SUPPLEMENTARY APPENDIX

Hematopoiesis specific loss of Cdk2 and Cdk4 results in increased erythrocyte size and delayed platelet recovery following stress

Senthil Raja Jayapal,[1] Chelsia Qiuxia Wang,[1,2] Xavier Bisteau,[1] Matias J. Caldez,[1,3] Shuhui Lim,[1] Vinay Tergaonkar,[1] Motomi Osato,[2] and Philipp Kaldis[1,3]

[1]Institute of Molecular and Cell Biology, A*STAR Agency for Science, Technology and Research; [2]Cancer Science Institute of Singapore, National University of Singapore; and [3]National University of Singapore, Department of Biochemistry, Republic of Singapore

 doi:10.3324/haematol.2014.106468
Manuscript received on February 24, 2014. Manuscript accepted on January 21, 2015.
Correspondence: kaldis@imcb.a-star.edu.sg

Supplemental Figures and Methods

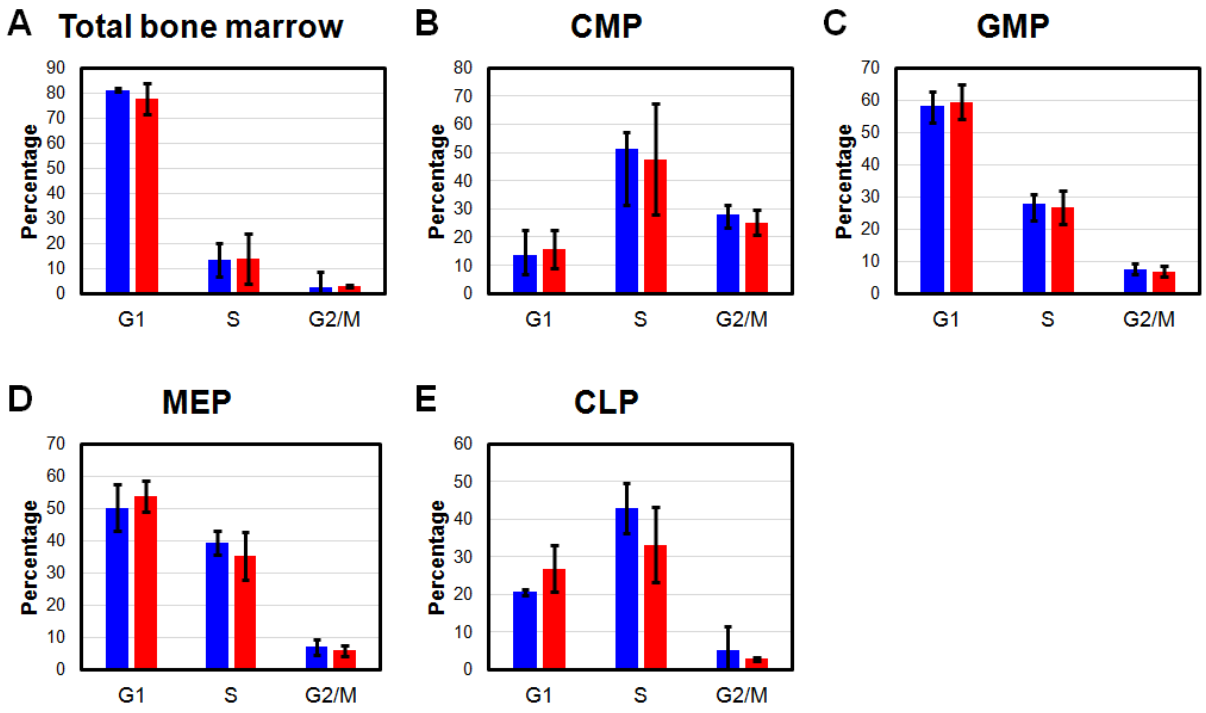

Figure S1. Cell cycle analysis of bone marrow hematopoietic progenitors. The percentage of cells in the G1, S, and G2/M phases of the cell cycle for total bone marrow (A), Common Myeloid Progenitor [CMP] (B), Granulocyte Macrophage progenitor [GMP] (C), Megakaryocyte-Erythroid progenitor [MEP] (D) and the Common Lymphoid Progenitor [CLP] (E) fractions are shown for $Cdk2^{fl/fl}Cdk4^{-/-}$vavCre DKO mice (red bars) and littermate controls (blue bars) (n=3). Error bars represent standard deviation. Two-tailed t-test results are indicated by asterisks. *, $p<0.05$.

Generation of Cdk2^{flox} mice. Mouse genomic DNA harboring the Cdk2 locus was sub-cloned from the clone pBeloBACII 192B20 (ResGEN, 96021), into the pBlight-TK vector as has been described previously[1]. LoxP recombination sites and a neomycin-selection cassette were introduced flanking the fourth and fifth coding exons of the mouse Cdk2 genomic locus using recombineering technique[2]. The resulting targeting vector (PKB630) was linearized by NotI digestion and electroporated into ES cells. Following positive and negative selection with Geneticin and ganciclovir, respectively, genomic DNA from surviving ES cell colonies was used to screen for homologous recombination by Southern hybridization. ES cell clones that were correctly targeted were identified and used for the generation of the Cdk2 conditional knockout mouse strain. To generate the Cdk2^{fl} allele, the neomycin cassette was removed by crossing Cdk2 conditional knockout mice with β-actin–Flpe transgenic mice [strain name: B6.Cg-Tg(ACTFLPe) 9205Dym/J; stock no.: 005703; The Jackson Laboratory].

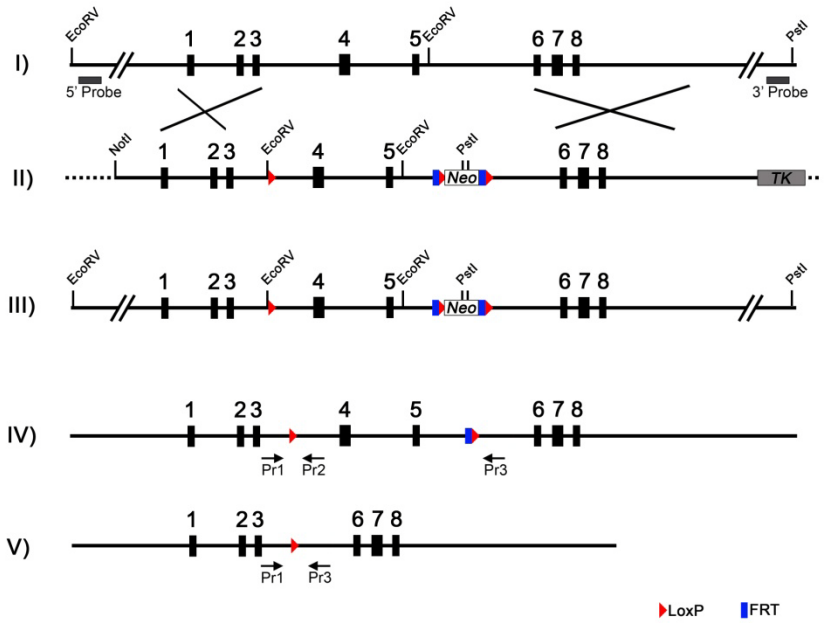

Figure S2. Generation of Cdk2 conditional knockout mice.

Genotyping primers. Mice were routinely genotyped by PCR using the following primers:

Mouse Strain	Genotyping primers	Band size
$Cdk2^{fl/fl}$	PKO1011: 5'- GCG CCA CCA CTG CTT GGC TCA GAT A -3' PKO1131: 5'- CAA GCA CGA ACC AAC ACA CAT AAG T -3'	WT : 342 bp Floxed : 441 bp
$Cdk4^{-/-}$	PKO0103: 5'- ATA TTG CTG AAG AGC TTG GCG G -3' PKO0104: 5'- CGG AAG GCA GAG ATT CGC TTA T -3' PKO0105: 5'- CCA GCC TGA AGC TAA GAG TAG CTG T -3'	WT : 195 bp Neo(null) : 315 bp
$Cdk2^{-/-}$	PKO1640: 5'- GTC TTC TTG TTC CCC AGG TG -3' PKO1641: 5'- CGC CTT CTA TCG CCT TCT TG -3' PKO1642: 5'- ATG GGT GGG CAG CAT AGA AC -3'	WT : 156 bp Neo(null) : 389 bp
$Rb^{fl/fl}$	PKO1677: 5'- TCC CCT AAT CCC TGT AAG TT -3' PKO1678: 5'- ACT AGG TTA AGT TGT GGC TC -3' PKO1679: 5'- GAG ACC TGC TTT TGT ACA AG -3'	WT : 145 bp Floxed : 193 bp Null : 243 bp
vavCre	PKO2258: 5'- CTC CCC GAG GGG CCA AGT GA -3' PKO2259: 5'- GCC AGG CCT CTG GCT TGC AG -3'	Cre positive: 500 bp

Flow cytometric analysis of bone marrow, spleen, and thymus. Flow cytometric analysis and sorting by LSRII or FACSAria (BD Biosciences) were performed using standard methods[3]. Briefly, blocking non-specific binding in mouse serum was followed by antibody staining on ice for 30 minutes. After washing, cells were suspended in 0.5 ml of phosphate-buffered saline (PBS) with 0.05 μg/ml Hoechst 33258 (Invitrogen, H3569) to exclude dead cells. For flow cytometry, a homogenous population in cell size [forward scatter–side scatter (FSC-SSC) window] and viable cells were gated and analyzed for individual antigen expressions. For identification of $cKit^{+}Sca1^{+}lineage^{-}$ (KSL) cells, positive selection for the markers c-Kit and Sca-1, and negative selection for markers of mature hematopoietic cell lineages (CD3, CD4, CD8, B220, Gr-1, Mac-1, Ter-119 and IL-7Rα) were used. Antibodies against CD34, Flt3 and FcγRII/III were used to further fractionate the KSL and myeloid progenitor ($cKit^{+}Sca1^{-}lineage^{-}$) populations. For identification of mature lineage cells, the following markers were used: myeloid, Mac-1 and Gr-1; B-cells, B220 and CD19; NK-cells,

Dx5 and CD3; MgK, CD41 and CD61; T-cells, CD4 and CD8; erythroid, CD71 and Ter119. For Ki-67 staining, BM cells were first stained for KSL, then fixed and permeabilized using BD Cytofix/Cytoperm Fixation/Permeabilization solution kit (BD Pharmingen, 555028) according to manufacturers' instructions, after which cells were stained with FITC-conjugated antibodies to Ki-67 (BD Pharmingen, 556003). For megakaryocyte polyploidy analysis, BM cells were sorted for lineage$^-$ population and cultured for 3 days in α-MEM supplemented with 10 ng/ml IL-3 (PeproTech, 213-13), 10 ng/ml SCF (PeproTech, 250-03) and 50 ng/ml TPO (PeproTech, 300-18). Cells were then harvested, followed by overnight fixation with ice-cold 70% ethanol at -20ºC. Cells were washed twice with PBS and stained with anti-CD41 FITC-conjugated antibody (BD Pharmingen, 553848) at 4ºC for 30 minutes. After washing, cells were incubated in propidium iodide (PI) staining buffer [50 µg/ml PI (Sigma, P4170), 100 µg/ml DNase-free RNase A (Roche, 10109142001) in PBS] for 30 minutes at room temperature prior to flow cytometric analysis. The antibodies used for flow cytometry are:

Antigen	**Clone**	**Fluorochrome**	**Company**	**Catalogue No:**
c-Kit	2B8	APC	eBioScience	17-1171-83
c-Kit	2B8	PE-cy7	BD Pharmingen	558163
Sca-1	D7	APC	eBioScience	17-5981-83
Sca-1	E13-161.7	FITC	BD Pharmingen	553335
Sca-1	E13-161.7	biotin	BD Pharmingen	553334
CD3e	145-2C11	PE	eBioScience	12-0031-85
CD4	H129.19	PE	BD Pharmingen	553653
CD8a	53-6.7	PE	eBioScience	12-0081-85
B220	RA3-6B2	PE	eBioScience	12-0452-83
B220	RA3-6B2	FITC	BD Pharmingen	553087
Mac-1	M1/70	PE	eBioScience	12-0112-85

Gr-1	RB6-8C5	PE	BD Pharmingen	553128
Gr-1	RB6-8C5	FITC	BD Pharmingen	553127
Ter119	TER119	PE	eBioScience	12-5921-83
IL-7Rα	A7R34	PE	eBioScience	12-1271-83
CD34	RAM34	FITC	BD Pharmingen	553733
Flt3	A2F10	biotin	eBioScience	13-1351-81
FcγRIII/II	2.4G2	APC-cy7	BD Pharmingen	560541
FcγRIII/II	2.4G2	APC	BD Pharmingen	558636
CD19	ID3	PE	BD Pharmingen	557399
Dx5 (CD49b)	Dx5	PE	BD Pharmingen	553858
CD3	145-2C11	FITC	BD Pharmingen	553062
CD41	MWReg30	FITC	BD Pharmingen	553848
CD61	2C9.G2	PE	BD Pharmingen	553347
CD4	H129.19	FITC	BD Pharmingen	553651
CD71	C2	FITC	BD Pharmingen	553266

Cumulative BrdU labeling for measurement of cell cycle length. Total bone marrow cells from $Cdk2^{fl/fl}Cdk4^{-/-}$vavCre (DKO) mice or littermate controls were cultured in erythroid differentiation medium containing 10μM BrdU for the indicated time periods, followed by fixing and staining for the detection of BrdU incorporation by flow cytometry using APC BrdU Flow Kit (BD Pharmingen, 552598). The cells were additionally immunostained with FITC-CD71 to specifically gate the $CD71^+$ erythroblasts during flow cytometry analysis of BrdU labeling. The cell cycle time (Tc) and length of S-phase (Ts) was calculated from the cumulative labeling index plot as described previously[4, 5].

Western blots. Cell pellets were lysed in Laemmli buffer (60mM Tris-HCl pH6.8; 10% Glycerol; 100mM DTT; 2% SDS) completed with Protease inhibitors (chymostatin, leupeptin

and pepstatin 10µg/ml), 50mM ß-glycerophosphate, 4mM NaF and 0.1mM sodium orthovanadate. Lysates were homogenized using a plastic pestle and boiled for 5 minutes. 25µg of whole lysates were resolved by SDS-PAGE, transferred to PVDF membranes and blotted using the following antibodies. Cdk2 affinity purified antibodies have been described previously[1]. Cdk4 (Cell signaling, 2906), phospho-Rb (BD Pharmingen, 554136), phospho-Rb pT821 (Biosource-Invitrogen, 44-582G), phospho-Rb pS807/811 (Cell Signaling, 9308), phospho-Rb pS795 (Cell Signaling, 9301), p130 (BD Transduction, 610261), p107 (eBioscience, 14-6744), Cdk1 (Santa Cruz, sc-954), cyclin A (Santa Cruz, sc-596), cyclin E (eBioscience, 14-6714), cyclin D3 (Santa Cruz, sc-6283), E2F4 (Santa Cruz, sc-866), p27 (BD Transduction, 610242), Cdk6 (Santa Cruz, sc-177), HSP90 (BD Transduction, 610419), or GAPDH (Cell Signaling, 2118).

References

1. Berthet C, Aleem E, Coppola V, Tessarollo L, Kaldis P. Cdk2 knockout mice are viable. Curr Biol. 2003;13(20):1775-85.

2. Lee EC, Yu D, Martinez de Velasco J, Tessarollo L, Swing DA, Court DL, et al. A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics. 2001 Apr 1;73(1):56-65.

3. Wang CQ, Motoda L, Satake M, Ito Y, Taniuchi I, Tergaonkar V, et al. Runx3 deficiency results in myeloproliferative disorder in aged mice. Blood. 2013 Jul 25;122(4):562-6.

4. Lim S, Kaldis P. Loss of Cdk2 and Cdk4 induces a switch from proliferation to differentiation in neural stem cells. Stem Cells. 2012;30(7):1509-20.

5. Nowakowski RS, Lewin SB, Miller MW. Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. J Neurocytol. 1989 Jun;18(3):311-8.