

Figure 3 Attenuated polyploidy in *Dedd*^{-/-} decidual cells. (A) In vitro decidualizing uterine stromal cells were stained with Hoechst to identify nuclei after different times in culture, and the number of polynuclear cells among 600 cells was counted microscopically. Analysis was performed by 3 independent researchers. (B and C) Quantification of DNA in in vitro decidualizing uterine stromal cells (B) or ex vivo stromal cells isolated from 4.5-dpc implantation sites (C). Cells were stained with Hoechst and analyzed by flow cytometry. Three independent experiments were performed, and a representative set of profiles is presented. In C, the average sizes for 2n, 4n, 8n, and ≥16n populations are also presented (right panel). ***P* < 0.01.

antiapoptotic effect, in addition to regulation of the cell cycle (29–31). It is also noteworthy that no reproductive defects have been reported in mice deficient in Akt-1, -2, or -3, with the exception of two reports that *Akt1*^{-/-} mice harbor a partial defect in follicular development within the ovary (32, 33). Thus, each Akt isoform may be functionally redundant in supporting fertility, given that *Dedd*^{-/-} mice in which levels of all isoforms of Akt are decreased show a dramatic uterine defect (11). Our finding that the back-expression of Akt-1 efficiently recovered polyploidy in *Dedd*^{-/-} stromal cells may support this idea. Unfortunately, mice doubly or triply deficient for multiple types of Akt die before they reach reproductive age (34). Future creation and analysis of conditional knockout mice in which various types of Akt are specifically deficient in the uterus will help to elucidate the involvement of Akt in fertility.

Perspectives. Our present findings identifying DEDD as an indispensable element in support of early pregnancy might shed light on the pathogenesis of infertility of unknown cause. Indeed, *DEDD* expression in uterine stromal cells increases along with decidualization both in mice and humans. In women experiencing implantation failure, one-third of the failures have been attributed to the embryo itself (35). Although the remaining two-

thirds of failures appear to be the result of an inadequate uterine environment, the precise defect is often unknown. Thus, it may be worth addressing whether DEDD dysfunction is present in infertile women, either genetically or functionally, in uterine stromal cells. This may contribute to the development of new therapeutic strategies for infertility, though association of DEDD function with polyploidization as well as Akt and cyclin D3 in humans certainly needs to be further assessed.

Methods

Mice and human specimens. *Dedd*^{-/-} mice had been cross-bred to C57BL/6 for 17 generations before being used for experiments. Female mice of 2–6 months of age were used. The day of the vaginal plug was considered as 0.5 dpc. Pseudopregnant mice were produced by mating female mice with vasectomized male mice of the CD-1 strain. All mice were maintained under specific pathogen-free conditions. All animals used in the experiments were cared for in accordance with institutional guidelines. Human endometrial tissue was obtained from women with benign diseases. The experimental procedures were approved by the institutional review board of the University of Tokyo, and all women provided written informed consent for the use of their endometrial tissue.

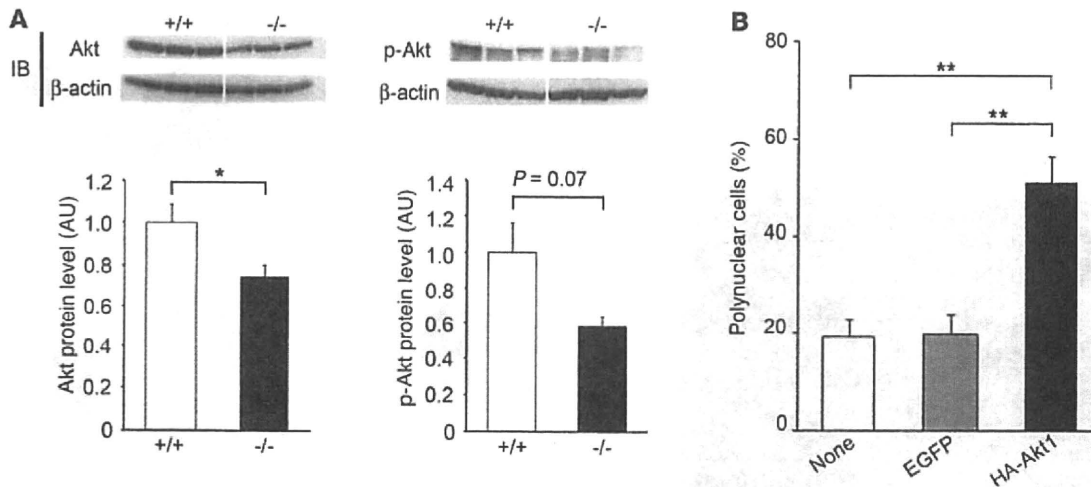


Figure 4

Involvement of Akt level in defective polyploidy in *Dedd*^{-/-} decidua cells. (A) Immunoblotting of *Dedd*^{+/+} and *Dedd*^{-/-} uteri at 5.5 dpc for total Akt (left) and phosphorylated Akt (at Thr308, right). Three mice were analyzed. Quantification was performed with NIH Image J software. Values are presented as protein levels relative to those from *Dedd*^{+/+} uteri. Error bars indicate SEM. (B) In vitro decidualizing *Dedd*^{-/-} stromal cells were transfected with expression vector for HA-Akt1 or control EGFP at day 2. At day 5, cells were stained for HA and Hoechst; thereafter, the proportion of polynuclear cells among more than 100 HA-positive cells was evaluated microscopically. Average results from 4 independent sets of experiment are presented. Error bars indicate SEM. **P* < 0.05, ***P* < 0.01.

Reagents and antibodies. Antibodies and reagents used were as follows. Primary antibodies were: anti-COX-2 (polyclonal, Cell Signaling Technology); anti-TIMP3 (W-18, Santa Cruz Biotechnology Inc.); anti-Akt (pan) (11E7, Cell Signaling Technology); anti-Ki67 (SP6, Thermo Scientific); anti-phospho-Akt (Thr308) (244F9, Cell Signaling Technology); anti-cyclin D3 (C-16, Santa Cruz Biotechnology Inc.) for immunohistochemistry; anti-cyclin D3 (DCS-22, Thermo Scientific) for immunoblotting and immunoprecipitation; anti-Cdk4 (DCS-35, Thermo Scientific); anti-Cdk6 (DCS-83, Thermo Scientific); anti-β-actin (ACTN05 [C4], Abcam); anti-HA high-affinity antibody (3F10, Roche); and anti-FLAG antibody (M2, Sigma-Aldrich). Secondary antibodies and related reagents were: Alexa Fluor 488-conjugated anti-rabbit IgG antibody (Invitrogen); Alexa Fluor 546-conjugated anti-goat IgG antibody (Invitrogen); Hoechst 33258 and Hoechst 33324 (Invitrogen); Protein Block Serum-Free (Dako Cytomation); normal goat serum (Wako); Mayer's hematoxylin (Muto Pure Chemicals Co. Ltd.); eosin Y solution (Sigma-Aldrich); HRP-conjugated anti-mouse IgG (Pierce); and HRP-conjugated anti-rabbit IgG (Pierce).

Analysis of ovulation and fertilization. Briefly, superovulation was induced by intraperitoneal injection of pregnant mare serum gonadotropin (PMSG) (at -2.5 dpc; 5 IU/mouse) and human chorionic gonadotropin (hCG) (at -0.5 dpc; 5 IU/mouse). Oviducts from mated females were flushed with M2 medium at 0.5 dpc. Under a microscope, numbers of eggs were determined, and fertilization efficiency was evaluated by morphology of the eggs (i.e., having 2 nuclei or a fused large nucleus).

Histologic and immunohistochemical analysis. Uterine specimens were fixed by infusion of 4% PFA. For immunohistochemistry, deparaffinized sections were boiled in 10 mM sodium citrate buffer, pH 6.0, by microwave for 10 minutes. After blocking, sections were incubated with primary antibodies at 4°C for 16 hours, followed by secondary antibodies at room temperature for 1 hour. Samples were counterstained with Hoechst 33258. The areas of TIMP3-positive region and edematous region were measured by ImageJ software (NIH). Similarly, the embryonic area was measured. The size of each embryo was calculated as the sum of the embryonic area multiplied by its thickness (10 μm/section).

In situ hybridization. A 433-bp DNA fragment corresponding to the nucleotide positions 81–513 of mouse *Dedd* was subcloned into pGEMT-Easy vector (Promega) and was used for generation of sense or antisense RNA probes. A 431-bp DNA fragment corresponding to the nucleotide positions 1082–1512 of mouse *cyclin D3* and a 694-bp DNA fragment corresponding to the nucleotide positions 1851–2544 of mouse *Akt1* were similarly prepared. Paraffin-embedded uterine sections (6 μm) were hybridized with digoxigenin-labeled RNA probes at 60°C for 16 hours. The bound label was detected using NBT-BCIP, an alkaline phosphatase color substrate. The sections were counterstained with Kernechtrot (Muto Pure Chemicals Co. Ltd.).

Primary culture of uterine stromal cells. The isolation and culture of mouse uterine stromal cells were performed as previously described (36). Briefly, uterine horns on day 3.5 of pseudopregnancy were cut and washed in HBSS (Invitrogen) without Ca²⁺/Mg²⁺ and phenol red but containing 100 U/ml penicillin and 100 μg/ml streptomycin (Pen Strep; Invitrogen) and 2.5 μg/ml amphotericin B (Sigma-Aldrich). Tissues were digested in HBSS containing 6 mg/ml dispase (Invitrogen) and 25 mg/ml pancreatin (Sigma-Aldrich) and subsequently treated with 0.5 mg/ml collagenase (Sigma-Aldrich). The digested cells were passed through a 70-μm nylon filter to eliminate clumps of epithelial cells and were plated at 5 × 10⁵ cells per 25 cm². For in vitro decidualization, the adherent cells were cultured in phenol red-free DMEM and Ham's F-12 nutrient mixture (DMEM/F-12, 1:1) (Invitrogen) with 1% charcoal-stripped FBS (Invitrogen) and antibiotics, 17β-estradiol (E2, 10 nM) (Sigma-Aldrich), P4 (1 μM) (Sigma-Aldrich), and HB-EGF (30 ng/ml) (Sigma-Aldrich). The induction of differentiation was continued for 7 days without changing medium. Isolation and differentiation of human endometrial stromal cells were performed as described previously (37). Cells were treated with phenol red-free DMEM/F-12 containing 5% charcoal-stripped FBS, antibiotics, E2 (10 ng/ml), and P4 (100 ng/ml) for 12 days. Media were replenished every 3 days.

Analysis of cell cycle and polyploidization. Cell cycle was analyzed using the Click-iT EdU kit (Invitrogen). Cultured uterine stromal cells were harvested at days 1, 3, and 7 after 10 μM EdU incorporation for 2 hours each. Trypsinized cells were fixed with 4% PFA and permeabilized with saponin. Incorporated EdU

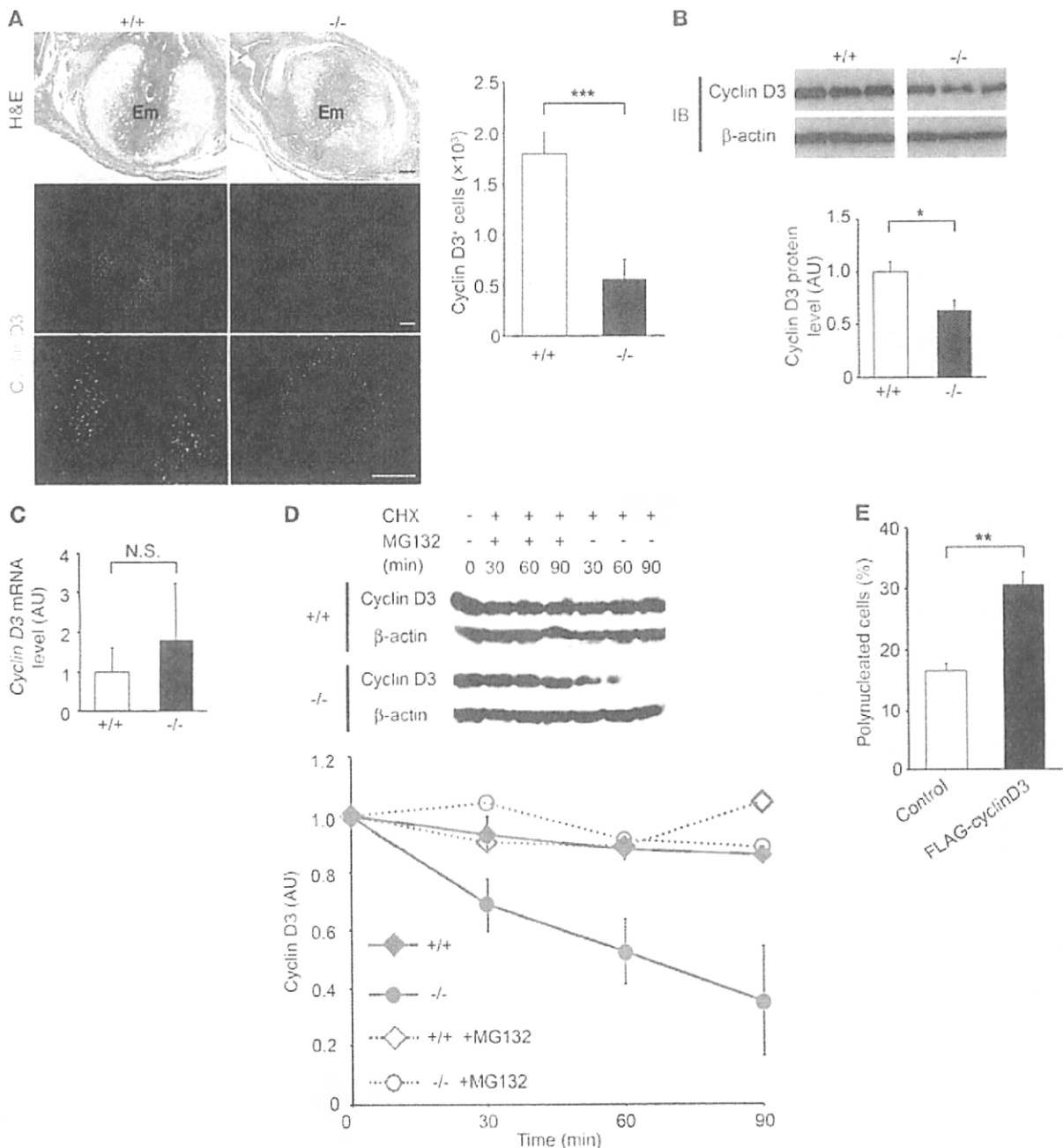


Figure 5

Decreased stability of cyclin D3 protein in *Dedd*^{-/-} decidua cells. (A) At 5.5 dpc, implantation sites in *Dedd*^{+/+} and *Dedd*^{-/-} uteri were immunostained for cyclin D3. Bottom panels show higher magnifications of the dotted area in the middle panels. Scale bars: 200 μm. Graph on the right: Cyclin D3-positive cells within an implantation site were counted and compared in at least 4 different sections, and the means are presented. Error bars indicate SEM. (B) Immunoblotting of *Dedd*^{+/+} and *Dedd*^{-/-} uteri at 5.5 dpc for cyclin D3 and quantification of results. Three mice were analyzed. Error bars indicate SEM. (C) mRNA level for *cyclin D3* in 5.5-dpc implantation sites. No significant decrease in mRNA level was detected in *Dedd*^{-/-} cells and tissues ($n = 3$ for each). (D) Protein degradation assay for cyclin D3. Uterine stromal cells at day 3 of in vitro decidualization were treated with cycloheximide (CHX; 50 μM) for the indicated periods in the presence or absence of proteasome inhibitor MG132 (10 μM). At each time point, cells were harvested, and the lysate was analyzed for cyclin D3 by immunoblotting. The amount of cyclin D3 protein at each time point was normalized to that of β-actin and is presented as relative level to that at pretreatment (0 minutes) in *Dedd*^{+/+} or *Dedd*^{-/-} cells. Three independent experiments were performed, and representative blots are presented. Error bars indicate SEM. (E) In vitro decidualizing *Dedd*^{-/-} stromal cells were transfected with expression vector for FLAG-cyclin D3 at day 2. At day 5, cells were stained for FLAG and Hoechst; thereafter, the proportion of polynuclear cells among more than 100 FLAG-positive cells was evaluated microscopically. Average results from 4 independent sets of experiment are presented. Error bar indicate SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

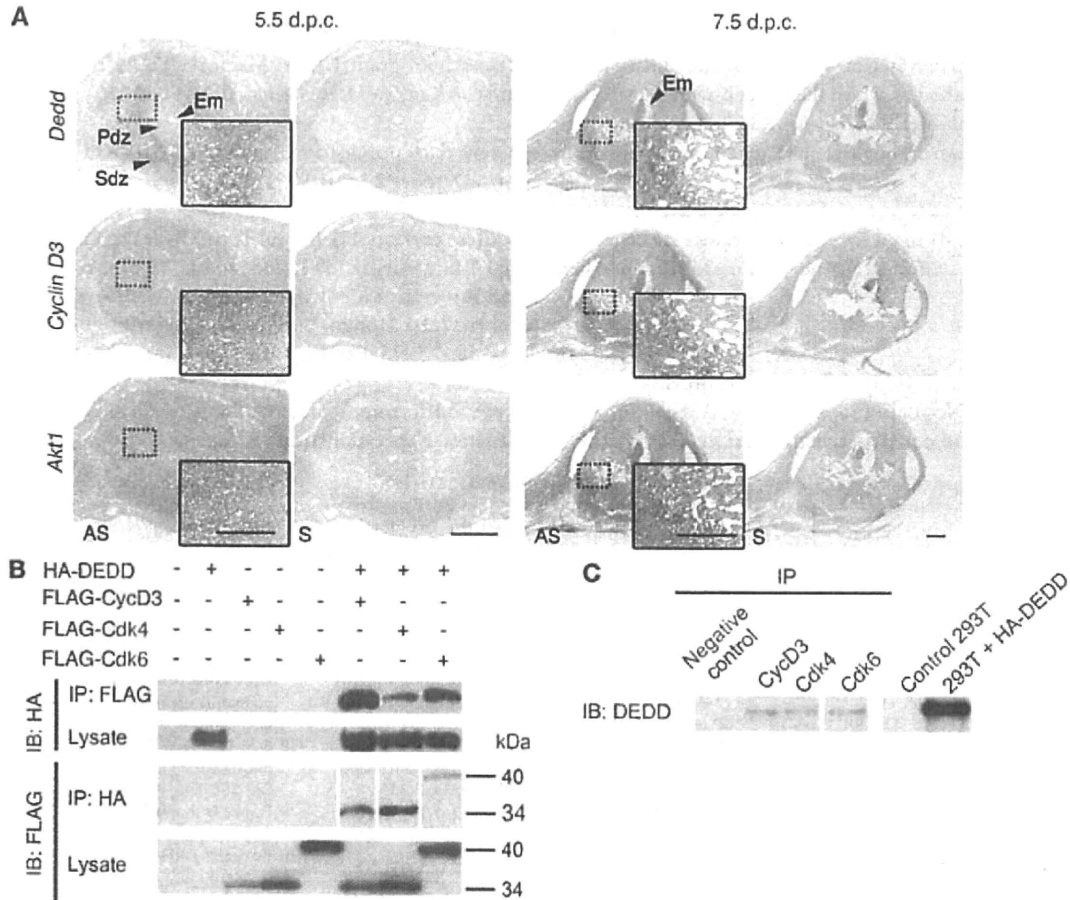


Figure 6

The association of DEDD with cyclin D3. (A) In situ mRNA analysis for *Dedd*, *cyclin D3*, and *Akt1* in 5.5- and 7.5- dpc wild-type mouse uteri. Microphotographs at a higher magnification are also presented. AS, antisense probe; S, sense control probe; PdZ, primary decidual zone; Sdz, secondary decidual zone. Scale bars: 500 μ m. (B) Binding study. HA-tagged DEDD and FLAG-tagged cyclin D3, Cdk4, or Cdk6 were expressed in HEK293T cells, and association of HA-DEDD and each FLAG-tagged protein was evaluated by a coimmunoprecipitation assay with anti-HA or anti-FLAG antibody. (C) Coprecipitation of endogenous DEDD with cyclin D3, Cdk4, or Cdk6 from lysates of 5.5-dpc uterine implantation sites. Precipitates were immunoblotted with an anti-DEDD antibody. HEK293T cells with or without transfection of HA-tagged DEDD were used as controls.

was detected by an alkylation reagent conjugated with Alexa Fluor 488. DNA content was quantitatively detected by subsequent staining with Hoechst 33342. At least 10,000 cells for each sample were analyzed by flow cytometry. For evaluation of polyploidy, cells were cultured in a 4-well chamber and stained with Hoechst after fixation with 4% PFA. Multinucleated cells were calculated under a fluorescence microscope and counted by 3 independent researchers in a blinded fashion from more than 600 total cells each.

Flow cytometry (analysis of cell DNA contents). In vitro decidualizing cells were stained with Hoechst 33342 and analyzed for DNA content by an LSR II flow cytometer (BD). At least 10,000 cells were analyzed for each. For ex vivo cells, 4.5-dpc uterine implantation sites were digested by a collagenase treatment, and purified single cells were stained with Hoechst 33342. The large stromal cell population was gated, and at least 1,000 cells were analyzed for DNA content.

Akt and cyclin D3 overexpression in decidual cells. Uterine stromal cells at day 2 of culture in the presence of E2, P4, and HB-EGF were transfected with a pCruz-HA-Akt1, pFLAG-cyclinD3, or a mock expression vector by using Lipofectamine 2000 (Invitrogen). At day 5, cells were fixed with 4% PFA and permeabilized with 0.25% Triton X-100. The transfected cells

were detected by anti-HA high-affinity antibody or anti-FLAG antibody conjugated with Cy3. Multinucleated cells were counted under a fluorescence microscope after Hoechst staining.

Protein degradation assay. Day 3 in vitro decidualizing stromal cells were treated with 50 μ M cycloheximide (Sigma-Aldrich) in the presence or absence of 10 μ M MG132 (Sigma-Aldrich) and were harvested at the indicated time points. Cells were lysed in a lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and Complete Mini [Roche]) and used for immunoblotting.

Coimmunoprecipitation assay. HEK293T cells were transfected with pCAG-DEDD-HA, pFLAG-cyclin D3, pFLAG-Cdk4, or pFLAG-Cdk6 by electroporation. Cells were lysed and incubated with anti-HA or anti-FLAG beads for 16 hours at 4°C. Precipitates were analyzed for the presence of coprecipitated molecule by immunoblotting using anti-FLAG or anti-HA antibody. For the binding of endogenous DEDD with cyclin D3, Cdk4, or Cdk6, 5.5-dpc uterine implantation sites were homogenized in lysis buffer and incubated with anti-cyclin D3, anti-Cdk4, or anti-Cdk6 antibody bound to protein G beads. Precipitates were immunoblotted with an anti-DEDD polyclonal antibody prepared in-house (8).

Quantitative and semiquantitative RT-PCR. QPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Results were analyzed by the $\Delta\Delta C_t$ method, in which the C_t s of β -actin were used for normalization. Primer sequences are detailed in Supplemental Table 1. For semiquantitative PCR shown in Supplemental Figure 3, primers are shown in Supplemental Table 2.

Statistics. All statistical analyses were performed using 2-tailed Student's *t* test. Standard deviation and variance of every data set were calculated, and the homoscedasticity was confirmed by *F* test. *P* values less than 0.05 were considered significant.

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