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C3G transgenic mouse models with specific expression in platelets reveal a new role for C3G in platelet clotting through its GEF activity

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Abstract

We have generated mouse transgenic lineages for C3G (tgC3G) and C3GΔCat (tgC3GΔCat, C3G mutant lacking the GEF domain), where the transgenes are expressed under the control of the megakaryocyte and platelet specific PF4 (platelet factor 4) gene promoter. Transgenic platelet activity has been analyzed through *in vivo* and *in vitro* approaches, including bleeding time, aggregation assays and flow cytometry. Both transgenes are expressed (RNA and protein) in purified platelets and megakaryocytes and do not modify the number of platelets in peripheral blood. Transgenic C3G animals showed bleeding times significantly shorter than control animals, while tgC3GΔCat mice presented a remarkable bleeding diathesis as compared to their control siblings. Accordingly, platelets from tgC3G mice showed stronger activation in response to platelet agonists such as thrombin, PMA, ADP or collagen than control platelets, while those from tgC3GΔCat animals had a lower response. In addition, we present data indicating that C3G is a mediator in the PKC pathway leading to Rap1 activation. Remarkably, a significant percentage of tgC3G mice presented a higher level of neutrophils than their control siblings. These results indicate that C3G plays an important role in platelet clotting through a mechanism involving its GEF activity and suggest that it might be also involved in neutrophil development.

1. Introduction

Rap1 is a small GTPase of the Ras family that is ubiquitously expressed [1]. Many studies support a role for the Rap1 isoform Rap1b (but not Rap1a) in critical aspects of platelet function, including aggregation, coagulation, adhesion and spreading, through the platelet integrin αIIbβ3 [2, 3]. Rap1b is highly expressed in mature megakaryocytes and platelets, accounting for about 0.1% of the total cellular proteins [3]. Recently, genetic evidences have demonstrated that Rap1b is involved in a common crucial step required for platelet activation [4]. Rap1b participates in the responses triggered by most platelet agonists, including ADP, collagen, epinephrine, thrombin and PMA [4-6]. In particular, ADP, thrombin and PMA activate platelet Rap1b through pathways involving an increase in intracellular calcium via Gαq-mediated PLC activation [2]. Rap1b can also be activated by Ca^{2+}-independent mechanisms involving Gαi/Gαz signaling and the subsequent activation of PI3K [7-9].

Rap proteins are activated upon binding to GTP, which is stimulated by guanine nucleotide exchange factors (GEFs) [3]. The RapGEFs described to have a significant expression in human platelets are CalDAG-GEFI/RasGRP2, CalDAG-GEFIII/RasGRP3 and PDZ-GEF1 [10, 11]. In mouse platelets, the most relevant Rap1 GEF described so far is CalDAG-GEFI, which function is crucial for signal integration in platelets. Mouse platelets lacking CalDAG-GEFI are severely compromised in the integrin-dependent aggregation as a consequence of their inability to activate Rap1 [12, 13], although a CalDAG-GEF-independent Rap1 activation has been described in response to thrombin [5, 14].

C3G is a Rap1/2 GEF that regulates Rap activation by tyrosine kinases [1, 3]. C3G-mediated Rap1 activation plays critical roles in adhesion. In fact, C3G-dependent Rap1 activation is essential during early mouse embryogenesis due to its role in integrin- and paxillin- mediated cellular adhesion and spreading [15]. Moreover, C3G is required for the formation and stabilization of integrin β1- and paxillin-positive focal adhesions [16]. C3G is

also an essential activator of Rap1 during junction formation, both in epithelial and endothelial cells [17]. In addition, C3G has been implicated in Rap1-dependent adhesion in many hematopoietic-cell types [18, 19]. Based on the critical role of C3G in adhesion processes regulated by Rap1, we can hypothesize that C3G, which is expressed at low level in mouse platelets [20], could participate in Rap1-mediated platelet functions. This is supported by previous data showing the involvement of C3G in thrombopoietin (TPO)-mediated Rap1 activation during megakaryocytic differentiation to platelets [3]. TPO binding to the MpI receptor induces CrkL phosphorylation and the recruitment of a Cbl/CrkL/C3G complex, which results in the sustained activation of ERKs [21].

Rap1 is rapidly activated after stimulation of human platelets with α-thrombin. Phospholipase C-mediated increase in intracellular calcium is necessary and sufficient for this activation, which suggests the participation of CalDAG-GEFs [5, 22]. Nevertheless, thrombin induces a second peak of Rap1 activation, which is mediated by protein kinase C (PKC), but independent of Ca^{2+} and CalDAG-GEF [5, 14]. The specific components of this second pathway, and in particular, the specific GEF that contributes to the second wave of Rap1 activation has not been identified so far.

To examine whether C3G might play a role in platelet functions, we have generated C3G and C3GΔCat (C3G mutant with a deletion in the GEF domain) transgenic mice with specific protein over-expression in megakaryocytes and platelets. *In vivo* and *in vitro* experiments revealed a higher activation and aggregation of C3G transgenic platelets than those from wild type animals. In contrast, platelets expressing the C3GΔCat mutant showed impaired activation and aggregation. Thus, our results indicate that the manipulation of C3G expression modifies platelets response and therefore, it could play an important role as a therapeutical target in thrombotic disorders through the modulation of Rap1 activity. Unexpectedly, C3G transgenic mice have a significant higher number of neutrophils than control mice, suggesting a possible C3G function in neutrophil development.

Research Ethics

This study was carried out in strict accordance with *EU Directive 2010/63/EU for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm; Uniform Requirements for manuscripts submitted to Biomedical journals http://www.icmje.org.* The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Salamanca, ID number: *SAF2010-20918-C02-02.* All surgery was performed under ketamine anesthesia, and all efforts were made to minimize suffering.

2. Materials and Methods

2.1. Genetic engineering of plasmids PF4-C3G-LN and PF4-C3GΔCat-LN

A BamHI fragment containing human C3G gene was excised from pLTR2C3G construct [23] and cloned into pBluescript SK+ (Stratagene) previously modified by *in vitro* mutagenesis to create a NcoI site (pBluescriptSK-NcoI), using oligonucleotides pSKNcoI-F (5'-TGCATATCAAGCTTATCGATACCATGGACCTCGAGG-´3) and pSKNcoI-R (5'-CCTCGAGGTCCATGGTATCGATAAGCTTGATATCGA-´3). The resulting construct was named pSKNco-C3G. PF4-Globin vector, containing the 1.1 Kb rat PF4 promoter followed by a 1.7 Kb fragment of the human β-globin gene [24, 25], was modified by cloning a SpeI linker (NEB, Ipswich, MA) into the restriction site BsgI. The fragment containing β-globin gene was removed by NcoI-SpeI digestion and substituted by the C3G fragment excised from pSKNco-C3G to generate plasmid PF4-C3G.

The DNA fragment containing mutant C3GΔCat was obtained from plasmid pLTR2C3GΔCat [23] by digestion with BamHI and ClaI and inserted into plasmid pSP72 (Promega). Then, the C3GΔCat fragment was removed from this construct with HindIII and

BglII and cloned into pBluescriptSK-NcoI to create pSKNcoI-C3GΔCat. C3GΔCat was finally excised from this construct with NcoI-SpeI and cloned into PF4-Globin containing the SpeI linker. The resulting construct was named PF4-C3GΔCat.

A 1765 bp HindIII-EcoICRI LoxP flanked, Eukaryotic Neomycin Selection Cassette (LoxP-PGK-Neo-LoxP) (supplied by GenOway, Lyon, France) was inserted into HindIII-SmaI sites of pSP72. From this construct, the LoxP-PGK-Neo-LoxP cassette was excised as a KpnI-XhoI fragment and inserted into the unique KpnI-XhoI restriction sites of PF4-C3G and PF4-C3GΔCat to generate final plasmids PF4-C3G-LN and PF4-C3GΔCat-LN (Fig. S1, 1B). The PF4-C3G-LN and PF4-C3GΔCat-LN constructs were linearized by digestion with PvuI/KpnI. In this process, a 1.5 kb fragment containing the bacterial ampicillin resistance gene was eliminated. The respective 8.2 kb and 7.8 kb resulting fragments were purified by electroelution followed by ethanol precipitation and used for ES cell electroporation. GenOway performed the generation of chimeras by blastocysts injection of stable ESC´s clones using GenOways´s Safe DNA transgenesis technology ™.

2.2. Genotyping of transgenic mice by PCR

Genomic DNA was purified from lysed tails by precipitation with 5M NaCl and ethanol (protocol based on [26]). Then, for Southern blot analysis it was digested with BamHI to obtain a fragment of 3.6 Kb or 5.3 Kb from C3G or C3GΔCat constructs respectively, which were detected using a radioactively labeled 740 bp EcoRI probe, produced by digestion of PF4-C3G-LN construct (Fig. 1B, C). In addition, genomic DNA amplification was performed by polymerase chain reaction (PCR) using the following primers: A1 (sense): 5'-ACCACATGGCAGTCAAACTCACAGC-3´ and C1 (antisense): 5'-TCTTCTGCCTTTGAGACCTGGAAGC-3´ to get a fragment of 847 bp (Fig. 1A, 1B and 1D). Primer A1 aligns within the PF4 promoter fragment, whereas primer C1 aligns within the second exon of C3G gene. PCR conditions were: 94ºC, 45s; 57ºC, 45s; 72ºC, 80s; 40 cycles.

2.3. Platelet purification for RNA analysis

Blood (0.5-1 ml) was collected by retro-orbital bleeds from anesthetized mice (156 mg/kg ketamine, 6 mg/kg valium and 150 μg/kg atropine in 0.9% NaCl) and used to prepare platelet–rich plasma (PRP) upon treatment with 200-400 μl of acid citrate-dextrose (ACD) anticoagulant solution (80 mM trisodium citrate, 52 mM citric acid, 180 mM glucose). The PRP was isolated by spinning the anticoagulated blood at 200 *g*, 20 min at RT. This PRP was centrifuged at 3160 *g* for 10 min to obtain the platelet pellet. Remaining red cells were eliminated by a brief incubation of the pellet with 1% ammonium oxalate. After two additional washes in PBS, the platelet pellet was resuspended in RLT lysis buffer from *RNeasy kit* (QIAGEN) following the manufacturer specifications for total RNA extraction.

2.4. Semiquantitative RT-PCR

cDNA from transgenic and control platelets was obtained from RNA using SuperScript® II Reverse Transcriptase (Invitrogen) and amplified by PCR to determine C3G expression using specific primers corresponding to different human C3G exons: Ex3 Forward (F): 5′-GCAACAGACAGATTTCTACCAG-3′; Ex4 Reverse (R): 5′-CTGTGCTGAATTCGAGGATC-3′; Ex5R: 5′-CTTCACTCCATCCAGCACA-3′; Ex7F: 5′-AGCAGAGCTCCCCCTGA-3′; Ex8F: 5′-CCTCCACCAGCATTGACAC-3′; Ex9R: 5′-CTAGTGTTTCACAGCTTGTGTT-3′; Ex10F: 5′-ACCACTATGATCCCGACTATG-3′; Ex12R: 5′-TCACTGCCGTCTCTGCTG-3′; Ex15F: 5′-GATTTGGTGTTGTACTGCGAG-3′; Ex17R: 5′-CCTGGCTGCTACCCCC-3′; Ex22F: 5′-GGCCTGGCCGAGTACT-3′; Ex24R: 5′-TGCTGGAAGCAGCGCATG-3′. The amplified fragments were normalized against the mouse gene CD41, a platelet specific internal control, using primers: mCD41-F1: 5’-ATGCGGGCTCTCAGCAACATTG-3’ and mCD41-R1: 5’-TTGCCACAGGCAACATCACGAC- 3’. The conditions for PCR were: 94°C, 45s; 55°C, 45s; and 72°C, 80 min for 35 cycles. Densitometric analysis of the bands was performed using

Image J (Image Processing and Analysis in Java) free software.

2.5. Megakaryocytic differentiation

Freshly isolated bone marrow cells from femurs of 6-8 week-old mice were cultured in RPMI supplemented with 20% horse serum and maintained at 5% CO_2 and 33 °C. Megakaryocytes were differentiated from bone marrow cultures by addition of 50 ng/ml thrombopoietin (TPO, CellGenix) to promote megakaryopoiesis. After 7 days, cells in suspension were collected and used to prepare protein extracts or total RNA as described above.

2.6. Bleeding time

Measurements were made in 3 week-old mice at weaning (before genotyping) and in the same mice at 6-month age. A 5 mm section of the tail tip from anesthetized mice was transected, and the tail was immersed in 37°C PBS to score time to cessation of blood flow.

2.7. Flow cytometric analysis of C3G expression and platelet activation

Platelet activation was determined by flow cytometry using FITC Rat anti-mouse CD62P antibody (BD Pharmingen) to detect P-selectin expression on the surface and Alexa Fluor® 488 labeled fibrinogen (Molecular Probes), which selectively binds to the high affinity conformation of the αIIbβ3 integrin. Alternatively, we measured integrin activation using PE-labeled Rat anti-mouse αIIbβ3 (JON/A clone) antibody (Emfret Analytics) [27]. Blood (50 µl) was collected from the retro-orbital plexus of anesthetized mice with a heparinized glass capillary into 2 ml tubes containing 200 µl of TBS/heparin (20 mM Tris-HCl, 137 mM NaCl, pH 7.3, 20 U/ml heparin). Heparinized blood was diluted 1:6 with modified Tyrode's Hepes buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM Hepes [N-2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid], pH 7.0) containing 5 mM glucose,

0.35% bovine serum albumin (BSA), and 1 mM $CaCl_2$. Diluted blood was washed by spinning 5 min at 1300 *g* and suspended in the same buffer. Finally, blood samples (25 μl out of 1.25 ml) were incubated with the agonists together with appropriate fluorophore-conjugated monoclonal antibodies or fibrinogen for 15 min at room temperature and directly analyzed on a FACSCalibur (Becton Dickinson). PMA (2 μM), α-thrombin (0.2 U/ml or 1U/ml) and ADP (10 μM) were used as agonists of platelet activation [28]. For analysis of C3G expression Tyrode's Hepes buffer-washed blood, prepared as above, was incubated with saturating amounts of FITC-labeled CD41 for 20 min in the dark. After two washes in PBS/1% BSA/0.1% Na-azide, blood was fixed in 200 μl of Leucoperm™ Reagent A (Abd Serotec) at room temperature for 15 min. After washing with PBS/1% BSA, fixed blood was permeabilized and stained for C3G by incubation with Leucoperm™ Reagent B (Abd Serotec) and rabbit anti-C3G antiserum #1008 [23, 29] for 30 min at RT. After washing twice with PBS/1% BSA, platelets were incubated with saturating amounts of anti-rabbit-Cy5 for 20 min in the dark, washed again twice and resuspended in 400 μl PBS before flow cytometric analysis. FlowJo 9.1 (Tree Star, Inc. Ashland, OR) software program was used for data analysis.

2.8. Platelet aggregation assays

Platelet aggregation was measured in a final volume of 800 μl of blood diluted to 1:2 with HEPES buffer, using a Chrono-Log aggregometer (Haverton, USA). Samples were incubated at 37ºC under constant stirring and, after 5 min, 5 μg/ml collagen (Chrono-Log) was added. The extent of platelet aggregation was defined arbitrarily as the percent of increase of electrical resistance between the two immersed wires of the electrode probe. For ADP-, PAR-4 receptor–specific agonist, GYPGKF (PAR4) and PMA-induced platelet aggregation, a Multiplate Aggregometer (Verum Diagnostica GmbH) was used. Citrated blood (175 μl) was diluted 1:2 in 0.9% saline solution, containing 62.5 mM $CaCl_2$, and incubated at 37ºC under

constant stirring. After 3 min, 6.6 μM ADP, 10 μM PMA, 1 μM PMA or 0,66 mM PAR4 was added and aggregometry monitorized for 6 min. The extent of platelet aggregation was measured as the increase in impedance generated by the attachment of platelets onto the Multiplate sensor, which is transformed into arbitrary aggregation units.

2.9. Rap1 activation assay

Activation of Rap1 was evaluated essentially as described previously [30] using GST-RalGDS RBD immobilized on Glutathione-Sepharose, which is known to bind specifically and selectively the GTP-bound form of Rap1B. Briefly, same volume of PRP per assay, containing about 5-10 x 10^7 mouse platelets, were stimulated at room temperature with 0.2 U/ml α-thrombin for 1 minute or 0.2 μM PMA for 5 minutes. When indicated, platelets were pretreated with 5 μM bisindolylmaleimide, 1 min before PMA stimulation. Platelets were then lysed for 10 minutes at 4°C with equal volume of 2X RIPA modified lysis buffer (100 mM Tris-HCl, pH 7.5; 400 mM NaCl, 2% NP-40, 5 mM $MgCl_2$, 2% glycerol, 2 mM Na_3VO_4, 50 mM NaF, 2mM PMSF, 2 μM aprotinin, 2 μM leupeptin). Glutathione- agarose beads prebound to RalGDS RBD fused to GST were washed with ice-cold lysis buffer and 20 μl bead volume was added to cell lysates and incubated for 45 min at 4°C. The precipitates were collected by brief centrifugation, washed three times with 1 X RIPA buffer, and finally resuspended in 25 μl of SDS sample buffer. Precipitated GTP-Rap1 was separated by SDS-PAGE and detected by immunoblotting with anti-Rap1mAb (610196) from BD Biosciences. Total Rap1 present in platelet lysates was determined in a 20 μl of lysate sample, removed prior to bead addition, by Western-blot analysis of Rap1. Fold stimulation of active Rap1 was determined by densitometry using Image J free software.

2.10. Flow cytometric analysis of neutrophil content

The percentage of neutrophils in whole blood was determined by flow cytometry using antibodies against the granulocyte specific markers Ly-6G/6C (Gr1) and CD11b (Mac1) (BD Pharmingen). Briefly, EDTA-anticoagulated blood (100-150 μl) was incubated for 30 min with 12 ml ice-cooled red blood cells lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 10 mM EDTA pH 7.4) to eliminate the erythrocytes. After a 10 min centrifugation, blood cells were washed with cold PBS containing 1% fetal calf serum (FCS). Cells were resuspended in 150-200 μl PBS/1% FCS and incubated with excess of antibodies for 20 min at 4ºC in the dark.

2.11. Evaluation of blood cellularity

White blood cells and platelet counting was determined from EDTA-anticoagulated blood (30 μl) using a haemocytometer (Hemavet Counter HV950FS).

2.12. Statistical analysis

Data are represented as mean ± SEM. As comparisons were made between two experimental groups, an unpaired Student's t-test was used. Results were considered significant when $p<0.05$.

3. Results

3.1. Generation of transgenic mouse models expressing C3G or mutant C3GΔCat under the PF4 gene promoter

C3G and C3GΔCat mutant (lacking the last 439 bp of full-length C3G catalytic domain) [23], were cloned into plasmid PF4-Globin to specifically direct transgene expression to megakaryocytes and platelets [24, 25, 31]. To do so, the fragment carrying the β-globin gene was replaced by C3G- or C3GΔCat-fragments. A LoxP-PGK-Neo-LoxP cassette was also inserted in both constructs. The resulting plasmids were named PF4-C3G-LN and PF4-C3GΔCat-LN, respectively (Fig. S1), where the transgenes were under the control of the PF4 gene promoter (Fig. 1A, B). The constructs also contain a Kozak consensus sequence to ensure a correct initiation of translation (Fig. 1A). Both constructs were used to generate transgenic mice through blastocyst injection of stable ESC´s (performed by GenOway Company, Lyon, France). We selected two C3G transgenic lines (2C1 and 6A6) and one C3GΔCat line (8A3), with correct integration of the transgenes (Fig. 1C, D).

3.2. Characterization of C3G transgene expression

Semiquantitative RT-PCR analysis of total RNA from purified platelets, using primers for different C3G exons, revealed a significant C3G over-expression in transgenic platelets as compared to those from wild type mice (Fig. 2A). Platelets from heterozygous animals also showed a clear increase in C3G expression (Fig. 2A). An evident C3G over-expression (~2-4 fold increase) was also detected in platelets from C3GΔCat transgenic animals. As expected, we did not observe any increase in the expression of exons 22-24 in C3GΔCat mutant, as this region was deleted (Fig. 2B). Platelet specific CD41 gene (GPIIb, encoding the αIIb subunit of platelet integrin αIIbβ3) was amplified as a control of platelet integrity and loading. C3G over-expression was also observed in TPO-differentiated megakaryocytes from the transgenic

C3G-2C1 line (Fig. 2C), but no C3G over-expression was detected in other tissues such as liver or kidney (Fig. S2), in agreement with the restricted expression of the transgenes to megakaryocytes and platelets [24].

Expression of C3G and C3GΔCat transgenic proteins was evident in lysates of TPO-differentiated megakaryocytes, as compared to wild type mice (Fig. 2D). Transgene expression was also detected in platelets by flow cytometry (cells positive for CD41 and C3G-Cy5), although expression levels showed great variability among individuals (Fig. 2E, F).

3.3. C3G and C3GΔCat transgenes alter the hemostatic function of platelets

To study the involvement of C3G on hemostasis, we performed tail-bleed assays in transgenic mice and their wild type siblings at weaning and 6 months later in the same animals. At weaning, tgC3G animals showed statistically significant shorter bleeding times than control animals (mean ± SEM: 44.0 ± 4.5 seconds *vs* 64.2 ± 6.7 seconds, respectively, p=0.024). In contrast, the mean time for bleeding cessation in tgC3GΔCat animals was significantly higher than in their corresponding sibling controls (125.8 ± 17.7 seconds *vs* 86.8 ± 7.5 seconds, respectively, p=0.0052, Fig. 3A). The differences were reinforced after 6 months, especially for tgC3GΔCat mice (74.1 ± 11.0 seconds in tgC3G *vs* 122.1 ± 17.3 seconds in wild type; p=0.0275 and 254.1 ± 24.8 seconds in tgC3GΔCat *vs* 89.3 ± 10.2 seconds in wild type; p= 0,0000085, Fig. 3B). The same tendency was observed in the blood mass obtained (data not shown). The hemostatic disorder observed in the transgenic animals was not due to defects in platelet formation, as the platelet number was similar in mice of the different genotypes (Table 1). Additionally, there were no significant changes in hematocrit and hemoglobin levels in transgenic animals (Table S1), which means that C3GΔCat transgenic animals do not suffer from spontaneous hemorrhaging or anemia. These results indicate that C3G participates in platelet clotting through a mechanism dependent on its catalytic domain, which might involve the participation of Rap1 in this effect.

3.4. C3G and C3GΔCat transgenes modify the response of platelets to agonists

To determine whether the hemostatic defects observed in the transgenic animals were caused by impaired platelet function, we induced platelet activation by using several agonists known to activate Rap1, including thrombin, PMA and ADP. We determined P-selectin expression on the surface, as a measure of agonist-induced degranulation, and integrin αIIbβ3 activation, as a measure of inside-out signaling. As shown in Figure 4A, platelets from transgenic C3G mice showed higher expression of P-selectin on the surface in response to thrombin and PMA, while a significant reduced response to these agonists was observed in platelets from C3GΔCat transgenic animals as compared to wild type platelets. Similarly, platelets from C3G transgenic mice showed higher αIIbβ3 binding to Alexa Fluor® 488-conjugated fibrinogen in response to thrombin and ADP, while integrin from C3GΔCat-expressing platelets was less responsive to these agonists than control platelets (Fig. 4B). Similar results were obtained with the JON/A antibody regarding thrombin activation (data not shown). Expression of αIIbβ3 was similar in transgenic and control platelets, as assessed by flow cytometry with anti-CD41-APC (data not shown). Under our experimental conditions, PMA barely stimulated αIIbβ3 integrin, while ADP hardly affected surface P-selectin levels, as previously observed by other authors [28]. Additionally, platelet aggregation was impaired in tgC3GΔCat platelets in response to 5 μg/ml collagen. In contrast, tgC3G platelets showed increased aggregation as compared with platelets from wild-type mice (Fig. 4C, D). Similar results were obtained in response to ADP, PMA and PAR4 peptide (Fig. 4E and Fig. S3). All these *in vitro* experiments are in agreement with the tail-bleed assay and suggest that C3G participates in PMA, thrombin, ADP and collagen-mediated platelet activation, possibly through Rap1. Moreover, fibrinogen-binding and aggregation assays indicate that C3G plays a role in the inside-out activation of αIIbβ3 integrin.

3.5. Activation of Rap1 in the transgenic platelets: Rap1 mediates the effect of C3G

All the above results indicate that C3G overexpression enhances Rap1-mediated integrin activation. In contrast, C3GΔCat transgene would behave as a C3G dominant negative mutant, inhibiting Rap1. To confirm this hypothesis, we performed a Rap1 activation assay on freshly isolated PRP stimulated with 0.2 U/ml α-thrombin for 1 min, conditions that have been widely validated [5, 12]. Basal Rap1 activation was increased in tgC3G mice as compared to wild type siblings, while tgC3GΔCat platelets showed lower Rap1-GTP basal levels than control animals (Fig. 5A). Similarly, Rap1 activation by thrombin was stronger in tgC3G platelets than in wild type platelets, while thrombin-stimulated tgC3GΔCat platelets showed lower Rap1-GTP levels than wild type siblings, in agreement with data in Figure 4.

It has been described that, under aggregating conditions, thrombin-induced Rap1 activation is rapidly downregulated [5]. When the experiment was performed under these conditions (platelets stirred during incubation), we observed a faster Rap1 downregulation in tgC3G platelets and a significant lower dowregulation in tgC3GΔCat platelets as compared to their respective controls (Fig. 5B). All these results indicate that transgenic C3G and C3GΔCat expression upregulates or downregulates Rap1 activation, respectively, which is in agreement with their effects in platelet aggregation.

3.6. C3G is a relevant mediator in the PKC pathway leading to Rap1 activation

Platelet aggregation stimulated by PMA was not affected in CalDAG-GEF1 KO platelets [12], which suggests the existence of other Rap1 GEFs playing a role in PKC-mediated Rap1 activation. To assess whether C3G could contribute to the PKC-Rap1 pathway in platelets, we performed a Rap1 assay on PRP stimulated with 0.2 μM PMA for 5 min in the presence or absence of the PKC inhibitor bisindolylmaleimide. Results in Figure 6A show that, PMA stimulates Rap1, in agreement with previous reports [5]. This stimulation was much higher in C3G transgenic platelets than in wild type platelets, which is in agreement with the

results shown in Figure 4 and suggests that C3G participates in the platelet PKC pathway. Furthermore, the increase in PMA-induced Rap1 activation observed in C3G transgenic platelets was almost completely abolished by bisindolylmaleimide, which indicates that PKC-induced Rap1 activation is mediated by C3G. This was confirmed by the absence of Rap1 activation in response to PMA in tgC3GΔCat platelets. These results are supported by platelet activation assays monitoring P-selectin expression on the surface. Figure 6B shows that the increase in P-selectin expression observed in C3G transgenic platelets stimulated with PMA was prevented upon pretreatment with bisindolylmaleimide. In contrast, plateletet activation by thrombin was only partially inhibited by bisindolylmaleimide (Fig. 6B), in agreement with the existence of thrombin-induced PKC-dependent and independent pathways [5]. Therefore, we have now identified C3G as the Rap1 GEF responsible for this Rap1 activation by PKC.

3.7. C3G transgenic mice showed an increase in neutrophils

As mentioned, we found no differences in the number of platelets between transgenic and control animals (Table 1). However, haemocytometric analysis showed a significant increase in neutrophils in tgC3G mice as compared with control siblings (Table 2). This result was verified by flow cytometry using granulocyte specific markers Ly-6G/6C (Gr1) and CD11b (Mac1) (Fig. 7A, B). In contrast, no significant effect was observed in C3GΔCat transgenic platelets. Moreover, no transgenic expression was detected in purified granulocytes obtained from hydrocortisone-driven bone marrow differentiation (data not shown), in agreement with the specific expression of the PF4 promoter in megakaryocytes/platelets. Therefore, the increase in the percentage of neutrophils must be an indirect effect of the over-expression of C3G in platelets.

4. Discussion and conclusions

In this paper we demonstrate for the first time the involvement of C3G in platelet function. To do so, we have generated C3G transgenic mice in which the human C3G gene is under the megakaryocytic/platelet specific PF4 gene promoter. The backbone plasmid used in the generation of the transgenic constructs has been widely validated for its specific expression in megakaryocytes and platelets [24, 25, 31].

Platelets are non-nucleated cell fragments. This guarantees that our RNA preparations are free of genomic DNA contaminant. In addition, in the PCR analysis each pair of primers amplified several exons to ensure a cDNA origin of the template. The oligonucleotides used in the genotyping and expression characterization of the transgenic animals correspond to the human C3G sequence. In fact, we have detected endogenous C3G expression in human platelets with our sets of primers (data not shown). Thus, it is likely that most of the detected C3G would correspond to the human transgene. Nevertheless, considering the high homology between human and mouse sequences (87%), the mouse C3G endogenous gene is also detected in the wild type controls. Over-expression of the C3G transgenic proteins is evident, both in megakaryocytes and platelets and their levels are sufficient to modify Rap1 activation and platelet function.

The results obtained with the full-length C3G transgene are strengthened by the use of two different chimeric lines. Furthermore, our results with this transgenic model are reinforced by the use of a dominant negative (DN) version of the transgene that blocks Rap1 activation and, consequently, platelet function. This DN function is clearly demonstrated by the decrease in Rap1-GTP levels observed in platelets from tgC3GΔCat (8A3) strain stimulated with thrombin (Fig. 5) and PMA (Fig. 6). This C3GΔCat mutant (which lacks the essential aminoacids for the GEF function) would act by sequestering upstream elements important for the activation of Rap1 by other GEFs, This hypothesis agrees with results from Figure 4, showing a lower response to agonists in C3GΔCat platelets, than in wild type platelets. This

C3G DN transgene (C3GΔCat) has been previously demonstrated to act as a negative mutant in other systems [29, 32]. In all of our experimental approaches, the C3GΔCat mutant effect was opposite to that of the full C3G transgene. This clearly demonstrates that the effect of the C3G transgene is a direct effect on Rap1 and not a random or artefactual effect.

Results derived from blood clotting experiments demonstrate that C3G plays a relevant role in hemostasis. Hence, while control mice stop bleeding within the first 2 minutes, some of the C3GΔCat chimeras bled longer than 8 minutes, limit time of the experiment. In contrast, most C3G transgenic mice ceased bleeding within the first minute. These results are in agreement with the role of Rap1b in the regulation of the affinity state of integrin αIIbβ3 in mouse megakaryocytes and platelets [4, 33]. Furthermore, our results indicate that C3G participates, at least, in thrombin, PMA, ADP and collagen-mediated platelet activation. In platelets, α-thrombin induces an early Rap1 activation, through a calcium-mediated pathway, independent of PKC activation. This is followed by a second activation phase mediated by PKC and, partially, by integrin αIIbβ3 [5, 34].

So far, CalDAG-GEFI is the only Rap1 GEF with a proved role in platelet function [12-14, 35]. CalDAG-GEFI knockout mice are severely deficient in platelet aggregation in response to low thrombin and partially impaired in response to low PMA concentration. However, CalDAG-GEFI knockout platelets are normally activated by high concentrations of these agonists (10X), and maintain a partial response to collagen, indicating the existence of CalDAG-GEF1-independent signaling pathways [12, 14, 36]. Here we show that, apart from CalDAG-GEFI, C3G is also endogenously expressed in platelets (see Fig. 2) and hence, it may play a physiological role. In fact, our results showing an increased response to thrombin in tgC3G platelets and an impaired response in tgC3GΔCat platelets, together with the opposite effects of both transgenes over thrombin-mediated Rap1 activation, indicate that C3G participates in a thrombin-Rap1 pathway in platelets.

Additionally, based on our results showing an increase in PMA-induced activation of

tgC3G platelets, together with the fact that platelet aggregation triggered by PMA is not impaired in CalDAG-GEF1 KO platelets [12], it can be proposed a specific involvement of C3G in the second (PKC-dependent) phase of Rap1 activation induced by thrombin [5, 14]. This is further reinforced by results in Figure 6 showing that PKC-mediated Rap1 activation is mainly dependent on C3G. Hence, based on the results presented here and on with previous results showing the existence of CalDAG-GEFI and PKC independent pathways for Rap1 activation [14], as well as participation of PKC in the second phase of thrombin-mediated Rap1 activation [5], it is possible to propose a model where C3G could be a mediator in the thrombin-stimulated pathways in platelets (Figure 8). We can not exclude the possibility that the increase in Rap1-GTP induced by thrombin in tgC3G platelets is, in part, a reflect of the higher basal Rap1-GTP levels in these platelets. However, the levels of Rap1-GTP in PMA-stimulated tgC3G platelets are higher than the sum of basal Rap-GTP in tgC3G platelets and Rap1-GTP induced by PMA in wild type platelets (figure 6A), which suggests an amplification of the thrombin response and hence, the participation of C3G in this pathway. This is better reflected in Figure 6B, where all controls have been normalized, and is also evident in the thrombin activation studies (Figure 4A-B). In addition, other RapGEFs with detectable expression in mouse platelets [20] could contribute to PKC-dependent Rap1 activation, as there is still some platelet aggregation in transgenic C3GΔCat platelets stimulated with PMA.

ADP activates Rap1, both in human and mouse platelets [7-9, 34]. So, our results showing an impaired response to ADP in tgC3GΔCat platelets indicate that C3G would mediate, at least in part, this ADP-induced Rap1 activation. We did not detect a significant ADP-induced activation of αIIbβ3 integrin with JON/A antibody (data not shown). Nevertheless, ADP clearly activated fibrinogen-binding to αIIbβ3 integrin, which is in agreement with a synergistic effect between fibrinogen and ADP [37]. The observed increase in integrin activation in tgC3G platelets stimulated with thrombin, ADP or collagen was not

due to an upregulation of αIIbβ3 gene expression induced by C3G, as we did not observe significant differences, either in total αIIbβ3 content (Fig. 2D), or in that present on the surface (data not shown derived from cytometric analysis). Fibrinogen-binding and collagen-mediated aggregation assays indicate that C3G participates in the inside-out activation of αIIbβ3 integrin. In addition, C3G contributes to collagen-mediated platelet activation probably through glycoprotein GPVI, the main collagen receptor that activates Rap1 in platelets [36, 38, 39].

It is well known that Rap1 protein levels increase during megakaryocyte maturation [40]. On the other hand, C3G silencing in the megakaryoblastic cell line MEG-01 results in a decrease in total Rap1 protein content (data not shown). This result is in agreement with the propose role for C3G in the differentiation and maturation of megakaryocytes [3, 41, 42]. Despite this putative role of C3G, we found no differences in platelet number between transgenic and wild type animals, although this result is in agreement with the phenotype observed in Rap1b-null mice [4]. One potential explanation would be the participation of C3G in the initial steps of the megakaryopoiesis, but not in the final maturation to platelets.

Transgenic full-length C3G, but not C3GΔCat mice showed a significant increase in the number of neutrophils. As expected, purified neutrophils do not express transgenic C3G. This raises the possibility of an indirect effect of C3G overexpression in platelets, which could lead to the release of platelet-derived factors able to stimulate neutrophil development. It is known that platelets secrete chemokines that attract neutrophils and activate neutrophil production of pro-inflammatory cytokines [43]. For example, CXCL4 (PF4) can activate neutrophils in the presence of appropriate co-stimuli such as tumor necrosis factor alpha (TNF-α). Therefore, it is reasonable to hypothesize that C3G could stimulate the production and release of one or several platelet factors that stimulate neutrophil production. In support of this hypothesis resting C3G transgenic platelets displayed a low, but significant activation, as compared to control platelets (see Fig. 4).

Results from our group and others support the involvement of C3G in chronic myeloid leukemia (CML) [44-46]. CML chronic phase is characterized by the presence of a high percentage of mature neutrophils in peripheral blood, which present high levels of CrkL phosphorylation, a constitutive C3G adaptor protein [47]. Moreover, platelet functions such as aggregation and clot retraction are often abnormal in chronic myelogenous leukemia (CML) patients [48, 49]. CrkL is also abnormally phosphorylated in platelets from CML patients [50], similarly to that observed in neutrophils. Interestingly, and in agreement with a putative role of C3G in neutrophils, Rap1a is activated by a variety of stimuli involved in neutrophil functions [51]. Rap1a interacts with neutrophil NADPH oxidase [52] and stimulates superoxide production during the oxidative burst [53]. Furthermore, another Rap1 GEF, CalDAG-GEFI, has also been shown to play an important role in neutrophil function [13, 35]. Based on that, our C3G transgenic models would be interesting tools in order to study, in a Bcr-Abl background, whether C3G plays a role in the initial steps of CML development by influencing abnormal neutrophil production and platelet function.

In conclusion, the work presented here shows for the first time that C3G plays a role in the regulation of platelet function and opens a new perspective on C3G as a potential therapeutic target in thrombotic disorders. Future studies will be conducted to determine whether C3G participates in inside-out or/and outside-in signaling and to dissect the specific signaling pathways that engage C3G in the regulation of Rap1 activity in platelets. Finally, the C3G transgenic mouse models will be useful to assess the potential role of C3G in neutrophil development and function as well as in CML development.

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Conflict of interest statement

The authors have declared that no competing interests exist.

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Author Contributions

CG was the principal investigator, designed the experiments and takes primary responsibility for the paper. SG-H, VM and JG-B performed most of the laboratory work for this study. NC and MS participated in the tail bleed assay and in the purification of platelets. SO-R participates in the Rap1 assays. CG-M, MP and JRG-P performed the aggregometry studies. MA contributed to the analysis of data. CG and AP co-ordinated the research and wrote the paper.

References

[1] J.L. Bos, All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral, EMBO J., 17 (1998) 6776-6782.

[2] S.J. Shattil, H. Kashiwagi, N. Pampori, Integrin signaling: the platelet paradigm, Blood, 91 (1998) 2645-2657.

[3] P.J. Stork, T.J. Dillon, Multiple roles of Rap1 in hematopoietic cells: complementary versus antagonistic functions, Blood, 106 (2005) 2952-2961.

[4] M. Chrzanowska-Wodnicka, S.S. Smyth, S.M. Schoenwaelder, T.H. Fischer, G.C. White, Rap1b is required for normal platelet function and hemostasis in mice, J. Clin. Invest., 115 (2005) 680-687.

[5] B. Franke, M. van Triest, K.M. de Bruijn, G. van Willigen, H.K. Nieuwenhuis, C. Negrier, J.W. Akkerman, J.L. Bos, Sequential regulation of the small GTPase Rap1 in human platelets, Mol. Cell. Biol., 20 (2000) 779-785.

[6] Z. Wang, S.P. Holly, M.K. Larson, J. Liu, W. Yuan, M. Chrzanowska-Wodnicka, G.C. White, L.V. Parise, Rap1b is critical for glycoprotein VI-mediated but not ADP receptor-mediated alpha2beta1 activation, J. Thromb. Haemost., 7 (2009) 693-700.

[7] D. Woulfe, H. Jiang, R. Mortensen, J. Yang, L.F. Brass, Activation of Rap1B by G(i) family members in platelets, The Journal of biological chemistry, 277 (2002) 23382-23390.

[8] P. Lova, S. Paganini, F. Sinigaglia, C. Balduini, M. Torti, A Gi-dependent pathway is required for activation of the small GTPase Rap1B in human platelets, J. Biol. Chem., 277 (2002) 12009-12015.

[9] S.M. Jung, M. Ohnuma, N. Watanabe, M. Sonoda, M. Handa, M. Moroi, Analyzing the mechanism of Rap1 activation in platelets: Rap1 activation is related to the release

reaction mediated through the collagen receptor GPVI, Thromb. Res., 118 (2006) 509-521.

[10] J. Schultess, O. Danielewski, A.P. Smolenski, Rap1GAP2 is a new GTPase-activating protein of Rap1 expressed in human platelets, Blood, 105 (2005) 3185-3192.

[11] M.J. Lorenowicz, J. van Gils, M. de Boer, P.L. Hordijk, M. Fernandez-Borja, Epac1-Rap1 signaling regulates monocyte adhesion and chemotaxis, J. Leukoc. Biol., 80 (2006) 1542-1552.

[12] J.R. Crittenden, W. Bergmeier, Y. Zhang, C.L. Piffath, Y. Liang, D.D. Wagner, D.E. Housman, A.M. Graybiel, CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation, Nat. Med., 10 (2004) 982-986.

[13] W. Bergmeier, T. Goerge, H.W. Wang, J.R. Crittenden, A.C. Baldwin, S.M. Cifuni, D.E. Housman, A.M. Graybiel, D.D. Wagner, Mice lacking the signaling molecule CalDAG-GEFI represent a model for leukocyte adhesion deficiency type III, J. Clin. Invest., 117 (2007) 1699-1707.

[14] S.M. Cifuni, D.D. Wagner, W. Bergmeier, CalDAG-GEFI and protein kinase C represent alternative pathways leading to activation of integrin alphaIIbbeta3 in platelets, Blood, 112 (2008) 1696-1703.

[15] Y. Ohba, K. Ikuta, A. Ogura, J. Matsuda, N. Mochizuki, K. Nagashima, K. Kurokawa, B.J. Mayer, K. Maki, J. Miyazaki, M. Matsuda, Requirement for C3G-dependent Rap1 activation for cell adhesion and embryogenesis, EMBO J., 20 (2001) 3333-3341.

[16] P.J. Stork, Does Rap1 deserve a bad Rap?, Trends Biochem. Sci., 28 (2003) 267-275.

[17] N. Dube, M.R. Kooistra, W.J. Pannekoek, M.J. Vliem, V. Oorschot, J. Klumperman, H. Rehmann, J.L. Bos, The RapGEF PDZ-GEF2 is required for maturation of cell-cell junctions, Cell. Signal., 20 (2008) 1608-1615.

[18] A. Arai, Y. Nosaka, H. Kohsaka, N. Miyasaka, O. Miura, CrkL activates integrin-mediated hematopoietic cell adhesion through the guanine nucleotide exchange factor C3G, Blood, 93 (1999) 3713-3722.

[19] R. de Jong, A. van Wijk, N. Heisterkamp, J. Groffen, C3G is tyrosine-phosphorylated after integrin-mediated cell adhesion in normal but not in Bcr/Abl expressing cells, Oncogene, 17 (1998) 2805-2810.

[20] J.W. Rowley, A.J. Oler, N.D. Tolley, B.N. Hunter, E.N. Low, D.A. Nix, C.C. Yost, G.A. Zimmerman, A.S. Weyrich, Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes, Blood, 118 (2011) e101-e111.

[21] M. Sattler, R. Salgia, Role of the adapter protein CRKL in signal transduction of normal hematopoietic and BCR/ABL-transformed cells, Leukemia, 12 (1998) 637-644.

[22] K. Eto, R. Murphy, S.W. Kerrigan, A. Bertoni, H. Stuhlmann, T. Nakano, A.D. Leavitt, S.J. Shattil, Megakaryocytes derived from embryonic stem cells implicate CalDAG-GEFI in integrin signaling, Proc. Natl. Acad. Sci. U S A, 99 (2002) 12819-12824.

[23] C. Guerrero, A. Fernandez-Medarde, J.M. Rojas, J. Font de Mora, L.M. Esteban, E. Santos, Transformation suppressor activity of C3G is independent of its CDC25-homology domain, Oncogene, 16 (1998) 613-624.

[24] K. Ravid, D.L. Beeler, M.S. Rabin, H.E. Ruley, R.D. Rosenberg, Selective targeting of gene products with the megakaryocyte platelet factor 4 promoter, Proc. Natl. Acad. Sci. U S A, 88 (1991) 1521-1525.

[25] Y. Zhang, Y. Nagata, G. Yu, H.G. Nguyen, M.R. Jones, P. Toselli, C.W. Jackson, M. Tatsuka, K. Todokoro, K. Ravid, 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, 103 (2004) 3717-3726.

[26] S.A. Miller, D.D. Dykes, H.F. Polesky, A simple salting out procedure for extracting DNA from human nucleated cells, Nucleic. Acids Res., 16 (1988) 1215.

[27] W. Bergmeier, V. Schulte, G. Brockhoff, U. Bier, H. Zirngibl, B. Nieswandt, Flow cytometric detection of activated mouse integrin alphaIIbbeta3 with a novel monoclonal antibody, Cytometry, 48 (2002) 80-86.

[28] B. Nieswandt, M. Moser, I. Pleines, D. Varga-Szabo, S. Monkley, D. Critchley, R. Fässler, Loss of talin1 in platelets abrogates integrin activation, platelet aggregation, and thrombus formation in vitro and in vivo, J. Exp. Med., 204 (2007) 3113-3118.

[29] C. Guerrero, S. Martin-Encabo, A. Fernandez-Medarde, E. Santos, C3G-mediated suppression of oncogene-induced focus formation in fibroblasts involves inhibition of ERK activation, cyclin A expression and alterations of anchorage-independent growth, Oncogene, 23 (2004) 4885-4893.

[30] P. Lova, S. Paganini, E. Hirsch, L. Barberis, M. Wymann, F. Sinigaglia, C. Balduini, M. Torti, A selective role for phosphatidylinositol 3,4,5-trisphosphate in the Gi-dependent activation of platelet Rap1B, J. Biol. Chem., 278 (2003) 131-138.

[31] N. Papadantonakis, M. Makitalo, D.J. McCrann, K. Liu, H.G. Nguyen, G. Martin, S. Patel-Hett, J.E. Italiano, K. Ravid, Direct visualization of the endomitotic cell cycle in living megakaryocytes: differential patterns in low and high ploidy cells, Cell cycle (Georgetown, Tex, 7 (2008) 2352-2356.

[32] S. Martin-Encabo, E. Santos, C. Guerrero, C3G mediated suppression of malignant transformation involves activation of PP2A phosphatases at the subcortical actin cytoskeleton, Experimental cell research, 313 (2007) 3881-3891.

[33] A. Bertoni, S. Tadokoro, K. Eto, N. Pampori, L.V. Parise, G.C. White, S.J. Shattil, Relationships between Rap1b, affinity modulation of integrin alpha IIbbeta 3, and the actin cytoskeleton, J. Biol. Chem., 277 (2002) 25715-25721.

[34] B. Franke, J.W. Akkerman, J.L. Bos, Rapid Ca2+-mediated activation of Rap1 in human platelets, EMBO J., 16 (1997) 252-259.

[35] R. Pasvolsky, S.W. Feigelson, S.S. Kilic, A.J. Simon, G. Tal-Lapidot, V. Grabovsky, J.R. Crittenden, N. Amariglio, M. Safran, A.M. Graybiel, G. Rechavi, S. Ben-Dor, A. Etzioni, R. Alon, A LAD-III syndrome is associated with defective expression of the Rap-1 activator CalDAG-GEFI in lymphocytes, neutrophils, and platelets, J. Exp. Med., 204 (2007) 1571-1582.

[36] B. Nieswandt, D. Varga-Szabo, M. Elvers, Integrins in platelet activation, J. Thromb. Haemost., 7 Suppl 1 (2009) 206-209.

[37] C. Gachet, A. Stierlé, P. Ohlmann, F. Lanza, D. Hanau, J.P. Cazenave, Normal ADP-induced aggregation and absence of dissociation of the membrane GP IIb-IIIa complex of intact rat platelets pretreated with EDTA, Thromb. Haemost., 66 (1991) 246-253.

[38] M.K. Larson, H. Chen, M.L. Kahn, A.M. Taylor, J.E. Fabre, R.M. Mortensen, P.B. Conley, L.V. Parise, Identification of P2Y12-dependent and -independent mechanisms of glycoprotein VI-mediated Rap1 activation in platelets, Blood, 101 (2003) 1409-1415.

[39] B. Nieswandt, C. Brakebusch, W. Bergmeier, V. Schulte, D. Bouvard, R. Mokhtari-Nejad, T. Lindhout, J.W. Heemskerk, H. Zirngibl, R. Fassler, Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen, EMBO J., 20 (2001) 2120-2130.

[40] A. Balduini, A. Pecci, P. Lova, N. Arezzi, C. Marseglia, F. Bellora, C. Perotti, C. Balduini, C. Balduini, M. Torti, Expression, activation, and subcellular localization of the Rap1 GTPase in cord blood-derived human megakaryocytes, Experimental cell research, 300 (2004) 84-93.

[41] H. Morita, T. Tahara, A. Matsumoto, T. Kato, H. Miyazaki, H. Ohashi, Functional analysis of the cytoplasmic domain of the human Mpl receptor for tyrosine-phosphorylation of the signaling molecules, proliferation and differentiation, FEBS Lett., 395 (1996) 228-234.

[42] F.K. Racke, K. Lewandowska, S. Goueli, A.N. Goldfarb, Sustained activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway is required for megakaryocytic differentiation of K562 cells, J. Biol. Chem., 272 (1997) 23366-23370.

[43] A. Zarbock, R.K. Polanowska-Grabowska, K. Ley, Platelet-neutrophil-interactions: linking hemostasis and inflammation, Blood Rev., 21 (2007) 99-111.

[44] Y.J. Cho, B. Hemmeryckx, J. Groffen, N. Heisterkamp, Interaction of Bcr/Abl with C3G, an exchange factor for the small GTPase Rap1, through the adapter protein Crkl, Biochem. Biophys. Res. Commun., 333 (2005) 1276-1283.

[45] J. Gutierrez-Berzal, E. Castellano, S. Martin-Encabo, N. Gutierrez-Cianca, J.M. Hernandez, E. Santos, C. Guerrero, Characterization of p87C3G, a novel, truncated C3G isoform that is overexpressed in chronic myeloid leukemia and interacts with Bcr-Abl, Experimental cell research, 312 (2006) 938-948.

[46] V. Maia, M. Sanz, J. Gutierrez-Berzal, A. de Luis, A. Gutierrez-Uzquiza, A. Porras, C. Guerrero, C3G silencing enhances STI-571-induced apoptosis in CML cells through p38 MAPK activation, but it antagonizes STI-571 inhibitory effect on survival, Cell. Signal., 21 (2009) 1229-1235.

[47] T. Oda, C. Heaney, J.R. Hagopian, K. Okuda, J.D. Griffin, B.J. Druker, Crkl is the major tyrosine-phosphorylated protein in neutrophils from patients with chronic myelogenous leukemia, J. Biol. Chem., 269 (1994) 22925-22928.

[48] Y. Sultan, J.P. Caen, Platelet dysfunction in preleukemic states and in various types of leukemia, Ann. N. Y. Acad. Sci., 201 (1972) 300-306.

[49] H. Gralnick, Hemostasis and thrombosis in acute and chronic leukemia, in Henderson ES, Lister TA (eds): Leukemia (ed 5). Philadelphia, PA, Saunders, (1990) p711.

[50] A. Oda, Y. Miyakawa, B.J. Druker, A. Ishida, K. Ozaki, H. Ohashi, M. Wakui, M. Handa, K. Watanabe, S. Okamoto, Y. Ikeda, Crkl is constitutively tyrosine

phosphorylated in platelets from chronic myelogenous leukemia patients and inducibly phosphorylated in normal platelets stimulated by thrombopoietin, Blood, 88 (1996) 4304-4313.

[51] L. M'Rabet, P. Coffer, F. Zwartkruis, B. Franke, A.W. Segal, L. Koenderman, J.L. Bos, Activation of the small GTPase rap1 in human neutrophils, Blood, 92 (1998) 2133-2140.

[52] M.T. Quinn, C.A. Parkos, L. Walker, S.H. Orkin, M.C. Dinauer, A.J. Jesaitis, Association of a Ras-related protein with cytochrome b of human neutrophils, Nature, 342 (1989) 198-200.

[53] Y. Li, J. Yan, P. De, H.C. Chang, A. Yamauchi, K.W. Christopherson, 2nd, N.C. Paranavitana, X. Peng, C. Kim, V. Munugalavadla, R. Kapur, H. Chen, W. Shou, J.C. Stone, M.H. Kaplan, M.C. Dinauer, D.L. Durden, L.A. Quilliam, Rap1a null mice have altered myeloid cell functions suggesting distinct roles for the closely related Rap1a and 1b proteins, J. Immunol., 179 (2007) 8322-8331.

Figure legends

Fig. 1 Genomic analysis of PF4-C3G-LN and PF4-C3GΔCat-LN constructs (A) Partial sequence of PF4 promoter and C3G gene (bold), common to both PF4-C3G-LN and PF4-C3GΔCat-LN constructs. Primers A1 and C1, used in mice genotyping, are shadowed and underlined. BamHI, XbaI, NheI and NcoI restriction sites are shadowed. Nucleotides in italic correspond to an intronic sequence present in the constructs. Consensus Kozak sequence is underlined. (B) Schematic representation of the linearized PF4-C3G-LN and PF4-C3GΔCat-LN constructs showing relevant restriction enzymes, the position of A1 and C1 primers and the location of the 740 bp EcoRI probe used in the Southern blot analysis. The size of the BamHI fragments detected by the probe is indicated. (C) Screening of transgenic clones by Southern blot with genomic DNA (20 μg) extracted from the indicated C3G and C3GΔCat clones. The arrows point to the position of the 3.6 kb and 5.3 kb bands detected with the radiolabeled EcoRI probe. Clones selected for this work are marked with an asterisk. (D) Screening of C3G-6A6 positive clones by PCR analysis of genomic DNA extracted from the indicated clones. Oligonucleotides A1/C1 detected a band of 847 bp in the transgenic clones, while oligonucleotide pair 2F/3R amplified a 252 bp band, corresponding to exons 2-3 of the human transgene. In addition, oligonucleotides 2F/3R amplified a 715 bp band in the controls, corresponding to mouse genomic DNA, which includes intron 2. Positive clones are highlighted with an asterisk.

Fig. 2 Expression of C3G transgenes. C3G expression was analyzed by RT-PCR in purified platelets from (A) wild type (-), homozygous (+) or heterozygous (±) full-length C3G (6A6 line) or (B) C3GΔCat transgenic mice (8A3 line) and their corresponding wild type siblings. Each panel shows the expression of the indicated C3G exons, which were amplified with forward and reverse oligonucleotides located in the first and last exons respectively. K: cDNA

from K562 cells, P1: pLTR2C3G plasmid, P2: PF4-C3GΔCat-LN plasmid, C: PCR mix without cDNA, M: male, F: female. Relative densitometric values are shown. wt: wild type, tg: transgenic. (C) TPO-differentiated bone marrow cells RNA, from a C3G-2C1 transgenic mouse and a wild type sibling, was isolated and reverse transcribed to generate cDNA. PCR was subsequently conducted using primer sets that specifically amplify exons 3 to 5. Expression of CD41 was determined as loading control in panels A to C. (D) TPO-differentiated bone marrow total cell lysates were obtained from wild type, transgenic C3G-2C1 and transgenic C3GΔCat-8A3 mice. The panel is a representative Western blot showing transgenic C3G and C3GΔCat protein expression, together with the endogenous C3G, detected with anti-C3G (H-300) antibody (Santa Cruz Biotechnologies, sc-15359) and normalized with anti-β-actin antibodies (Sigma, A5441) and anti-Integrin αIIb (B-10) antibodies (Santa Cruz Biotechnologies, sc-166599). (E) Specificity of the intracellular C3G detection in platelets by flow cytometry; the plot represents a double labeling, CD41-FITC/anti-rabbit-Cy5, of peripheral blood cells incubated with or without rabbit anti-serum C3G #1008. (F) Representative histograms showing double CD41-FITC/C3G-Cy5-labeled blood cells from a transgenic 2C1 (left panel), 6A6 (middle panel) or 8A3 (right panel) mouse and their corresponding sibling controls. The percentage of C3G expression in the platelets gated by SSC/CD41-FITC is shown. Graphs are representative of 5 independent experiments. wt: wild type, tg: transgenic.

Fig. 3 Transgenic mice showed altered hemostasis. (A) Tail-bleed assays performed on transgenic C3G (tgC3G) and transgenic C3GΔCat (tgC3GΔCat) mice and their wild type controls (wt1 and wt2 respectively) at weaning. (B) The experiment was repeated in the same animals after 6 months. The box plots represent the bleeding times of 30-40 animals of each genotype. The median values are shown.

Fig. 4 Transgenic platelets are differently activated in response to agonists. Washed blood from transgenic and wild type mice were stimulated with PMA (2 μM), α-thrombin (0.2 U/ml) or ADP (10 μM) and incubated with (A) anti-CD62P-FITC antibody to determine the percentage of platelets expressing P-selectin on the surface or (B) with Alexa Fluor® 488-conjugated fibrinogen to determine the percentage of platelets with activated αIIbβ3 integrin. The histograms represent the mean ± SEM of 10 independent experiments. *p<0.05, **p<0.01; values are relative to the corresponding control platelets for each genotype. wt: wild type, tg: transgenic. (C) Representative aggregation traces from wild type, tgC3G and tgC3GΔCat mouse platelets treated with collagen (5 μg/ml). Arrows indicate the addition of agonist. (-) Lines: non-stimulated platelets, (+) lines: collagen-stimulated platelets. (D) Histograms represent the mean ± SEM of the aggregation percentage (as a measure of electrical resistance) of collagen-treated platelets from 3 mice of each genotype. *p<0.05, **p<0.01 *vs* wild type. Values for wild type animals include one animal per line (2C1, 6A6 and 8A3). (E) Histograms represent the mean ± SEM of the aggregation values (relative units) of ADP (6.6 μM), PMA (10 μM and 1 μM) and PAR4 (0,66 mM)-treated platelets from 3-5 mice of each genotype. *p<0.05; **p<0.01; ***p<0.001 *vs* wild type.

Fig. 5 Rap1 is differentially activated in C3G and C3GΔCat transgenic platelets. Platelets from tgC3G (2C1 and 6A6) or tgC3GΔCat (8A3) mice and their corresponding wild types were unstimulated (-) or stimulated (+) with 0.2 U/ml of α-thrombin for 1 min under (A) nonaggregating or (B) aggregating conditions. Platelets were then lysed, and activated Rap1 was isolated by binding to GST-RalGDS-RBD and detected by immunoblotting with anti-Rap1 antibodies. The results shown are representative of three experiments with similar results. The ration Rap1-GTP/Total Rap1 is indicated beneath the blots. wt: wild type, tg: transgenic, TH: thrombin.

Fig. 6 C3G participates in PKC-mediated Rap1 activation. (A) Platelets from tgC3G (6A6) or tgC3GΔCat (8A3) mice and their corresponding wild types were unstimulated (-) or stimulated (+) with 0.2 μM PMA for 5 min in the presence or absence of 5μM bisindolylmaleimide, which was added 5 min prior PMA stimulation. Platelets were then lysed, and activated Rap1 was isolated by binding to GST-RalGDS-RBD and detected by immunoblotting with anti-Rap1 antibodies. The ration Rap1-GTP/Total Rap1 is indicated beneath the blots. wt: wild type, tg: transgenic. BIS: bisindolylmaleimide. (B) Washed blood from transgenic and wild type mice were stimulated with PMA (2 μM), α-thrombin (1U/ml) and incubated with anti-CD62P-FITC antibody to determine the percentage of platelets expressing P-selectin on the surface. The histograms represent the mean ± SEM *(n=5)*. **p*<0.05, ***p*<0.01 *versus* unstimulated CT; †p<0.05, ††p<0.01 *versus* the corresponding bar in WT; ‡‡ p<0.01 *versus* tg6A6-PMA. Control values were normalized to 1 for a better appreciation of the effect of the treatments within the different lines. wt: wild type, tg: transgenic. BIS: bisindolylmaleimide.

Fig. 7 C3G transgenic mice showed increased neutrophil counts. (A) Representative contour plots showing the percentage of double Gr1-FITC/Mac1-PE labeled cells in lysed blood of a 6A6 transgenic mouse and a wild type sibling. (B) Histograms represent the superimposed profiles of Gr1-FITC- (left panel) and Mac1-PE- (right panel) labeled cells in the 6A6-tg and 6A6-wt mice from the above plots. Numbers indicate the percentage of Gr1- and Mac1-expressing cells respectively. Graphs are representative of the analysis of more than 20 different mice from each genotype. Similar results were found with the 2C1 transgenic line. wt: wild type, tg: transgenic.

Fig. 8 Schematic representation of thrombin-triggered pathways leading to αIIbβ3 activation in mouse platelets. C3G would participate in the PKC-mediated activation of Rap1. PLC-β2: phospholipase C β2; PAR4: protease activated receptor 4 BIS: bisindolylmaleimide, DAG: diacylglycerol; IP3: Inositol trisphosphate.

Figure 1
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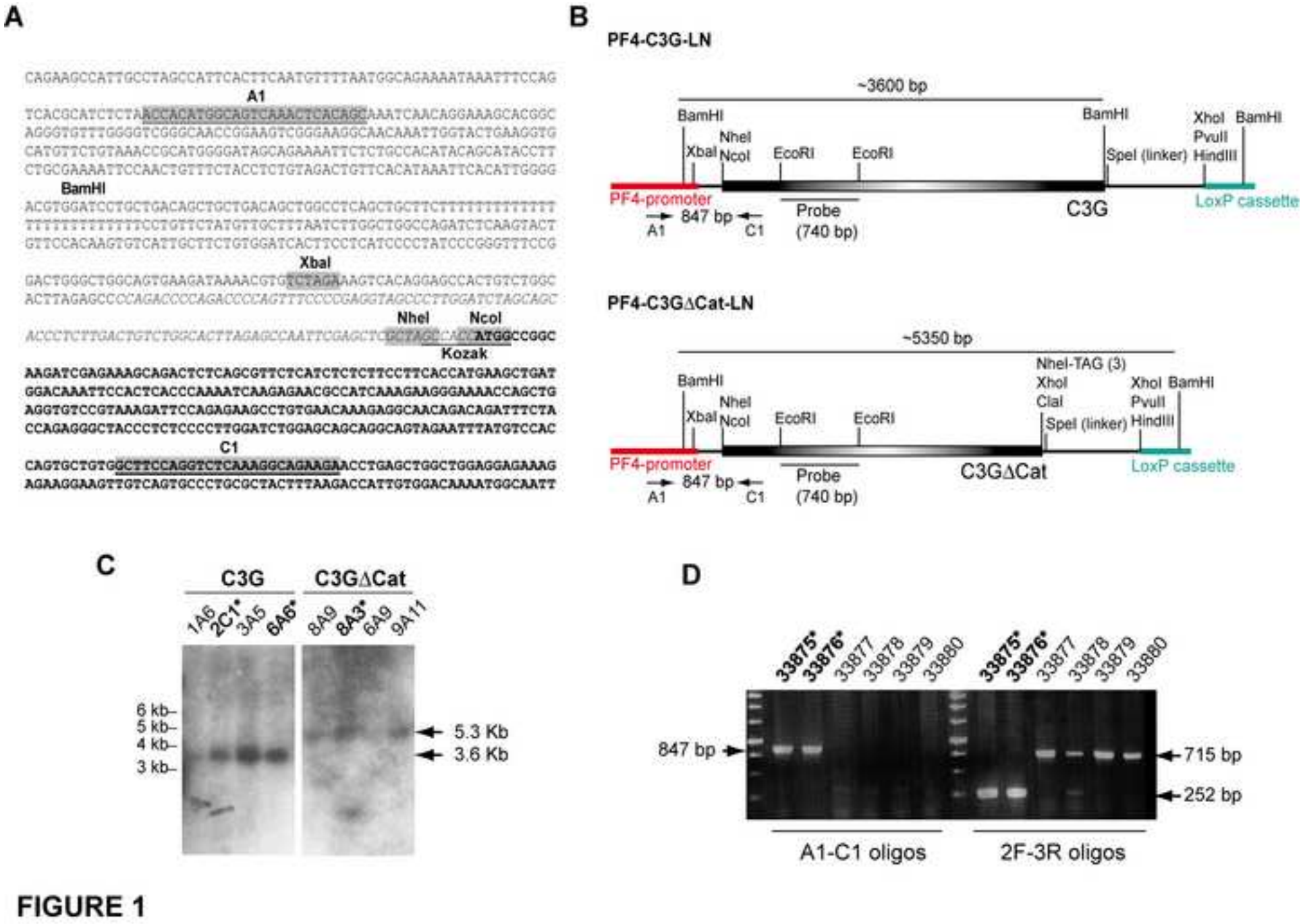

FIGURE 1

Figure 2

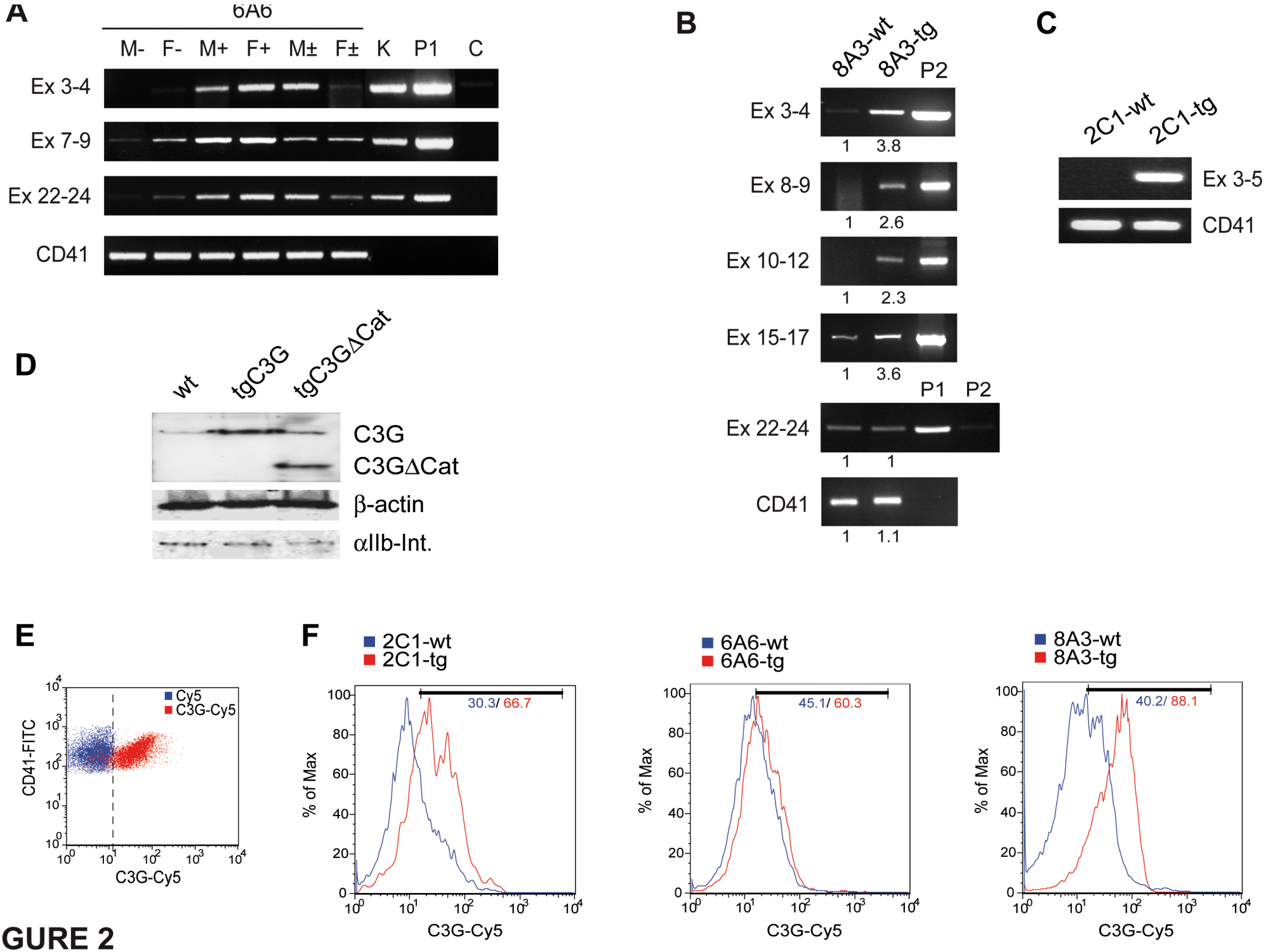

FIGURE 2

Figure 3
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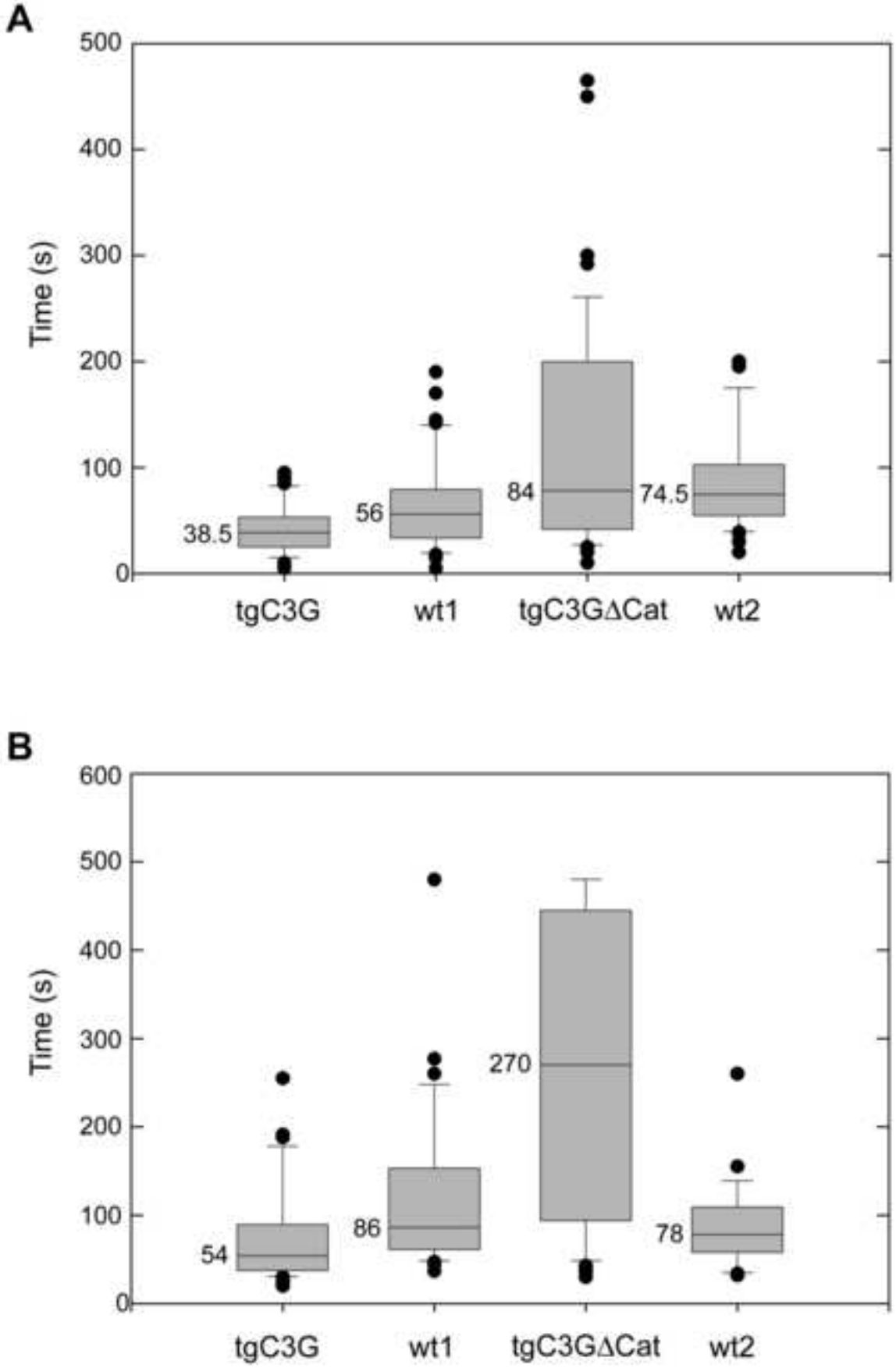

FIGURE 3

Figure 4

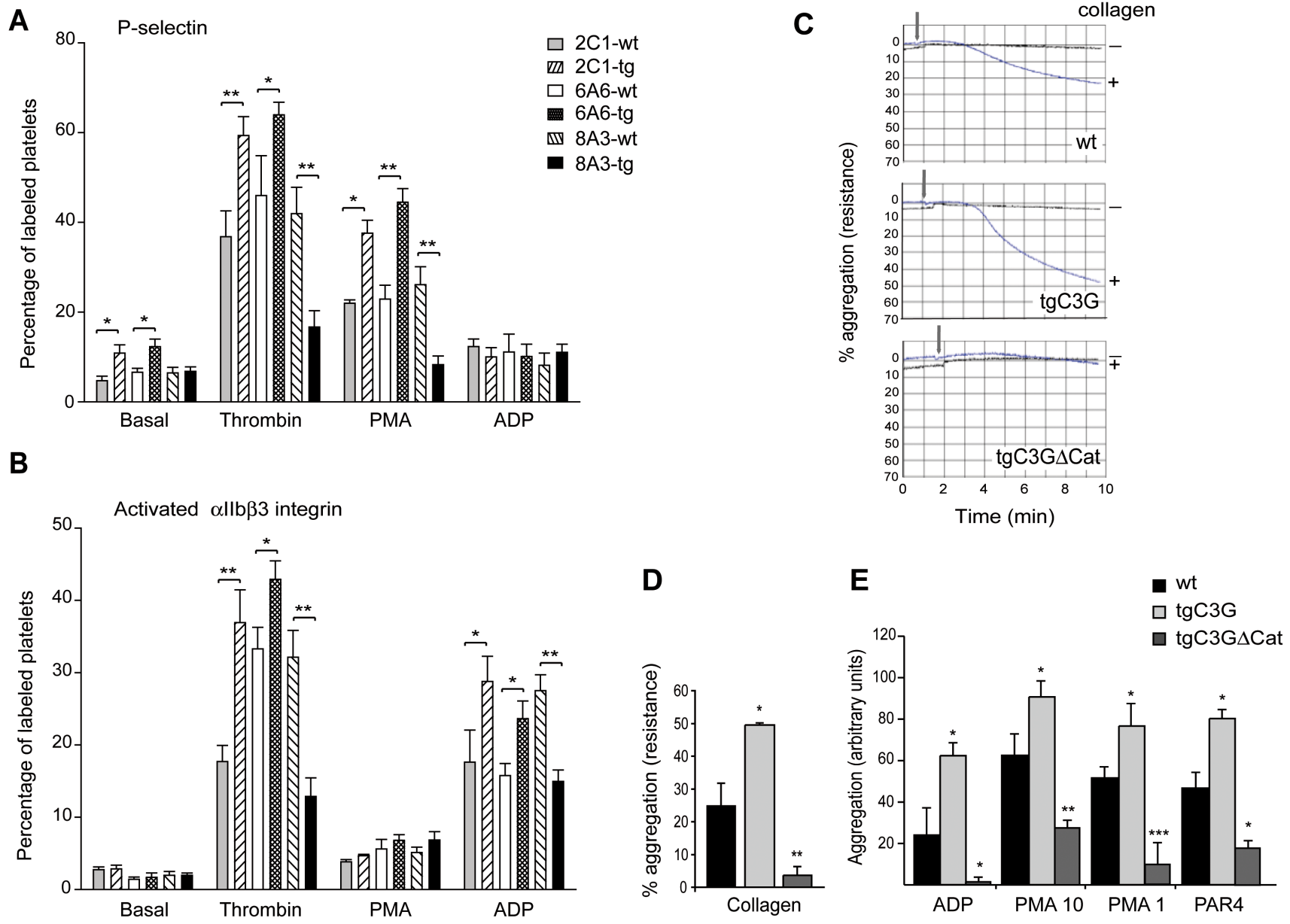

FIGURE 4

Figure 5

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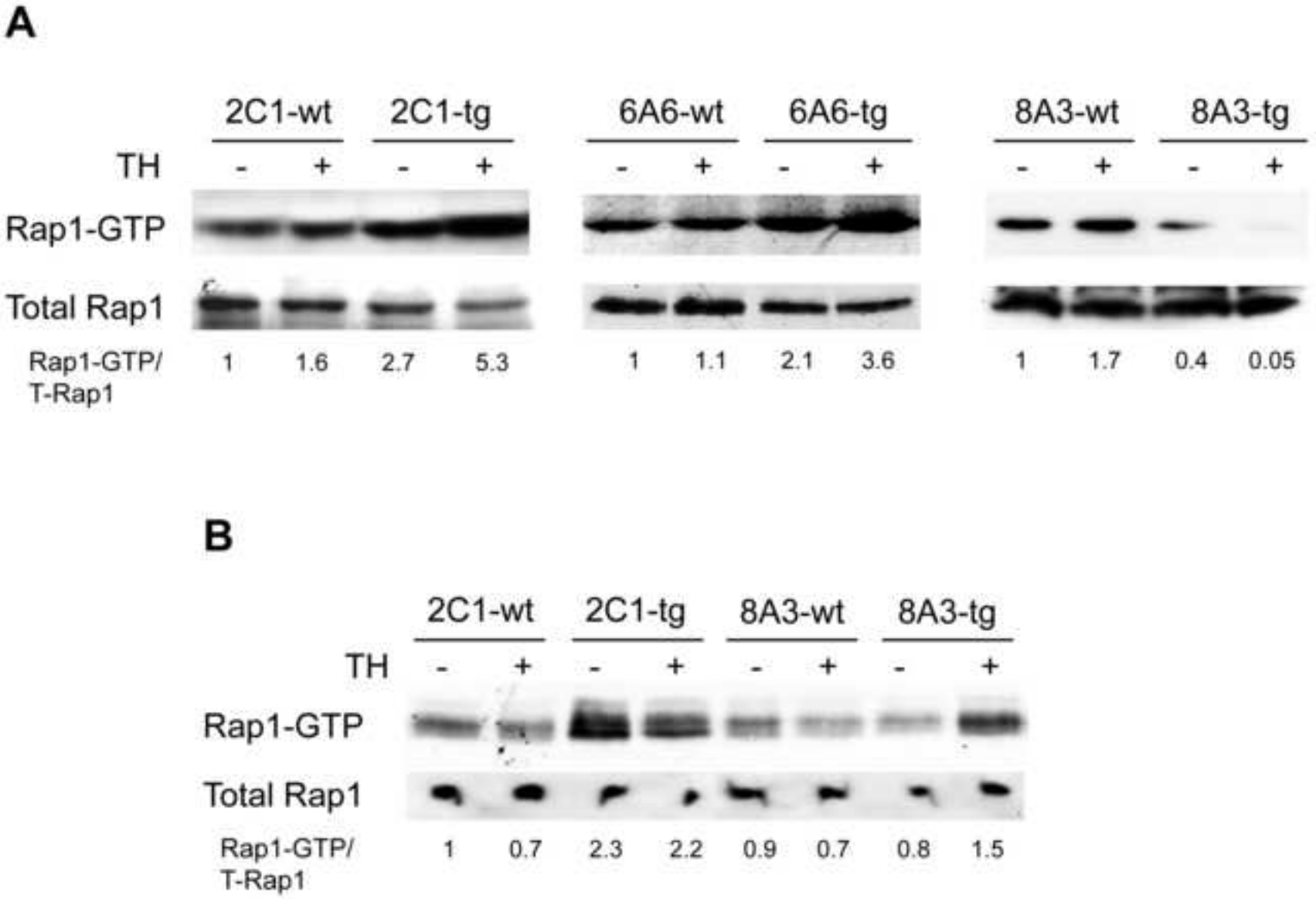
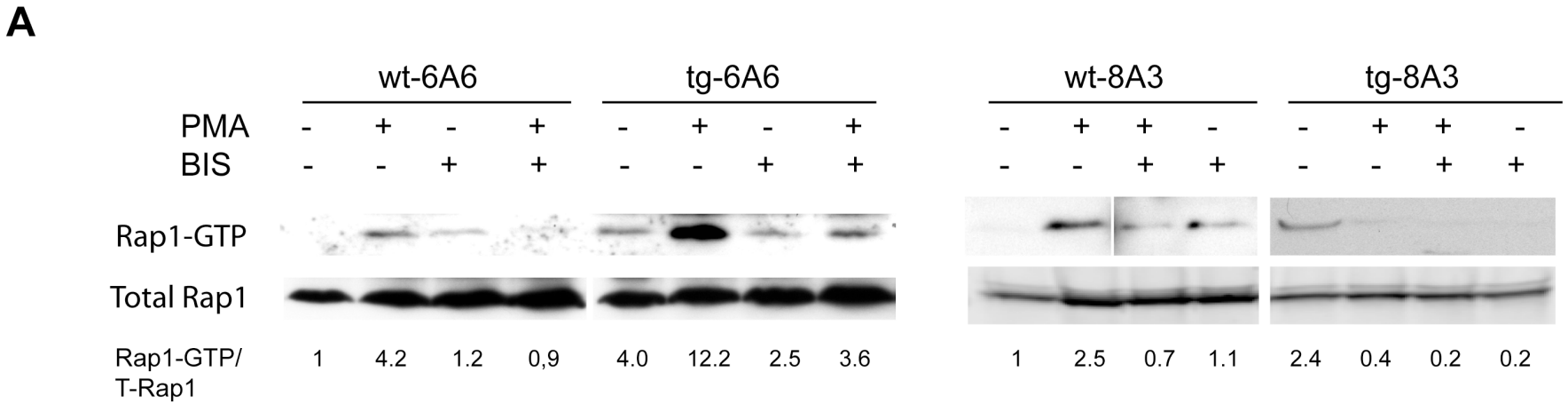


FIGURE 5

Figure 6



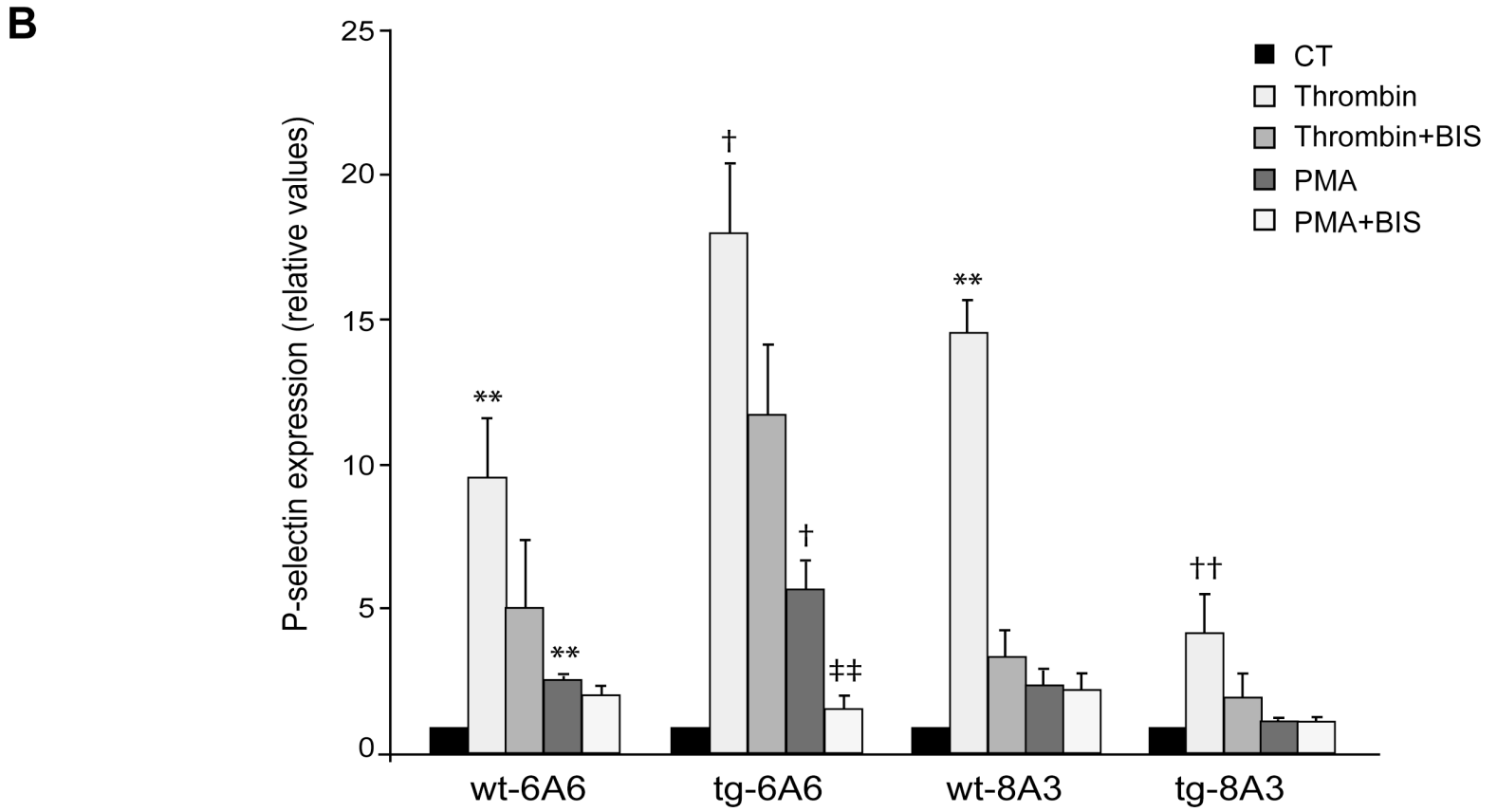


FIGURE 6

Figure 7

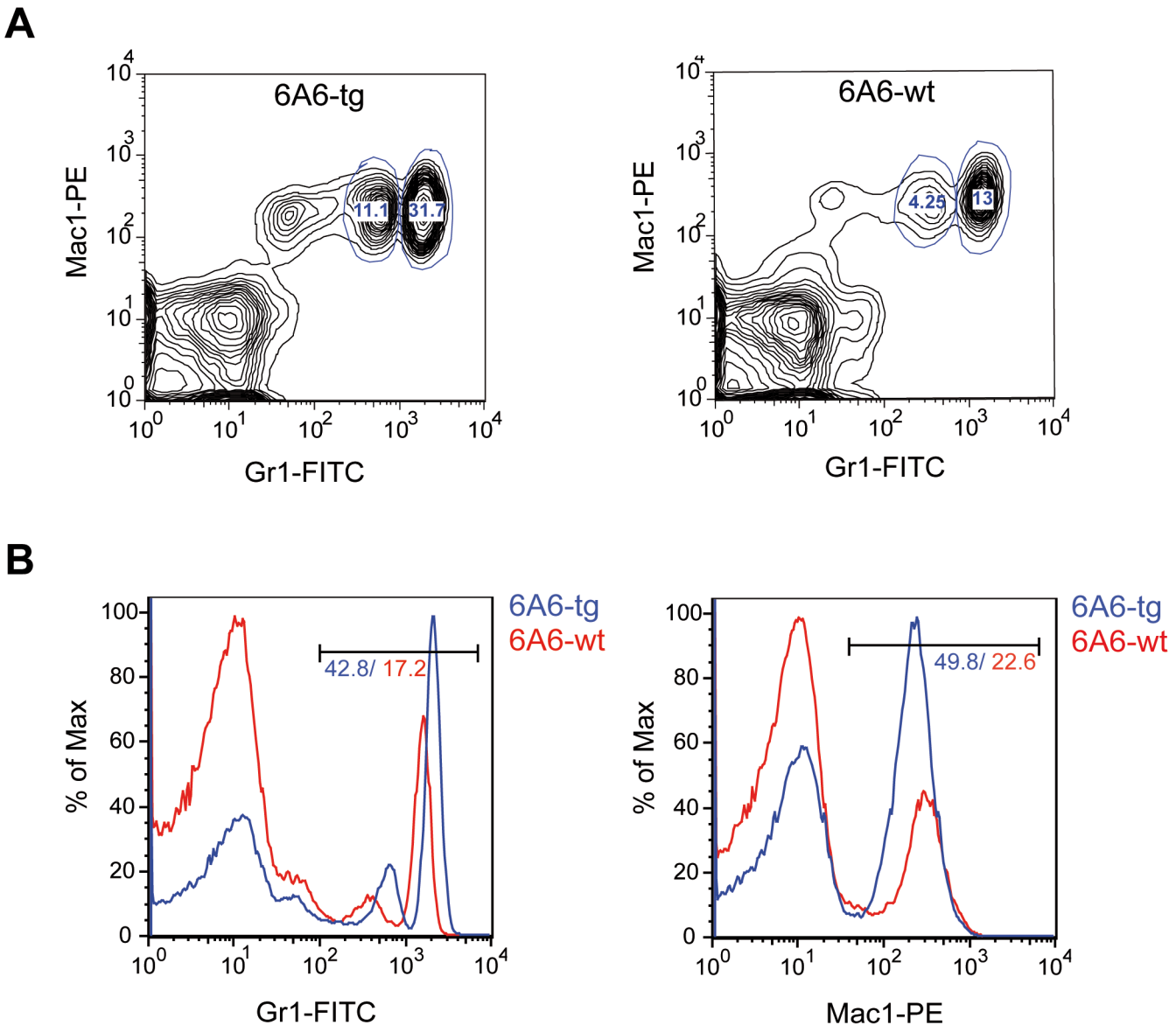


FIGURE 7

Figure 8

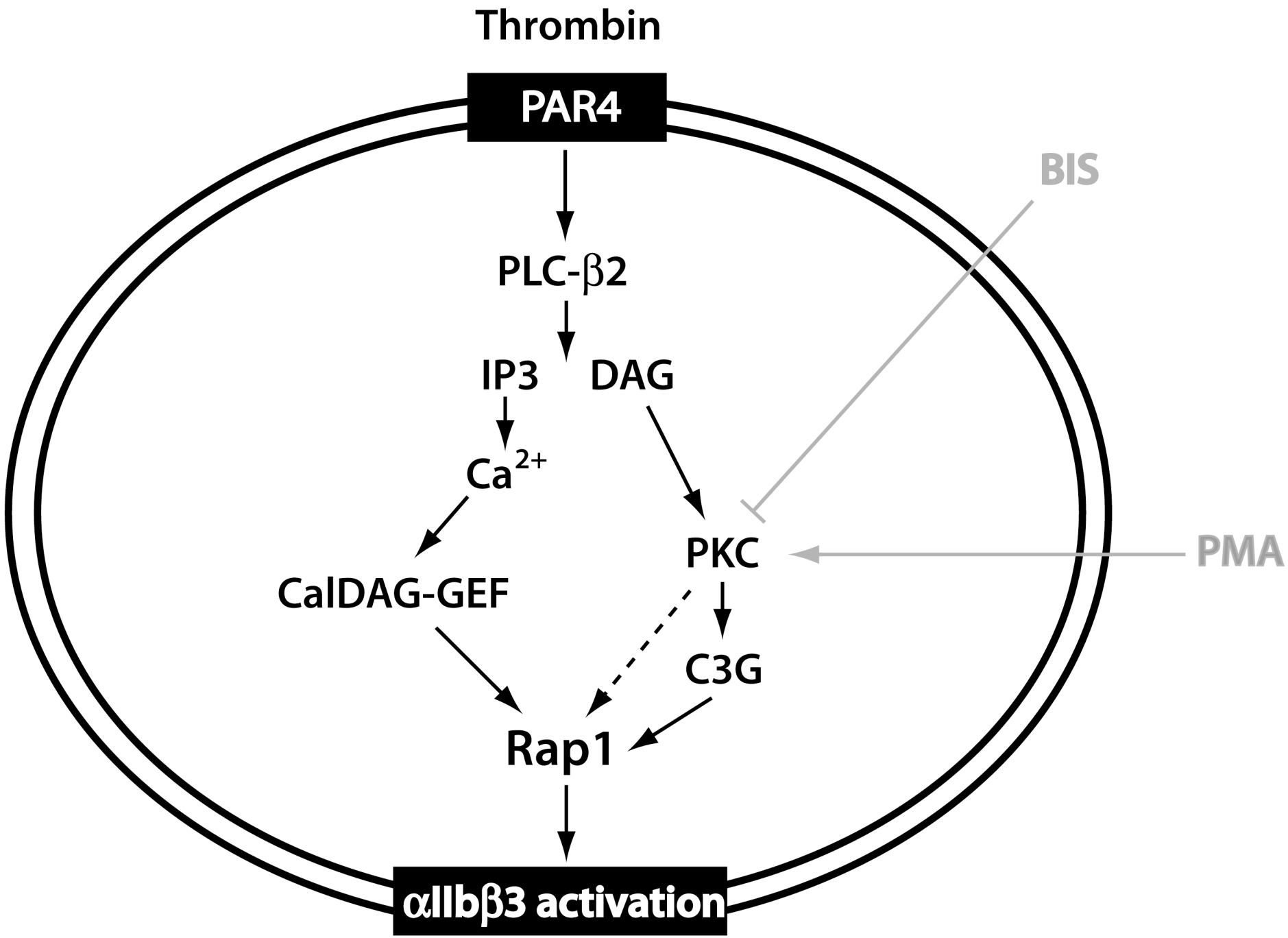


FIGURE 8

Supplemental Figure 1
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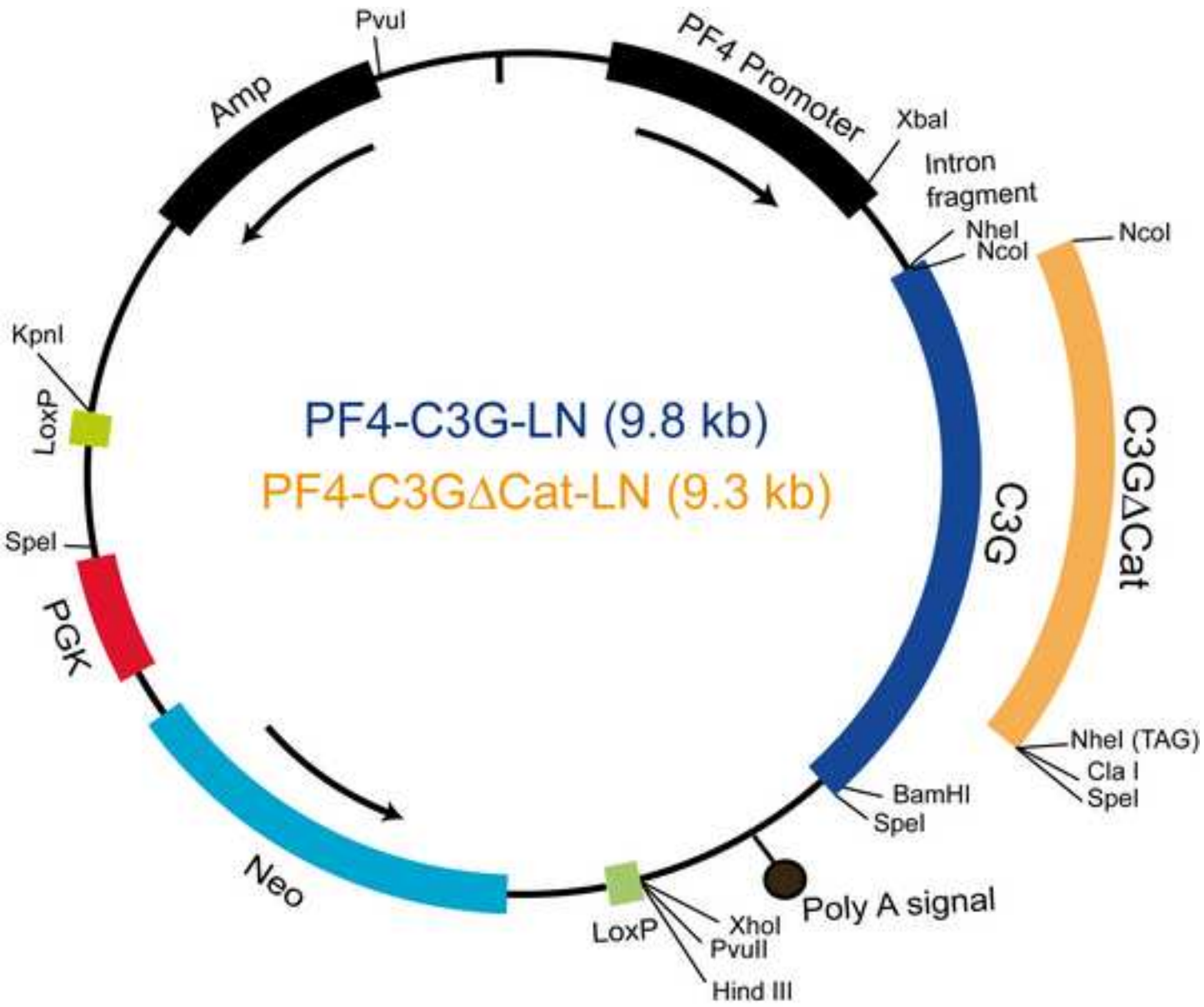

Figure S1

Supplemental Figure 2
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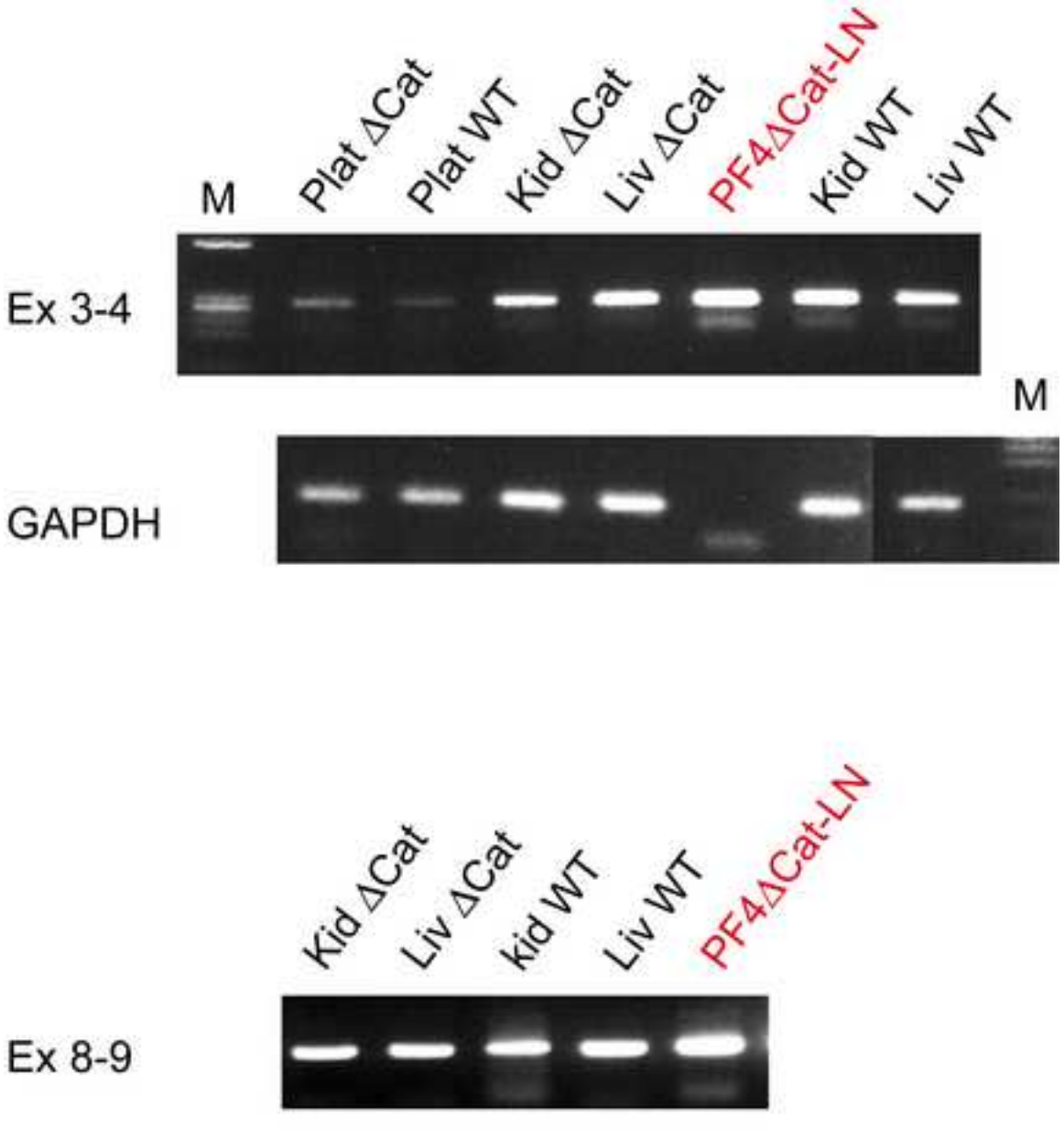

Figure S2

Supplemental Figure 3

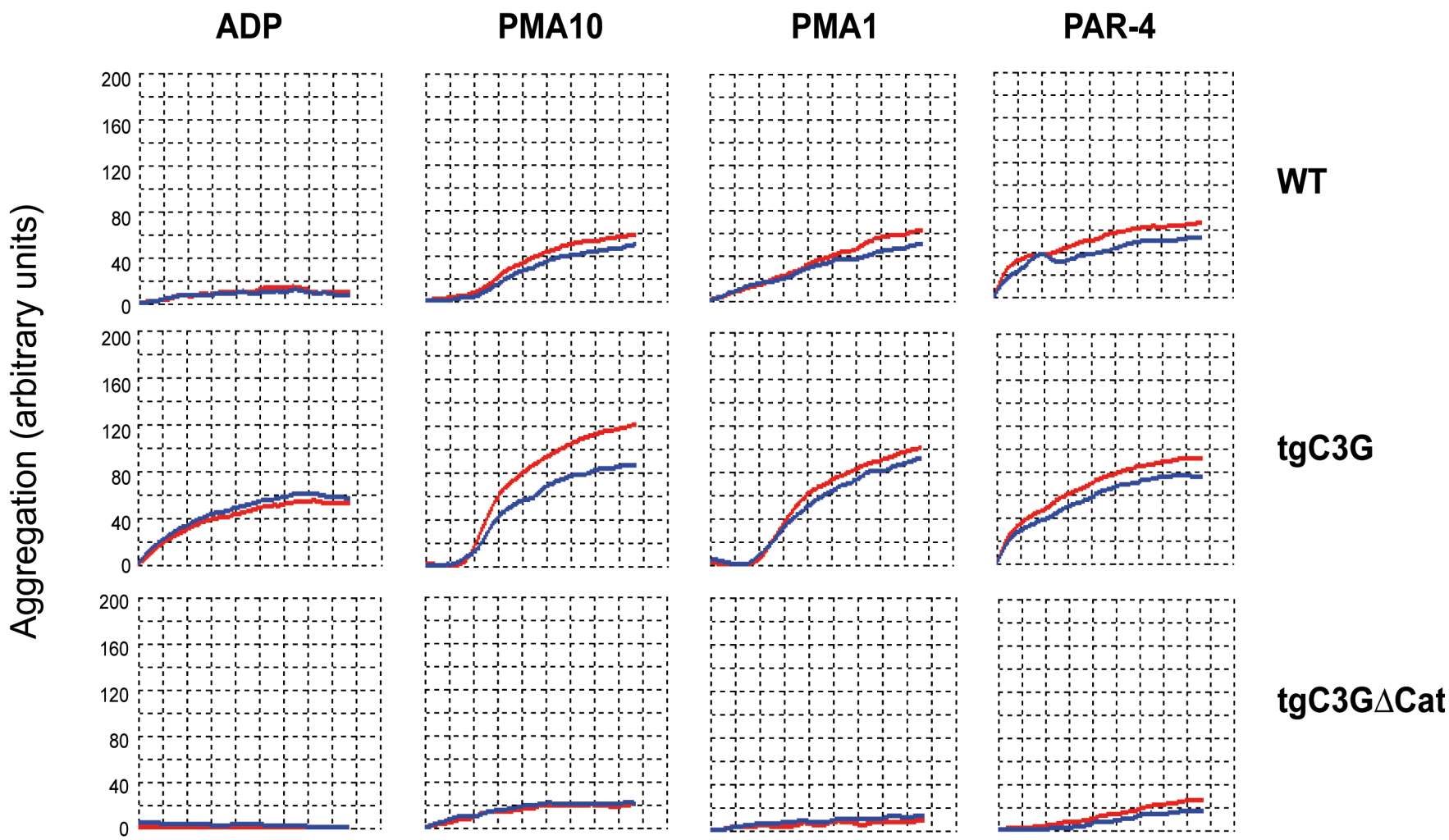

FIGURE S3

Table 1 C3G transgenes do not modify platelet number

Genotype	Sex	NA	Plt (x $10^3/\mu l$) mean ± SEM
2C1+	M	25	1145 ± 101
	F	21	1059 ± 59
2C1-	M	11	961 ± 32
	F	12	1020 ± 51
6A6+	M	31	1038 ± 31
	F	37	996 ± 35
6A6-	M	33	1120 ± 48
	F	29	974 ± 35
8A3+	M	20	1194 ± 49
	F	24	1063 ± 51
8A3-	M	13	1191 ± 68
	F	14	1000 ± 56

Platelet counts in transgenic mice and wild type siblings of the different genotypes. Counts were performed using a Hemavet Counter HV950FS. *p* values for transgenic animals versus wild type siblings are as follows: 0.12 for 2C1 males, 0.33 for 2C1 females, 0.08 for 6A6 males, 0.34 for 6A6 females, 0.48 for 8A3 males, 0.27 for 8A3 females. NA: number of animals. Plt: platelets.

Table 2 C3G transgene increases the number of neutrophils in peripheral blood

Age (months)	tgC3G	Wild type	P value
4	25.35 ± 8.49	20,96 ± 5.18	0.033
6	27.06 ± 7.94	22.07 ± 5.06	0.024
8	29,59 ± 7.68	25.24 ± 5.94	0.033
10	31.00 ± 8.54	24.76 ± 6.27	0.064

Percentage of neutrophils (mean ± SEM) in C3G transgenic mice and wild type siblings. Measures were performed at different ages in 35 mice positive for C3G transgene, (2C1 and 6A6 lines) and 21 wild type mice, using a Hemavet Counter HV950FS. *p* values for transgenic *versus* wild type neutrophil percentage are indicated.

Supplementary Material (for online publication)
Click here to download Supplementary Material (for online publication): Supplementary data-BBAMCR-11-328.doc

Supplementary data

Table S1 Red blood cell (RBC) counts in C3G and C3GΔCat transgenic mice

	Hematocrit (%)	Hemoglobin (gm/dL)	RBC (M/ml)
Wild type 6A6 (n=19)	44.68 ± 9.92	13.26 ± 1.45	8.99 ± 1.04
Transgenic 6A6 (n=19)	47.47 ± 6.96	13.96 ± 1.06	9.25 ± 0.50
Wild type 2C1 (n=22)	44.76 ± 7.51	13.77 ± 0.75	9.02 ± 0.35
Transgenic 2C1 (n=29)	46.34 ± 5.08	13.90 ± 0.46	9.12 ± 0.36
Wild type 8A3 (n=20)	47.81 ± 7.46	13.92 ± 1.02	9.34 ± 0.50
Transgenic 8A3 (n=28)	45.69 ± 3.12	14.21 ± 0.68	9.45 ± 0.37

Values represent the mean ± SEM. *p* values for transgenic mice *versus* wild type sibling controls were as follows: Hematocrit: p=0.16 for 6A6, p=0.16 for 2C1, p=0.09 for 8A3; Hemoglobin: p=0.051 for 6A6, p=0.24 for 2C1, p=0.12 for 8A3; RBC: p=0.16 for 6A6, p=0.17 for 2C1 and p=0.19 for 8A3.

Figure legends

Fig. S1 Schematic representation of plasmids PF4-C3G-LN (9.8 kb) and PF4-C3GΔCat-LN (9.3 kb) used to generate respectively the C3G and C3GΔCat transgenic mice (see details in Materials and Methods). Arrows indicate the direction of transcription. Relevant restriction enzymes are shown. Neo: neomycin resistance gene; Amp: ampicillin resistance gene; PGK: eukaryotic promoter for expression of neomycin resistance in mammalian cells.

Fig. S2 Transgenic expression in tissues. Total RNA was isolated from transgenic C3GΔCat and wild type kidney and liver homogenates, as well as from purified platelets. Reverse transcription was conducted and the resulting cDNA amplified by PCR using oligonucleotides 3F/4R and 8F/9R. GAPDH was used to normalize. M: DNA ladder.

Fig. S3 Aggregation assays. Aggregation traces from wild-type, tgC3G and tgC3GΔCat platelets treated with 6,6 μM ADP, 10 μM PMA or 1 μM PMA and 0.66 mM TRAP4 peptide, using a Multiplate aggregometer (Verum Diagnostica GmbH). These results were confirmed in 4 separate experiments.