

Rasa3 is a GTPase activating protein of the GAP1 family which targets Ras and Rap1. Rasa3 catalytic inactivated mice (*Rasa3*^{-/-}) results in early embryonic lethality¹. Here, we show that nude mice reconstituted with *Rasa3*^{-/-} hematopoietic cells (*SCID-R3*^{-/-}) results in a lethal syndrome characterized by severe defects during megakaryopoiesis, thrombocytopenia and a predisposition to preleukemia. This *Rasa3*^{-/-} megakaryocytes (MKs) present an increased active Rap1 and constitutive integrin activation, recapitulated in *Rasa3*^{+/-} platelets, that lead to defects in migration, adhesion and differentiation into proplatelet megakaryocytes².

1. Survival, splenomegaly, thrombocytopenia and megakaryocyte alterations in *SCID-Rasa3*^{-/-} mice.

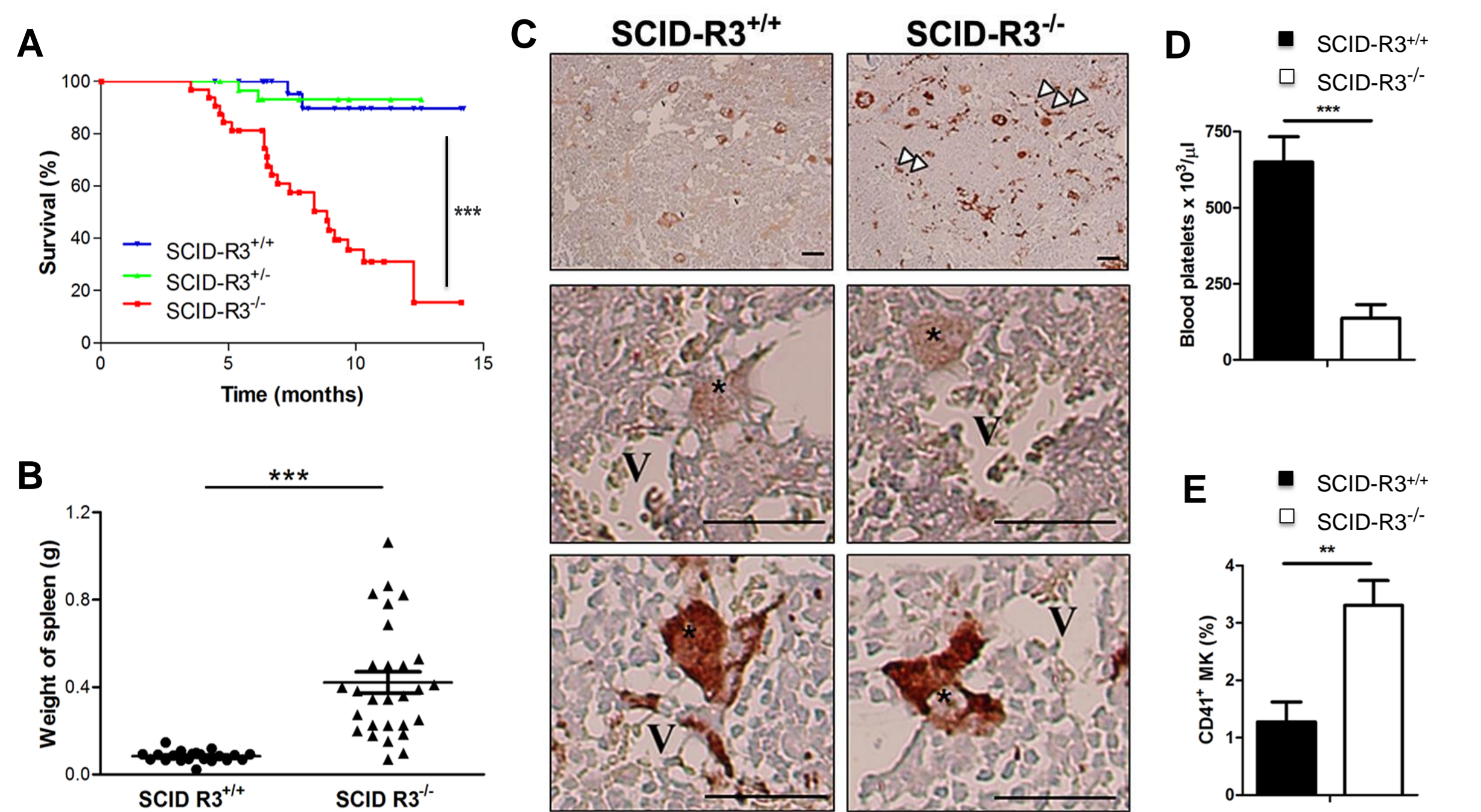


Fig. 1. A. Survival of irradiated SCID mice reconstituted with *Rasa3*^{+/+} (*SCID-R3*^{+/+}), *Rasa3*^{+/-} (*SCID-R3*^{+/-}) or *Rasa3*^{-/-} (*SCID-R3*^{-/-}) fetal liver cells (FLC). B. Spleen weight from age-matched *SCID-Rasa3*^{+/+} and moribund *SCID-Rasa3*^{-/-} mice. C. vWF-stained bone marrow sections of *SCID-R3*^{+/+} and *SCID-R3*^{-/-} femurs 3 months after SCID mice reconstitution. V: vessel; *: megakaryocyte; arrowheads: abnormal vWF deposits. Scale bars: 50 μ m. D. Blood platelet counts in age-matched *SCID-Rasa3*^{+/+} and moribund *SCID-Rasa3*^{-/-} mice. E. Mean \pm SEM of CD41⁺ megakaryocyte (MK) percentages in the bone marrow detected by flow cytometry. Statistics (unpaired t test): *: P<0.05; **: P<0.01; ***: P<0.001.

3. Abnormal adhesion of *Rasa3*^{-/-} megakaryocyte

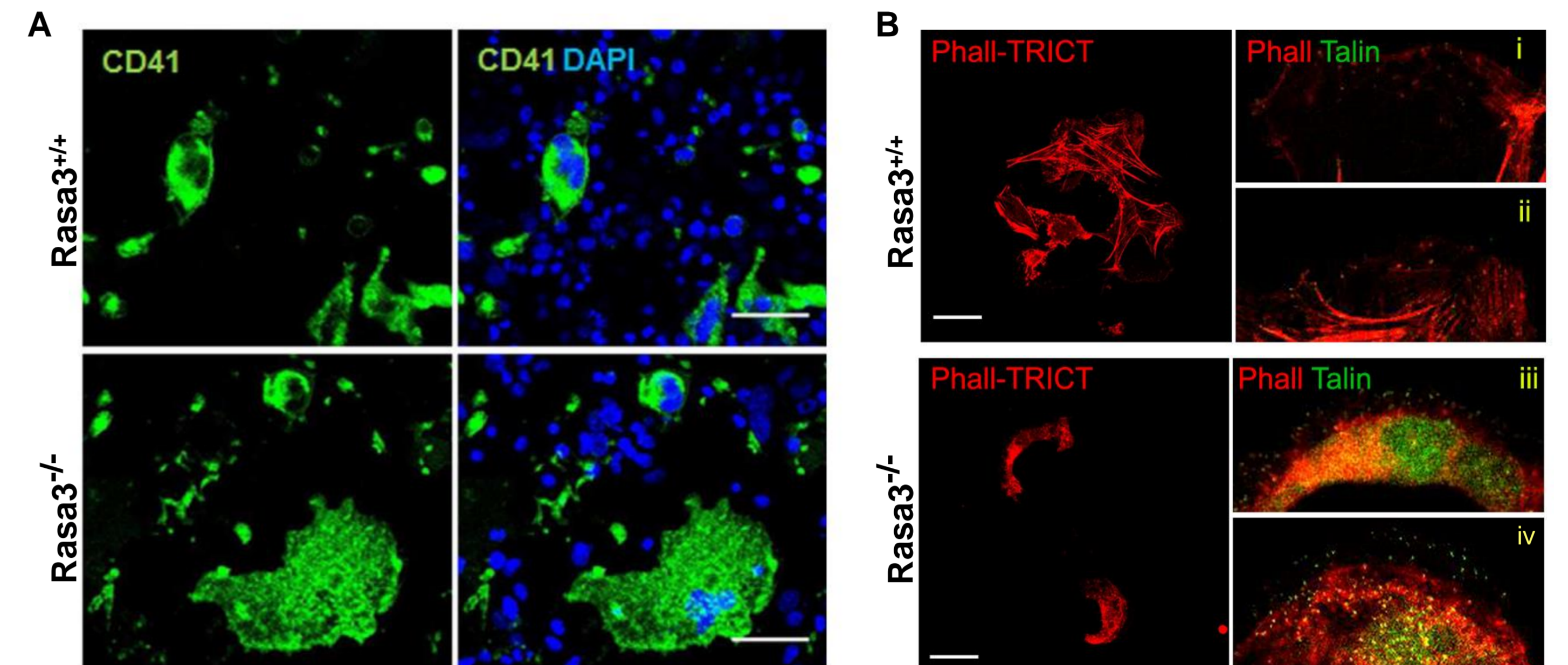


Fig. 3. FLC were isolated from E12.5 *Rasa3*^{+/+} and *Rasa3*^{-/-} embryos. A. *Rasa3*^{+/+} (upper panels) and *Rasa3*^{-/-} (lower panels) FLC cultures after 6 days with TPO were stained with the megakaryocyte marker CD41 antibody (green) and DAPI (blue). B. *Rasa3*^{+/+} and *Rasa3*^{-/-} adherent megakaryocytes were stained with phalloidin-TRITC (actin, red) and Talin-FITC (green). Confocal images were obtained from the bottom of the cells. (i-iv): 4 \times Digital magnification of phalloidin-TRITC and Talin-FITC merge. An increased Talin staining is observed in *Rasa3*^{-/-} megakaryocytes (iii and iv), as compared with *Rasa3*^{+/+} megakaryocytes (i and ii). Scale bar: 50 μ m.

4. Constitutively activated Rap1 and GPIIb/IIIa in *Rasa3*^{-/-} MKs

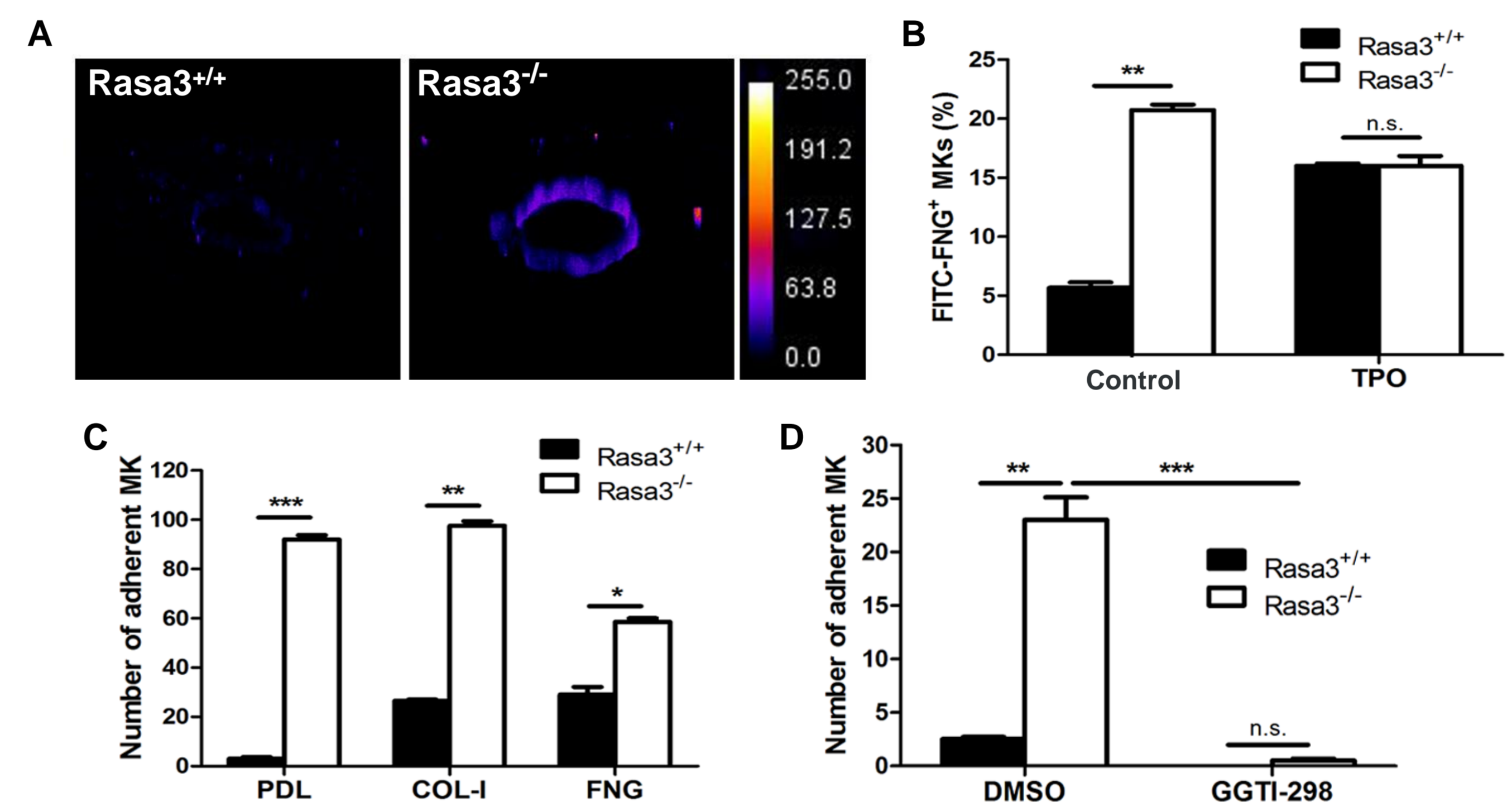


Fig. 4. Megakaryocytes from FLC cultured with TPO were enriched on a BSA-gradient A. Non adherent megakaryocytes on day 6 of culture were analyzed for active, GTP-bound Rap1 by immunofluorescence. 3D reconstructions of *Rasa3*^{+/+} (left panel) and *Rasa3*^{-/-} (right panel) megakaryocytes are shown in pseudocolor fluorescence intensity lookup. B. Inside-out GPIIb/IIIa integrin signaling was investigated in megakaryocytes by quantifying soluble FITC-fibrinogen (FITC-FNG) bound to the CD41⁺ cell surface by flow cytometry. Megakaryocytes were treated with or without 100 ng/ml TPO for 30 min. C. Megakaryocytes were cultured on Poly-D-Lysine- (PDL), collagen-I- (COL-I) and fibrinogen- (FNG) coated plates in medium containing 10% FBS. Adherent megakaryocyte were quantified after 18h. D. Addition of the Rap1 inhibitor GGTI-298 (3 μ M) abolished the abnormal adherent phenotype of *Rasa3*^{-/-} megakaryocytes. Statistics (unpaired t test): n.s. not significant; * P<0.05; ** P<0.01; *** P<0.001.

5. *Rasa3*^{+/-} platelets have the same defects as *Rasa3*^{-/-} MKs

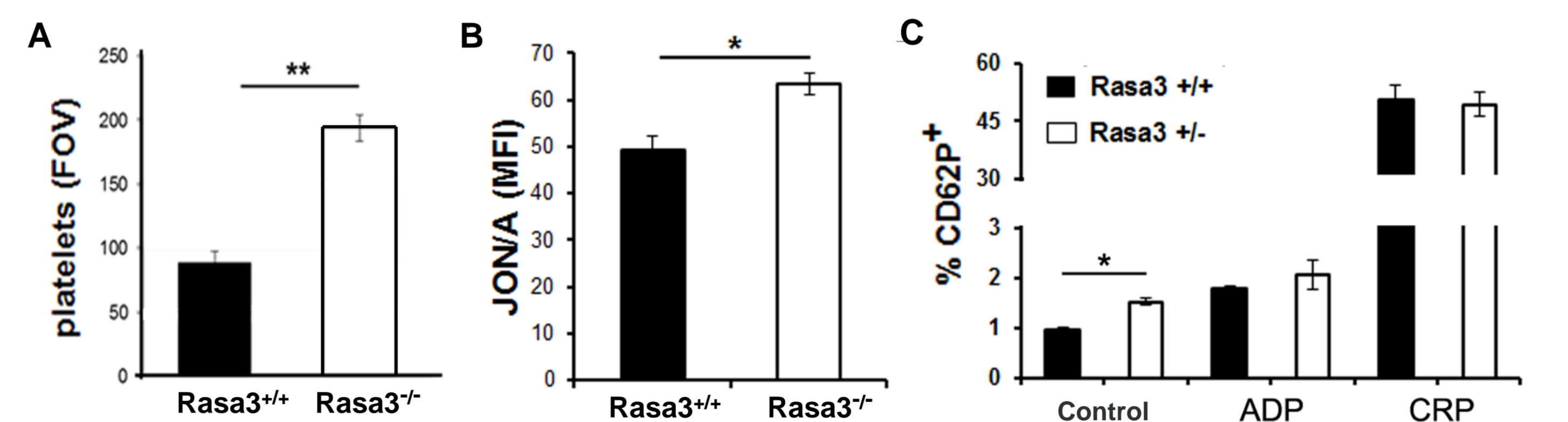


Fig. 5. Unstimulated platelets were isolated from 8 week-old *Rasa3*^{+/+} and *Rasa3*^{+/-} mice. A. After 45 min, an increased number of *Rasa3*^{+/-} platelets adhered to BSA-coated plates, as compared with *Rasa3*^{+/+} platelets. Mean \pm SEM of platelet counts per field of view (FOV). B. Mean fluorescence intensity (MFI) of the JON/A antibody binding to the high affinity conformation of the integrin α IIb β 3 on *Rasa3*^{+/+} and *Rasa3*^{+/-} platelets in resting condition. C. Mean \pm SEM of the percentage of CD62P⁺ platelets in non stimulated condition (control) and after ADP (25 μ M) or CRP (1 μ g/ml) stimulation. Statistics (unpaired t test): *: P<0.05; **: P<0.01

2. Altered *SCID-Rasa3*^{-/-} megakaryocyte motility, adhesion and differentiation

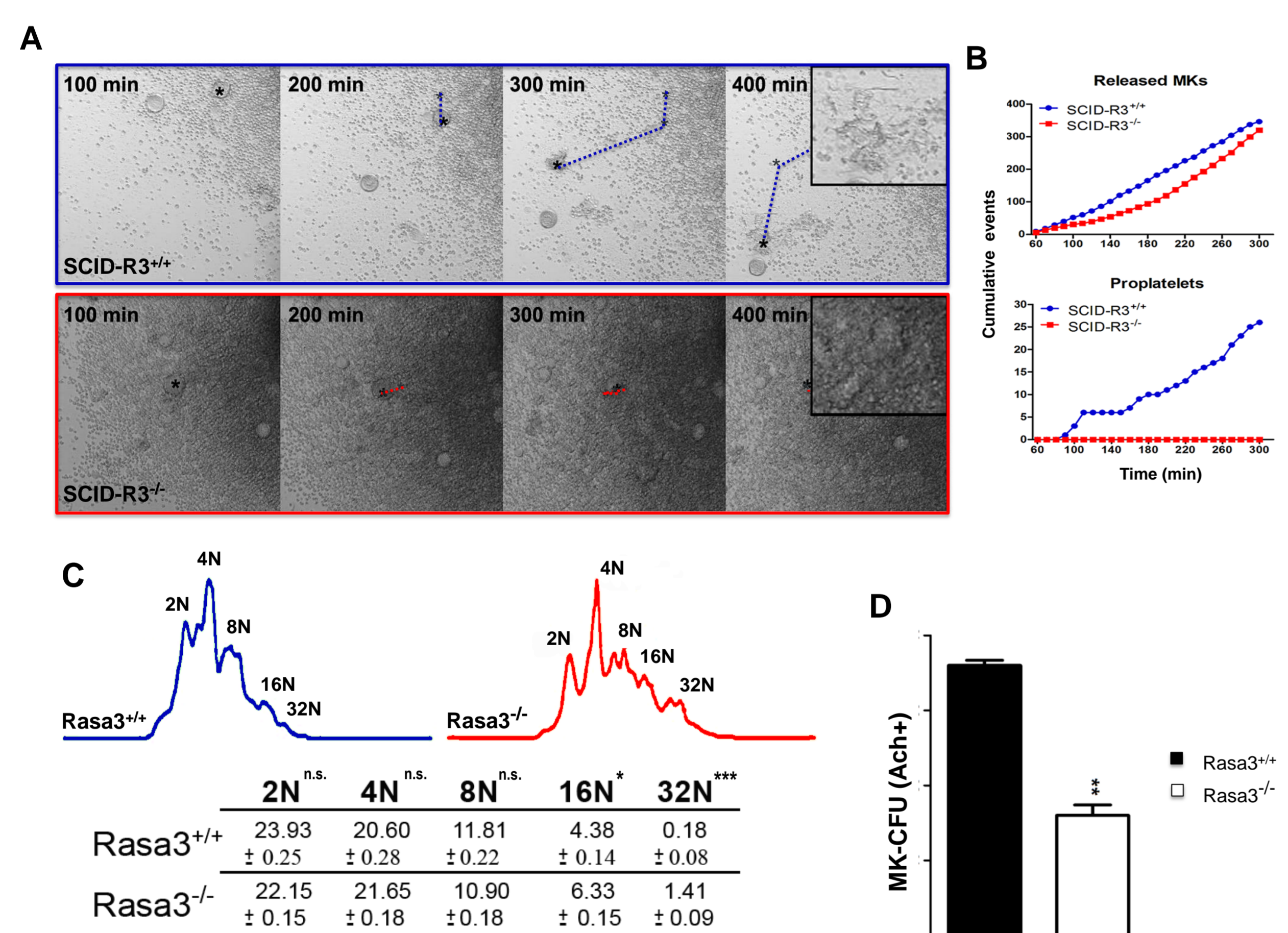


Fig.2. Bone marrow explants were isolated from *SCID-R3*^{+/+} and *SCID-R3*^{-/-} mice 2 months after irradiation/reconstitution and cultured *ex vivo*. Images were taken every 10 min. A. Time lapse representative images. The asterisk indicates the same megakaryocyte that finally develops into a proplatelet forming megakaryocyte (*SCID-R3*^{+/+} explants) or that adheres to the culture plate and fail to form proplatelets (*SCID-R3*^{-/-} explants). Insets: higher magnification shows the proplatelet forming megakaryocyte in the *SCID-Rasa3*^{+/+} explants and the adherent megakaryocyte in the *SCID-Rasa3*^{-/-} explants. Scale bars: 50 μ m. B. Number of megakaryocytes released from the explants (upper panel) and of proplatelet (lower panel). C. Increased megakaryocyte ploidy in FLC culture at day 2 after TPO treatment. Representative images of DNA content in *Rasa3*^{+/+} and *Rasa3*^{-/-} CD41⁺ megakaryocytes. The table show the percentages of individual ploidy classes in FLC-derived CD41⁺ megakaryocytes (mean \pm SEM). D. The graph represents the number of MK-CFU (Megakaryocyte Colony Formation Unit) formed by *Rasa3*^{+/+} and *Rasa3*^{-/-} FLC cultures after 3 days (mean \pm SEM). Statistics (unpaired t test): n.s. not significant; *: P<0.05; **: P<0.01; ***: P<0.001

References:

- Iwashita S, et al. (2007) J Biol Chem 282: 3413-7.
- Molina-Ortiz P, Polizzi S, et al (2014). PLoS Genet. 10: e1004420.
- Bury, L et al (2012). PLoS One, 7(4), e34449.

Conclusions

- Catalytic inactivation of Rasa3 in the hematopoietic system causes a lethal syndrome characterized by severe thrombocytopenia, splenomegaly and increased number of megakaryocyte in the spleen and the bone marrow.
- Rasa3*^{-/-} megakaryocytes present defective migration, adhesion, differentiation and proplatelets formation. Human megakaryocyte from patient with constitutively activated-GPIIb/IIIb mutated protein resemble *Rasa3*^{-/-} megakaryocyte defects³.
- Rasa3*^{-/-} megakaryocytes have constitutively active GPIIb/IIIa integrin and increased levels of Rap1-GTP.
- Rasa3*^{+/-} platelets results suggest that Rasa3 has the same role in platelets and in megakaryocyte.