the impaired hematopoiesis was cell autonomous and not caused by a defect in the hematopoiesis-supporting environment in the BM.

The strong growth inhibition of the mDomino-deficient hematopoietic cells prompted us to investigate the cell proliferation using embryonic fibroblasts from *mDom^{fl/fl}* mice. The induced deletion of the mDomino gene led to an acute and strong inhibition of the cellular proliferation of MEFs. Cell-cycle analysis revealed that the mDomino-deficient MEFs exhibited pleiotropic cell-cycle defects, including delayed and insufficient entry into the S phase, increase in the G₂/M phase,

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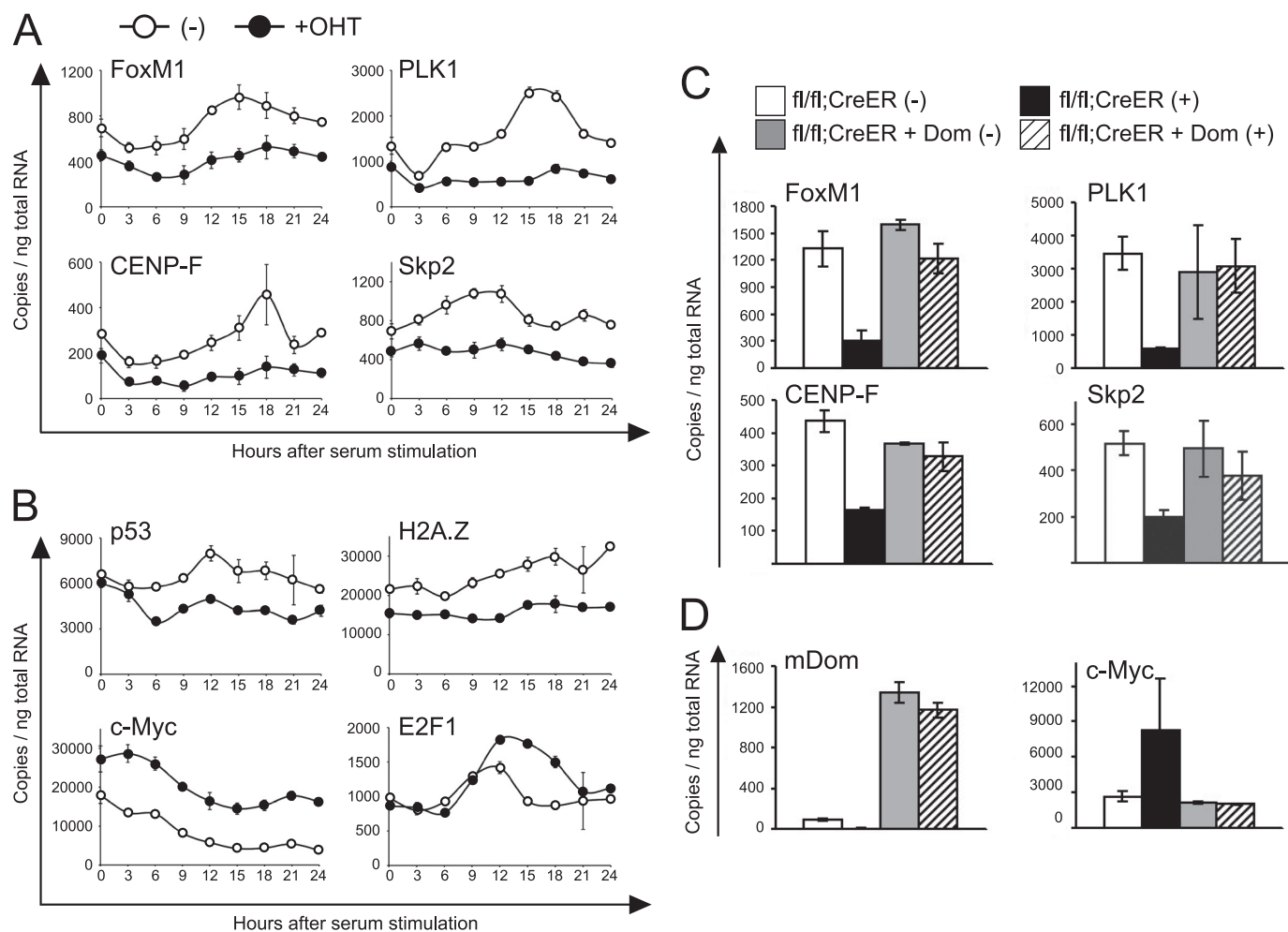


FIGURE 7. Expression of cell-cycle-related genes in mDomino-deleted MEFs. A and B, *mDom^{fl/fl};CreER* MEFs were untreated (-) or treated (+) with OHT, serum-starved for 24 h, and then re-stimulated with 10% fetal bovine serum. At the indicated times, the RNA was extracted and analyzed for expression of the indicated genes by quantitative RT-PCR. C and D, parental *mDom^{fl/fl};CreER* MEFs and mDomino-expressing transformants (*fl/fl;CreER + Dom*) were untreated (-) or treated (+) with OHT, cultured for 3 days, and then subjected to expression analysis by quantitative RT-PCR.

and the accumulation of polyploid and/or multinucleated cells with micronuclei. These phenotypes are very similar to those of *Trrap*-deleted MEFs, in which a spindle checkpoint failure leads to mitotic catastrophe (33), suggesting that the polyploid and/or multinucleated population of mDom-deficient cells might arise from a failure in mitotic checkpoint followed by endoreduplication of the DNA without cytokinesis. In *Saccharomyces cerevisiae*, the Swr1 complex is involved in chromosome segregation and plays an important role in chromosome stability (34). In addition, the TRRAP-Tip60 complex, which also contains p400/mDomino, is known to be required for the recruitment and accumulation of DNA repair proteins at sites of DNA double-strand break (35). Thus, p400/mDomino may also be involved in maintaining chromosome integrity.

In the human osteosarcoma U2OS cell line and primary fibroblasts, the shRNA-mediated knockdown of p400/mDomino results in the induction of cell-cycle inhibitor p21, cell-cycle arrest at G₁, and premature senescence (21, 22). However, the mDomino-deleted MEFs, which also showed strong growth inhibition, did not show up-regulated *p21* expression (data not shown), suggesting that the loss of mDomino can block the cell cycle by a mechanism other than the p53-p21

pathway. Our DNA microarray analysis showed that the expression levels of many cell-cycle-related genes were significantly reduced in the mDomino-deleted MEFs. Interestingly, among the strongly affected genes, *CENP-F*, *Nek2*, and *Plk1* are representative G₂/M-specific genes that are regulated by the transcription factor FoxM1 (36–38), which was also among the strongly reduced genes. This finding indicates that the cell-cycle phenotypes of the mDomino-deficient MEFs were at least partly due to the impaired expression of FoxM1 and its target genes. In this regard, it is noteworthy that *FoxM1*-deficient MEFs exhibit mitotic malfunction similar to our results (36). However, the exogenous overexpression of FoxM1 cDNA in *mDom^{fl/fl};CreER* MEFs failed to rescue the cell-cycle phenotype (data not shown), suggesting that p400/mDomino is responsible for the regulated expression not only of FoxM1 targets but also of other cell-cycle-related genes. A candidate transcription factor of note is the oncoprotein c-Myc, which interacts with the Tip60-TRRAP-p400/mDomino complex and regulates the expression of various genes positively or negatively, possibly through the histone-acetyl-transferring and/or the H2A.Z-exchanging activities of the complex (3, 17, 19). This hypothesis is consistent with the results of our microarray analysis

(supplemental Table 1), in which the strongly affected genes included several Myc targets, such as *E2F2*, *Mybl2*, *Cdc6*, *Cyclin A*, and *FoxM1* (39–43). To elucidate whether p400/mDomino is directly involved in the regulatory expression of these genes, we need to investigate physical and functional interactions of the p400/mDomino chromatin-remodeling complex with the c-Myc and/or FoxM1 on the promoter region of their target genes by chromatin immunoprecipitation analyses using cell-cycle-synchronized fibroblasts, in our future experiments.

Taking our present results together with our previous studies, we have shown that p400/mDomino is essential for hematopoiesis of both the yolk sac and the adult BM. The acute extinction of hematopoietic cells is probably due to the strong growth inhibition and apoptotic cell death of the mDomino-deleted cells. We also provide evidence that p400/mDomino plays an essential role in the cell-cycle progression of fibroblasts, probably through its transcriptional activation of cell-cycle-regulatory genes, especially the targets of FoxM1 and c-Myc. Because p400/mDomino exerts its chromatin-remodeling function via its H2A.Z-exchange activity, the interaction of p400/mDomino with c-Myc (and possibly with FoxM1) might regulate the deposition and/or eviction of the H2A.Z variant at the promoter nucleosome of cell-cycle-regulatory genes (14, 16, 44). Future studies on the interaction between transcription factors and the p400/mDomino complex and their role in the H2A.Z-positioning mechanism will provide insight into the biological significance of the H2A.Z-exchanging machinery in cell growth and differentiation.

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