Selective dependency of CALR-mutant myeloproliferative neoplasms on TYK2 signaling

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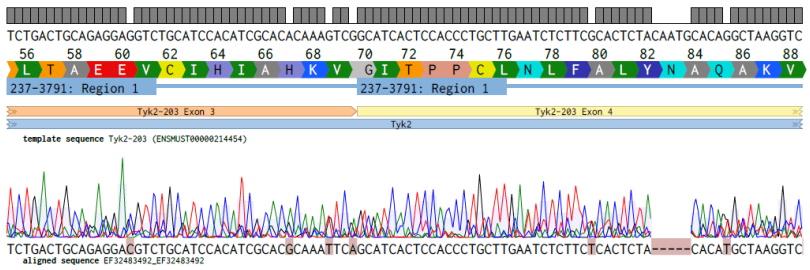
Supplementary Figures – Summary

Supplementary Figure S1

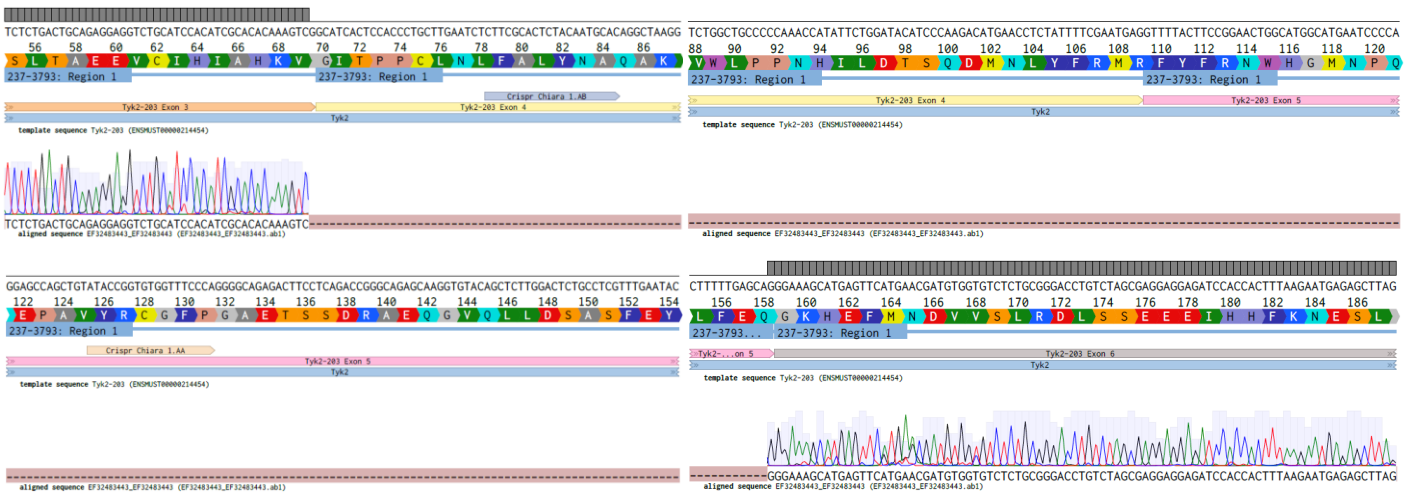
A

Strategy 1 – 32D^{MPL} JAK2V617F

Small deletion



Large deletion



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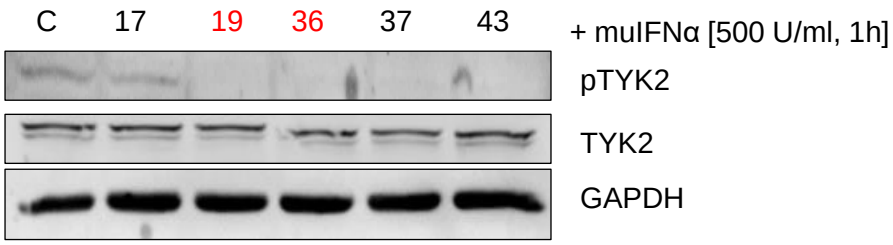
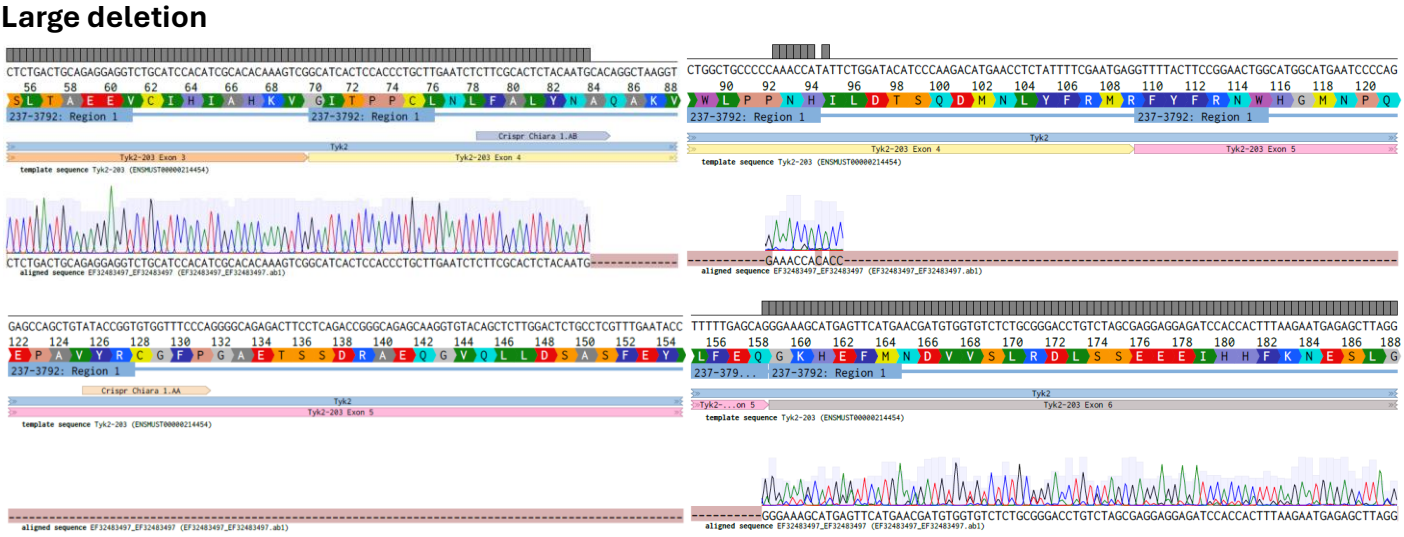


C

Strategy 1 – 32D^{MPL} CALRdel52

Large deletion



D

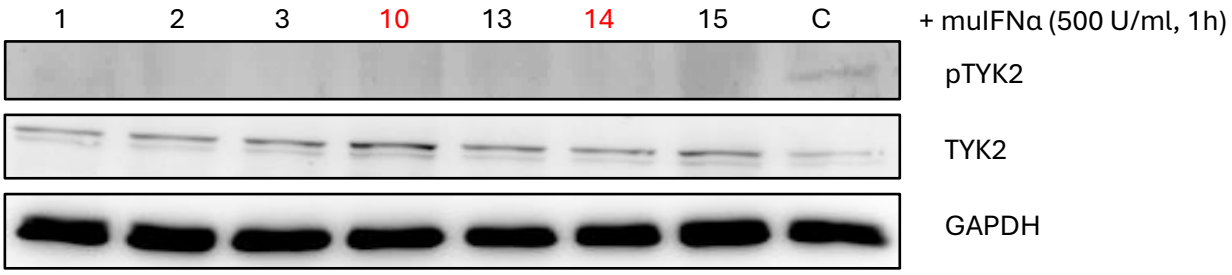


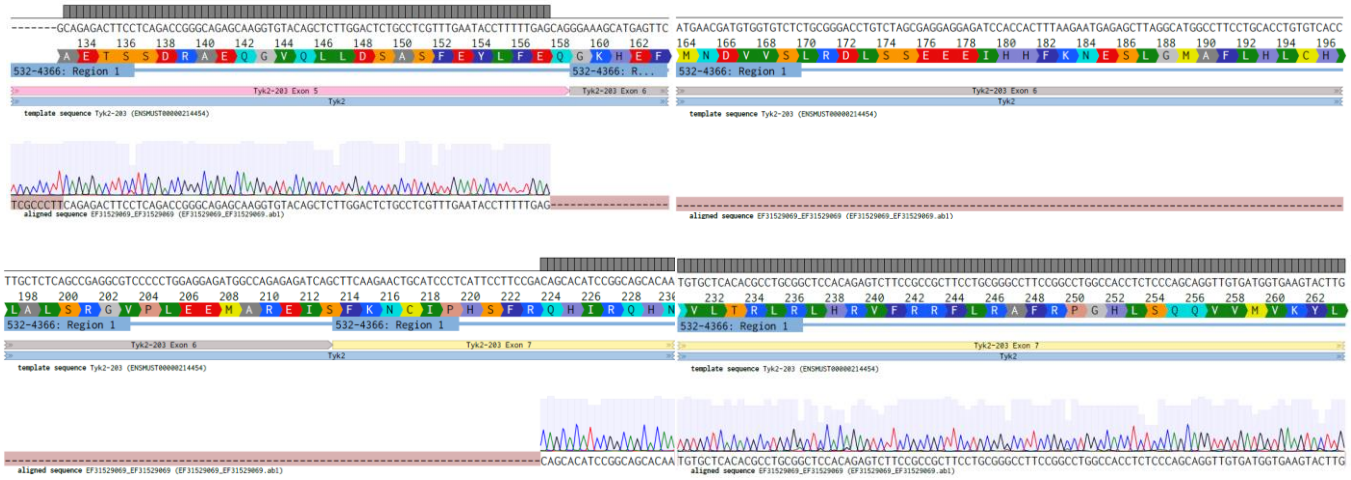
Figure S1. Analysis of clones resulting from CRISPR strategy 1. After electroporation with two guides and the nuclease, single cell dilutions of 32D^{MPL} JAK2V617F (**A-B**) and CALRdel52 (**C-D)** cells were made. Representative 32D^{MPL} JAK2V617F (**A**) and CALRdel52 (**C**) clones, respectively. cDNA of single clones was isolated and the *TYK2* gene was amplified using specific PCR primers spanning the guide locations. Fragments were analyzed via gel electrophoresis, purified and sent for Sanger sequencing. **B** and **D** Western blot of potentially interesting clones. Each clone was stimulated with murine IFNα (500 U/ml) for 1 h to induce TYK2 phosphorylation as positive control. Analyzed clones were highlighted in red. P indicates parental TYK2 WT cells. GAPDH was used as loading control.

Supplementary Figure S2

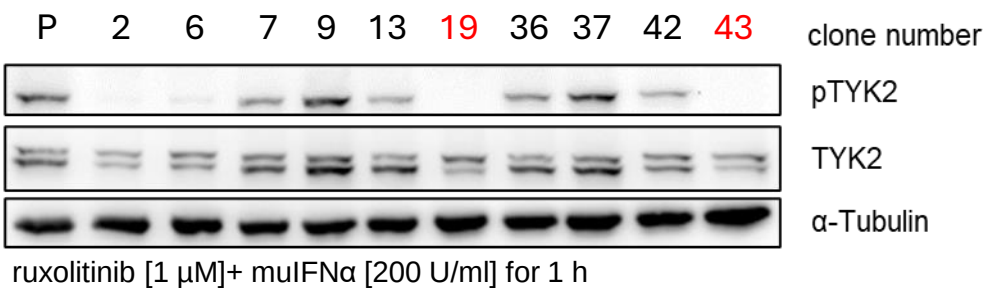
A

Strategy 2 – 32D^{MPL} JAK2V617F

Large deletion



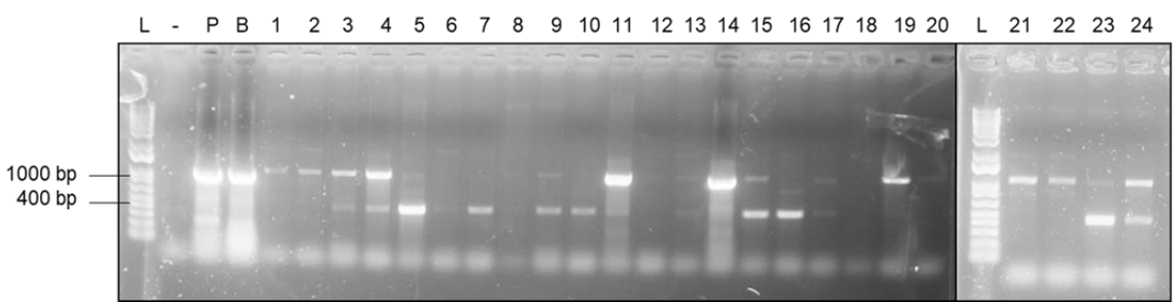
B



ruxolitinib [1 μM]+ muIFNα [200 U/ml] for 1 h

C

Strategy 2 – 32D^{MPL} CALRdel52



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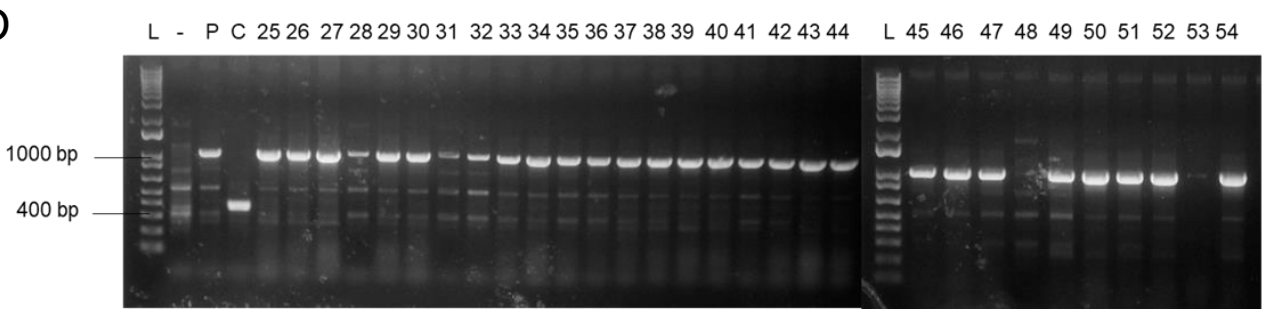
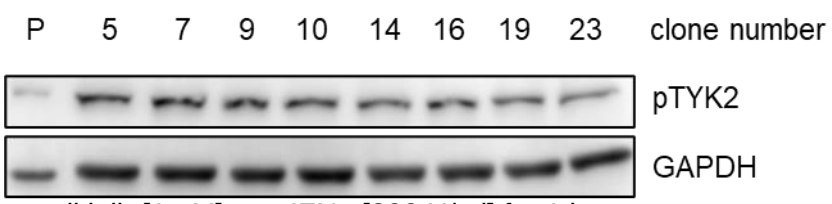


E



ruxolitinib [1 μM]+ muIFNα [200 U/ml] for 1 h

Figure S2. Analysis of clones resulting from CRISPR strategy 2. A After electroporation of 32D^{MPL} JAK2V617F cells with 4 guides and nickase, single cell dilutions were made. cDNA of single clones was isolated and cloned into the TOPO vector for subsequent Sanger sequencing. Large deletions were detected. **B** Clones of interest were treated with IFNα and ruxolitinib to induce phosphorylated TYK2 by IFNα and conserve pTYK2 as type I TKI lead to a paradoxical hyperphosphorylation of the regulatory tyrosines. Lysates were applied to immunoblotting and all clones retained whole TYK2 protein while some were phosphorylation deficient. Clones 19 and 43 are highlighted, showing the same deletion as illustrated in A. **C, D** 32D^{MPL} CALRdel52 cells were electroporated with 4 guides and nickase and single cells were grown in single cell dilution directly after electroporation (C) and two weeks later (D). Resulting clones were analyzed for TYK2 gene sequence via PCR and gel electrophoresis. No clone with deletion of TYK2 has been stabilized. **E** Resulting clones were treated with IFNα and ruxolitinib to induce phosphorylated TYK2, lysates were prepared and applied to immunoblotting. pTYK2 was retained in all clones.

Supplementary Figure S3

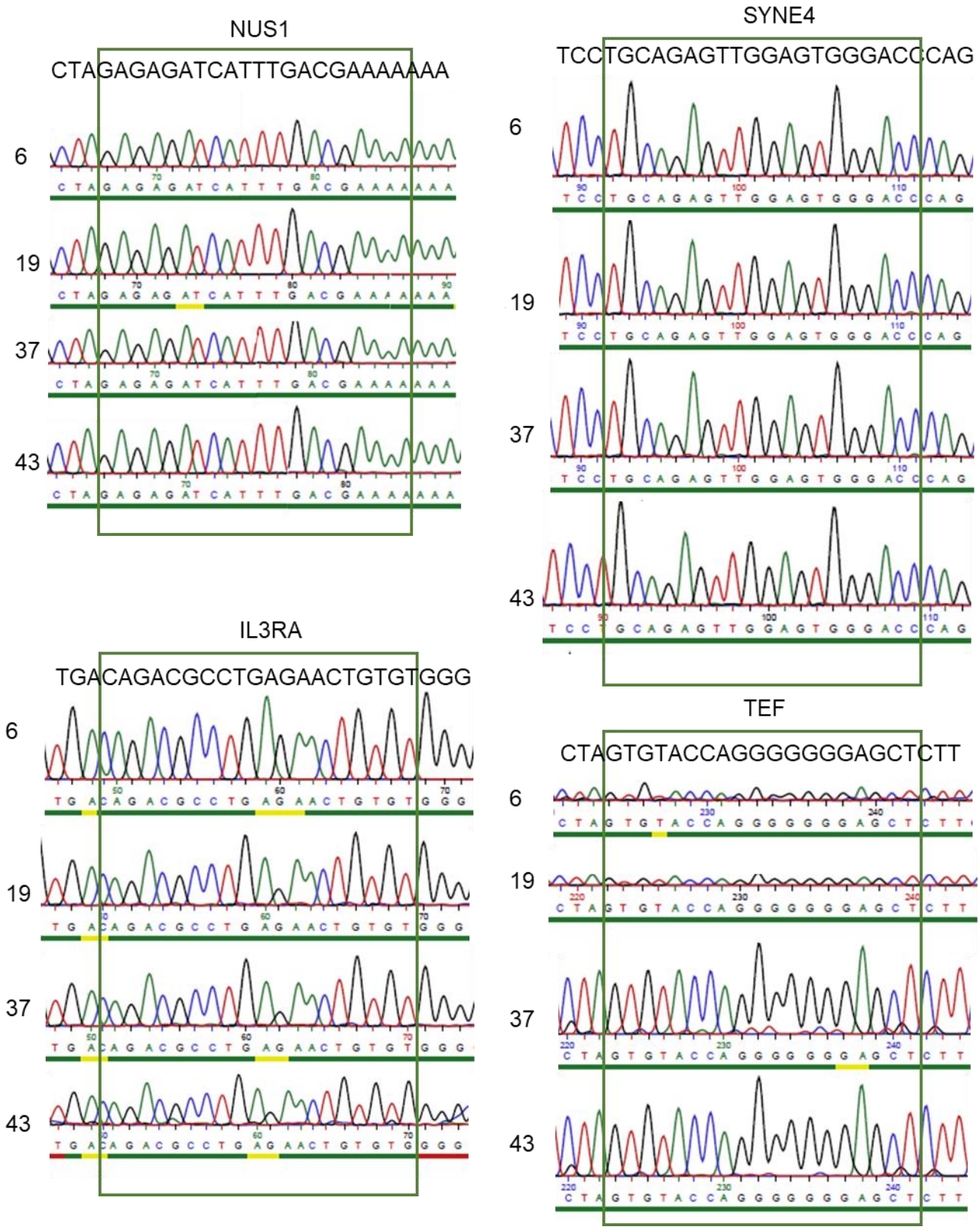
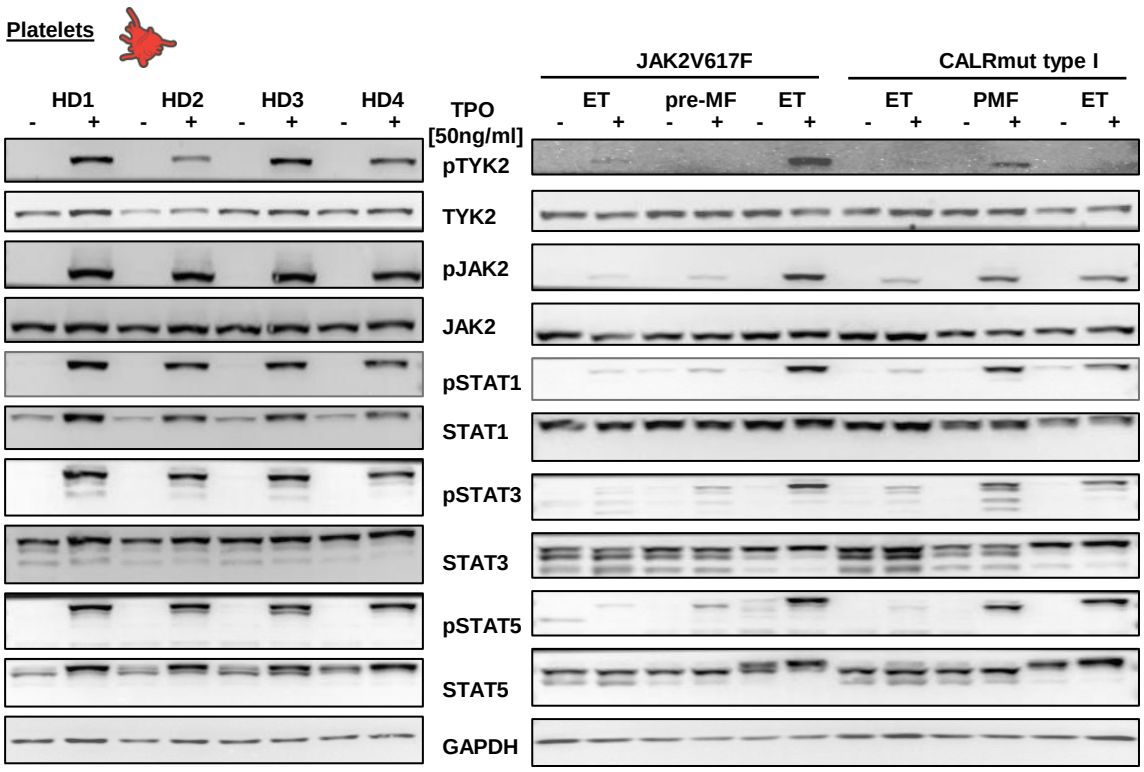


Figure S3: Off-target screening in CRISPR clones. *NUS1*, *SYNE4, IL3RA* and *TEF* were identified as possible off-targets for the CRISPR guides. Regions of interest were amplified from genomic DNA via PCR, the resulting gene fragment was purified and analyzed via Sanger sequencing. Results of the sequencing are shown for each clone, none of the clones showed alterations in their gene sequence

Supplementary Figure S4

A



B

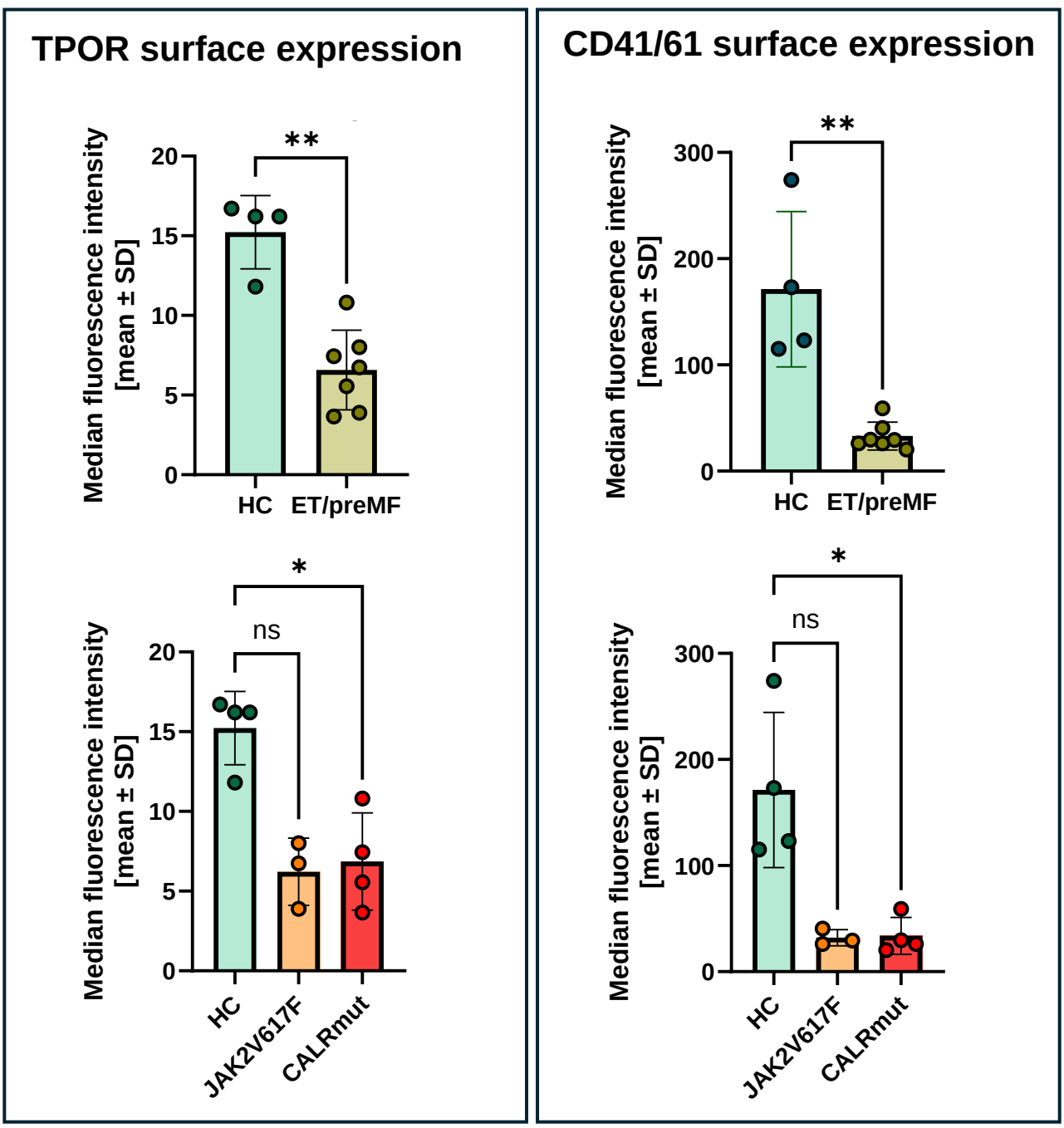


Figure S4: Analysis of primary platelets from healthy donors (HD) or ET and preMF patients. A Platelets from HD and MPN patients were isolated from peripheral blood (Supplementary Table S1) and stimulated with 50 ng/ml human TPO for 1 h. Lysates of the stimulated and unstimulated samples were applied to immunoblotting. **B** TPOR (left) and CD41/61 (right) surface levels were assessed on HD vs ET/preMF patients using flow cytometry. Statistical analysis was done using unpaired t-test. * p<0.05; ** p<0.01

Supplementary Figure S5

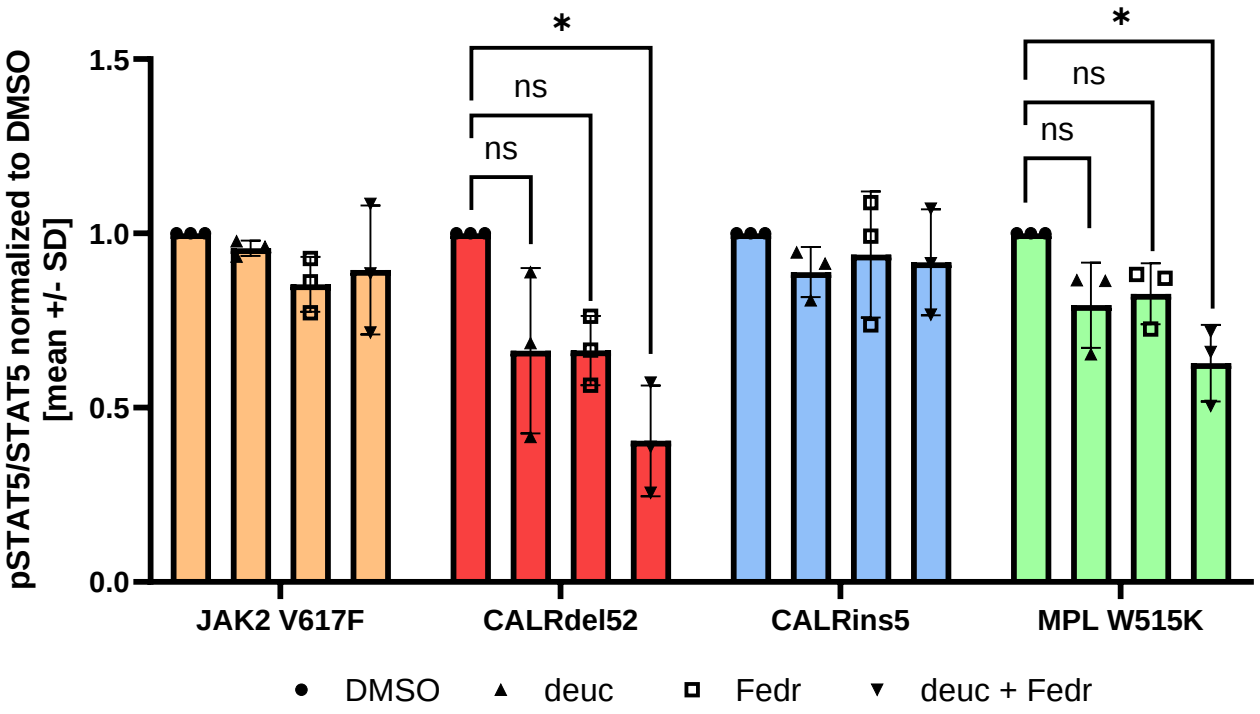


Figure S5: Significant downregulation of downstream signaling in combination treatment of deucravacitinib and fedratinib. 32D^{MPL} cells carrying a JAK2V617F, CALRdel52, CALRins5 or 32D MPLW515K were treated with 1 µM deuc or 0.5 µM fedratinib or a combination of both for 4 h**,** cells were harvested and their lysates applied to immunoblotting. Densitometry analysis was done using GelAnalyzer (ver. 23.1.1) software and pSTAT5 was compared with whole protein STAT5 and values were normalized to DMSO control. Statistical analysis was done using one-way ANOVA (Kruskal-Wallis test + multiple comparison) ns $p > 0.05$; *$p < 0.05$

Supplementary Figure S6

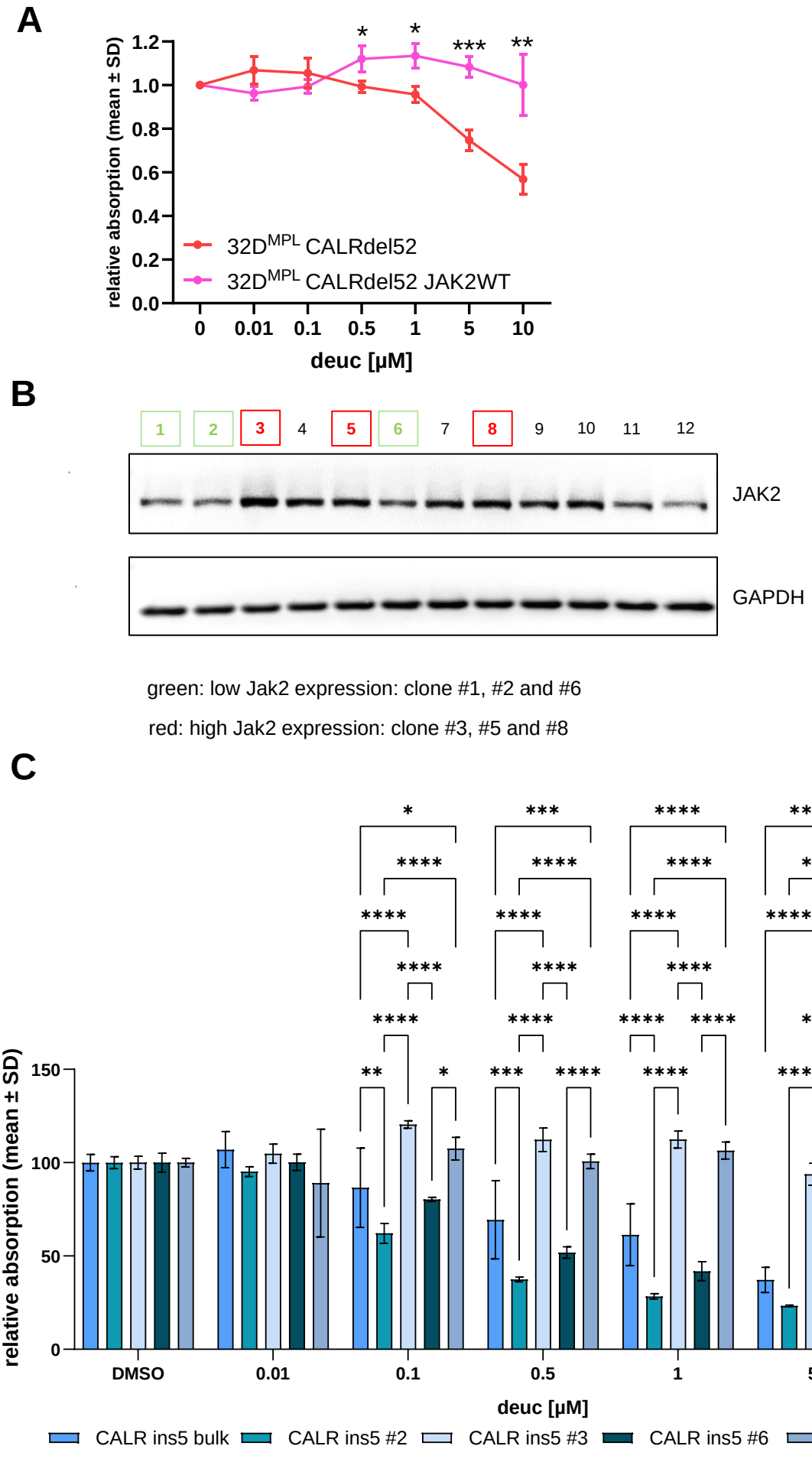


Figure S6: High JAK2 protein levels protect CALR-mutant cells from TYK2 dependence. A 32D^{MPL} CALRdel52 and 32D^{MPL} CALRdel52 JAK2 WT cells were treated with increasing concentrations of deucravacitinib and metabolic activity was assessed after 72 h of cultivation in an MTT assay. Statistical analysis was assessed using unpaired t-test. **B** Single clones of 32D^{MPL} cells were grown after single cell dilution and analyzed for JAK2 protein levels. Differences in basal JAK2 expression were noted, and three clones with high and three with low levels were selected. **C** 32D^{MPL} CALRins5 parental cells (bulk) and clones with low (#3 and #8) and high (#2 and #6) were seeded with increasing concentrations of deucravacitinib. Metabolic activity was measured after 72 h using MTT and statistical analysis was done using two-way ANOVA. * p<0.05; ** p<0.01; *** p<0.001; ****p<0.0001

Supplementary Figure S7

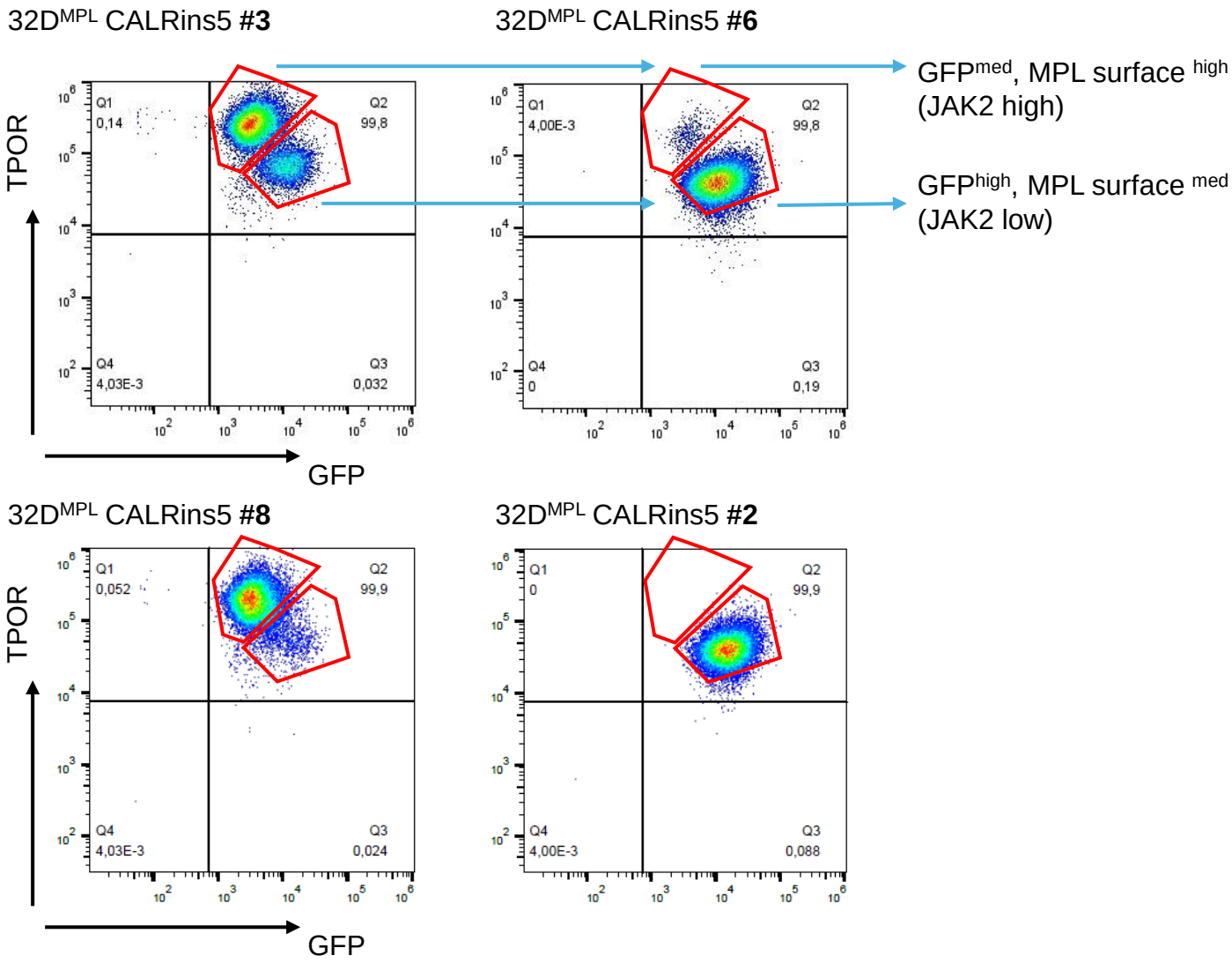


Figure S7: Clonal differences in TPOR surface expression. GFP and TPOR surface levels were assessed in 32D^{MPL} CALRins5 clones via flow cytometry. Two populations with differential ratios were observed: for #3 and #8 (left) , the main population was GFP^{low} (CALRins5^{low}) and TPOR^{high} while the main population in clones #2 and #6 (right) was GFP^{high} (CALRins5^{high}) and TPOR^{med}.

Supplementary Figure S8

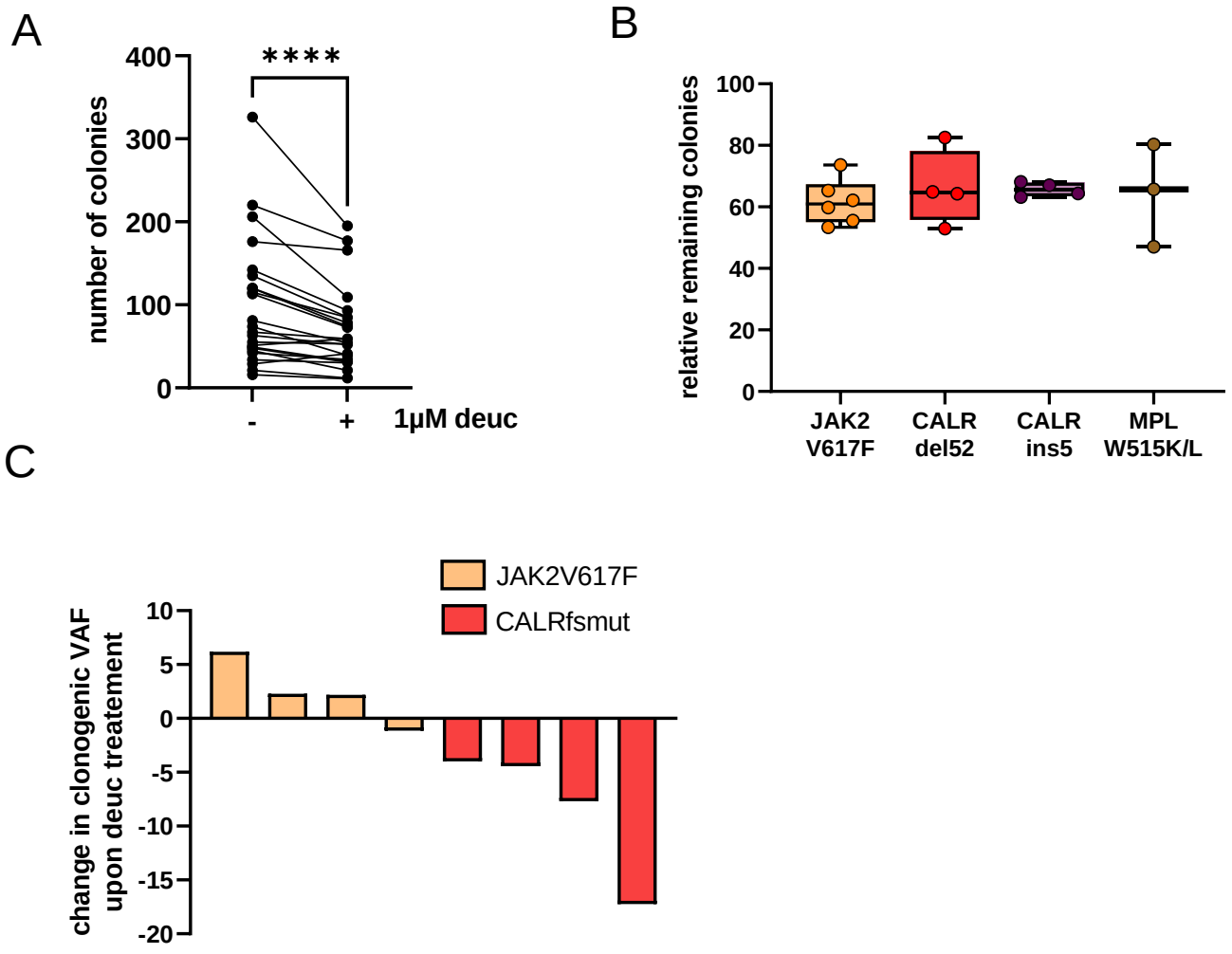


Figure S8: Analysis of primary MPN patient-derived cells. A PBMC were isolated from patients with MPN (n = 24) and seeded in methylcellulose containing 1 µM deucravacitinib (deuc). Colonies were counted after 10-13 days of cultivation. Statistical analysis was done using Wilcoxon matched-pairs signed-rank test. **B** Relative colony numbers after deucravacitinib treatment are shown for ET and PMF patients only. Colony counts were normalized to the respective untreated controls. **C** Clonogenic variant allele frequency (VAF) was assessed by analyzing the fraction of mutated colonies as described before (Kalmer et al., 2022). Clonogenic VAF was compared in untreated and deuc-treated condition. Negative change in clonogenic VAF indicates reduction of mutated colonies upon treatment. ****p <0.0001