

# Modifiers of von Willebrand factor identified by natural variation in inbred strains of mice

Jordan A. Shavit,<sup>1</sup> Ani Manichaikul,<sup>2</sup> Heidi L. Lemmerhirt,<sup>3</sup> Karl W. Broman,<sup>2</sup> and David Ginsburg<sup>1,3,4</sup>

<sup>1</sup>Department of Pediatrics, University of Michigan, Ann Arbor; <sup>2</sup>Department of Biostatistics, Johns Hopkins University, Baltimore, MD; <sup>3</sup>Department of Human Genetics, and <sup>4</sup>Howard Hughes Medical Institute and Department of Internal Medicine, University of Michigan, Ann Arbor

**Type 1 von Willebrand disease (VWD) is the most common inherited human bleeding disorder. However, diagnosis is complicated by incomplete penetrance and variable expressivity, as well as wide variation in von Willebrand factor (VWF) levels among the normal population. Previous work has exploited the highly variable plasma VWF levels among inbred strains of mice to identify 2 major regulators, *MvWF1* and *MvWF2* (modifier of VWF).**

***MvWF1* is a glycosyltransferase and *MvWF2* is a natural variant in *Vwf* that alters biosynthesis. We report the identification of an additional alteration at the *Vwf* locus (*MvWF5*), as well as 2 loci unlinked to *Vwf* (*MvWF6-7*) using a backcross approach with the inbred mouse strains WSB/EiJ and C57BL/6J. Through positional cloning, we show that *MvWF5* is a *cis*-regulatory variant that alters *Vwf* mRNA expression. A similar mechanism**

**could potentially explain a significant percentage of human VWD cases, especially those with no detectable mutation in the VWF coding sequence. *MvWF6* displays conservation of synteny with potential VWF modifier loci identified in human pedigrees, suggesting that its ortholog may modify VWF in human populations. (Blood. 2009;114:5368-5374)**

## Introduction

von Willebrand factor (VWF) is a central component of hemostasis, serving as the adhesive link between platelets and the injured blood vessel wall, as well as the carrier for factor VIII. Deficiencies in VWF result in von Willebrand disease (VWD), the most common inherited bleeding disorder in humans. Elevated VWF levels may also be an important risk factor for thrombosis, both through a direct role in platelet adhesion,<sup>1</sup> as well as indirectly by causing elevated levels of factor VIII.<sup>2-4</sup> Diagnosis of VWD is elusive in many cases because of its variable expressivity and incomplete penetrance<sup>5</sup> and the nonspecific nature of bleeding symptoms.<sup>6</sup> VWF plasma protein levels also display a broad distribution in the normal human population. Thus, it is often difficult to determine whether a person has VWD and is at risk for pathologic hemorrhage or simply has VWF levels in the low range of normal.

Levels of plasma VWF have been shown to be largely determined by genetic factors, with estimates of heritability in humans ranging from 25% to 32% by pedigree analysis,<sup>7,8</sup> to 66% to 75% in twin studies.<sup>9,10</sup> ABO blood group is responsible for one-third of the genetic variability in VWF plasma levels.<sup>11</sup> However, the loci responsible for the remaining two-thirds of this genetic component are unknown. Recent evidence from European and Canadian cooperative studies on type 1 VWD have found that disease diagnosis does not segregate with VWF genotype in approximately 50% of families, supporting the existence of additional genetic factors.<sup>12-16</sup>

Laboratory mice display wide variation in VWF levels with 65% heritability in a cross between the strains A/J and CASA/RkJ,<sup>17</sup> strikingly similar to the estimates for humans derived from twin studies.<sup>9,10</sup> This variability among inbred mouse strains has been

used to identify genetic loci modifying VWF levels, including *MvWF1* (modifier of *Vwf*), a mouse glycosyltransferase (*B4galnt2*) that alters clearance of VWF.<sup>18</sup> A similar mechanism probably explains the modification of human VWF levels by ABO blood group and some cases of type 1 VWD.<sup>19,20</sup> A natural variant of the murine *Vwf* gene has also been identified (*MvWF2*).<sup>17</sup>

We now report a backcross between 2 additional inbred mouse strains, WSB/EiJ (WSB) and C57BL/6J (B6), with relatively high and low levels of plasma VWF, respectively. Genetic analysis identified 3 significant loci regulating VWF levels. The first is a novel *cis*-regulatory allele of *Vwf*, whereas the others map to novel loci on chromosomes 5 and 10.

## Methods

### Mouse strains and bleeding

Mice were purchased from The Jackson Laboratory. Each individual mouse was bled on 3 separate occasions, at least 1 week apart. Bleeds were performed after isoflurane-induced anesthesia by retro-orbital technique on alternating eyes from week to week, removing approximately 75  $\mu$ L whole blood with each bleed into heparinized capillary tubes (Thermo Fisher Scientific). For the strain survey, 3 females of each strain were bled between 4 and 8 weeks of age. Further analysis of the C57BL/6J (B6) and WSB/EiJ (WSB) strains were as follows: 2 each of WSB males and females, 3 males and 4 females of B6, and 4 males and 5 females of (B6  $\times$  WSB) F1 mice were bled between 3 and 8 weeks of age. For the backcross study, WSB males were crossed to B6 females to generate F1 progeny. Both male and female F1s were backcrossed to B6 to produce the N2 generation. Two hundred seven N2 mice were also bled in the same manner described above, with the first bleed at weaning, the second between 3.5 and

Submitted July 15, 2009; accepted August 15, 2009. Prepublished online as *Blood* First Edition paper, September 29, 2009; DOI 10.1182/blood-2009-07-233213.

An Inside *Blood* analysis of this article appears at the front of this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2009 by The American Society of Hematology

6.5 weeks, and the third from 5 to 8.5 weeks. The mice were then exsanguinated by cardiac puncture after pentobarbital-induced anesthesia, and blood was collected into 0.5M EDTA pH 8.0 at a dilution of 1:40. Organs were harvested for genomic DNA preparation. Mice were housed in microisolator cages, and all procedures were approved by and performed according to the University of Michigan's Committee on Use and Care of Animals guidelines.

### VWF plasma protein quantitation and analysis

Platelet-poor plasma was isolated from whole blood by centrifugation at 2000g and stored at  $-70^{\circ}\text{C}$  before analysis. VWF levels were quantitated by enzyme-linked immunosorbent assay (ELISA) essentially as previously described.<sup>17</sup> A pool of male and female adult B6 plasma (age, 6-8 weeks) was used to generate a standard curve, and a mean VWF level was calculated for each mouse from the 3 retro-orbital bleeds. Because of age and sex differences between the standard pool and experimental groups, the B6 parental strain levels are slightly higher than the value of 10 that was arbitrarily assigned to the standard. Analysis of variance was performed on natural log-transformed VWF values to assess the ELISA assay variance (variation in replicate measurements on the same plasma sample), environmental variance (variation among replicate measurements taken on the same animal), genetic variance (variation among animals), and total backcross population variance. Heritability was estimated by comparing genetic variance with total backcross population variance after correcting for assay variance. Assay variance was determined to be approximately 2% of total variance.

### Genotyping

Genomic DNA was isolated from liver by digestion in buffer containing 0.1 mg/mL proteinase K, 0.1M Tris (pH 8.0), 0.2M NaCl, 0.2% SDS, and 5mM EDTA at  $55^{\circ}\text{C}$  overnight, followed by phenol/chloroform extraction, isopropanol precipitation, washing with 70% ethanol, and resuspension in 10mM Tris-Cl, pH 8.0. Genotyping on DNA from 200 mice was performed by the Mammalian Genotyping Service of the National Heart, Lung, and Blood Institute. Seven mice with VWF levels in the middle of the population were removed from the 207 N2 progeny, and 157 simple sequence length polymorphisms (SSLPs) were examined across the genome. At the University of Michigan standard genotyping was performed by polymerase chain reaction (PCR) and agarose gel electrophoresis as described<sup>17</sup> to type an additional 28 SSLPs and 4 single nucleotide polymorphisms (SNPs) on all 207 N2 mice, 33 SSLPs selectively applied to mice with the 5% highest and lowest VWF levels, and 7 SSLPs on the entire N2 population excluding the 5% highest and lowest VWF groups. All map positions are noted in millions of base pairs (Mbp), obtained from NCBI (National Center for Biotechnology Information) Build 37 of the mouse genome, unless otherwise noted.

### Genome scan analysis and QTL identification

A genome scan was performed by standard interval mapping<sup>21</sup> with the natural log of the mean VWF level (averaged across the 3 plasma samples) as a quantitative trait and sex as an additive covariate. LOD (logarithm of odds) scores were obtained as the log-10 likelihood ratio comparing a model with a single QTL (quantitative trait locus) to a model with no QTLs. Because the effect size of the QTL on chromosome 6 was so strong, 2 additional genome scan analyses were also performed with the marker *D6Mit366* included as either an additive or interactive covariate, keeping the sex adjustment in both cases. The choice of *D6Mit366* was arbitrary because *D6Mit329* and *D6Mit366* are both at the peak of the chromosome 6 QTL and only 1 Mbp apart. Significance thresholds were obtained by permutation testing, whereby the genome scan analysis was repeated 50 000 times on the autosomes and 1 000 000 times on the X chromosome,<sup>22</sup> randomly shuffling phenotypes while keeping genotypes fixed.<sup>23</sup> The LOD scores for genomewide significance at levels  $\alpha$  equal 0.01 (highly significant), 0.05 (significant), and 0.25 (suggestive) were taken as quantiles of the corresponding permutation distributions, partitioning the type I error rate across the autosomes and the X chromosome, according to the

method of Broman et al.<sup>22</sup> Note that sample sizes in analyses of the X chromosome were reduced from 207 to 78 individuals, because analysis was restricted to individuals from crosses for which the X chromosome was segregating.<sup>22</sup> Finally, 95% confidence intervals were obtained as 96.5% Bayes credible intervals.<sup>24,25</sup> Data management and genome scan analyses were performed with the use of R<sup>26</sup> and the R/qtl package.<sup>27</sup>

### Allele-specific primer extension analysis

Three male and 3 female 9-week-old (B6  $\times$  WSB) F1 mice were anesthetized with isoflurane and pentobarbital and humanely killed. Lungs were dissected and immediately frozen in liquid nitrogen, and total mRNA was isolated with Trizol (Invitrogen). mRNA was treated with DNase I (Invitrogen) and subjected to reverse transcription (RT)-PCR with SuperScript One-Step (Invitrogen) with the use of *Vwf* internal exon 7 primers 5'-GGGAGCAATGCCAGCTACT-3' and 5'-GGCACTGTGGTCAGTCAG-3', at  $50^{\circ}\text{C}$  for 30 minutes,  $94^{\circ}\text{C}$  for 2 minutes, 35 cycles of  $94^{\circ}\text{C}$  for 30 seconds,  $56^{\circ}\text{C}$  for 30 seconds,  $72^{\circ}\text{C}$  for 1 minute, concluding with  $72^{\circ}\text{C}$  for 10 minutes. Relative allele-specific mRNA accumulation was determined using a primer extension assay with fluorescently labeled primers as previously described,<sup>17,28</sup> using a standard curve composed of B6 and WSB genomic DNA quantitated with PicoGreen (Invitrogen) and mixed in various ratios. The RT-PCR product was treated with ExoSAP-IT (US Biochemical Corp) to remove free nucleotides and primers. Primer extension was carried out with Thermo Sequenase (US Biochemical Corp), a 6-FAM-labeled primer (5'-TGGATCCCGAGTCCTTTGTGGCTC-3') and a mixture of nucleotides containing ddCTP and run at  $94^{\circ}\text{C}$  for 2 minutes, 30 cycles of  $94^{\circ}\text{C}$  for 30 seconds,  $65^{\circ}\text{C}$  for 30 seconds,  $72^{\circ}\text{C}$  for 1 minute, resulting in differentially sized products because of a T/C SNP +758 bp from the A of the initiation methionine of *Vwf*. Products were diluted 1:13 in Hi-Di formamide (Applied Biosystems), separated on an Applied Biosystems 3730XL DNA Analyzer at the University of Michigan Sequencing Core, and quantitated with GeneMarker 1.51 (SoftGenetics LLC). The standards were fit to a line resulting in an  $R^2$  value of 0.996, calculated by linear regression with Microsoft Excel 2003 (Microsoft Corporation).

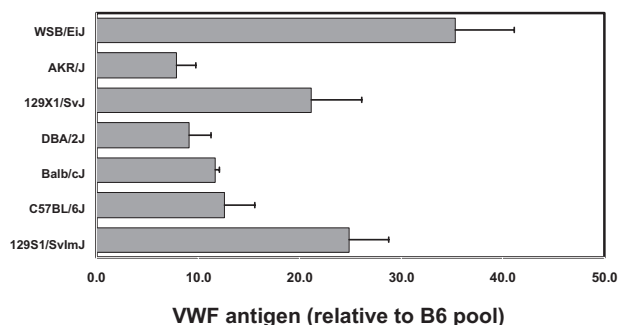
### Calculation of *Vwf* locus contribution to plasma VWF levels in the N2 population

The following calculation was used to assess the relative contribution of the *Vwf* locus to the level of plasma VWF. The N2 population was subdivided into 2 groups based on genotype at a G/T SNP in exon 5 of *Vwf* (+365 base pairs from the A of the initiation methionine) that produces an *XhoI* restriction fragment polymorphism that cuts WSB, but not B6 (amplified with the primers 5'-GGCAAGAGAATGAGCCTGTGC-3' and 5'-TGAAATCACAGAATCAATGGACTA-3'), and the average VWF level was calculated for the resulting B6:B6 and B6:WSB groups. Any minor modifier loci would be expected to be evenly divided among these 2 groups by Mendelian inheritance; therefore, their effects on plasma VWF levels should be evenly distributed.

## Results

### VWF plasma levels in WSB/EiJ mice are 3.5-fold higher than C57BL/6J

VWF levels were quantitated by ELISA on plasma obtained from 6 laboratory inbred strains as well as the wild-derived inbred strain WSB. VWF plasma protein levels were determined as the average of data from 3 separate retro-orbital bleeds, done at least 1 week apart. WSB exhibited the highest plasma VWF, with levels 3.5-fold greater than most of the common inbred strains, including B6 (Figure 1). Two 129/Sv substrains tested each displayed intermediate levels of VWF, which were 2-fold greater than B6.



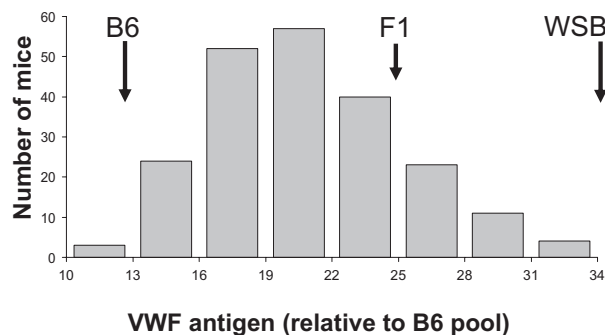
**Figure 1. Inbred mouse strain survey for VWF plasma protein levels.** Individual mice were bled, and the results were averaged and normalized to an adult B6 plasma pool arbitrarily assigned a value of 10. Error bars represent SD.

### (B6 × WSB) N2 progeny display a wide range of VWF levels

B6 was chosen to cross with WSB because it has been used as a standard strain in many previous hemostasis and other disease models, and it is also the reference strain for the mouse genome.<sup>29,30</sup> Importantly, neither B6 nor WSB contain allelic variants associated with the previously reported modifier loci *Mvfw1* and *Mvfw2*.<sup>17,31</sup> (B6 × WSB) F1 plasma VWF levels were intermediate between the 2 parental strains but closer to WSB (Figure 2). Therefore, an outcross/backcross approach with B6 as the backcross parent was chosen. Plasma VWF levels were determined on 207 N2 progeny generated from a (B6 × WSB) F1 backcross to B6 (Figure 2). The observed levels in N2 animals encompassed the entire phenotypic range, extending to the pure parental strains and peaking just below the level of F1 mice. Heritability of VWF levels in the (B6 × WSB) N2 population was determined to be approximately 71%. There was also a statistically significant difference in population averages among bleeds (population averages: bleed 1, 24.5; bleed 2, 18.2; bleed 3, 16.4;  $P < .001$ ).

### Genotyping of N2 backcross progeny identifies 3 major QTLs for VWF plasma levels

Two hundred N2 progeny were genotyped with 189 markers spaced across the genome. Additional markers were used in several subsets of mice (see “Genotyping”). Standard interval mapping at 1-centimorgan (cM) intervals using the R/qtl package<sup>27</sup> was performed on the natural log-transformed mean VWF levels for the N2 population with adjustment for sex. Three QTLs were identified with significant linkage to VWF levels, with peak markers at



**Figure 2. Histogram of N2 generation VWF plasma levels.** Individual N2 mice were bled, and the results were averaged and normalized to an adult B6 plasma pool that was arbitrarily assigned a value of 10. The VWF antigen levels were binned and plotted as indicated on the x-axis. The locations of the parental strain and (B6 × WSB) F1 hybrid values are indicated by ↓. These are 13.1, 24.8, and 35.3, for B6, F1, and WSB, respectively.

*D6Mit329* (*Mvfw5*), *D10Mit269* (*Mvfw6*), and *D5Mit66* (*Mvfw7*) and LOD scores of 11.65, 3.72, and 3.22, respectively (Figure 3A; Table 1).

To detect additional minor QTLs masked by the extremely strong effect of the chromosome 6 locus, the data were reanalyzed with the marker *D6Mit366* (115.2 Mbp) as an additive or interactive covariate. The results were very similar to the initial analysis, without significant changes in the statistical significance of the LOD scores for *Mvfw6* and *Mvfw7* (Figure 3B-C). No additional loci, including the known modifier loci *B4galnt2* (*Mvfw1*), *Mvfw3-4*,<sup>32</sup> and the human ABO blood group ortholog, *Abo* (chromosomes 11, 4, 13, and 2, respectively), surpassed the 0.05 significance threshold in any of these analyses (as determined by permutation testing).

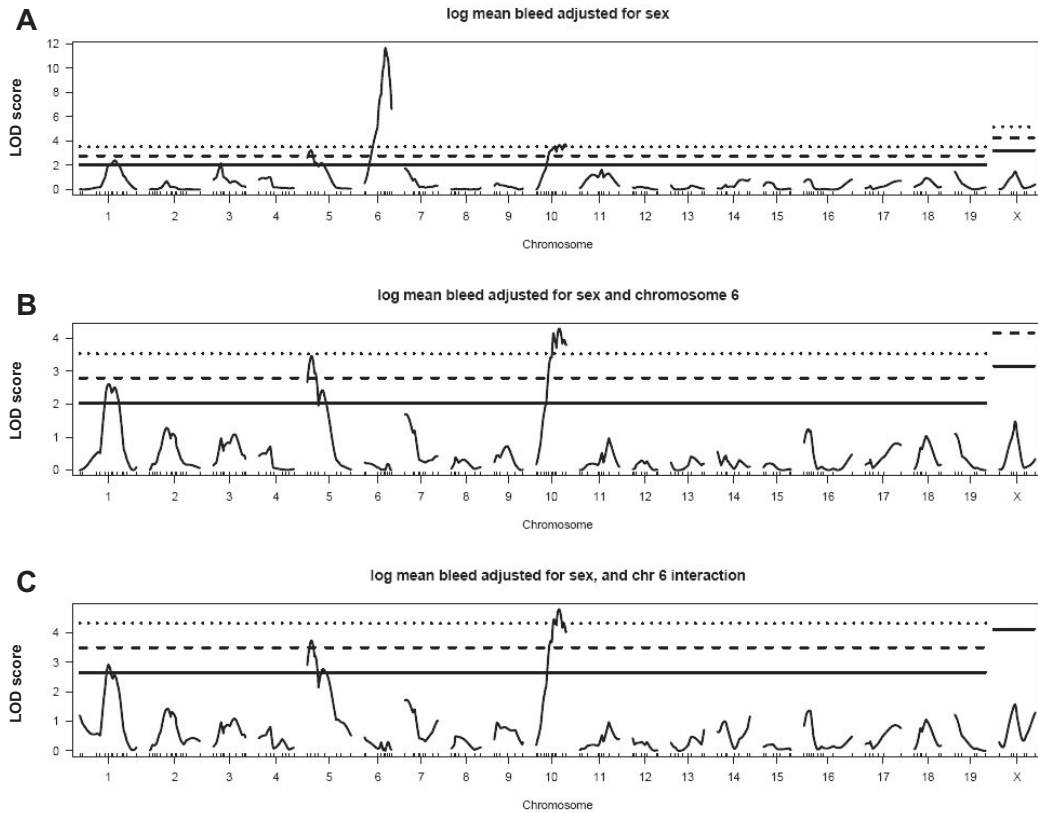
Examination of candidate genes in the peak of the *Mvfw5* QTL showed that the *Vwf* gene itself is located in this region. (B6 × WSB) N2 mice that were BW (B6:WSB) at both the *D6Mit329* and *D6Mit339* markers (114.0 and 136.3 Mbp, respectively, flanking the *Vwf* locus) were backcrossed to B6 to the N4 and N5 generations, with selection for heterozygosity at both markers. VWF levels were measured as described above on mice that were BB (B6:B6, non-BW littermates derived during backcrossing) or BW at both markers (Figure 4 inset). There was a statistically significant increase in VWF levels in the BW mice (19.6 vs 14.6 in BB mice), confirming that *Mvfw5* is a true QTL and narrowing the candidate region to 11.6 cM, which includes *Vwf* (Figure 4).

### Strain-specific differences at the *Vwf* locus alter steady state *Vwf* mRNA levels

Sequencing of the WSB *Vwf* cDNA followed by comparison to published B6 sequence revealed 9 nonsynonymous SNPs, all localized to the VWF propeptide (Table 2). Eighteen synonymous SNPs were discovered, with a large block devoid of any variation at the 3' end (Table 2). This represents a greater level of divergence between B6 and WSB, 2 strains that are thought to be primarily *mus musculus domesticus* in origin, versus A/J and CASA/RkJ (the strains used to identify *Mvfw2-4*), which are *mus musculus domesticus* and *mus musculus castaneus*, respectively.<sup>17</sup>

To evaluate potential *cis*-regulatory differences in mRNA expression or stability, lung mRNA was isolated from (B6 × WSB) F1 mice and subjected to RT-PCR for *Vwf* mRNA, followed by primer extension analysis that distinguishes the B6 and WSB alleles because of an exonic SNP (Figure 5A). A similar approach has been successful in distinguishing expression from maternal and paternal alleles in cases of human VWD.<sup>33</sup> Quantitation showed that 38.7% ( $\pm 0.6\%$ ) of transcripts were derived from the B6 allele and 61.3% from the WSB locus (Figure 5B).

Subdivision of the N2 population into 2 groups based on genotype at *Vwf* (see “Methods”) resulted in an average VWF level of 18.5 in the B6:B6 group and 22.7 in the B6:WSB group. The B6:B6 group represents the contribution of 2 B6 alleles of *Vwf* (or 9.25 per B6 allele), whereas B6:WSB represents 1 B6 and 1 WSB allele (or  $22.7 - 9.25 = 13.45$  for WSB). Thus, the B6 allele contributes 41% of plasma VWF in B6:WSB heterozygotes ( $9.25/22.7$ ), versus 59% for the WSB allele ( $13.45/22.7$ ). The results of these calculations are indistinguishable from the mRNA data determined by primer extension (38.7% B6 and 61.3% WSB;  $P > .6$  by  $\chi^2$  analysis with the plasma values as the expected values). Therefore, we conclude that the *Vwf* locus contribution to VWF plasma protein levels in the B6xWSB N2 population can be fully explained by differential mRNA accumulation from the 2 alleles. Additional minor contributions from the *Vwf* gene to the



**Figure 3. R/qtl analysis of N2 progeny shows 3 QTLs that modify VWF plasma levels.** Interval mapping was performed on natural log-transformed mean VWF levels. (A) LOD scores with adjustment for sex show 3 QTLs on chromosomes 6, 10, and 5 (*Mvwf5*, *Mvwf6*, and *Mvwf7*, respectively). No additional loci were identified by analysis for additive (B) or epistatic (C) effects upon adjustment for *Mvwf5*. Solid, dashed, and dotted lines indicate  $\alpha = 0.25$  (suggestive), 0.05 (significant), and 0.01 (highly significant) thresholds, respectively, obtained by permutation testing.

difference in plasma VWF levels between these strains, such as biosynthesis or clearance resulting from the nonsynonymous SNPs, cannot be completely excluded but are probably small.

***Mvwf6* overlaps with human VWF QTL**

The 96.5% Bayes credible intervals were constructed for *Mvwf6* and *Mvwf7* (Figure 6), as previously described.<sup>25</sup> Human orthologous regions were determined from comparative orthology maps<sup>34</sup> and aligned to potential VWF modifier regions identified in human populations. Souto et al (from the Genetic Analysis of Idiopathic Thrombophilia or GAIT study)<sup>35</sup> analyzed VWF levels as a quantitative trait in a group of 21 Spanish families and found potential linkage to several regions unlinked to the human *VWF* locus. The linkage identified in the GAIT study included 22q11.1, which displays orthology to *Mvwf6* (Figure 6). Similar comparisons to linkage data from a large Amish pedigree phenotyped by ristocetin cofactor activity<sup>36</sup> reveal orthology for *Mvwf6* with human loci at 12q12 and 21q22.3 (Figure 6).

**Discussion**

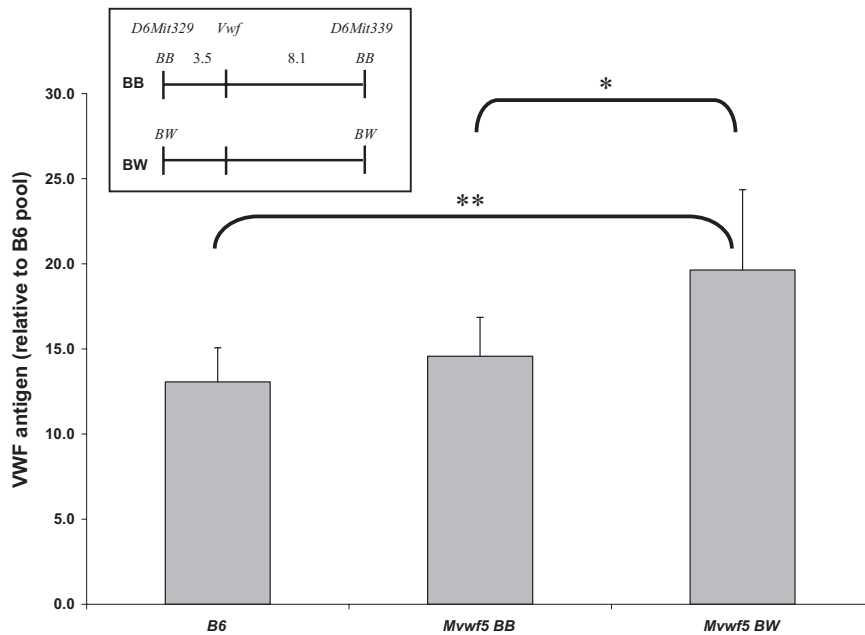
Previous analyses of laboratory strains of mice have identified both major and minor loci regulating VWF levels.<sup>17,18,32</sup> We now report a second strong *Vwf* variant associated with regulation of VWF plasma protein levels, as well as 2 additional minor loci that are unlinked to the *Vwf* locus. Using 2 previously unexamined strains, we found the heritability of VWF plasma levels to be 71%, similar to the value of 65% found in an (*A/J* × *CASA/RkJ*) F2 population<sup>17</sup> and also similar to estimates of VWF level heritability in human populations.<sup>9,10</sup> The (*B6* × *WSB*) N2 population displayed a statistically significant decrease in VWF levels over time, falling by 49% from weaning (3 weeks) to adulthood (5-8.5 weeks). This may parallel the pattern in humans, in which plasma VWF at birth decreases by 43% to near adult levels by 6 months of age.<sup>37</sup> In contrast, the (*A/J* × *CASA/RkJ*) F2 population<sup>17</sup> did not show a similar age-dependent decrease in VWF. These data show strain-specific differences in the regulation of VWF levels over time in mice and raise the possibility of similar factors in humans.

**Table 1. QTLs identified with significant linkage to plasma VWF**

| Locus        | Chromosome | Position, Mbp | Peak marker      | LOD   | P      |
|--------------|------------|---------------|------------------|-------|--------|
| <i>Mvwf5</i> | 6          | 114.1         | <i>D6Mit329</i>  | 11.65 | < .001 |
| <i>Mvwf6</i> | 10         | 110.0         | <i>D10Mit269</i> | 3.72  | .006   |
| <i>Mvwf7</i> | 5          | 32.0          | <i>D5Mit66</i>   | 3.22  | .018   |

Standard interval mapping was performed on natural log-transformed mean VWF levels from the *B6* × *WSB* N2 population at 1-cM intervals by using the R/qtl package with adjustment for sex. P values represent the genomewide significance of LOD scores as determined by permutation testing. QTL indicates quantitative trait locus; VWF, von Willebrand factor; and LOD, logarithm of odds.





**Figure 4. VWF levels in congenic mice.** VWF plasma levels were determined. B6 represents C57BL/6J mice purchased from The Jackson Laboratory ( $n = 7$ ). *MvWF5* BW mice were backcrossed to B6 to the N4 or N5 generation, and VWF levels on littermates that were BB ( $n = 18$ ) or BW ( $n = 11$ ) at both *D6Mit329* and *D6Mit339* were determined (see inset; numbers indicate centimorgan [cM] distances between markers and *Vwf* indicates the *XhoI* restriction fragment polymorphism described in "Methods"). \*The difference between these 2 groups is statistically significant ( $P < .001$  by *t* test). \*\*The difference between these 2 groups is statistically significant ( $P < .004$  by *t* test). There was no significant difference between the B6 and BB groups ( $P > .1$  by *t* test). Error bars represent SD.

*MvWF5* is a natural mouse allele altering plasma VWF because of a *cis*-regulatory mutation in the *Vwf* gene itself. This is the second example of a natural variant *Vwf* allele among inbred strains of mice, as *MvWF2* is due to a VWF gene-coding mutation.<sup>17</sup> The relative elevation in steady-state levels of *Vwf* mRNA derived from the WSB *MvWF5* allele could be due to an alteration in

transcriptional regulation, splicing, or mRNA stability, mechanisms identified in many common human genetic disorders, such as the thalassemias.<sup>38</sup>

Although most human type 1 VWD mutations identified to date affect protein function, transport, secretion, or clearance, this probably represents an ascertainment bias because most studies only examine exons, exon/intron junctions, and the proximal promoter and would miss more distant regulatory sequence changes. Consistent with this hypothesis, comprehensive exon sequencing in patients with type 1 VWD has identified mutations in only 67% of persons.<sup>12,16,39,40</sup> Of those with identified mutations, 7% altered splice sites and 11% were localized to the promoter. Although not tested directly, both classes of mutation would be expected to alter allelic expression at the mRNA level, as shown here for murine *MvWF5*. In addition, a subset of the larger class of exonic mutations could also potentially affect mRNA levels through alterations in mRNA stability. Distant regulatory mutations not covered in the human sequence analysis performed to date could also affect mRNA expression, potentially accounting for a significant percentage of the above patients for whom no mutations were identified. Recent data suggest that long-range effects of distant regulatory elements could be particularly important for *VWF* gene expression.<sup>41-43</sup> Thus, a significant contribution from the large quantity of VWF intergenic and intronic sequences remains to be explored.

The presence of SNPs within the murine *Vwf* mRNA sequence permitted the dissection of relative mRNA expression from each *Vwf* allele in this and a previous study.<sup>17</sup> An analogous approach has been used for peripheral blood platelet *VWF* mRNA in humans.<sup>33</sup> Future studies applying similar RNA SNP analysis to human patients could potentially define novel subgroups of type 1 VWD.

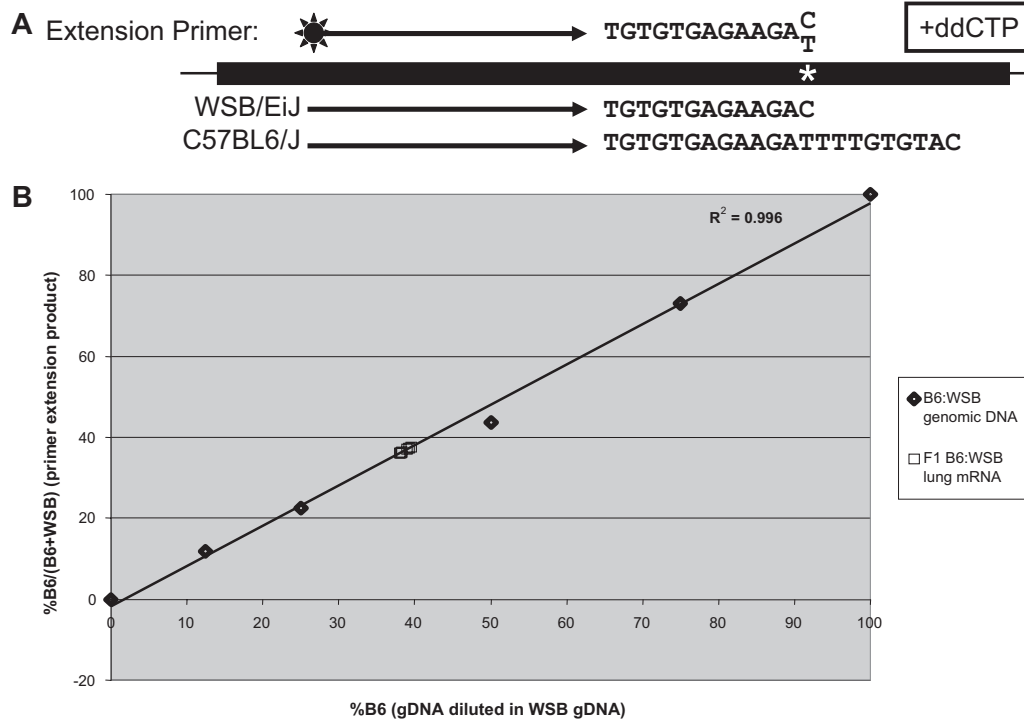
Our findings, taken together with previous work,<sup>17,18,32</sup> provide genetic analysis of 5 mouse strains in 3 different crosses and has identified 2 independent, naturally occurring *Vwf* gene mutations leading to altered plasma VWF levels. These data suggest that the equivalent of type 1 VWD is remarkably common in mice, as well as in humans. Reports of VWD in horse, cat, pig, rabbit, and dog<sup>5,44</sup> suggest that a high prevalence

**Table 2. Location of SNPs in coding sequence of mouse *Vwf***

| Position | VWF domain | B6 | WSB | Amino acid $\Delta$ |
|----------|------------|----|-----|---------------------|
| 308      | Propeptide | C  | T   | T103M               |
| 365      | Propeptide | G  | T   | R122L               |
| 516      | Propeptide | T  | C   | —                   |
| 596      | Propeptide | G  | A   | R199Q               |
| 600      | Propeptide | T  | C   | —                   |
| 758      | Propeptide | T  | C   | I253T               |
| 767      | Propeptide | C  | T   | T256M               |
| 774      | Propeptide | T  | C   | —                   |
| 1110     | Propeptide | T  | C   | —                   |
| 1236     | Propeptide | T  | C   | —                   |
| 1404     | Propeptide | T  | C   | —                   |
| 1581     | Propeptide | C  | T   | —                   |
| 1678     | Propeptide | T  | C   | S560P               |
| 1754     | Propeptide | C  | A   | A585E               |
| 1833     | Propeptide | T  | C   | —                   |
| 1852     | Propeptide | A  | G   | S618G               |
| 1965     | Propeptide | C  | T   | —                   |
| 2231     | Propeptide | T  | C   | L744P               |
| 2373     | D'         | G  | A   | —                   |
| 2487     | D'         | C  | T   | —                   |
| 2493     | D'         | T  | C   | —                   |
| 2502     | D'         | A  | G   | —                   |
| 2583     | D'         | T  | C   | —                   |
| 3969     | A1         | T  | C   | —                   |
| 4311     | A1         | T  | C   | —                   |
| 7148     | B3         | T  | C   | —                   |
| 8390     | CK         | C  | T   | —                   |

mRNA was isolated from WSB lung and reverse transcribed, and polymerase chain reaction was performed to isolate fragments of *Vwf* for sequencing. B6 sequence was obtained from NCBI Build 37 of the mouse genome. VWF domains were deduced by comparison to the human amino acid sequence.

— indicates there is no amino acid change caused by a SNP.



**Figure 5. Allele-specific expression analysis of *Vwf* in (B6 × WSB) F1 mice.** Adult lung cDNA was prepared from F1 mice (n = 3 males and 3 females). Polymerase chain reaction was performed with exonic primers flanking a T/C SNP, followed by primer extension with a fluorescently labeled primer and dATP, dGTP, dTTP, and ddCTP, which results in differentially sized products in each strain (A). Fluorescent primer extension products were separated, quantitated, and compared with genomic standards (B). B6 and WSB genomic DNA were mixed in various proportions to produce the standard curve. Results are expressed as a percentage of B6 transcripts from total transcripts. Linear regression was performed with Microsoft Excel.

of *Vwf* gene mutations may be common among all mammalian species. These observations, together with the highly variable levels of plasma VWF in humans<sup>45</sup> and mice (Lemmerhirt et al<sup>17</sup> and Johnsen et al<sup>31</sup>; Figure 1), might be explained by common selective pressures, potentially including interactions with infectious pathogens. Consistent with this hypothesis, analysis of *B4galnt2* (*Mvwl1*) in wild mouse populations suggests that this locus is under selective pressure.<sup>46</sup> These observations also suggest that the genetic regulation of plasma VWF in the inbred

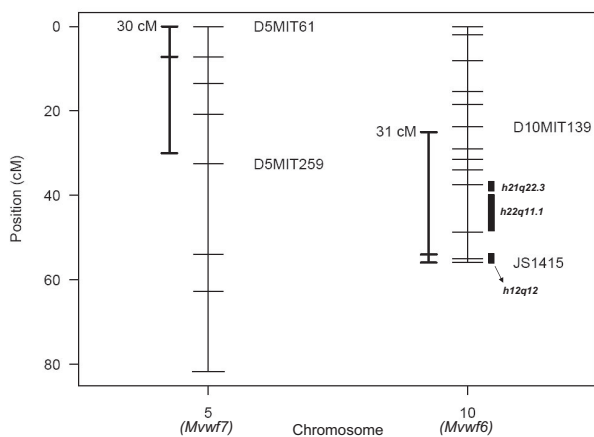
laboratory mouse is complex and probably involves a large number of genes. The corresponding picture in the outbred human population is probably more complex and even more difficult to approach experimentally.

The orthologous region of *Mvwl6* overlaps with a significant VWF QTL identified in the GAIT study,<sup>35</sup> as well as regions identified as potential human VWD modifiers in a large Amish pedigree.<sup>36</sup> These human loci are distinct from those showing potential conservation of synteny with *Mvwl3* and *Mvwl4*.<sup>32</sup> Thus, analyses of inbred mouse strains have identified 5 potential modifier loci outside of the *Vwf* gene, *Mvwl1*,<sup>18</sup> *Mvwl3-4*,<sup>32</sup> and *Mvwl6-7* (this report), 3 of which display conservation of synteny with potential human modifier loci. Although the overlapping regions are still quite large, positional cloning of these VWF modifier genes in the mouse should identify candidate genes for the modification of bleeding and thrombotic risk in humans.

### Acknowledgments

We thank the National Heart, Lung, and Blood Institute Mammalian Genotyping Service; the University of Michigan Sequencing and Genotyping Core; and Dave Siemieniak, Tricia Hayes, Diana Keung, Jennifer Myaeng, Susan Spaulding, Beverly Twiss, and Alok Swaroop for technical assistance.

This work was supported by the National Institutes of Health (R37-HL39693 and P01-HL057346, D.G.; and R01-GM074244, K.W.B.), the American Heart Association (0675025N, J.A.S.), the National Hemophilia Foundation Clinical Fellowship Program (J.A.S.), the National Institute of Child Health and Human Development (HD028820, J.A.S.), and 2 National Science Foundation Graduate Fellowship Awards (H.L.L. and A.M.). D.G. is an investigator of the Howard Hughes Medical Institute.



**Figure 6. *Mvwl* QTL 96.5% Bayes credible intervals.** Chromosomes are represented as vertical bars, and ticks represent markers used in mapping. To the left of each chromosome is the 96.5% Bayes credible interval constructed as described.<sup>25</sup> To the right of chromosome 10 are the regions with human homology of synteny to significant regions identified in human studies 22q11.1,<sup>35</sup> 21q22.3, and 12q12.<sup>36</sup> JS1415 is a marker derived from a B6/WSB T/G SNP downstream from the *Vwf* gene (128.3 Mbp) that produces an *MspI* restriction fragment polymorphism.

## Authorship

Contribution: J.A.S. participated in research design, performed the research, and wrote the paper; A.M. aided in data analysis and wrote the paper; H.L.L. participated in research design and performed the research; K.W.B. aided in data analysis and wrote the paper; and D.G. participated in research design and wrote the paper.

## References

1. Vischer UM. von Willebrand factor, endothelial dysfunction, and cardiovascular disease. *J Thromb Haemost*. 2006;4(6):1186-1193.
2. Koster T, Blann AD, Briët E, Vandenbroucke JP, Rosendaal FR. Role of clotting factor VIII in effect of von Willebrand factor on occurrence of deep-vein thrombosis. *Lancet*. 1995;345(8943):152-155.
3. Kraaijenhagen RA, Anker PSI, Koopman MMW, et al. High plasma concentration of factor VIII is a major risk factor for venous thromboembolism. *Thromb Haemost*. 2000;83(1):5-9.
4. Rosendaal FR. High levels of factor VIII and venous thrombosis. *Thromb Haemost*. 2000;83(1):1-2.
5. Levy GG, Ginsburg D. Getting at the variable expressivity of von Willebrand disease. *Thromb Haemost*. 2001;86(1):144-148.
6. Sadler JE. Von Willebrand disease type 1: a diagnosis in search of a disease. *Blood*. 2003;101(6):2089-2093.
7. Vossen CY, Hasstedt SJ, Rosendaal FR, et al. Heritability of plasma concentrations of clotting factors and measures of a prethrombotic state in a protein C-deficient family. *J Thromb Haemost*. 2004;2(2):242-247.
8. Souto JC, Almasy L, Borrell M, et al. Genetic determinants of hemostasis phenotypes in Spanish families. *Circulation*. 2000;101(13):1546-1551.
9. de Lange M, Snieder H, Ariens RA, Spector TD, Grant PJ. The genetics of haemostasis: a twin study. *Lancet*. 2001;357(9250):101-105.
10. Orstavik KH, Magnus P, Reiser H, Berg K, Graham JB, Nance W. Factor VIII and factor IX in a twin population. Evidence for a major effect of ABO locus on factor VIII level. *Am J Hum Genet*. 1985;37(1):89-101.
11. Gill JC, Endres-Brooks J, Bauer PJ, Marks WJ, Montgomery RR. The effect of ABO blood group on the diagnosis of von Willebrand Disease. *Blood*. 1987;69(6):1691-1695.
12. Cumming A, Grundy P, Keeney S, et al. An investigation of the von Willebrand factor genotype in UK patients diagnosed to have type 1 von Willebrand disease. *Thromb Haemost*. 2006;96(5):630-641.
13. Eikenboom J, Van Marion V, Putter H, et al. Linkage analysis in families diagnosed with type 1 von Willebrand disease in the European study, molecular and clinical markers for the diagnosis and management of type 1 VWD. *J Thromb Haemost*. 2006;4(4):774-782.
14. James PD, Paterson AD, Notley C, et al. Genetic linkage and association analysis in type 1 von Willebrand disease: results from the Canadian type 1 VWD study. *J Thromb Haemost*. 2006;4(4):783-792.
15. Lanke E, Johansson AM, Hallden C, Lethagen S. Genetic analysis of 31 Swedish type 1 von Willebrand disease families reveals incomplete linkage to the von Willebrand factor gene and a high frequency of a certain disease haplotype. *J Thromb Haemost*. 2005;3(12):2656-2663.
16. Goodeve A. Genetics of type 1 von Willebrand disease. *Curr Opin Hematol*. 2007;14(5):444-449.
17. Lemmerhirt HL, Shavit JA, Levy GG, Cole SM, Long JC, Ginsburg D. Enhanced VWF biosynthesis and elevated plasma VWF due to a natural variant in the murine Vwf gene. *Blood*. 2006;108(9):3061-3067.
18. Mohlke KL, Purkayastha AA, Westrick RJ, et al. Mvfw1, a dominant modifier of murine von Willebrand factor, results from altered lineage-specific expression of a glycosyltransferase. *Cell*. 1999;96(1):111-120.
19. Haberichter SL, Castaman G, Budde U, et al. Identification of type 1 von Willebrand disease patients with reduced von Willebrand factor survival by assay of the VWF propeptide in the European study: molecular and clinical markers for the diagnosis and management of type 1 VWD (MCMMD-1VWD). *Blood*. 2008;111(10):4979-4985.
20. Millar CM, Riddell AF, Brown SA, et al. Survival of von Willebrand factor released following DDAVP in a type 1 von Willebrand disease cohort: influence of glycosylation, proteolysis and gene mutations. *Thromb Haemost*. 2008;99(5):916-924.
21. Lander ES, Botstein D. Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics*. 1989;121(1):185-199.
22. Broman KW, Sen S, Owens SE, Manichaikul A, Southard-Smith EM, Churchill GA. The X chromosome in quantitative trait locus mapping. *Genetics*. 2006;174(4):2151-2158.
23. Churchill GA, Doerge RW. Empirical threshold values for quantitative trait mapping. *Genetics*. 1994;138(3):963-971.
24. Sen S, Churchill GA. A statistical framework for quantitative trait mapping. *Genetics*. 2001;159(1):371-387.
25. Manichaikul A, Dupuis J, Sen S, Broman KW. Poor performance of bootstrap confidence intervals for the location of a quantitative trait locus. *Genetics*. 2006;174(1):481-489.
26. Ihaka R, Gentleman R. R: a language for data analysis and graphics. *J Comput Graph Stat*. 1996;5:299-314.
27. Broman KW, Wu H, Sen S, Churchill GA. R/qtl: QTL mapping in experimental crosses. *Bioinformatics*. 2003;19(7):889-890.
28. Buchner DA, Trudeau M, Meisler MH. SCNM1, a putative RNA splicing factor that modifies disease severity in mice. *Science*. 2003;301(5635):967-969.
29. Battey J, Jordan E, Cox D, Dove W. An action plan for mouse genomics. *Nat Genet*. 1999;21(1):73-75.
30. Waterston RH, Lindblad-Toh K, Birney E, et al. Initial sequencing and comparative analysis of the mouse genome. *Nature*. 2002;420(6915):520-562.
31. Johnsen JM, Levy GG, Westrick RJ, Tucker PK, Ginsburg D. The endothelial-specific regulatory mutation, Mvfw1, is a common mouse founder allele. *Mamm Genome*. 2008;19(1):32-40.
32. Lemmerhirt HL, Broman KW, Shavit JA, Ginsburg D. Genetic regulation of plasma von Willebrand factor levels: quantitative trait loci analysis in a mouse model. *J Thromb Haemost*. 2007;5(2):329-335.
33. Nichols WC, Lyons SE, Harrison JS, Cody RL, Ginsburg D. Severe von Willebrand disease due to a defect at the level of von Willebrand factor mRNA expression: detection by exonic PCR-restriction fragment length polymorphism analysis. *Proc Natl Acad Sci U S A*. 1991;88(9):3857-3861.
34. The Jackson Laboratory. Mammalian Orthology. <http://www.informatics.jax.org/orthology.shtml>. Accessed January 29, 2009.
35. Souto JC, Almasy L, Soria JM, et al. Genome-wide linkage analysis of von Willebrand factor plasma levels: results from the GAIT project. *Thromb Haemost*. 2003;89(3):468-474.
36. Di Paola J, Rickard M, Murray J, Burns T, Wang K, Shapiro A. A genome-wide linkage scan of a large Amish pedigree with von Willebrand disease (VWD) identified several chromosomal regions that may contain potential modifiers of von Willebrand factor (VWF) levels and disease variability [abstract]. *Blood*. 2006;108(11):Abstract 176.
37. Andrew M, Paes B, Milner R, et al. Development of the human coagulation system in the full-term infant. *Blood*. 1987;70(1):165-172.
38. Cunningham MJ, Sankaran VG, Nathan DG, Orkin SH. The thalassemias. In: Orkin SH, Nathan DG, Ginsburg D, Look AT, Fisher DE, Lux SE, eds. *Nathan and Oski's Hematology of Infancy and Childhood*. 7th ed. Philadelphia, PA: Saunders/Elsevier; 2009:1015-1108.
39. Goodeve A, Eikenboom J, Castaman G, et al. Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMMD-1VWD). *Blood*. 2007;109(1):112-121.
40. James PD, Notley C, Hegadorn C, et al. The mutational spectrum of type 1 von Willebrand disease: results from a Canadian cohort study. *Blood*. 2007;109(1):145-154.
41. Bernat JA, Crawford GE, Ogurtsov AY, Collins FS, Ginsburg D, Kondrashov AS. Distant conserved sequences flanking endothelial-specific promoters contain tissue-specific DNase-hypersensitive sites and over-represented motifs. *Hum Mol Genet*. 2006;15(13):2098-2105.
42. Kleinschmidt AM, Nassiri M, Stitt MS, et al. Sequences in intron 51 of the von Willebrand factor gene target promoter activation to a subset of lung endothelial cells in transgenic mice. *J Biol Chem*. 2008;283(5):2741-2750.
43. Hough C, Cameron CL, Notley CR, et al. Influence of a GT repeat element on shear stress responsiveness of the VWF gene promoter. *J Thromb Haemost*. 2008;6(7):1183-1190.
44. Ginsburg D, Bowie EJW. Molecular genetics of von Willebrand disease. *Blood*. 1992;79(10):2507-2519.
45. Nichols WC, Ginsburg D. von Willebrand disease. *Medicine (Baltimore)*. 1997;76(1):1-20.
46. Johnsen JM, Teschke M, Pavlidis P, et al. Selection on cis-regulatory variation at B4galnt2 and its influence on von Willebrand factor in house mice. *Mol Biol Evol*. 2009;26(3):567-578.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

The current affiliation for A.M. is Department of Biomedical Engineering, University of Virginia, Charlottesville, VA. The current affiliation for K.W.B. is Department of Biostatistics and Medical Informatics, University of Wisconsin, Madison, WI.

Correspondence: David Ginsburg, Howard Hughes Medical Institute, University of Michigan, 210 Washtenaw Ave, Life Sciences Institute, Rm 5036, Ann Arbor, MI 48109; e-mail: ginsburg@umich.edu.