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GRK5, a platelet function modulator of thrombin activation, is causally linked to cardiovascular disease and affects PAR4 receptor signaling

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Abstract

Dual antiplatelet therapy reduces ischemic events in cardiovascular disease, but increases bleeding risk. Thrombin receptors PAR1 and PAR4 are drug targets, but the role of thrombin in platelet aggregation remains largely unexplored in large populations. We performed a GWAS of platelet aggregation in response to full-length thrombin, followed by clinical association analyses, Mendelian Randomization, and functional characterization including iPSC-derived megakaryocyte and platelet experiments. We identified a single sentinel variant in the *GRK5* locus (rs10886430-G, $P=3.0x10^{-42}$) associated with increased thrombin-induced platelet aggregation (*ß*=0.70, SE=0.05). We show disruption of platelet *GRK5* expression by rs10886430-G is associated with enhanced platelet reactivity. The proposed mechanism of a GATA1-driven megakaryocyte enhancer is confirmed in allele-specific experiments. Utilizing further data, we demonstrate the allelic effect is highly platelet- and thrombin-specific, and not likely due to effects on thrombin levels. The variant is associated with increased risk of cardiovascular disease outcomes in UK BioBank, most strongly pulmonary embolism. The variant associates with increased risk of stroke in the MEGASTROKE, UK BioBank and FinnGen studies. Mendelian Randomization analyses in independent samples support a causal role for rs10886430-G increasing risk for stroke, pulmonary embolism, and VTE through its effect on thrombin-induced platelet reactivity. We demonstrate that GRK5 promotes platelet activation specifically via PAR4 receptor signaling. GRK5 inhibitors in development for heart failure and cancer could have platelet off-target deleterious effects. Common variants in *GRK5* may modify clinical outcomes with PAR4 inhibitors, and upregulation of GRK5 activity or signaling in platelets may have therapeutic benefits.

Introduction

Activated platelets provide the link between inflammation, thrombosis, and atherosclerotic cardiovascular disease [1]. Platelet reactivity is highly heritable [2; 3], but the limited number of previously identified loci explain only a small portion of the estimated heritability [4]. Despite thrombin being the principal enzyme of hemostasis and viewed as the strongest platelet agonist [5], the genetics of thrombin-induced platelet reactivity is not well understood and heretofore has not been investigated on a genome-wide scale. Many of thrombin's cellular effects are initiated by protease-activated receptors (PARs) which are G protein-coupled receptors (GPCRs) [6]. PAR1 (MIM: 187930) and PAR4 (MIM: 602779) are the receptors primarily responsible for mediating the effects of thrombin in human platelets [5].

Dual antiplatelet therapy (DAPT) reduces the occurrence of both stent-related and spontaneous myocardial infarction (MI) after acute coronary syndrome (ACS), but with concomitant increase in bleeding risk [7]. Thus, there is a need for milder DAPT targets in order to maintain or increase efficacy while reducing bleeding, given the narrow therapeutic window of most antiplatelet treatments[8]. Development of more effective strategies could potentially expand anti-platelet therapy into primary prevention where it generally is not recommended due to bleeding risks. Given their role in platelet biology PAR1 and PAR4 have both been the focus of antithrombotic drug development. Targeting PAR1, the high affinity thrombin receptor, led to vorapaxar, approved for preventing thrombotic events in patients with MI when used in combination with standard-of-care DAPT [9]. Of limited clinical utility vorapaxar is associated with increased risk of major bleeding events [9] where the mortality risk due to bleeding can be comparable or greater than that due to MI [10]. Compared to PAR1 there is evidence that targeting PAR4 is associated with a lower bleeding risk and can achieve an effective antithrombotic profile, though large trials of PAR4 inhibition are still lacking [11-13].

While prospective studies have demonstrated the association of platelet function with cardiovascular disease (CVD) events in patients with established cardiovascular disease, there is less existing evidence that

platelet function predicts CVD or CVD outcomes in the healthy population [14]. Clinical trials have demonstrated a relationship between high on-treatment platelet reactivity and adverse clinical ischemic events, but tailoring therapy based on platelet reactivity remains uncertain[15]. Platelet function traits remain relatively unexplored in large populations, in particular for thrombin and PAR1/PAR4 platelet activation. In order to address this broad knowledge gap—to discover genes that may mediate CVD or bleeding risk, classify treatment subpopulations or suggest new therapeutic targets, we performed the first genome-wide association study (GWAS) of thrombin-induced platelet aggregation.

Subjects and Methods

PARTICIPANTS AND GENOMEWIDE ANALYSES

We conducted and present analyses from the following methodological approaches: 1) a GWAS of platelet thrombin activation, 2) platelet and other cell and tissue eQTL analyses, including Mendelian Randomization (MR), for our lead locus, and signal co-localization analyses, 3) two sample MR for cardiovascular disease outcomes from multiple Consortia and biobanks, 4) integration of megakaryocyte and other epigenetic data at our lead locus, 5) site-directed mutagenesis and regulatory enhancer assays in 3 cell backgrounds, and 6) iPSC-derived megakaryocyte, and platelet, siRNA and chemical inhibitor experiments to dissect functional effects on platelets. The samples and resources utilized are described in **Table S1**. The demographic characteristics of the GWAS study sample are shown in **Table S2**.

The Caerphilly Prospective Study assessed platelet aggregation induced by full-length thrombin (0.056 units/mL, Sigma Aldrich) in middle aged males using light transmission aggregometry (LTA) [16]. All participants provided written informed consent. Genotyping of 1248 samples was performed with the Affymetrix UK BioBank Axiom array. Following sample and genotyping quality control, imputation was done on 1184 samples

using the Haplotype Reference Consortium (HRC) 1.1 panel. We conducted a GWAS using a linear mixed model adjusting for age and medication usage. A significance threshold of $P < 7 \times 10^{-9}$ was adopted to account for all variants tested. Conditional analyses adjusting for the strongest peak SNP in *GRK5* (MIM: 600870), rs10886430, were conducted by adding SNP dosage as a covariate to the base model. Our methods for platelet aggregation, genotyping, quality control procedures, imputation, and GWAS are further described in the Supplementary Appendix.

CAUSAL ANALYSIS AND MULTI-TRAIT COLOCALIZATION FOR GRK5 LOCUS

Testing for causal association with expression quantitative trait loci (eQTL) was conducted with the use of the platelet RNA and expression 1 (PRAX1) platelet eQTL dataset [17]. We investigated whether thrombin-induced aggregation and platelet cell traits share a common association signal at the *GRK5* locus by performing a co-localization analysis with quantitative traits derived from multiple blood cell lineages: platelets (mean platelet volume, MPV; platelet count, PLT; platelet distribution width, PDW), red blood cell count (RBC), and white blood cell count (WBC) [18]. Furthermore, we conducted similar genetic colocalization analyses for the *GRK5* locus using genome-wide study data for thrombin generation potential [19], platelet aggregation to ADP, collagen and epinephrine [4; 20], and eQTLs from 44 cells and tissues from the GTeX Project version 7, five white blood cell types and platelets from the CEDAR project [21], and aortic endothelial cells [22]. Two sample Mendelian Randomization (MR) analysis was conducted using the rs10886430-G allele as the genetic instrument and thrombin-induced platelet aggregation as exposure in separate analyses for nine pulmonary, stroke, or heart disease outcomes from the UK BioBank [23], four stroke outcomes from the MEGASTROKE consortium [24], and cardiovascular disease codes in the FinnGen Biobank (version 4)[25]. Only this SNP was used in MR analyses because in conditional analyses of chromosome 10 this was the only independently significant

SNP associated with thrombin reactivity or platelet *GRK5* expression levels. Details regarding these analyses are given in the Supplementary Appendix.

REGULATORY FUNCTION

We integrated epigenetic regulatory maps of chromatin accessibility, enhancer RNA (eRNA), histone marks, enhancer elements, and DNA-binding proteins assessed in megakaryocyte-erythroid lineage models (ENCODE)[26; 27] and cultured primary megakaryocytes (BLUEPRINT) to annotate potential functional impacts of the intronic *GRK5* rs10886430 variant [28; 29]. Protein network analysis of transcriptional regulators that bind the rs10886430 variant was performed with STRING 10.5. We used site-directed mutagenesis to investigate the impact of the rs10886430 variant on enhancer activity *in vitro* in *GATA1*- (MIM: 305371) and *GATA2*- (MIM: 137295) overexpressing HEK293 cells, as well as HUVEC and K562 cells. Details regarding the data integration, network analysis, enhancer reporter luciferase assays and conditional overexpression of *GATA1* and *GATA2* are provided in the Supplementary Appendix.

PLATELET FUNCTION siRNA AND INHIBITOR EXPERIMENTS

The role of GRK5 in platelet function was assessed by siRNA as well as pharmacologic inhibition of GRK activity. Immortalized megakaryocyte progenitor cell lines (imMKCLs) were generated from human induced pluripotent stem cells and maintained as previously described [30]. For siRNA experiments imMKCLs were transfected with *GRK5* or control eGFP siRNA for 48 h, then analyzed by qRT-PCR and *in vitro* flow cytometric analysis of platelet function(via P-selectin and PAC-1) following stimulation with either 20 μM ADP plus 20 μM thrombin receptor-activating peptide 6 (TRAP-6 that activates platelets via PAR1) or control. Pharmacologic inhibition of GRK activity was investigated with the pan-GRK inhibitor CCG215022 (MedChemExpress) which

exhibits nanomolar IC_{50} values against both GRK2 as well as GRK5 and selectivity against PKA[31]. Platelet-rich plasma (PRP) samples obtained from healthy donors (n=3) were treated with 0.78μM CCG215022 or DMSO vehicle for 45 min prior to stimulation with increasing concentrations of one of three platelet agonists: PAR4 Activating Peptide (PAR4-AP, 1, 20, 50 μM), TRAP-6 (1, 10, 20 μM), ADP (1, 10, 20 μM) or vehicle control followed by flow cytometric analysis of platelet function (via P-selectin and PAC-1). Details regarding the siRNA experiments in imMKCL, GRK inhibition in PRP samples, and platelet function assessment by flow cytometry are provided in the Supplementary Appendix.

Results

We performed a GWAS of thrombin-induced platelet aggregation in the Caerphilly Prospective Study including >7.75 million common and low-frequency (minor allele frequency (MAF) > 0.01) SNPs imputed via the HRC panel. There was no evidence for inflation of test statistics (λ = 1.005) (**Figure S1A**). We observed 17 variants that surpassed the genome-wide significance threshold ($P < 7 \times 10^{-9}$) for association with thrombin-induced aggregation, all localized to 10q26.11 (**Figure 1A**). Conditional analysis identified no additional signals independent of the sentinel variant located in the first intron of the *GRK5* locus (*GRK5*, rs10886430, P = 3.0 x 10^{-42}), ~ 43-kb downstream from the transcription start site (TSS) within consensus intron 1 (**Figure S1B**, **Table S3**). The minor G allele (MAF 0.136) of the *GRK5* SNP was associated with increased platelet reactivity to thrombin (β = 0.70, SE = 0.05; with other covariates fixed this is ~3.9% per allele increase in thrombin reactivity) (**Figure 1B**). Variance component analysis indicates this single variant explained 18.3% of variation in the thrombin phenotype. There was no significant population structure in Caerphilly, being overwhelmingly European ancestry based on principal components clustering with multi-ethnic samples in the UK BioBank (**Figure S2**). Likewise, we found little relatedness in Caerphilly with only n=303 having any 1st, 2nd or 3rd degree

relations. Inclusion of the genetic relatedness matrix in the GWAS accounted for this. Nonetheless, we conducted a sensitivity analysis removing the n=303 individuals and analyzing chromosome 10 and rs10886430 remained highly significant ($P = 2.01 \times 10^{-31}$), indicating the results are population associations rather than strong family effects.

We next asked whether thrombin-induced aggregation and platelet, red and white cell count traits share a common association signal at the *GRK5* locus. We performed a Bayesian test for co-localization between the Caerphilly thrombin GWAS and multiple blood cell lineage traits from the UK BioBank/INTERVAL study meta-analysis [18], interrogating shared variants in an ~1.8 Mb independent linkage disequilibrium block containing the *GRK5* lead SNP. We observed strong evidence for co-localization (posterior probability > 0.99) between thrombin reactivity and platelet cell traits (MPV, PLT, PDW) but not WBC or RBC, supporting the hypothesis a single variant affects these traits in platelets (**Figure 2A**). The *GRK5* lead SNP (rs10886430) was identified as the shared, potentially causal variant in each case (**Table S4**). With the association limited to platelets, we next asked whether the rs10886430 variant affects platelet reactivity mediated by other agonists. We conducted GWAS on aggregation to ADP and Collagen in Caerphilly participants and performed colocalization analyses with the thrombin GWAS. No evidence of colocalization was observed between thrombin reactivity and either agonist (**Table S5**). Further, no colocalization was observed with aggregation to ADP, Collagen, or Epinephrine in the largest such GWAS published to date in independent cohorts [4] (**Table S6**). We next asked whether the effect on thrombin reactivity was limited to platelet activation or was also observed in thrombin generation potential traits which could indicate an effect mediated via thrombin levels. No colocalization was observed between platelet reactivity to thrombin and three phenotypic markers of thrombin generation [19] (**Table S7**).

To determine potential regulatory impacts of the rs10886430 variant, we first examined its association with functional expression changes cataloged in the platelet-specific PRAX1 study eQTL dataset [17; 32]. We utilized summary data-based Mendelian Randomization (SMR) analysis with the PRAX1 eQTL dataset to test for association between platelet gene expression and platelet reactivity to thrombin. We identified *GRK5* as the only gene at a genome-wide significance level ($ß_{SMR}$ = -1.54, SE_{SMR} = 0.20, P_{SMR} = 3.67 x 10^{-14}) (**Figure 2B**, **Table S8**). Finding no evidence to suggest the SMR association could be due to genetic linkage (P_{HEIDI} = 0.16), we concluded expression of *GRK5* is associated with platelet reactivity driven by the rs10886430 variant. As predicted by our SMR analysis, the minor rs10886430-G allele is a strong *cis*-eQTL for decreased platelet *GRK5* expression ($ß_{eQTL}$ = -0.456, P_{eQTL} = 8.27 x 10^{-20}) in the PRAX1 study [17]. We further replicated this strong *GRK5* eQTL in the independent CEDAR platelet dataset [21] ($ß_{eQTL}$ = -0.429, P_{eQTL} = 1.11 x 10^{-20}). To investigate the tissue specificity of the genetic effect of rs10886430-G on *GRK5* expression we conducted co-localization analyses between the Caerphilly thrombin GWAS in the *GRK5* locus and 51 tissue or cell types. We observed no evidence of co-localization between thrombin reactivity and *GRK5* expression among 44 tissues profiled by the Genotype-Tissue Expression (GTEx) Project (**Table S9**), five other white blood cell types [21] (**Table S10**), or vascular endothelial cells [22] (**Table S11**).

Having observed the strong association of rs10886430-G with lower *GRK5* expression exclusively in platelets, we asked whether the genetic effect of the variant on platelet reactivity was further associated with relevant cardiovascular or cerebrovascular disease pathology. We utilized GWAS statistics for nine pulmonary, stroke, or heart disease diagnoses in the UK BioBank cohort. There was evidence for an association of rs10886430-G with several diagnoses, most strongly pulmonary embolism (OR = 1.25, SE = 1.03, P = 8.1 x 10^{-13}) (**Table S12**). We performed 2-sample MR to test for a causal relationship between thrombin reactivity (exposure) and

disease outcome for the nine UK BioBank diagnoses. We observed strong, positive association between thrombin-induced platelet reactivity at the *GRK5* locus and multiple disease outcomes, most significantly with diagnosis of pulmonary embolism (OR_{MR} = 1.38 [1.26 – 1.52], P_{MR} = 2.40 x 10^{-11}), also with diagnoses of cerebral infarction (OR_{MR} = 1.19 [1.08 – 1.32], P_{MR} = 7.43 x 10^{-4}) and acute myocardial infarction (OR_{MR} = 1.14 [1.07 – 1.21], P_{MR} = 6.67 x 10^{-5}), but not heart failure (OR_{MR} = 1.04 [0.96 – 1.12], P_{MR} = 0.39) (**Figure 2C**, **Table S12**). Next we investigated the rs10886430-G variant in stroke subtypes from MEGASTROKE [24], the cohorts of which are independent of UK BioBank. There was evidence for a stronger association in cardioembolic stroke (OR = 18.42, SE = 2.96, P = 6.16 x 10^{-4}) than ischemic stroke (OR = 1.61, SE = 1.15, P = 2.29 x 10^{-4}), and no association for large artery stroke (OR = 4.32, SE = 5.09, P = 0.39) (**Table S13**). In MR analyses we observed strong, positive association between thrombin-induced platelet reactivity at the rs10886430-G variant and cardioembolic stroke (OR_{MR} = 64.36 [3.09 – 1340.44], P_{MR} = 7.18 x 10^{-3}), all stroke (OR_{MR} = 1.79 [1.19 – 1.27], P_{MR} = 7.69 x 10^{-4}), and ischemic stroke (OR_{MR} = 1.97 [1.22 – 1.33], P_{MR} = 7.17 x 10^{-4}), but not large artery stroke (OR_{MR} = 10.24 [0.08 – 773.88], P_{MR} = 0.37) (**Table S13**). Finally, the G allele was again associated with increased risk of multiple cardiovascular disease outcomes in the FinnGen study: DVT of lower extremities (OR_{MR} = 1.25 [1.11– 1.41], P_{MR} = 2.28 x 10^{-4}), ischemic stroke (OR_{MR} = 1.15 [1.06 – 1.25], P_{MR} = 5.75 x 10^{-4}), portal vein thrombosis (OR_{MR} = 2.51 [1.30-4.85], P_{MR} = 6.31 x 10^{-3}), right bundle branch block (OR_{MR} = 1.68 [1.14 – 2.48], P_{MR} = 8.51 x 10^{-3}), myocardial infarction (OR_{MR} = 1.12 [1.03 – 1.22], P_{MR} = 1.09 x 10^{-2}), atrioventricular block (OR_{MR} = 1.24 [1.04– 1.47], P_{MR} = 1.64 x 10^{-2}), and reduced risk for hypertension (OR_{MR} = 0.94 [0.89 – 0.98], P_{MR} = 7.49 x 10^{-3}) and cardiomyopathies (OR_{MR} = 0.79 [0.68 – 0.91], P_{MR} = 1.85 x 10^{-3}) (**Table S14**).

A GRK5 gain of function coding variant (p.Gln41Leu) leading to enhanced β-adrenergic receptor (βAR) desensitization of excessive catecholamine signaling has been proposed to provide a 'genetic β-blockade' that

improves survival in African Americans with heart failure [33]. Functional studies have also shown β2-ARs can inhibit platelet aggregation and adhesion [34]. In our study, this variant was not associated with thrombin reactivity (P = 0.51, data not shown). Having observed associations with multiple pathologic cardiopulmonary traits, we asked whether the *GRK5* variant's effect on platelet reactivity was modified by pharmacological β-blockade. A small subset of Caerphilly participants were taking cardiac-specific, β1-AR selective (5.1%) or non-selective drugs (3.1%) at the time platelet reactivity was measured. Fitting linear models with specific and non-specific beta-blockers as additional covariates, we observed a negative interaction effect on platelet reactivity between the *GRK5* variant and β1-AR selective drugs (β = -0.60, SE = 0.24, P = 0.01) (**Figure S3**).

To study the regulatory function of the *GRK5* variant we next integrated cell type-specific epigenome maps derived from primary megakaryocyte cells [28; 29]. The variant localizes to a region of open chromatin (DNAse hypersensitivity peak site) in a predicted enhancer region characterized by broad enrichment of active marks H3K27Ac and H3K4me1 (**Figure 3A**). Active enhancers are often characterized by short, unstable bi-directional transcripts termed enhancer RNAs (eRNAs). Integrating nascent transcription maps in K562 cells we observed the *GRK5* variant localizes to the predicted TSS region of a eRNA [27] (**Figure S4**). Having observed multiple lines of evidence the variant is in an enhancer element, we asked whether the position was occupied by transcriptional regulators *in vivo*, as this could provide a clear testable mechanism of action in a non-coding DNA context. To this end, we scanned transcription factor (TF) binding datasets from mega-erythroid cell models generated by the ENCODE consortium [26]. The variant position was bound by 27 factors, including the master hematopoietic transcription factor GATA1 in peripheral blood erythroblasts and the histone acetyltransferase p300 (MIM: 602700) in K562 cells (**Table S15**).

We verified binding of the *GRK5* variant by GATA1 is also observed in primary MK cells [35] and hematopoietic stem/progenitor-derived erythroid precursors [36]. The GATA1 binding motif is highly enriched at active eRNA in K562 cells [37]. We next considered whether there was functional connectivity or association between the factors binding the *GRK5* variant locus. Using the STRING algorithm we constructed a highly connected network model incorporating 24 of 26 mappable transcriptional regulators (Protein-Protein interaction enrichment p-value: $< 1.0 \times 10^{-16}$) (**Figure 3B**). Clustering of the network revealed several properties: SWI/SNF chromatin remodeling complex clustering with GATA1 (red cluster), transcriptional repressors (including one cluster of polycomb-related proteins) (green cluster), cAMP responsive factors (blue cluster), p300 and lineage factor IKZF1 (MIM: 603023) (khaki cluster), and NF-E2 (MIM: 601490) complex (light green cluster). These included p45 and MAFK (MIM: 600197), subunits of the heterodimeric NF-E2 complex required for megakaryocyte maturation and platelet production *in vivo* [38]. Collectively, these results suggest the *GRK5* non-coding variant affects platelet reactivity by modulating a functional megakaryocyte lineage enhancer, leading to platelet populations with altered *GRK5* expression and function.

We confirmed the locus drives enhancer activity *in vitro* by luciferase reporter assay, through binding of GATA1 and not through binding of GATA2 (**Figure S5A-C**). Enhancer activity in endogenous GATA1-expressing cells is largely abolished upon deletion of the GATA1 core binding motif ($GATA1^{del}$ **Figure S5B**). Given its close proximity to the GATA1 motif, we hypothesized the rs10886430-G could reduce the regulatory potential of the enhancer by interfering with binding of GATA1. Thus, we investigated the impact of the GRK5 variant on enhancer activity, both by targeted four-base deletion as well as single-base substitution of the minor "G" allele (**Figure 4**). In K562 cells introduction of the GRK5 variant effect allele ($AtoG^{mut}$) repressed enhancer activity 1.5-fold ($P < 0.001$). Targeted deletion of the base position ($AGTG^{del}$) produced nearly identical results.

Endothelial HUVEC cells, which express lower levels of *GATA1* (confirmed by qRT-PCR, data not shown), exhibited weak enhancer activity and a diminished capacity for the GRK5 variant effect allele to repress enhancer activity (1.3-fold, $P < 0.05$) (**Figure 4**).

Having established a potential mechanism by which the DNA variant regulates *GRK5* expression, we next investigated the role of GRK5 in platelet physiology. To this end we utilized iPSC-derived immortalized MK cell lines (imMKCLs) which produce functional platelets expressing cell markers CD42b (MIM: 606672)and VWF (MIM: 613160) [30]. We first assessed the impact of reducing *GRK5* expression in imMKCL by siRNA, achieving a knockdown efficiency of ~75% (**Figure S6A**). We performed platelet function testing of GRK5-depleted and control platelet progeny cells stimulated with agonists ADP/TRAP-6 by flow cytometry, measuring platelet surface activated GPIIb-IIIa (PAC1 antibody) and platelet surface P-Selectin (CD62P). Transient knockdown of GRK5 increased markedly the percentages of platelets positive for P-Selectin 1.6-fold ($P < 0.05$) and activated GPIIb-IIIa 1.2-fold ($P < 0.05$) (**Figure 5A**). The amounts of P-Selectin and activated GPIIb-IIIa exposed on the surface of each platelet, as judged by the geometric mean fluorescence intensity (MFI), were largely unchanged (**Figure 5A**).

We next investigated the effect of repressing GRK activity on platelet function in the specific context of PAR1- or PAR4-activated signaling *ex vivo* via CCG215022, a pan-specific small molecule inhibitor exhibiting nanomolar IC_{50} values against both GRK2 (MIM: 109635) and GRK5. To this end, we performed platelet function testing on treated and control PRP stimulated with either TRAP-6 (PAR1 activator) or PAR4-AP by flow cytometry. Treatment with the GRK inhibitor increased the percentages of platelets positive for P-Selectin up to 2.3-fold in the presence of PAR4-AP but not TRAP-6 (**Figure S6B**). Given we observed activation in siRNA experiments upon ADP/TRAP-6 co-stimulation, the absence of an effect by PAR1 signaling alone in healthy

donor PRP treated with GRK inhibitor led us to consider whether stimulation with ADP alone would have an effect. We performed additional platelet function testing with a range of concentrations of TRAP-6, PAR4-AP, or ADP following GRK inhibition (**Figure 5B, Figure S7**). Significant increases in P-Selectin positive cells were again observed for PAR4-AP (20uM: 2.7-fold, $P < 0.01$), to a lesser extent ADP (1 uM: 1.2-fold, $P < 0.05$), but not TRAP-6 (**Figure 5B, Figure S7**). Since TRAP-6 activation may have been saturated at doses between 1 uM to 20 uM, we ran independent experiments at lower doses of TRAP-6 in donor PRP (0.001 uM, 0.01 uM, 0.10 uM) and again found no effect of GRK inhibition on platelet activation (**Figure S8**). Together, these results suggest that inhibition of platelet GRK5 promotes PAR4-mediated platelet activation and to a lesser extent ADP-mediated activation, but not PAR1-mediated platelet activation.

Discussion

In a GWAS of thrombin-induced platelet aggregation, we identify a *GRK5* non-coding variant (rs10886430-G) strongly associated with increased reactivity to thrombin. We observed that thrombin-induced aggregation shares a common association signal at the *GRK5* locus with three platelet cell indices (MPV, PLT, and PDW), the rs10886430 SNP being the shared causal variant in each case. Interestingly, the SNP had previously been identified as a sentinel variant positively associated with two indices: MPV and PDW [18] which are suggested by some as partial surrogates for platelet activation. Our study suggests a direct role for GRK5 in platelet activation, with an overall mechanism outlined in **Figure 6**.

To determine the regulatory potential of the rs10886430 variant we first applied SMR to test for association between platelet gene expression and reactivity to thrombin. The analysis supported a model

whereby decreased *GRK5* expression is associated with increased platelet reactivity through disruption by the rs10886430-G variant. The strong rs10886430-G eQTL for *GRK5* replicated in two independent platelet datasets [17; 21] but none of the other 44 tissues in GTEx, 5 white blood cell subtypes or aortic endothelial cells indicating significant platelet specificity. Utilizing megakaryocyte and erythroid epigenetic datasets we found evidence for an active cell lineage enhancer at the SNP site bound by master hematopoietic transcription factor GATA1. GATA1 plays a critical role in megakaryocyte maturation and platelet formation *in vivo* [39]. We supported the disruption of *GRK5* expression by multiple experiments. We further investigated the role of GRK5 in platelet function using an iPSC-derived megakaryocyte and platelet production model. Knockdown of GRK5 by siRNA increased functional markers of platelet activation in agonist stimulated cells, including α-granule release (surface P-Selectin) and inside-out activation of the platelet integrin complex GPIIb-IIIa. These results indicate GRK5 plays an important functional role in negatively regulating thrombin-induced platelet reactivity.

Thrombin is the most potent endogenous platelet activator and plays an important role in clot promotion and inhibition, and cell signaling, as well as additional processes that influence fibrinolysis and inflammation. The role of GRK5 could be mediated through several mechanisms. First, through canonical GRK GPCR desensitization of signal initiators PAR1 or PAR4 [6]. Second, GPCR desensitization of numerous downstream effectors of PAR signaling such as β2-AR (MIM: 107941) [34; 40], Akt (MIM: 164730), or the SRC family kinases [41]. To further determine whether GRK5 affected PAR1 or PAR4 signaling, we conducted experiments that showed GRK5 inhibitory effects are mediated via inhibition of PAR4 driven platelet activation.

PAR4 is involved in sustained platelet activation, and invokes sustained intracellular calcium response in platelets, phosphyltidylserine exposure, thrombin generation and fibrin deposition [13]. Thus, PAR4 has been

suggested as a novel anti-platelet therapeutic target, with primate models and other studies indicating PAR4 inhibition could provide superior inhibition with reduced bleeding diatheses [11; 12]. Our results suggest that a significant genetically determinable fraction of the population could potentially receive greater benefit from PAR4 inhibition. Interestingly, a missense variant p.Ala120Thr in PAR4 has been described as affecting activation [42]. However, this variant was not associated with full thrombin reactivity in our study (P=0.07, data not shown) and responses to a monoclonal antibody directed at PAR4 did not vary by genotype in another study [43]. Taken together, this suggest that *GRK5* variant rs10886430 may have stronger implications for thrombin-based platelet activation than the previously reported *PAR4* coding variant. Our study does have some limitations. Given the modest sample size false negatives are expected. Since there are no other reported thrombin, PAR1 or PAR4 platelet reactivity GWAS we cannot yet assess replication of those findings.

Given the importance of thrombin in clot formation we used 2-sample MR to determine whether the genetic effect of the variant on platelet reactivity may play a putatively causal role in cardiovascular or cerebrovascular disease pathology. Whereas the role of platelets in arterial thrombosis is well established, our MR analysis utilizing the strong GRK5 instrumental variable also suggest thrombin-driven platelet reactivity contributes to the trajectory of venous thromboembolism (VTE), both in deep venous thrombosis (DVT) as well as pulmonary embolism (PE). Recently, the variant was independently tied to increased VTE risk in the INVENT Consortium and replicated in the Million Veteran's Project [44]. Critical for DVT propagation *in vivo*, platelets are recruited to developing venous thrombi where they support leukocyte accumulation and promote formation of procoagulant neutrophil extracellular traps [45]. Markers of platelet activation are elevated in acute PE, correlate with the severity of right ventricular dysfunction, and can persist for several months [46]. The contributing role of platelets in VTE is further supported by the observation that aspirin therapy reduces the risk of DVT and PE in patients undergoing orthopedic surgery [47]. Thrombosis and excess

platelet activation are common pathological features of pulmonary arterial hypertension [48], another cause of pulmonary heart disease. While the etiologic heterogeneity characterizing ischemic stroke make it difficult to assign a causative role for platelet reactivity to any given subtype, the platelet content of embolized thrombi is twice that of in situ thrombi [49], suggesting the platelet aggregate increases propensity for embolization. The importance of platelets in pathogenesis of acute MI is supported by both clinical and *in vivo* animal studies which show the initial thrombus (following endothelial injury) is primarily composed of activated platelets [50].

In our investigation of the rs10886430-G variant among stroke subtypes available in MEGASTROKE we observed a markedly strong effect in prevalence of cardioembolic stroke, though not large vessel or small vessel disease. Compared to cardioembolic stroke, the large artery atherosclerosis and small vessel occlusion subtypes have vastly different etiologies [24]. MR analysis supported a causal role for thrombin-driven platelet reactivity specifically in the cardioembolic subtype. Causal associations with pulmonary embolism and cardioembolic stroke suggest the rs10886430-G variant is particularly enriched in emboli forming distally to the site of vascular occlusion. More broadly, our results underline the importance of thrombin-driven platelet reactivity in both venous and arterial disease. It remains to be seen whether rs10886430-G is an important variant to segment populations at risk relative to treatment for either venous or arterial disease.

Beta-blockers are a common preventative therapy following MI and a mainstay for the management of heart failure [33]. Functional studies have shown β2-ARs to inhibit platelet aggregation and adhesion through activation of platelet nitric oxide synthase [34]. Given the role of GRK5 in desensitizing β-AR signaling we investigated whether the *GRK5* variant's effect on platelet reactivity was modified by beta-blocker therapy in a subset of Caerphilly participants. We observed a negative interaction effect on platelet reactivity between the *GRK5* variant and β1-AR selective drugs and no effect with non-selective drugs. The absence of an appreciable effect with the latter may in part be explained by low sample sizes in the model (selective n = 56, non-selective

n = 34) as well as differences in chemistry which may affect platelet uptake [51]. Future work could potentially examine the genotypic effect of the *GRK5* variant on multiple drug classes in larger samples including direct thrombin inhibitors, beta-adrenergic blockers, and other anticoagulant and anti-platelet therapies. Over-expression of cardiac *GRK5* leads to early heart failure after pressure overload in mouse models [52]. Also upregulated in human heart failure, GRK5 is being investigated as a therapeutic target with selective small molecule inhibitors under development [53]. Our work highlights the potential for significant platelet-driven off-target effects with this or other strategies seeking to inhibit GRK5. Notably, in platelet RNA-sequencing data *GRK5* is by far the most expressed member of the GRK family. The next highest expressed GRK family members are *GRK6* (MIM: 600869) (~18% expression level of *GRK5*) and *GRK4* (MIM: 137026) (~2% expression level of *GRK5*), suggesting GRK5 is likely to be the critical protein family member active in platelets.[54] Given the role of GRK5 in controlling PAR4-mediated platelet activation and the association of the rs10886430-G genetic effect on platelet reactivity with cardiovascular and cerebrovascular embolic events we suggest that finding a mechanism to maintain GRK5 activity in platelets could prove beneficial in preventing venous and arterial cardiovascular disease.

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Declarations

The authors have no interests to declare.

Data and Code Availability

Caerphilly genotype and phenotypes are available by request to the study steward at University of Bristol: https://www.bristol.ac.uk/population-health-sciences/projects/caerphilly/. Other code and data is either freely available at the websites listed below or in the Supplement or its references, or by request to the corresponding author.

Web Resources

STRING https://string-db.org/

SMR https://cnsgenomics.com/software/smr/#Overview

COLOC https://cran.r-project.org/package=coloc

LD Blocks https://bitbucket.org/nygcresearch/ldetect-data/overview

Gene ATLAS http://geneatlas.roslin.ed.ac.uk/

EMMAX https://genome.sph.umich.edu/wiki/EMMAX

Haplotype Reference Consortium http://www.haplotype-reference-consortium.org/

Michigan Imputation Server https://imputationserver.sph.umich.edu

ENCODE https://www.encodeproject.org/summary/?type=Experiment

BLUEPRINT http://dcc.blueprint-epigenome.eu

GRASP Full GWAS Results (for Astle et al.)

https://grasp.nhlbi.nih.gov/downloads/FullResults/2016/2016_Astle/AstleREADME.txt

OMIM http://omim/org

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Figure Titles and Legends

Figure 1. **Regional and SNP associations of *GRK5* with thrombin-induced platelet aggregation.** A, Locus Zoom plot of the lead SNP (rs10886430) from genome-wide association analysis of thrombin-induced platelet aggregation in Caerphilly Prospective study (n = 1184). The plot depicts the 600-kb window flanking the rs10886430 (purple) variant which is located in the first intron of *GRK5*. B, Box plot of the effect of rs10886430-G variant dosage on thrombin-induced platelet aggregation (0.056 U/mL). Data points are plotted

as red circles. The bold horizontal line represents the median. The upper and lower hinges indicate the 25th and 75th percentiles, respectively. The whiskers extending from the hinges represent the values no further than 1.5 * interquartile range.

Figure 2. ***GRK5* rs10886430-G is linked to platelet traits and causally effects platelet *GRK5* expression and multiple cardiovascular disease outcomes.** The rs10886430 variant regulates platelet cell traits, *GRK5* platelet gene expression, as well as both cardiovascular and cerebrovascular disease pathologies. A, Heatmap of posterior probabilities from Bayesian colocalization analyses of thrombin reactivity and 5 blood cell traits in the 1.8 Mb LD block containing the rs10886430 variant (10q26.11). Shared SNP, probability of one shared SNP associated with both traits; Independent SNPs, probability of two independent SNPs associated with each trait; Trait 2 Only, probability of association with the blood cell trait and not with Thrombin-induced aggregation; Thrombin Only, probability of association with Thrombin-induced aggregation and not with the blood cell trait; No Association, probability of no association with either trait. B, Manhattan plot depicting summary data-based Mendelian Randomization (SMR) analysis of association between platelet gene expression and platelet reactivity to thrombin (0.056 U/mL). C, Mendelian Randomization analysis of thrombin reactivity (rs10886430G instrument) and cardiopulmonary phenotypes in UK BioBank (Outcomes). Plotted are the OR of the causal estimates (circles) and associated 95% confidence intervals (error bars), color of circle indicates -log transformed P-value of estimate. Further description of medical outcomes codes and statistics is found in the Supplement and **Table S12**.

Figure 3. ***GRK5* rs10886430 overlaps an active meta-erythroid lineage enhancer bound by interconnected factors.** The rs10886430 variant resides in a megakaryocyte enhancer element. A, Epigenetic regulatory maps

of primary-derived MK and K562 cells. B, STRING Protein Network Analysis of DNA binding factors recruited to the rs10886430 variant in ENCODE mega-erythroid cell models. Proteins are represented as circles. Colors indicate network cluster membership. Solid lines indicate interactions within a network cluster. Dotted lines indicate interactions between proteins in different clusters. Line colors indicate type of evidence: cyan (known interaction from curated database), magenta (known interaction experimentally determined), yellow-green (text mining), black (co-expression).

Figure 4. **Mutagenesis causing deletion or rs10886430 A to G transition disrupts enhancer activity in multiple cell backgrounds.** The rs10886430 variant modulates megakaryocyte enhancer activity. Top, diagram of GRK5 enhancer constructs: row 1, WT allele; row 2, four-base deletion including allele position; row 3, substitution with 'G' effect allele. Bottom, normalized luciferase activity in mega-erythroid K562 cells and endothelial HUVEC. Results are the mean of 3 independent experiments performed in quadruplicate (n total=12), error bars represent SEM. * $P < 1 \times 10^{-4}$, ** $P < 5 \times 10^{-5}$).

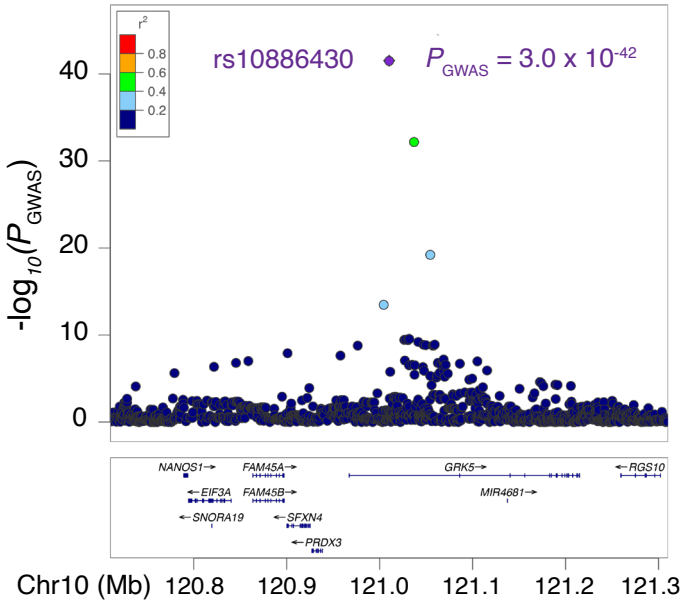
Figure 5. **Disrupting platelet GRK5 via siRNA or chemical inhibitor causes increased thrombin activation via a PAR4 receptor signaling process.** GRK5 perturbation promotes platelet activation. A, Change in platelet activation markers P-Selectin and activated GPIIb-IIIa (PAC1) in response to 20 μM ADP/TRAP6 stimulation (compared to untreated) following siRNA knockdown of *GRK5* expression (or Green Fluorescent Protein (GFP) siRNA negative control) in imMKCL cells. Data represent mean +/- SEM of 4 independent experiments. Differences assessed by Student's T-Test *, $p < 0.05$, **, $p < 0.01$. B, Change in P-Selectin in response to specific activation of either ADP, PAR1 (TRAP-6) or PAR4 (PAR4-AP) signaling (compared to untreated) following pharmacologic inhibition of GRK activity with 0.78 μM CCG215022 (GRKi) or vehicle control (Con) in

PRP samples from healthy donors. Plotted data represent mean +/- SEM (n=3) difference in percentages of marker-positive platelets following treatment with the indicated concentrations of agonist. Differences between PRP treated with GRK inhibitor versus control assessed by Student's T-Test *, $p < 0.05$, **, $p < 0.01$.

Figure 6. **Suggested mechanism of *GRK5* regulatory variant in influencing PAR4 platelet activation and thrombosis risk.** Schematic depicting the platelet-specific effects of *GRK5* variant rs10886430 via a GATA1 enhancer and modified suppression of platelet PAR4 signaling on thrombin activation. PAR4 has been studied as a drug target (BMS-986120). The model is supported by thrombin platelet reactivity association in PRP, a strong platelet eQTL in 2 independent samples, a lack of eQTL co-localization in other cells and tissues, mutagenesis and enhancer assays, siRNA and inhibitor (CCG215022) experiments in iPSC-derived megakaryocytes and platelets, and multiple population genetic studies for cardiovascular disease outcomes (UK BioBank, MEGASTROKE, FinnGen, INVENT, MVP).

Figure 1

A


r²
0.8
0.6
0.4
0.2
rs10886430
P_{GWAS} = 3.0 x 10^{-42}
-log$_{10}$(P_{GWAS})
40
30
20
10
0
NANOS1
FAM45A
GRK5
RGS10
EIF3A
FAM45B
MIR4681
SNORA19
SFXN4
PRDX3
Chr10 (Mb)
120.8
120.9
121.0
121.1
121.2
121.3

B

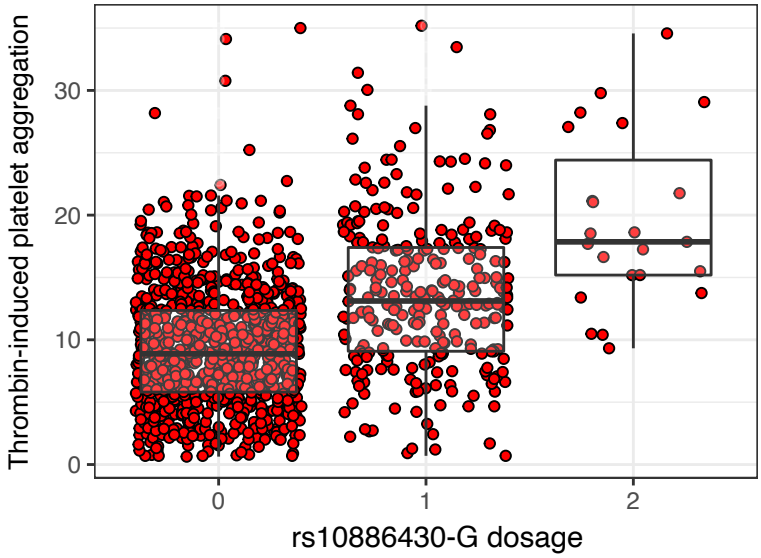

Thrombin-induced platelet aggregation
30
20
10
0
0
1
2
rs10886430-G dosage

Figure 2

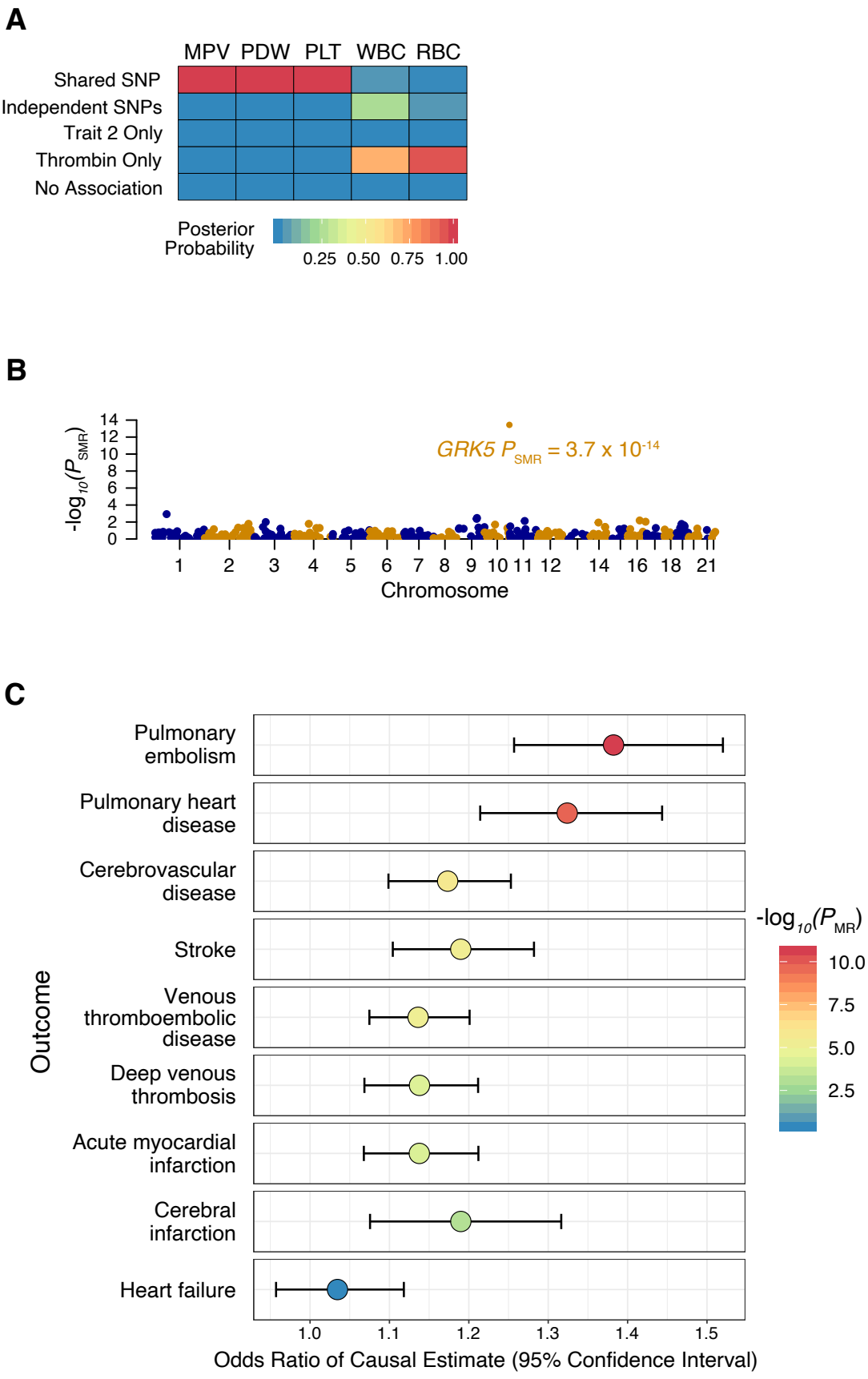

A
MPV PDW PLT WBC RBC
Shared SNP
Independent SNPs
Trait 2 Only
Thrombin Only
No Association
Posterior Probability
0.25 0.50 0.75 1.00
B
GRK5 P_SMR = 3.7 x 10^-14
-log10(P_SMR)
0 2 4 6 8 10 12 14
1 2 3 4 5 6 7 8 9 10 11 12 14 16 18 21
Chromosome
C
Pulmonary embolism
Pulmonary heart disease
Cerebrovascular disease
Stroke
Venous thromboembolic disease
Deep venous thrombosis
Acute myocardial infarction
Cerebral infarction
Heart failure
Outcome
-log10(P_MR)
10.0
7.5
5.0
2.5
1.0 1.1 1.2 1.3 1.4 1.5
Odds Ratio of Causal Estimate (95% Confidence Interval)

Figure 3

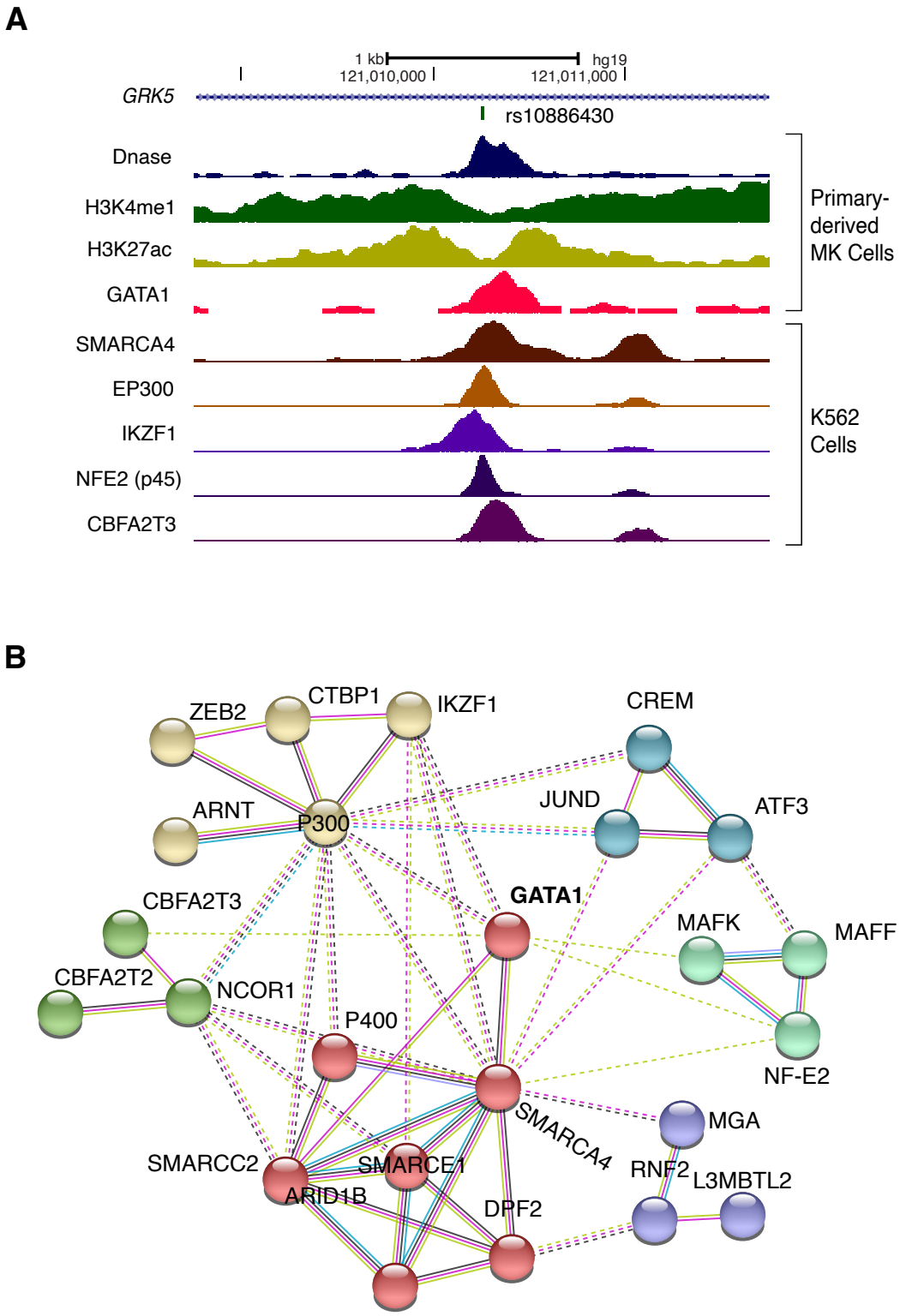

A
1 kb
hg19
121,010,000
121,011,000
GRK5
rs10886430
Dnase
H3K4me1
H3K27ac
GATA1
Primary-derived MK Cells
SMARCA4
EP300
IKZF1
NFE2 (p45)
CBFA2T3
K562 Cells
B
ZEB2
CTBP1
IKZF1
CREM
ARNT
P300
JUND
ATF3
CBFA2T3
GATA1
MAFK
MAFF
CBFA2T2
NCOR1
P400
NF-E2
SMARCA4
MGA
SMARCC2
SMARCE1
RNF2
L3MBTL2
ARID1B
DPF2

Figure 4

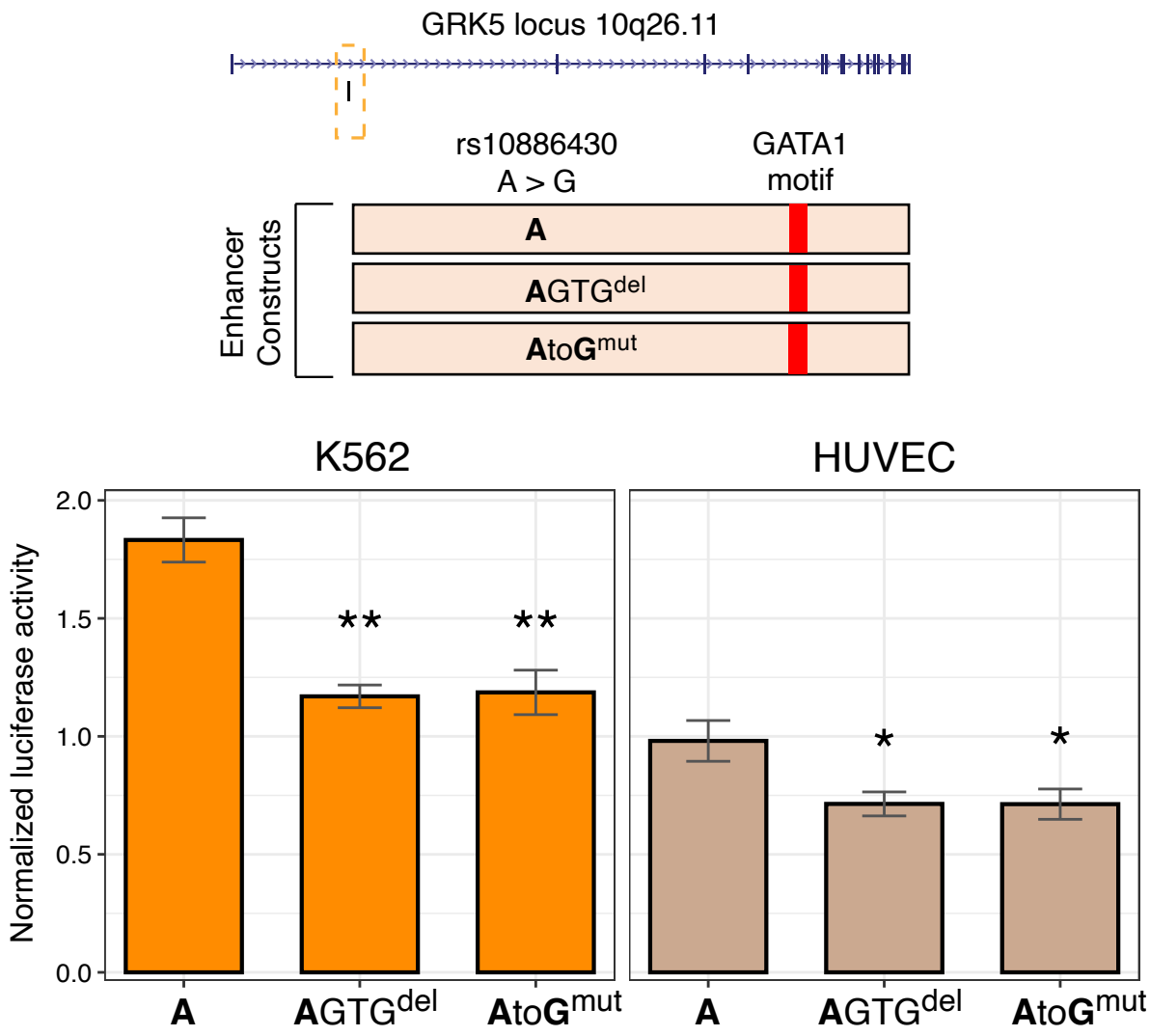

GRK5 locus 10q26.11
rs10886430
A > G
GATA1
motif
Enhancer
Constructs
A
AGTGdel
AtoGmut
K562
HUVEC
Normalized luciferase activity
2.0
1.5
1.0
0.5
0.0
**
**
*
*
A
AGTGdel
AtoGmut
A
AGTGdel
AtoGmut

Figure 5

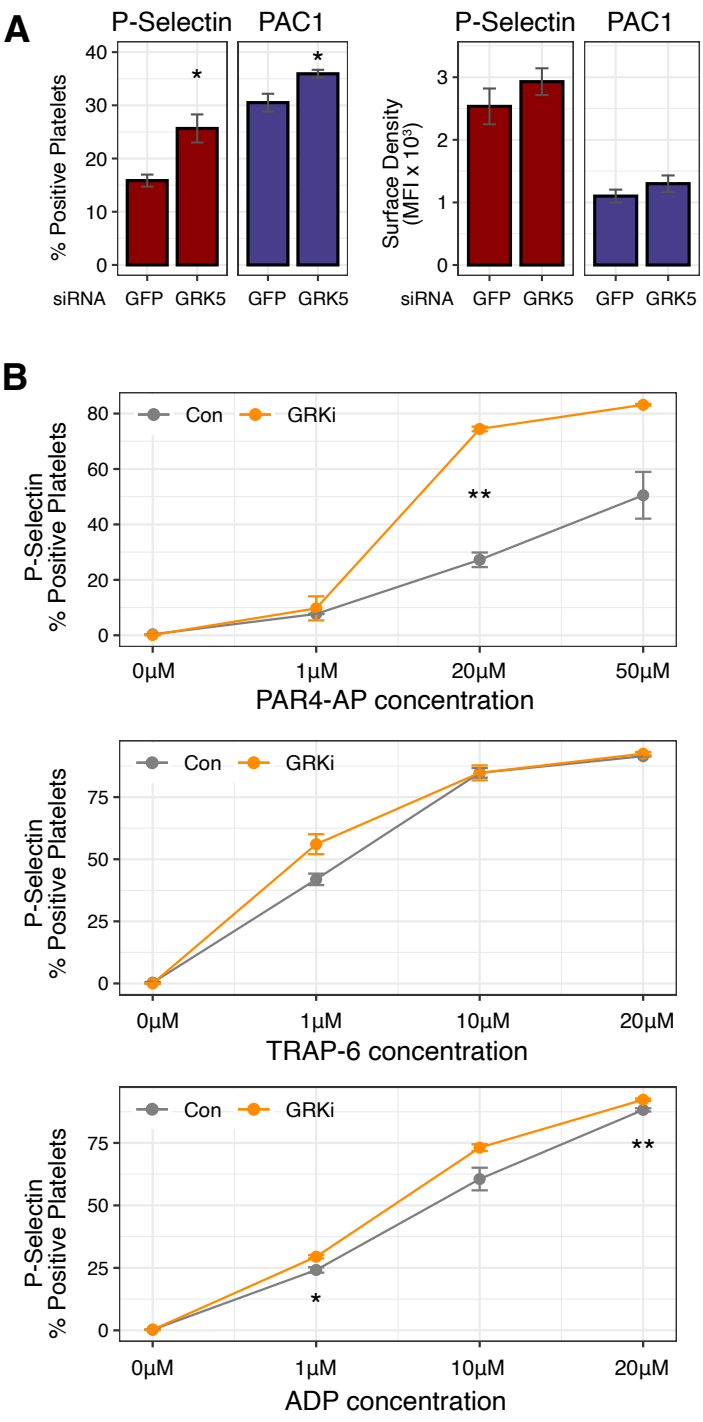
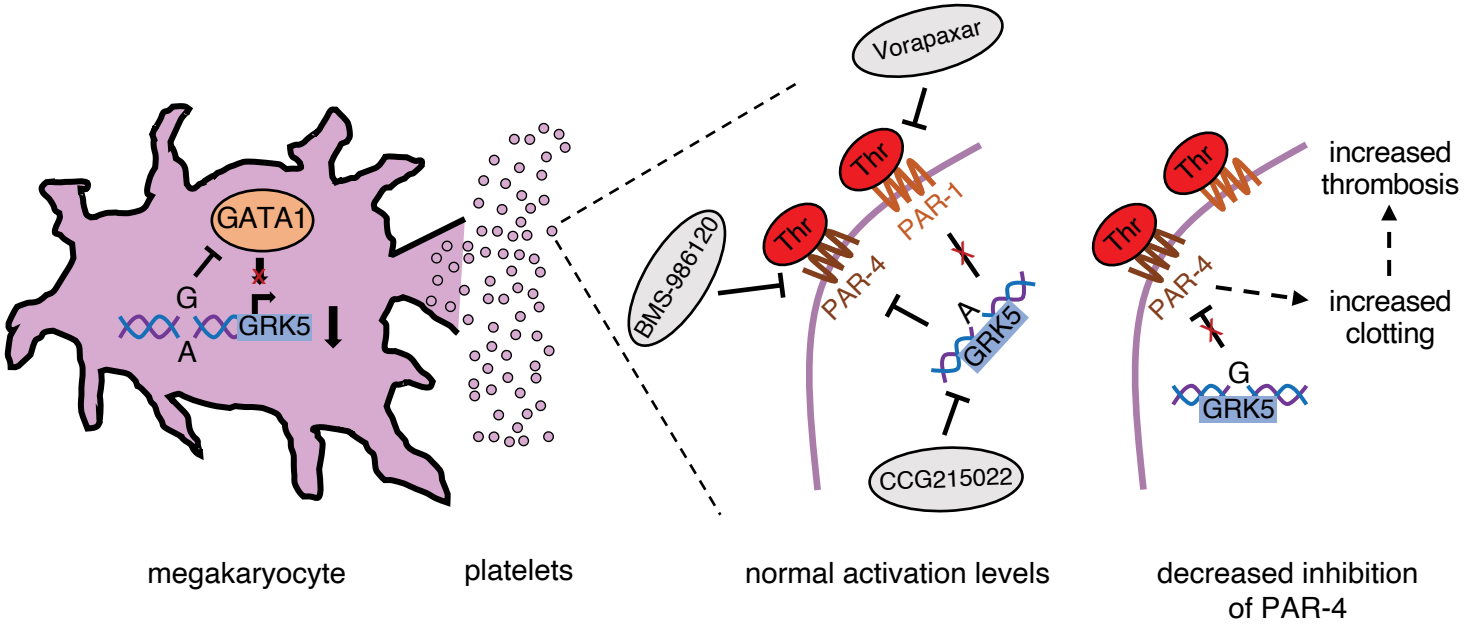

A
P-Selectin
PAC1
% Positive Platelets
Surface Density (MFI x 10³)
siRNA
GFP
GRK5
B
Con
GRKi
P-Selectin % Positive Platelets
PAR4-AP concentration
0μM
1μM
20μM
50μM
TRAP-6 concentration
10μM
ADP concentration

Figure 6


GATA1
G
A
GRK5
Vorapaxar
Thr
PAR-1
BMS-986120
Thr
PAR-4
A
GRK5
CCG215022
Thr
Thr
PAR-4
increased
thrombosis
increased
clotting
G
GRK5
megakaryocyte
platelets
normal activation levels
decreased inhibition
of PAR-4

Supplement