A normal cell cycle in most eukaryotic cells consists of a tightly regulated sequence of phases including DNA synthesis (S) followed by a gap (G2), mitosis (M), and a gap (G1). In the megakaryocytic lineage, the cells undergo endomitosis, which involves DNA synthesis in the absence of mitosis, thus giving rise to polyploid cells. We aimed at defining whether the megakaryocytic cell cycle consists of a continuous S phase or of G1/S phases and at determining which cyclins are involved in this process. Studies were performed in primary cultures of mouse bone marrow cells. DNA synthesis in megakaryocytes was followed by determining incorporation of a DNA precursor, bromodeoxyuridine (BrDU), into the cells by in situ staining for BrdU. These experiments showed that no more than 15% of the recognizable megakaryocytes in normal bone marrow are in the process of endomitosis, including S phases interrupted by short gaps. Using immunohistochemistry, we showed that mature megakaryocytes express the G1 phase cyclin and cyclin D3, but not the mitotic cyclin, cyclin B1. Under culture conditions that selectively promote megakaryocytopenesis, antisense oligonucleotides designed to suppress cyclin D3 expression, but not sense oligonucleotides or antisense oligonucleotides to cyclin B1, dramatically suppress endomitosis and abrogate megakaryocyte development. Our results indicate that endoreduplication in megakaryocytes is associated with low levels of or the absence of cyclin B1, whereas progression through this process depends on the G1 phase for which cyclin D3 is crucial.

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Cyclin D3 Is Essential for Megakaryocytopenesis
By Zhengyu Wang, Ying Zhang, Dimitry Kamen, Emma Lees, and Katya Ravid

THE MEGAKARYOCYTE is unique among bone marrow (BM) cells in possessing a polyploid nucleus. This cell is characterized by its large size, is rare in the BM (about 0.1% of total BM cells), and has a nucleus that contains up to 32 times the diploid amount of DNA. At some stage after commitment to the megakaryocytic lineage, megakaryocyte precursors stop cell division but continue to replicate their DNA, a process called endomitosis, followed by cytoplasmic maturation and platelet fragmentation. Little is known about the nature of the cell cycle in this unique cell type and what regulates it.

In all eukaryotic cells, progression through the cell cycle is regulated by cyclin-dependent protein kinases (CDKs), which are essential for the start of the S phase, and Cdc2, which is essential for the start of mitosis. B-type cyclins are synthesized during the S and G2 phases. The binding of cyclin B to Cdc2 induces phosphorylation and activation of the complex that is essential for the G2 to M transition. Activation of Cdc2 at the end of G2 is mediated by the phosphatase Cdc25. This process is followed by activation of the cyclin destruction system that results in the degradation of cyclin B at the end of mitosis, the release of inactive Cdc2, and entry into interphase. Cdk2, like Cdc2, is regulated by its association with cyclins. Cdk2 associates with cyclin E in the middle of G1, followed by formation of a complex with cyclin A at the start of S phase. Other studies also show that cyclin D, which is differentially expressed in different cell types, can regulate S phase progression via interaction with Cdk2, Cdk4, or Cdk5.

In the present study, we have used immunohistochemistry to follow DNA synthesis in primary BM megakaryocytes. Our results indicated that only a small portion of BM megakaryocytes were actively involved in endomitosis, including a short G1 phase followed by an S phase. We have determined the presence of different cyclins in cells undergoing endomitosis and used antisense oligonucleotides to show their role in promoting this unique cell cycle.

MATERIALS AND METHODS

Reagents and animals. FVB mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). Acetylthiocholine iodide, bromodeoxyuridine, fluorodeoxyuridine, adenosine, sodium citrate, theophylline, and antirat IgG tetramethylrhodamine isothiocyanate (TRITC) conjugate were from Sigma (St Louis, MO). Coumarin-3-carboxylic acid was from Molecular Probes Inc (Junction City, OR). Nylon mesh screens were from Spectremesh, Spectrum Medical, Inc (Los Angeles, CA). Goat antirat IgG conjugated to fluorescein isothiocyanate (FITC), culture media, and sera were purchased from Gibco Laboratories (Grand Island, NY). Oligonucleotides and S-oligonucleotides were from Midland Laboratories (Midland, TX). Mouse antibody to bromodeoxyuridine (BrDU) and BrDU staining kit were from Zymed Laboratories (South San Francisco, CA). Taq polymerase and polymerase chain reaction (PCR) reagents were obtained from Perkin Elmer Labs (Foster City, CA). Crystal Mount was from Fisher Scientific (Pittsburgh, PA). Mouse monoclonal antibody (MoAb) to cyclin B1 was from Pharmingen (San Diego, CA). Rat MoAbs to cyclin D3 or cyclin D1 (both recognizing mouse proteins) were from Oncogene Science (Uniondale, NY). Hematopoietic growth factors were from R&D Systems (Minneapolis, MN).

Cultures of BM cells. Mouse BM cells were isolated from the femurs as described before, except that deoxyribonuclease was omitted from the preparation buffer. Cells were cultured in 5% CO2 at 37°C in a liquid culture under conditions that were shown before to support maturation and ploidy of primary megakaryocytes. The cells were cultured in the presence of Iscove's modification of Dulbecco's medium (IMDM) and 20% horse serum, unless otherwise indicated, supplemented with penicillin (2,000 U/mL), streptomycin (200 μg/mL), and L-glutamine (0.592 mg/mL). DNA synthesis. Mouse BM cells were cultured for overnight, as described above. Subsequently, 10 μmol/L BrdU and 1 μmol/L fluorodeoxyuridine were added to the medium. At different time points, cells were collected and spun down with a cytopsin on a polysylane-coated slide. Exposure of cells to BrdU at this concentration for 24 hours is not toxic to cells. The cells were fixed in 75% methanol-isobutanol and air-dried on a glass slide. The slides were then stained with 0.1% crystal violet (solution A) for 5 minutes and rinsed in water. Subsequently, the slides were stained with 0.1% aqueous DAB solution (solution B) for 5 minutes. They were then rinsed in water and mounted with Crystal Mount (Fisher Scientific). The slides were observed under a light microscope equipped for differential interference contrast. The number of BrdU-positive cells was determined by scoring at least 20 fields in each experiment.
ethanol for 15 minutes at 4°C and then washed twice with phosphate-buffered saline (PBS; 136 mmol/L NaCl, 5 mmol/L Na$_2$HPO$_4$, 2.6 mmol/L KCI, 1.4 mmol/L KH$_2$PO$_4$, pH 7.4). Incorporation of BrdU to the cells was determined by using the Zymed BrdU staining kit (Zymed Laboratories). Fixed cells were stained with an antibody to BrdU followed by staining with a streptavidin-biotin system, as described by the manufacturer. This method was shown before to be sensitive, detecting DNA synthesis in normal or malignant cells in S phase.\(^{17}\) We used as positive controls intestine in S phase (as described by Ishibashi et al.\(^{19}\)) or by in-situ staining of cells spun down on slides using acetylcholinesterase iodide as a substrate.\(^{19}\)

**Immunohistochemistry.** Cytospun BM cells were fixed and treated, as described by the Zymed BrdU staining kit, with 5% goat serum for 15 minutes at room temperature to block nonspecific binding. The serum was drained (not washed) and the cells were incubated with 200 \(\mu\)L of PBS containing one of the following first antibodies, as indicated: 1 \(\mu\)L of mouse MoAb to human cyclin B1; 2 \(\mu\)L of rabbit polyclonal antibody to human cyclin B (gift of Dr Tony Hunter); 1 \(\mu\)L of mouse MoAb to BrdU; 1 \(\mu\)g of rat MoAb to mouse cyclin D3 or D1. All of these antibodies cross-react with the corresponding proteins from mouse (Tony Hunter [The Salk Institute, San Diego, CA], personal communication [November 1994]) and as tested by the manufacturers). Cross-reactivity of human cyclin B1 antibody with mouse cyclin B was also tested by us on a mouse cell line (data not shown). The cells were incubated for 1 hour at room temperature with the first antibody, washed three times with PBS, and incubated for 1 hour at room temperature with a secondary antibody, either antirat, antirabbit, or antimouse IgG conjugated to a fluorochrome. The slides were washed three times with PBS, mounted with Crystal Mount, and examined with phase and fluorescent microscopes.

**Antisense oligonucleotides in cultures of BM cells.** Phosphoethanoater oligonucleotides (S-oligos) were ethanol-precipitated, washed twice with 70% ethanol, and dissolved in sterile PBS at a concentration of about 10 mmol/L. BM cells were depleted of megakaryocytes by filtration through a 250-pm mesh filter followed by passing the eluent through a 17-pm mesh filter. This procedure resulted in depletion of more than 90% of the megakaryocytes in BM, as indicated twice with 70% ethanol, and dissolved in sterile PBS at a concentration of about 10 mmol/L. BM cells were depleted of megakaryocytes by filtration through a 250-pm mesh filter followed by passing the eluent through a 17-pm mesh filter. This procedure resulted in depletion of more than 90% of the megakaryocytes in BM, as indicated by filtration through a 250-pm mesh filter followed by passing the eluent through a 17-pm mesh filter. This procedure resulted in depletion of more than 90% of the megakaryocytes in BM, as indicated by depletion of more than 90% of the megakaryocytes in BM, as indicated by staining with the megakaryocyte-specific marker, acetylcholinesterase.\(^{19}\) The BM cells were cultured for 16 hours at a concentration of 3 \(\times 10^6\) cells/mL medium in 1-cm diameter plates in the presence of IMDM and dialyzed and heat-inactivated (30 minutes at 56°C) 20% fetal bovine serum supplemented with penicillin (2,000 U/mL), streptomycin (200 \(\mu\)g/mL), and L-glutamine (0.592 mg/mL). Under these culture conditions, high ploidy megakaryocytes are easily identified 3 days after culturing.\(^3\) S-oligos were added to the culture at a concentration of 15 pmol/L; 24 hours after the first addition, additional oligomers were added at a concentration of 10 pmol/L. Mouse BM cells cultured for 3 days in the presence or absence of the indicated oligomers were collected and stained for viability with trypan blue, counted with a hemocytometer, and subjected to acetylcholinesterase assay or to immunohistochemistry. Protein was determined as described before.\(^{19}\)

**Acetylcholinesterase activity.** Acetylcholinesterase activity was determined fluorometrically in cell lysate using acetylthiocholine iodide as a substrate in conjunction with coumarinylmaleimide, as described by Ishibashi et al.\(^{19}\) or by in-situ staining of cells spun down on slides using acetylthiocholine iodide as a substrate.\(^{19}\)

**RESULTS**

**DNA synthesis in BM megakaryocytes.** DNA synthesis in BM megakaryocytes was followed by determining incorporation of a DNA precursor, BrdU, into the cells. To this end, mouse BM cells were cultured in the presence of BrdU for up to 24 hours in a liquid culture system that promotes megakaryocyte Progeniess.\(^{14,15}\) The BM cells were stained at different time points in situ with an antibody to BrdU and the percentage of megakaryocytes actively synthesizing DNA was determined. Megakaryocytes were easily identified on the basis of size and morphology. These experiments were performed on nonsynchronized cells because any attempt to synchronize primary BM cells, eg, serum starvation or lovastatin,\(^{21}\) resulted in major cell death. To ascertain whether DNA synthesis in megakaryocytes is continuous or interrupted by a gap, we determined the percentage of cells that were BrdU-positive as a function of time of exposure to BrdU. After 30 and 60 minutes of incubation, 7% and 10%, respectively, of the megakaryocytes were BrdU-positive. After 90 minutes of incubation and at following successive time points (3, 6, 16, or 24 hours of incubation), a maximum of 1% of recognizable megakaryocytes were BrdU-positive, whereas most of the large mature cells were BrdU-negative (Fig 1). The time taken to observe a maximal percentage of cells that are BrdU-positive is approximately the length of the gap phase preceding the S phase. Cells in

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**Fig 1.** DNA synthesis in BM megakaryocytes. Mouse BM cells were cultured in a liquid culture for 24 hours in the presence of BrdU, all as described in the Materials and Methods. Cells were spun down on a slide and stained with antibody to BrdU followed by a peroxidase staining. Results are from one representative experiment of three performed (A). In every experiment, two slides were analyzed, each containing about 1.5 \(\times 10^6\) cells. Arrows point to some megakaryocytes of different sizes. Megakaryocytes were identified on the basis of morphology with a phase-contrast microscope under high magnification (B). Bar: 100 \(\mu\)m (A), 20 \(\mu\)m (B).

**Fig 2.** Expression of cyclin proteins. Cytospun BM cells (A) were subjected to double immunofluorescence staining with a mixture of rat antibody to cyclin D3 and mouse antibody to cyclin B, followed by reaction with a mixture of antirat Ig (IgG) conjugated to TRITs (B; cyclin D3) and antimouse IgG conjugated to FITC (C; cyclin B). In a separate experiment, a reaction with rabbit polyclonal antibody to cyclin B1 followed by staining with antirabbit Ig (IgG) yielded a result identical to the one presented in (C) (data not shown). As a control, cells were stained only with antirat IgG conjugated to TRITs (D). Cytospun BM cells (E) were also subjected to immunofluorescence staining with a rat antibody to cyclin D1 followed by a reaction with antirat IgG conjugated to TRITs (F; cyclin D1). Cells were analyzed with a phase-contrast microscope (A and E) and a fluorescent microscope (B, C, D, and F). The results are representatives of examination of two experiments, with each having 0.8 to 2.0 \(\times 10^6\) cells analyzed per slide in triplicate. The arrows point to a megakaryocyte identified on the basis of morphology. Bar: 40 \(\mu\)m.

**Fig 3.** Effect of cyclin D3 antisense and sense oligomers on megakaryocytes. A comparison analysis of cDNA sequences of murine cyclins D1, D2, and D3 on the GenBank database\(^{13,44,45}\) showed few regions with low homology. Sequences in a low-homology region (A) are presented 5' to 3', with the number on the left indicating the position in relation to the translation start. Mouse BM cells were cultured in the absence (B) or presence (C) of cyclin D3 antisense oligomer (5'-CACGCGGCTAAGACTCT-3'), which was designed to hybridize to mRNA in the region shown in (A), or in the presence of the corresponding cyclin D3 sense oligomers (D). The cells were cytospun and stained for acetylcholinesterase activity (giving rise to a brown product), followed by staining with hematoxylin. Culture conditions and other details are described in the Materials and Methods. The results are from one representative experiment of five performed. Bar, 100 \(\mu\)m.
Cyclin D1 211, GAGCCATGCTTAAGACT
Cyclin D2 81, AGAACCTGTTGACCATC
Cyclin D3 159, AGAGTTTACTCCGCCTG
the early stage of the gap phase will join the BrdU-positive population at the end of G1 phase, upon which a maximal number of cells will become BrdU-positive. Based on this rationale, we conclude that the G1 phase is less than 90 minutes in length. We also conclude that the majority of the mature megakaryocytes in normal BM are beyond the process of DNA synthesis and ploidization. These results represent averages derived from six preparations, each of about 150,000 cells, from three different experiments, with a standard deviation of up to 2%. A total of approximately 900 megakaryocytes were examined. In other experiments, in which the mature megakaryocytes were filtered out of the BM (see Materials and Methods) before culturing in the presence of BrdU, BrdU was incorporated into the majority of the newly developing megakaryocytes (data not shown).

Determination of expression of different cyclins in megakaryocytes. D-type cyclins regulate G1 progression, whereas cyclin B is essential for mitosis in different eukaryotic cells. Because of the rarity of megakaryocytes in BM, we used immunohistochemistry to detect the presence of these cyclins in individual cells. As shown in Fig 2, megakaryocytes stained strongly with an antibody to cyclin D3 but not with cyclin B antibody. About 10% of the megakaryocytes displayed staining with anticyclic D3, a value corresponding to the percentage of cells undergoing endomitosis (Fig 1). Positive staining with either cyclin B or cyclin D3 antibodies was occasionally observed in nonmegakaryocytes (Fig 2B and C). An antibody to cyclin D1 weakly stained about 10% of the megakaryocytes (Fig 2F). The intensity of staining observed in different preparations reacted with anticyclic D1 varied, but was consistently weaker than the staining displayed with anticyclic D3. These results represent the examination of several fields, each containing 0.8 to 2 x 10^3 cells, as detailed in Fig 2. The unavailability of an antibody for cyclin D2 that exhibits staining in immunohistochemistry studies prevented us from exploring the presence of this cyclin in megakaryocytes.

The use of antisense oligonucleotides to determine the role of cyclin D3 in endomitosis. Because cyclin D3 appeared to be highly expressed in megakaryocytes, antisense oligomers were used to determine its role in promoting megakaryocytopoiesis. A synthetic 17-mer oligonucleotide designed to hybridize with unique sequence elements in cyclin D3 mRNA (Fig 3A) was added to a culture of primary BM cells depleted of megakaryocytes. Mature megakaryocytes were removed from the culture before the addition of the antisense oligomers to follow-up potential effects on early and later stages of endomitosis. Under these culture conditions, the megakaryocytes develop from precursors and undergo endomitosis to form high ploidy cells that survive in the culture for more than 5 days. Immunohistochemistry of BM cells was used to establish that cyclin D3 protein was inhibited by treatment with the antisense oligomer. Quantitative analyses by Western or Northern blots were not practical to perform, because megakaryocytes represent less than 1% of total BM cells.

Megakaryocytes were identified by morphology and by in-situ staining for acetylcholinesterase, which was uniquely expressed in murine megakaryocytes. Antisense to cyclin D3 (ASD3) caused a 75% decrease in the total number of megakaryocytes and resulted in the emergence of small megakaryocytes, as compared with nontreated cells or cells cultured with sense oligonucleotides (SD3) (Fig 3B, C, and D). Also, pulse-labeling experiments with BrdU (for 2 hours) in BM cells cultured for 48 hours in the presence or absence of ASD3 showed that 2% and 12%, respectively, of the recognizable megakaryocytes synthesized DNA. These results indicated that cyclin D3 is essential for the normal progression through the cell cycle in megakaryocytes. The level of acetylcholinesterase activity in cell lysates prepared from BM cells correlated well with the in-situ staining for this enzyme, i.e., acetylcholinesterase activity was significantly reduced in cells treated with ASD3 but not in cells exposed to an antisense oligonucleotide designed to suppress expression of cyclin B (Table 1). Results similar to those described above were obtained with a different cyclin D3 antisense oligonucleotide designed to hybridize with mouse cyclin D3 mRNA along 17 bp immediately downstream to the translation start (results not shown). The antisense oligonucleotides did not significantly affect the total number of BM cells in culture. Because our culture conditions were originally selected to selectively promote megakaryocytopoiesis and less the proliferation of other BM cells, these studies described above did not exclude the possibility that ASD3 or ASB had the potential to abrogate the cell cycle of other subtypes of BM cells. Indeed, in the presence of hemopoietic growth factors (interleukin-3 [IL-3], IL-6, erythropoietin, and granulocyte-macrophage colony-stimulating factor) that support growth of myeloid and erythroid cells in culture, the total number of BM cells decreased when exposed to ASD3 or ASB (Table 1), indicating that the mitotic cell cycle in some other lineages depends on cyclins D3 or B. As also noticed in Table 1, in the presence of hemopoietic growth factors, ASB abrogated megakaryocytopoiesis to some extent. This is likely to be caused by an inhibitory effect of ASB on growth factors-induced conversion of precollapsed cells (undergoing a cyclin B-dependent mitotic cell cycle) to megakaryocytes.

DISCUSSION

The megakaryocyte is an unusual BM cell characterized by its ability to synthesize DNA in the absence of mitosis, thus forming a polyploid nucleus. Ploidy analyses of normal murine BM cells show a reproducible pattern of ploidy with a fixed ratio between megakaryocytes of different ploidy classes, including less than 5% 2N (diploid), 17% 8N, 61% 16N, 19% 32N, and 0.7% 64N cells. It is not certain yet whether polyploid megakaryocytes enter prophase as well as metaphase, which involves chromosome condensation and spindle formation in the absence of anaphase, or rather skip most of the stages in M phase to directly enter a G1 phase. Early ultrastructural studies suggested that megakaryocytes undergo metaphase during endomitosis; however, there was no clear reference to nuclear breakdown. Thus, although further electron microscopic analyses of different stages of mitosis in megakaryocytes are underway, we aimed at examining the role of different cyclins in promoting endomitosis.

Basic questions regarding cell cycle regulation in the megakaryocytic lineage have been difficult to address because of the lack of a pure megakaryocytic cell line that mimics the full developmental process of the megakaryocytic lineage. The
currently available cell lines exhibit multilineage properties and/or low ploidy states or require addition of multipotent agents such as phorbol esters to induce ploidy. In the current study, we used primary cultures of mouse BM cells under conditions found before to promote megakaryocyte ploidy. Because megakaryocytes are rare in the BM, comprising only 0.1% of total BM cells, we used in-situ staining to observe DNA synthesis. Experiments involving the usage of the DNA precursor BrdU showed that only a maximum of 15% of all mouse BM megakaryocytes were actively synthesizing DNA. The rest of the megakaryocytes, which were mostly large mature cells, were in a permanent resting phase. It is not clear yet which gene product is involved in turning off DNA synthesis in these mature megakaryocytes in normal BM. Cell cycle inhibitors in other systems, such as the p27 protein that mediates signals of transforming growth factor β and inhibits entry into S phase or the p21 protein, may play a role in the megakaryocytic cell cycle. We are currently pursuing a line of investigation to explore the potential role of such cell cycle inhibitors in megakaryocytes.

Odent et al determined the labeling index of megakaryocyte endomitotic figures after a single injection of radiolabeled thymidine to rats. These investigators concluded that the length of the S phase in megakaryocytes is about 7 to 8 hours and that the generation cycle time, which was defined as the time required for one complete round of DNA synthesis and endomitosis, was 9.3 hours. These findings are consistent with our estimation of the length of the gap phase preceding DNA synthesis in mouse BM megakaryocytes as being less than 90 minutes in length. It is of interest to note that a shortened cell cycle consisting of G1/S phases was also observed in some cells of Drosophila embryos. In the current study, we aimed at exploring the role of different cyclins in promoting the endomitotic cell cycle in megakaryocytes. Immunohistochemistry was used to show that the G1 phase cyclin, cyclin D3, was highly expressed in megakaryocytes. Experiments involving the use of antisense oligonucleotides were performed to determine the dependency of the megakaryocytic cell cycle on cyclin D3 and, hence, on the G1 phase. Antisense oligonucleotides to different cyclins were successfully used to suppress cyclins in other systems.

Our data indicated that antisense oligomers to cyclin D3 abrogated megakaryocyte development. These results further pointed to the importance of the G1 phase for normal progression through the endomitotic cell cycle in megakaryocytes. According to a preliminary report, cyclin D3 was highly expressed also in a human erythroleukemia cell line (HEL) induced to ploidize by phorbol esters. D-type cyclins are differentially expressed in various cell lineages in a highly growth-factor-dependent manner. Thus, it would be interesting to explore the inducibility of cyclin D3 in megakaryocytes by the potent endomitotic factor, thymopoietin, which was recently purified and characterized.

During a normal cell cycle, S phase progression depends on completion of the M phase. However, certain treatments, such as inhibitors of protein kinases in mammalian cells or high levels of the protein encoded by runt, which inhibits the mitotic kinase in fission yeast, block M phase and induce repeats of S phase. In certain Drosophila cells, endoreplication appears to be associated with the lack of cyclin B protein. It has been suggested that the absence of cyclin B in these Drosophila cells prevents M phase and, as a consequence, redirects the cell back to a repeated round of S phase. As also recently reported, the metaphase II arrest in mouse oocytes is controlled by destruction of cyclin B. In accordance with these reports is our observation that mouse megakaryocytes undergoing endomitosis either lack or contain a low level of cyclin B. It is yet to be explored whether low-level transcription/translation or rather rapid degradation regulates the level of cyclin B in polyploid megakaryocytes. Although we were unable to directly determine Cdc2 kinase activity in megakaryocytes, in all eukaryotic cells tested so far the activation of this mitotic kinase depends on availability of high levels of cyclin B. O’Connell et al suggested that different stages of mitosis may be regulated by different kinases, ie, condensation of chromatin is regulated by a NIMA kinase homologue whereas other mitotic events are regulated by Cdc2 kinase. It is plausible then that early stages of mitosis occur in megakaryocytes undergoing endomitosis. Because resting cells of all lineages lack cyclin B and yet the cells do not ploidize, we suggest that reduced levels of cyclin B alone may not be sufficient to drive endomitosis, but, in conjunction with an increase in the G1 phase-promoting cyclin, cyclin D3, endomitosis in megakaryocytes progresses normally.

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Cyclin D3 is essential for megakaryocytopoiesis

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