Supplementary Methods

Immunocytochemistry staining

Immunocytochemistry staining was conducted by the avidin-biotin-peroxidase complex (ABC) method. Bone marrow cells were cytospun onto poly-L-lysine–coated slides, air dried and fixed with 4% paraformaldehyde in 1×PBS (pH7.4) for 20 minutes at room temperature. Endogenous peroxidase of the cells was inactived by treatment with 3% hydrogen peroxide for 15 minutes at room temperature. The cells were incubated with normal blocking serum at room temperature for 30 minutes to reduce nonspecific binding. The primary antibodies (1:1000 dilution) were applied overnight at 4°C in a moist chamber. Next, the cells were incubated with the biotinylated second antibodies at room temperature for 30 minutes. After washing three times with PBS, the cells were incubated with streptavidin-peroxidase complex at room temperature for 30 minutes. Peroxidase activity was visualized with diaminobenzidine and the cells were counterstained with hematoxylin. After dehydrated in ethanol and cleared with xylene, the slides were mounted with Canada balsam (Sigma) for observation. Rat anti-KLH IgG_1 antibody and nonimmune goat sera were using as negative controls for the primary monoclonal antibody (against Tyro 3) and polyclonal antibodies (against Axl and Mer) respectively.

RT-PCR

Total RNA was prepared from enriched MKs using TRIzol® reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA), followed by DNase I treatment to remove potential contamining genomic DNA. Total RNA (0.5 μg) was reverse transcribed into cDNA in 20 μl of reverse transcription reaction mixture containing 2.5 μM random hexamers, 2 mM dNTPs, 5 U RNase inhibitor (Takara, Dalian, China), and 200 U M-MLV reverse transcriptase (Promega, Madison). PCRs (cycles of 94°C for 30 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 45 seconds) were performed for a number of cycles corresponding to the high end of the range in which a linear increase in products could be detected. The number of PCR cycles was 28, 32 and 35 for Tyro 3 subfamily receptors, and 22, 25

and 28 for GAPDH. The PCR primers were as follows: *Axl*, forward 5′-AGG CTC ATT GGC GTC TGT T-3′ and reverse 5′-ATC GCT CTT GCT GGT GTA G-3′; *Tyro 3*, forward 5′-TGA AGC CCG CAA CAT AAA-3′ and reverse 5′-TCC CAT TCC AGG ATA AGG-3′; *Mer*, forward 5′- CCT GGT TTT GAT GGC TAC TC-3′ and reverse 5′- GGA GGC TTC GTC CAT CTA AT-3′; *GAPDH*, forward 5′-CCA TCA CCA TCT TCC AGG AG-3′ and reverse 5′- CCT GCT TCA CCA CCT TCT TG-3′.

Counts of MKs in bone marrow and spleen

MKs numbers were scored based on a positive signal in acetylcholinesterase assay as previously reported.[1] Briefly, bone marrow cells were cytospun onto poly-L-lysine–coated slides (2×10^5 each slide) at 1000 rpm for 1 minute. The slides were then air dried and fixed with 4% paraformaldehyde in PBS at room temperature for 20 minutes. After washing twice with PBS, the slides were treated with the substrate staining solution (0.5 mg/ml acetylthiocholine iodide, 0.1 M sodium phosphate, pH 6.0, 5 mM sodium citrate, 3 mM CuSO4, and 0.5 mM K3Fe(CN)6) at room temperature for 2 hours. The number of AchE-positive cells was counted as MKs.

Fresh spleens were embedded in Tissue-Tek OCT compound and snap frozen in liquid nitrogen. Six μm thick sections were cut in a cryostat, and mounted on slides coated with poly-L-lysine. The slides were fixed in 4% paraformaldehyde in PBS at room temperature for 20 minutes, washed and staining for AchE described above. The number of MKs per square millimeter of was measured to evaluate the relative MK density in spleens.

Platelet function assays

Platelet-rich plasma (PRP) was prepared as previously reported.[2] In brief, blood was collected by cardiac puncture into 10% volume of HEPES/Tyrodes buffer (129 mM NaCl/8.9 mM $NaHCO_3$/2.8 mM KCl/0.8 mM KH_2PO_4/5.6 mM dextrose/10 mM Hepes/0.8 mM $MgCl_2$, pH 7.4) containing150 U/ml heparin and centrifuged at 100 *g*

for 10 minutes to collect PRP. Platelet-poor plasma (PPP) was prepared by centrifugation of the remaining blood sample at 2000 *g* for 10 minutes. Red blood cells remaining in the PRP were pelleted at 100 *g* for 5 minutes. The platelet number was adjusted to 3×10^8/ml with PPP. PRP or PPP was a pool from 3 mice.

Platelet aggregation was performed in an aggregometer (Chrono-Log, Havertown, PA). The platelet samples (250 μl) were incubated at 37°C for 5 minutes with stiring before platelet stimulants were given. Percent light transmission was measured compared with a PPP blank. For each concentration of agonist, an average percent aggregation was calculated as a mean ± SD, respectively, of 5 experiments.

Adenosine trisphosphate (ATP) secretion was measured using luciferin/luciferase reagent (Chrono-lume) (Chrono-Log, Havertown, PA) according to the manufacturer's protocol. Luciferin/luciferase (12μl) was added to 238 μl of PRP within 2 minutes before stimulation. One nmole of ATP (Chrono-Log) was used as internal standard. The average concentrations (nmoles) of released ATP were calculated as a mean ± SD of 5 experiments.

References

1. Zhang Y, Nagata Y, Yu G, Nguyen HG, Jones MR, Toselli P, et al. Aberrant quantity and localization of Aurora-B/AIM-1 and survivin during megakaryocyte polyploidization and the consequences of Aurora-B/AIM-1-deregulated expression. Blood 2004;103:3717-26.

2. Chrzanowska-Wodnicka M, Smyth SS, Schoenwaelder SM, Fischer TH, White GC, 2nd. Rap1b is required for normal platelet function and hemostasis in mice. J Clin Invest 2005;115:680-7.

Supplementary Tables

Table 1. Hematological Parameters

Parameter	Ctrl	T	A	M	TA	AM	TM	TAM
WBC (10^9/L)	8.59±2.2	8.77±1.97	8.34±1.31	8.47±1.81	13.4±5.9	8.26±0.59	11.7±4.0	14.0±3.0
RBC (10^{12}/L)	11.2±0.6	11.3±0.3	10.8±0.6	11.6±0.5	12.5±0.7	10.9±0.5	12.4±0.3	11.8±0.8
HGB (g/L)	162±8	174±16	168±9	167±11	173±17	163±13	167±8	172±14
PLT (10^9/L)	1196±87	1171±124	1180±65	1155±86	1164±56	1195±87	1120±192	591±46*
Differential counts (10^9/L)								
Lymphocytes	7.46±2.41	6.95±2.21	6.24±1.46	6.13±1.95	10.68±3.8	5.90±0.4	9.34±2.5	10.2±2.10
Monocytes	0.78±0.22	0.76±0.31	1.14±0.29	1.23±0.84	1.53±0.81	1.62±0.89	1.46±0.79	1.44±0.53
Neutrophils	0.24±0.11	0.37±0.145	0.46±0.27	0.38±0.17	0.92±0.34	0.27±0.11	0.56±0.28	0.48±0.16
Eosinophils	0.052±0.01	0.086±0.03	0.062±0.02	0.053±0.01	0.093±0.06	0.041±0.03	0.08±0.05	0.085±0.02

Counts of peripheral blood cells from adult (10-week-old) control (Ctrl) and mutant mice. All values are expressed as means ± SD for 8 female mice per genotype. RBC, red blood cell; WBC, white blood cell; HGB, hemoglobin; PLT: platelet. *P<0.05

Table 2. DNA ploidy of MKs in different genotype mice

Mouse	Percent of MKs in ploidy class					
	2N	4N	8N	16N	32N	64N
Ctrl	14.3±1.1	7.7±0.6	11.6±0.4	48.6±3.1	14.3±0.5	2.6±0.2
T	15.2±1.3	7.8±0.4	10.6±0.3	45.9±3.5	14.6±.6	3.0±0.1
A	13.7±1.2	7.2±0.8	12.4±0.6	47.3±4.2	13.2±1.2	3.3±0.4
M	14.1±1.6	7.2±0.7	11.0±0.7	46.7±5.1	13.9±1.3	3.6±0.5
TA	14.9±0.9	7.3±0.5	10.3±0.6	45.6±3.8	15.8±2.1	3.1±0.2
TM	12.2±1.5	6.2±0.7	11.8±0.7	48.5±4.2	14.3±0.7	3.6±0.6
AM	23.1±1.8	19.6±0.3	10.5±0.4	29.0±2.6	13.7±1.4	2.4±0.1
TAM	29.6±2.1	14.8±0.4	9.4±0.5	28.5±2.6	13.0±1.6	2.9±0.2

Data are the mean±standard deviation from analysis of 3 mice of each genotype

Supplementary Figures

Figure 1. Platelet aggregation and ATP release. (A) Representative profiles of platelet aggregation in response to 2 μM ADP in normal (Ctrl), single (T), double (TA) and triple mutant (TAM) mice. (B) The mean percents of platelet aggregation in response to 2 μM ADP for all genotype mice. Single mutant mice display mild decrease in aggregation rates (about 75% of normal control), while double and triple mutant mice exhibit evident decrease in aggregation rates (about 50% and 28% that of normal control respectively). (C) Representative profiles of platelet aggregation upon stimulation of 4 μg/ml collagen. (D) The mean percents of platelet aggregation at the concentration of 4 μg/ml collagen. The platelets from single mutant mice display aggregation rates of 36.6-42.2%. However, platelets of double mutants exhibit very low aggregation rates (less than 8%), and no evident aggregation was observed in platelets of TAM mice. (E) Representative profiles of ATP release. (F) ATP release in response to 5 μM ADP for all genotype mice. The values represent the means ± SD of 5 independent experiments using PRP pooled from 3 mice. The values for all single, double and triple mutants were decreased significantly compared with that of control mice, as determined by Student's *t* test ($P<0.05$).

Figure 2. Expression of Tyro 3 family receptors in megkaryocytes. (A) Immunostaining for Tyro 3, Axl and Mer. Bone marrow cells were cytospun onto glass slides and immunostained with specific antibodies. The cells were counterstained with hematoxylin. MKs were positively immunostained for all three receptors. Neutrophil (N) cells were negative. Some other cells were also positively stained (arrows). MKs were not positive in negative control though nonspecific staining was observed in some cells (arrowhead). (B) RT-PCR confirmed the expression of Tyro 3 family receptors in RNA level. Total RNAs were extracted from enriched MKs of wild type mice. All RT-PCRs were performed in independent RNA samples from three mice.

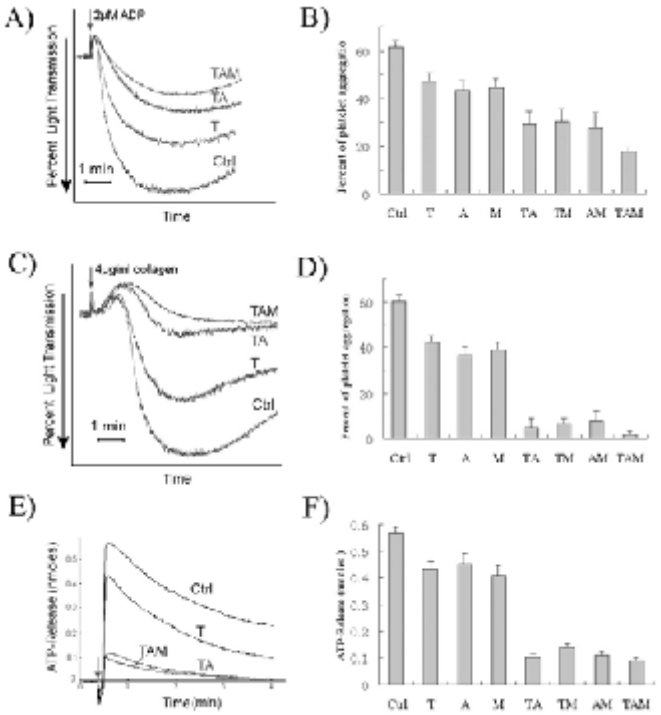
Figure 3. Counts of MKs in bone marrow. Bone marrow cells (2×10^5) were

cytospun onto poly-L-lysine–coated slides for acetylcholinesterase staining. Positively stained cells were scored under a microscope. A decrease of approximately 35% and 40% of MK numbers in bone marrow of AM and TAM mice respectively was observed compared to that of control mice. Other genotype mice showed no significant difference with control (Ctrl) mice in MK counts. The values represent the means ± SD for 5 female mice at 10 weeks postnatal. (*P<0.05)

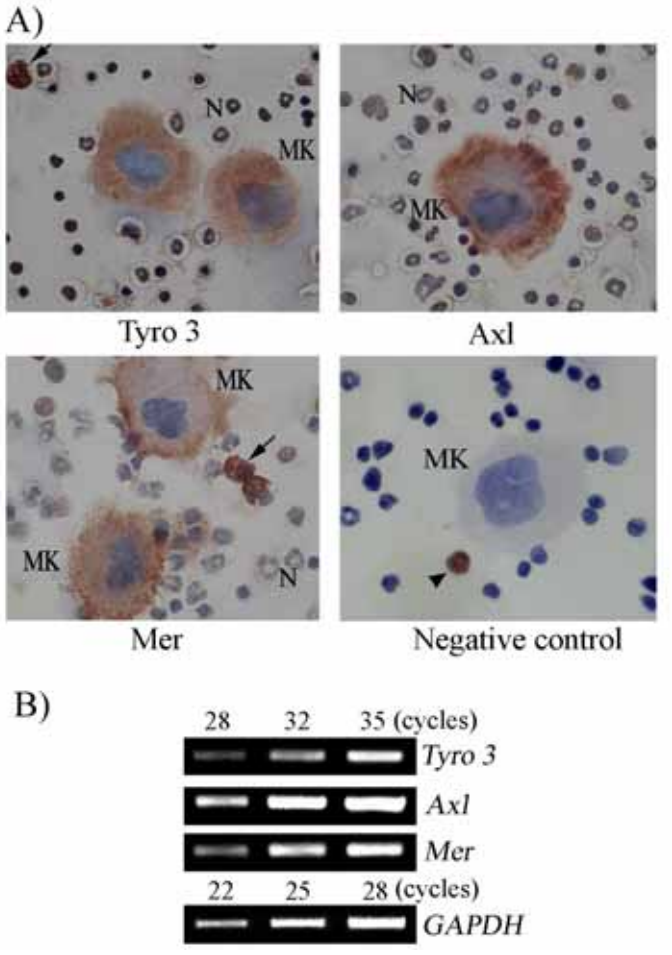
Figure 4. Splenic MKs counts. (A) Frozen spleen sections of control (Ctrl) and TAM mice at 10 weeks postnatal were stained for acetylcholinesterase, exhibiting dramatic increase numbers of MK (arrows) in spleen of TAM mice. (B) The numbers of MKs per square millimeter were counted in spleens of 10-wk-old different genotype mice. The values represent the means± SD for 5 female mice (*P<0.05).

Figure 5. Splenic MK ploidy analysis. Splenic MKs were enriched by Percoll and BSA density gradient and ploidy profiles were analyzed. (A) Most splenic MKs of MT mice are either 2N or 4N (61.5% and 20.7% respectively), however, those of AM and TAM mice show apparent increase in the proportion of cells greater than 8N, 23% and 46.2% of splenic MKs in AM and TAM mice respectively. B) AchE stains show many mature splenic MKs from TAM mice, which are similar to mature marrow MKs from control (Ctrl) mice (Original magnification, ×200).

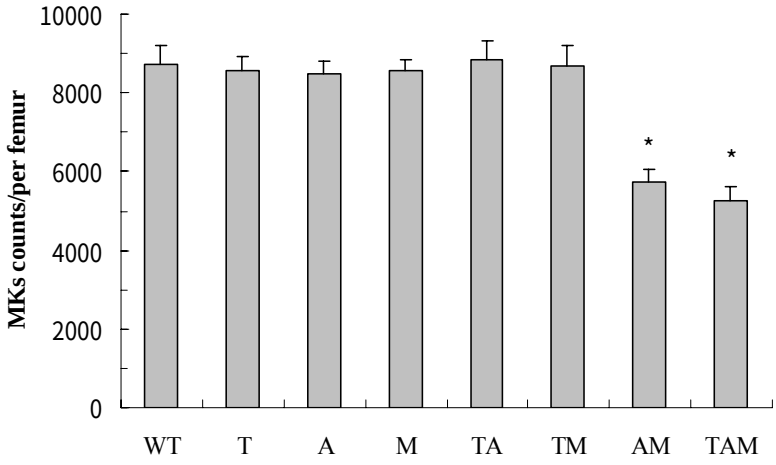
Supplementary Fig. 1



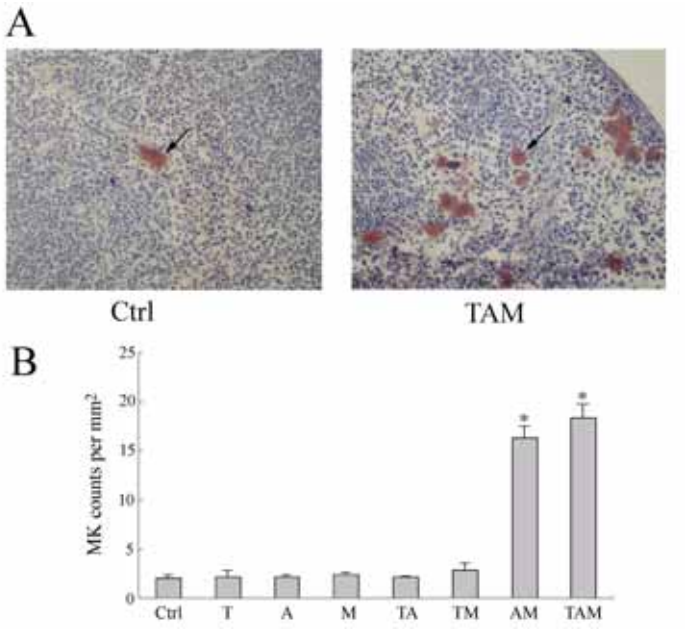
Supplementary Fig. 2



Supplementary Fig. 3



Supplementary Fig. 4



Supplementary Fig. 5