Analysis of Platelet Activation by Stable Isotope-Resolved Metabolomics (SIRM)

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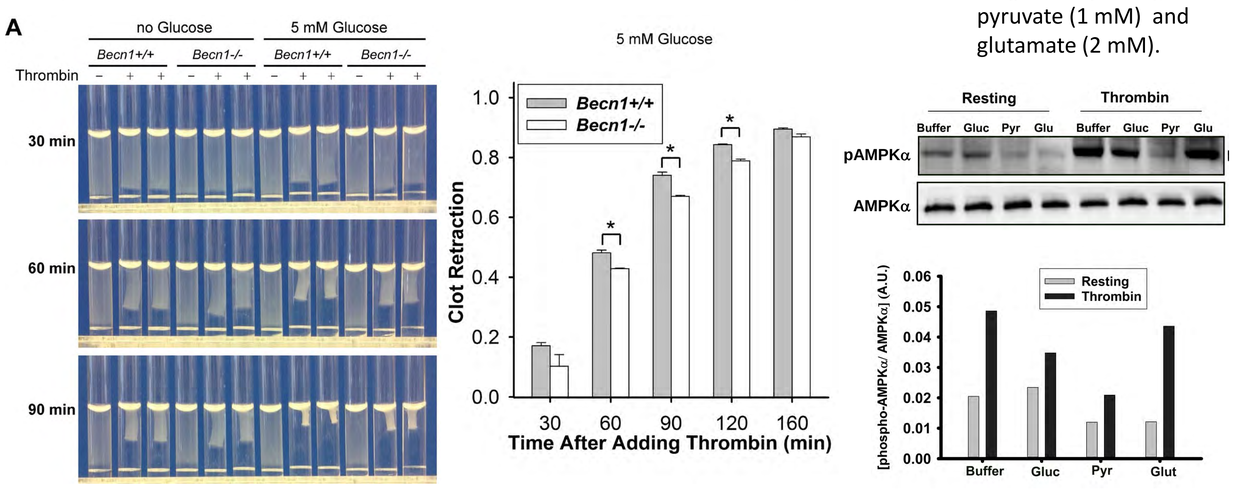
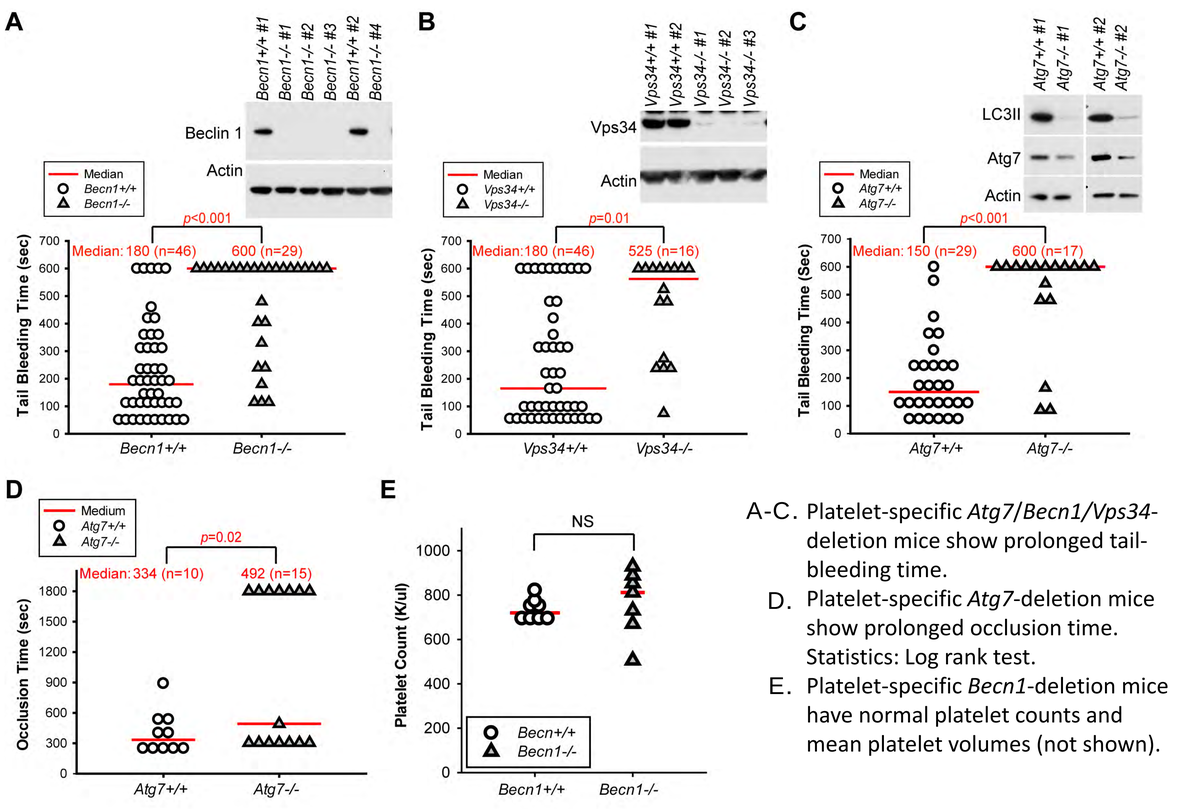
1. Glucose deprivation or platelet-specific loss of autophagy impairs clot retraction of washed mouse platelets *ex vivo*.

A. **Clot retraction of *Becn1$^{f/f}$* (labeled *Becn1$^{+/+}$*) and *Becn1$^{f/f}$;PF4-Cre/+* (labeled *Becn1$^{-/-}$*) mouse platelets in the absence and presence of glucose.** Platelets (3×10^8/ml) were re-suspended in glucose-free HEPES-Tyrode buffer (pH 7.4) supplemented +/- 5 mM D-glucose. After addition of 0.5 mg/ml human fibrinogen and 1 mM $CaCl_2$, clot retraction was initiated by the addition of 0.1 U/ml thrombin and recorded at the indicated times. * p<0.05

B. Phospho-AMPK levels increased during clot retraction, which was damped by supplement of glucose (5 mM), pyruvate (1 mM) and glutamate (2 mM).



2. Platelet-specific autophagy deficiency leads to robust bleeding diatheses



A-C. Platelet-specific *Atg7*/*Becn1*/*Vps34*-deletion mice show prolonged tail-bleeding time.

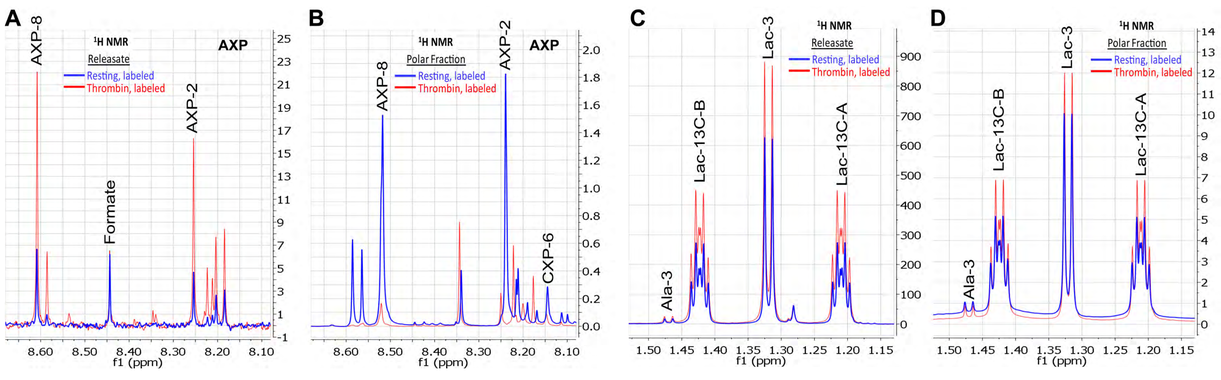
D. Platelet-specific *Atg7*-deletion mice show prolonged occlusion time. Statistics: Log rank test.

E. Platelet-specific *Becn1*-deletion mice have normal platelet counts and mean platelet volumes (not shown).

3. Stable Isotope Resolved Metabolomics (SIRM) shows activation-induced ATP secretion & lactate production/excretion in $^{13}C_6$-glucose-labeled human platelets

A-B. ^{1}H NMR spectra show AXP (*i.e.*, ATP/ADP/AMP.) in both (A) releasates and (B) polar extracts from cell pellets.

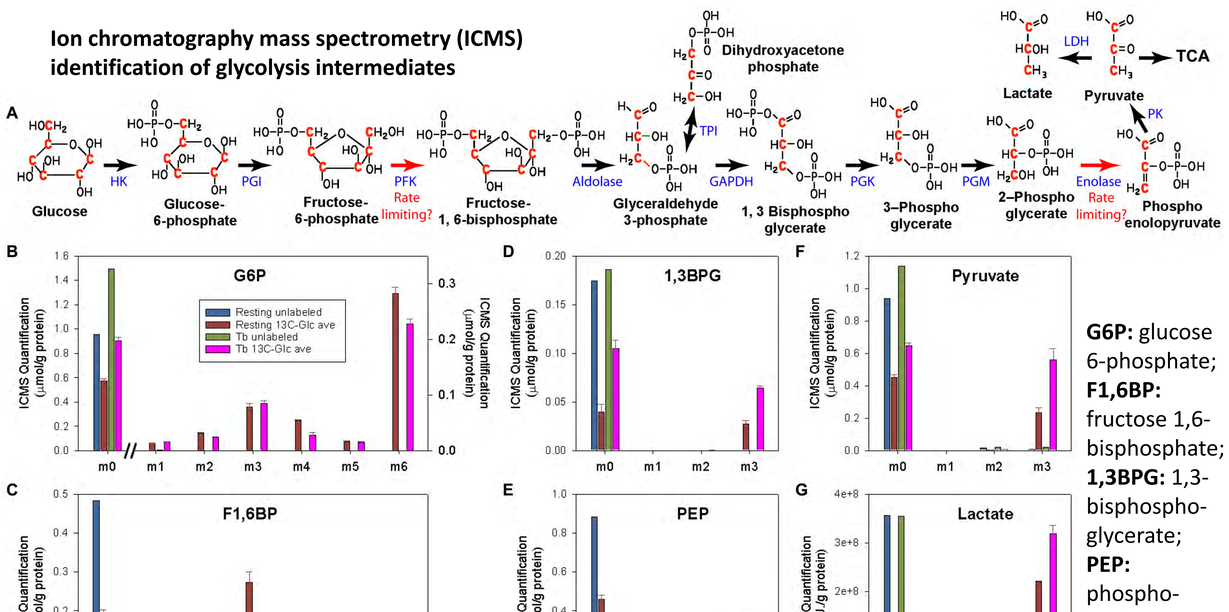
C-D. ^{1}H NMR spectra show lactate in both (C) releasates and (D) polar extracts from cell pellets.



Washed platelets (5×10^9 cells) were labeled with 15 mM $^{13}C_6$-glucose for 2 h before treated with 0.5 U/ml thrombin for 30 min (37°C). Signals were normalized to the amount of DSS NMR standard and then mg of total proteins.

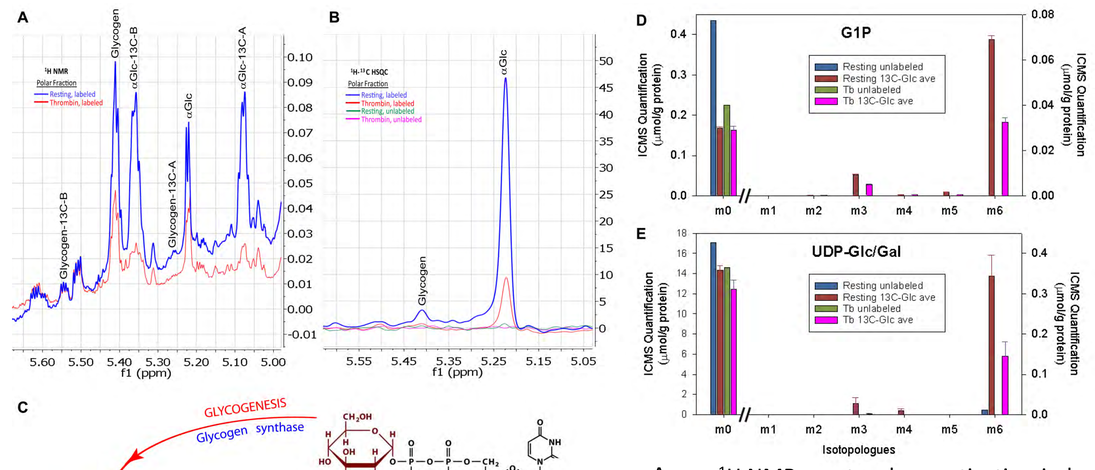
4. Following glycolysis in $^{13}C_6$-glucose-labeled human platelets with SIRM

Ion chromatography mass spectrometry (ICMS) identification of glycolysis intermediates



G6P: glucose 6-phosphate; **F1,6BP:** fructose 1,6-bisphosphate; **1,3BPG:** 1,3-bisphospho-glycerate; **PEP:** phospho-enolpyruvate.

5. Following glycogen metabolism in $^{13}C_6$-glucose-labeled human platelets

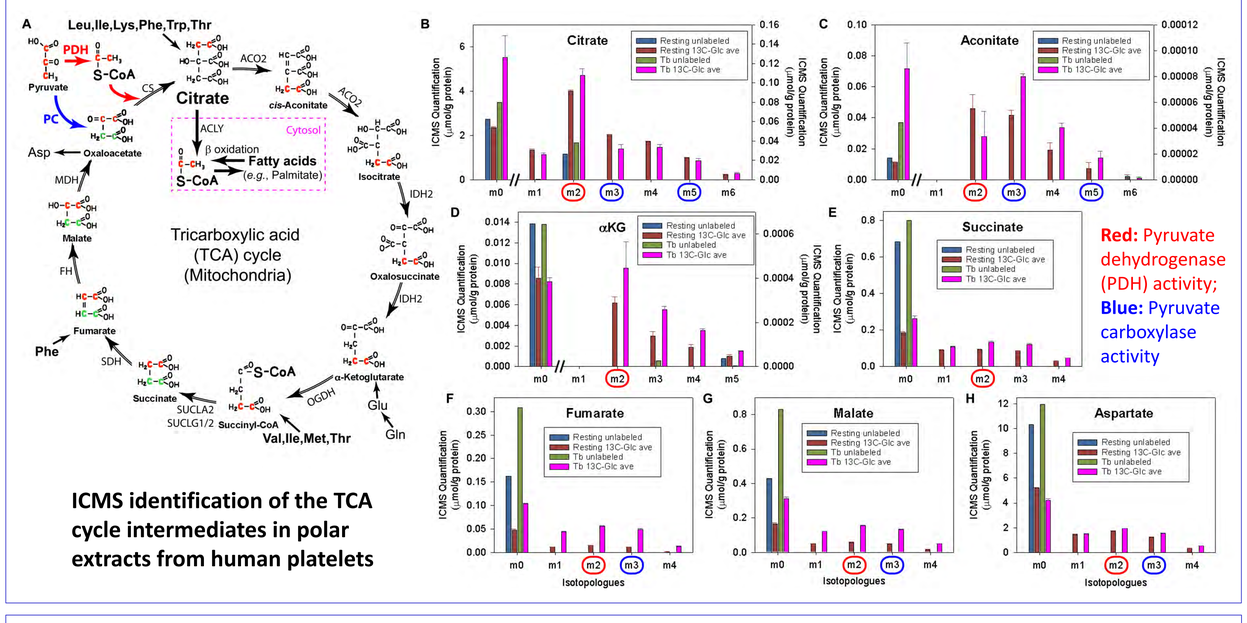


A. ^{1}H NMR spectra show activation-induced decreases in unlabeled and ^{13}C-labeled glucose and glycogen in the polar extracts from platelet pellets.

B. ^{1}H-^{13}C HSQC NMR spectra of polar fractions show glycogenesis in resting platelets and activation-induced glycogen breakdown.

D-E. ICMS shows newly synthesized (**D**) glucose-1 phosphate (G1P) and (**E**) UDP-Glucose or UDP-Galactose from $^{13}C_6$-labeled glucose.

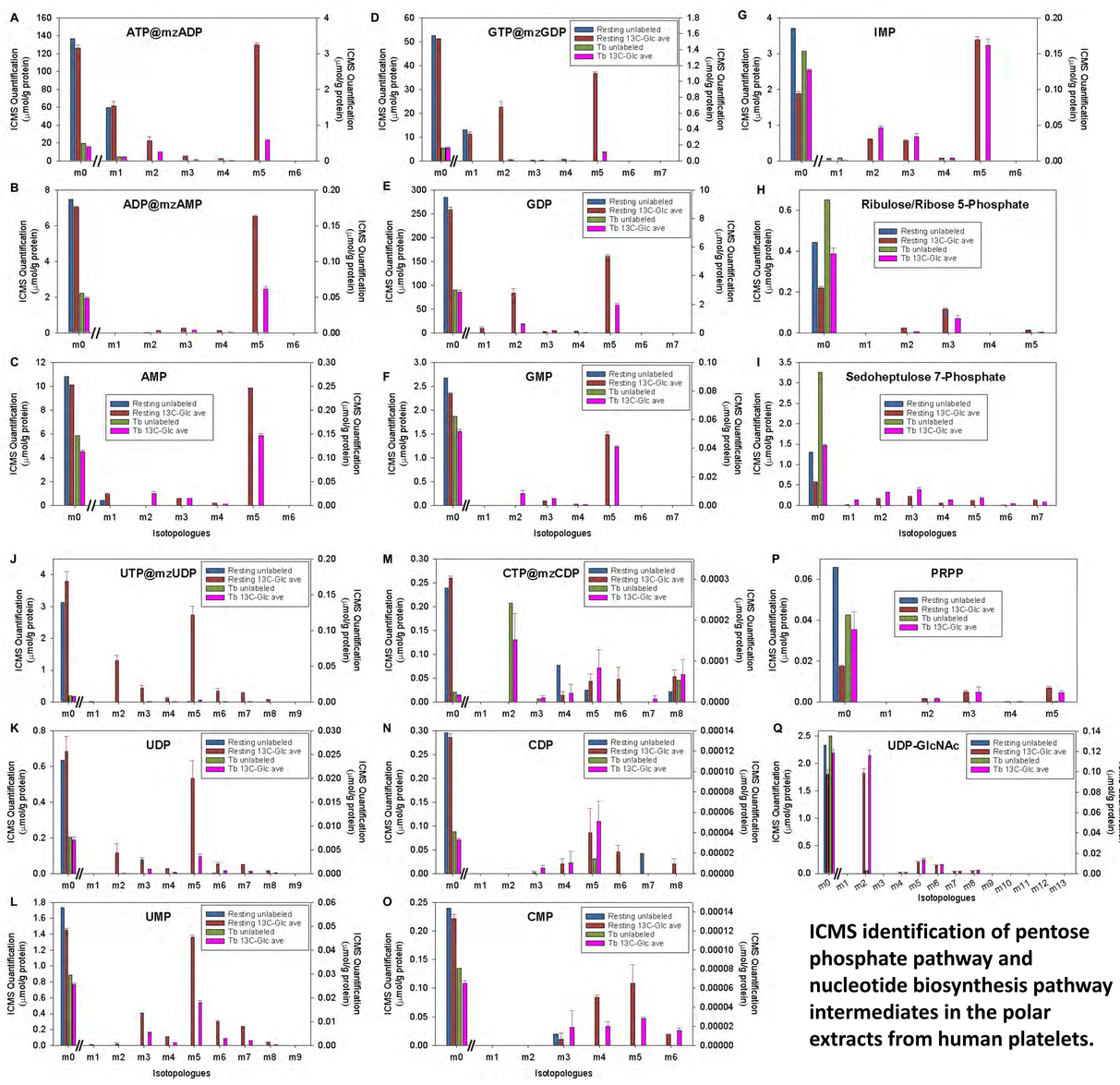
6. Following the TCA cycle in $^{13}C_6$-glucose-labeled human platelets



ICMS identification of the TCA cycle intermediates in polar extracts from human platelets

Red: Pyruvate dehydrogenase (PDH) activity; **Blue:** Pyruvate carboxylase activity

7. SIRM shows nucleotide biosynthesis in $^{13}C_6$-glucose-labeled human platelets



ICMS identification of pentose phosphate pathway and nucleotide biosynthesis pathway intermediates in the polar extracts from human platelets.

Conclusions & Significance

- Platelets require glucose and autophagy for clot retraction.
- Platelet autophagy is essential for hemostasis and thrombosis.
- Stable Isotope-Resolved Metabolomics (SIRM) reveals extensive reprogramming of glycolysis, glycogen metabolism, TCA cycle and nucleotide metabolism upon platelet activation.
- Our work depicts **the first comprehensive picture of platelet metabolism**, as compared to early studies that relied on crude analytics and inhibitors. This picture will lead to a better understanding of platelet energy utilization and yield critical insights into the causes of platelet dysfunction associated with *e.g.*, metabolic disorders.

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