

ORIGINAL ARTICLE

Novel factor VIII variants with a modified furin cleavage site improve the efficacy of gene therapy for hemophilia A

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Essentials

- Factor (F) VIII is an inefficiently expressed protein.
- Furin deletion FVIII variants were purified and characterized using *in vitro* and *in vivo* assays.
- These minimally modified novel FVIII variants have enhanced function.
- These variants provide a strategy for increasing FVIII expression in hemophilia A gene therapy.

Summary. *Background:* The major challenge for developing gene-based therapies for hemophilia A is that human factor VIII (hFVIII) has intrinsic properties that result in inefficient biosynthesis. During intracellular processing, hFVIII is predominantly cleaved at a paired basic amino acid cleaving enzyme (PACE) or furin cleavage site to yield a heterodimer that is the major form of secreted protein. Previous studies with B-domain-deleted (BDD) canine FVIII and hFVIII-R1645H, both differing from hFVIII by a single amino acid at this site, suggested that these proteins are secreted mainly in a single polypeptide chain (SC) form and exhibit enhanced function. *Objective:* We hypothesized that deletion(s) of the furin site modulates FVIII biology and may enhance its function. *Methods:* A series of recombinant hFVIII-furin deletion variants were introduced into hFVIII-BDD [Δ 1645, 1645-46(Δ 2), 1645-47(Δ 3), 1645-48(Δ 4), or Δ 1648] and characterized. *Results:* *In vitro*, recombinant purified Δ 3

and Δ 4 were primarily SC and, interestingly, had 2-fold higher procoagulant activity compared with FVIII-BDD. *In vivo*, the variants also have improved hemostatic function. After adeno-associated viral (AAV) vector delivery, the expression of these variants is 2–4-fold higher than hFVIII-BDD. Protein challenges of each variant in mice tolerant to hFVIII-BDD showed no anti-FVIII immune response. *Conclusions:* These data suggest that the furin deletion hFVIII variants are superior to hFVIII-BDD without increased immunogenicity. In the setting of gene-based therapeutics, these novel variants provide a unique strategy to increase FVIII expression, thus lowering the vector dose, a critical factor for hemophilia A gene therapy.

Keywords: factor VIII; furin; genetic therapy; hemophilia A; dependovirus.

Introduction

Deficiencies in factor VIII (FVIII) cause hemophilia A (HA), an X-linked bleeding disorder. The current treatment for hemophilia is protein replacement therapy, given either prophylactically or in response to bleeding. Gene-based approaches that would result in continuous FVIII expression have several advantages, including prevention of bleeding episodes, which would decrease the morbidity of the disease. Although the clinical trials for adeno-associated viral (AAV) vector-mediated delivery of factor IX (FIX) for hemophilia B show promising results with sustained FIX expression, the studies demonstrate that the vector dose may be limiting because of an anti-AAV immune response [1,2]. Thus, the safety of AAV-mediated clinical studies for HA will also depend on minimizing the AAV vector dose. Early studies of AAV-canine FVIII delivery in HA dogs suggest that the vector dose required to achieve therapeutic levels of FVIII may be higher than

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that used for AAV-FIX [3,4]. FVIII gene transfer has been challenging because of the relative inefficient expression of the FVIII protein. Thus, it is anticipated that success in HA gene therapy will be achieved with combined improvements in the transgene and vector that will result in improved expression. Identification of novel FVIII variants that have increased secretion and/or procoagulant activity to overcome these challenges may provide a strategy for lowering the vector dose in the setting of AAV delivery of FVIII.

FVIII is a cofactor for FIX that activates FX in the intrinsic pathway. FVIII has a domain structure of A1-A2-B-C1-C2. During intracellular processing, full length human FVIII (hFVIII) undergoes proteolysis at multiple sites, with the most predominant cleavage at the paired basic amino acid cleaving enzyme (PACE) or furin recognition motif (R-X-X-R) at the carboxy-terminus of the B-domain, giving rise to two polypeptide chains, the heavy chain and the light chain (Fig. 1). Through a metal ion-dependent association, these chains form a heterodimer that is the major secreted form of the hFVIII protein [5]. FVIII is cleaved by thrombin (IIa) at Arg372, Arg740 and Arg1689 to produce the active form of the protein [6,7]. Because the B-domain is not essential for the biological activity of the protein, a B-domain-deleted (BDD)

version of FVIII with only 14 residual amino acids that contain the furin cleavage site has been developed and used clinically over the last few decades [6,8].

Although human FVIII-BDD is secreted predominantly as a heterodimer [8,9], we observed that B-domain-deleted canine FVIII (cFVIII-BDD) is secreted primarily as a single-chain (SC) molecule [10]. In addition, cFVIII-BDD is more stable and has higher biological activity. Amino acid sequence analysis revealed that among the many differences between the canine and human FVIII amino acid sequences, a single amino acid difference resides between cFVIII (1645-HHQR-1648) and hFVIII (1645-RHQR-1648) at the furin cleavage recognition site that is highly conserved as RHQR in all other species analyzed. Characterization of hFVIII-R1645H demonstrated that this residue was important for secretion predominantly as a single polypeptide chain and contributes to its increased specific activity, which resembles, in part, the findings of cFVIII [11]. These data suggest that there is suboptimal cleavage of cFVIII and hFVIII-R1645H by furin. Furthermore, hFVIII-R1645H had enhanced hemostatic effects upon vascular injury and in the setting of AAV delivery was associated with increased circulating FVIII levels compared with wild-type hFVIII-BDD. To further investigate the role of this furin recognition site, we

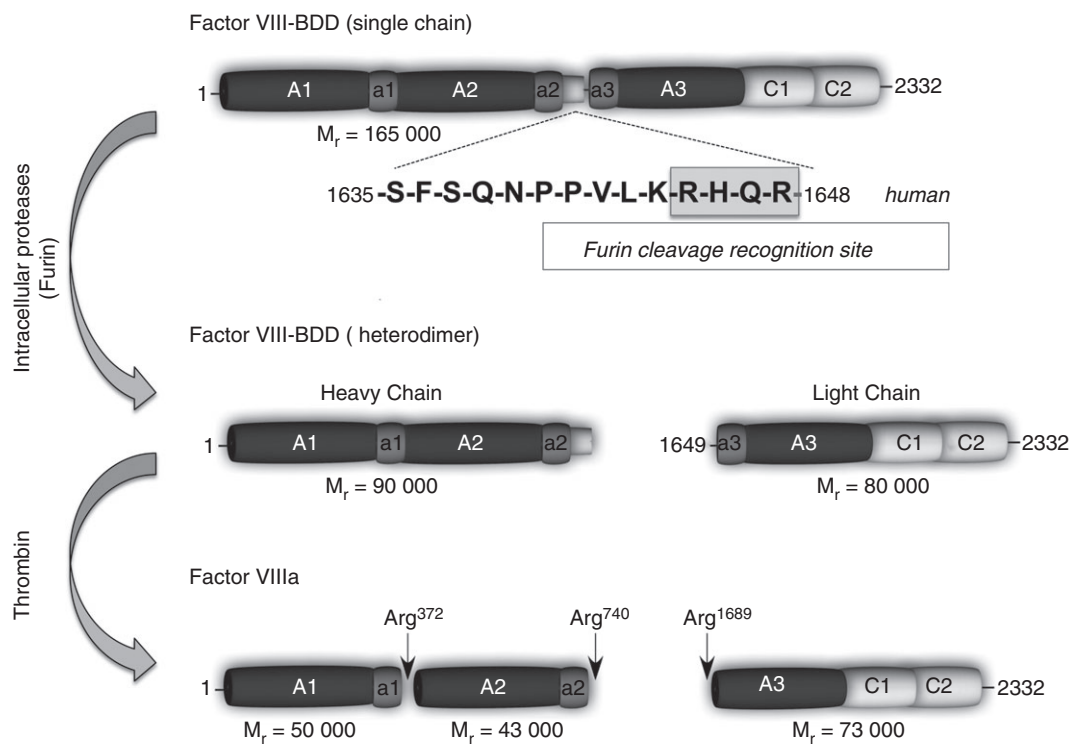


Fig. 1. Factor (F) VIII processing. FVIII has a domain structure that includes the A1-A2-B-A3-C1-C2 domains. The B domain is not required for procoagulant activity. In the B-domain-deleted form of FVIII (FVIII-BDD) there are 14 residual amino acids and within that region is the furin recognition motif (R-X-X-R). Furin cleaves after the arginine residue (R1648) to give rise to two polypeptide chains, the heavy chain (HC) and the light chain (LC). The HC and LC form a heterodimer that is the predominantly secreted form of FVIII in humans. Cleavage by thrombin (IIa) generates a heterotrimer, the activated form of the protein.

hypothesized that deletion of part or all of the furin cleavage recognition sequence may further alter the efficiency of cleavage at the site, resulting in a larger portion of single-chain molecules, and may lead to increased expression compared with hFVIII-R1645H. In this study, a series of furin deletion variant proteins were characterized *in vitro* and *in vivo* to further understand the role of this site in FVIII processing. These systematic studies reveal the critical residues at the furin site, deletion of which is essential for conferring higher biological activity and increased expression levels. The potential immunogenicity of these variants was studied with recombinant protein challenges in naïve and AAV-treated HA mice that are tolerant to hFVIII-BDD. Introduction of these variants into an AAV-hFVIII expression cassette demonstrates the advantage of these variants in a gene therapy setting.

Methods

Generation of stable baby hamster kidney (BHK) cell lines and protein purification

The FVIII variants were generated and purified as previously described [11–13].

Factor VIII activity

FVIII activity was determined by one- or two-stage activated partial thromboplastin time (APTT) [14]. A2 domain dissociation and intrinsic Xase assay were performed as previously described [10,11].

Surface plasmon resonance for assessment of affinity for VWF

The affinities of hFVIII variants for human plasma-derived von Willebrand factor (VWF) were determined by surface plasmon resonance (SPR) using a Biacore T200 (GE Healthcare, Piscataway, NJ, USA) at 25 °C. VWF [15] was immobilized on a CM5 sensor chip (GE Healthcare) via amine-coupling. hFVIII (0–40 nM) diluted in running buffer (140 mM NaCl, 20 mM HEPES, 10 mM CaCl₂ and 0.05% Tween 20, pH 7.4) was flowed through at 30 µL min⁻¹ for 300 s. hFVIII and VWF complex dissociation was observed for 600 s, before the surface was regenerated with 10 mM of glycine at pH 2.0 for 30 s, followed by a final 30-s buffer re-equilibration. Equilibrium dissociation constants (K_D) were determined by fitting all background-subtracted binding curves into a 1 : 1 binding model (Langmuir isotherm) using BIAevaluation Software (GE Healthcare).

N-terminal sequencing

N-terminal sequencing was performed by Dr Jan Pohl at the Biotechnology Core Facility Branch, Center for

Disease Control, Atlanta, GA, USA, using samples prepared as previously described [16].

Animal models

HA C57Bl6/129 (HA) mice [17,18] were used for the *in vivo* hemostatic challenge. Male HA/CD4 knockout mice (HA/CD4, C57BL/6) or HA/platelet hFVIII (HA/hF8, C57BL6/129) transgenic mice [19] were used for AAV studies. All procedures were approved by the Institutional Animal Care and Use Committee at The Children's Hospital of Philadelphia.

Administration of recombinant AAV vectors

The variants were introduced into a previously described pAAV-FVIII expression cassette [3]. Recombinant AAV serotype 8 vectors were produced using a triple transfection protocol as previously described [20]. Vector titers (vector genomes mL⁻¹; vg mL⁻¹) were determined by silver staining and quantitative PCR.

FVIII antigen and activity levels in vivo

FVIII antigen and activity levels in the mice were determined as previously described [11]. The variant proteins were tested alongside hFVIII-BDD on this ELISA and were determined to have similar affinity for these antibodies.

Results

Stable BHK cell lines expressing the human FVIII furin site deletion variants

Five deletion variants of the furin cleavage recognition site (1645–1648) were introduced into B-domain-deleted human FVIII (hFVIII-BDD) (Table 1). Stable BHK cell lines were established for each deletion variant and compared with a BHK cell line expressing wild-type hFVIII-BDD [10]. Recombinant protein was purified from the conditioned media using ion exchange chromatography. For hFVIII-BDD the yield was between 0.05 and 0.1 mg L⁻¹, whereas it was generally higher for the furin variants (Δ 1645, 0.27 mg L⁻¹; Δ 2, 0.16 mg L⁻¹; Δ 3, 0.34 mg L⁻¹; Δ 4, 0.31 mg L⁻¹; Δ 1648, 0.21 mg L⁻¹).

Following electrophoresis, the deletion variants migrated in a similar manner to hFVIII-BDD with the 160 kDa single-chain form of the protein, 90 kDa heavy chain and 80 kDa light chain (Fig. 2A). However, based on optical densitometry the ratio of the single chain vs. the heterodimer form was very different. The wild-type protein was predominantly a heterodimer (85%) with a small portion of the protein in the SC form (15%). The proportion of the protein in the SC form was 3- to 4-fold higher for the Δ 2 (57%), Δ 3 (53%) and Δ 4 (48%)

Table 1 Summary of furin deletion variants

hFVIII variant	PACE-furin site (1645-1648)	One-stage APTT ($\times 10^{-3}$ U mg ⁻¹)	Two-stage APTT ($\times 10^{-3}$ U mg ⁻¹)	Half-life of FVIIIa (min)	VWF affinity (K _D , nM)
hFVIII-SQ (WT)	R H Q R	9.6 \pm 0.6	361.1 \pm 24.6	1.0	0.7
hFVIII-del 1645 (Δ 1645)	- H Q R	8.2 \pm 0.3	436.2 \pm 11.8	1.1	1.4
hFVIII-del 1645-46 (Δ 2)	- - Q R	8.3 \pm 0.3	595.1 \pm 39.8	1.4	1.2
hFVIII-del 1645-47 (Δ 3)	- - -R	8.8 \pm 0.4	710.2 \pm 26.7	1.5	1.0
hFVIII-del 1645-48 (Δ 4)	- - - -	8.8 \pm 0.2	554.1 \pm 21.0	1.4	2.2
hFVIII-del 1648 (Δ 1648)	R H Q -	9.0 \pm 0.2	374.0 \pm 26.6	1.2	0.7

hFVIII, human FVIII; PACE, paired basic amino acid cleaving enzyme; APTT, activated partial thromboplastin time; VWF, von Willebrand factor.

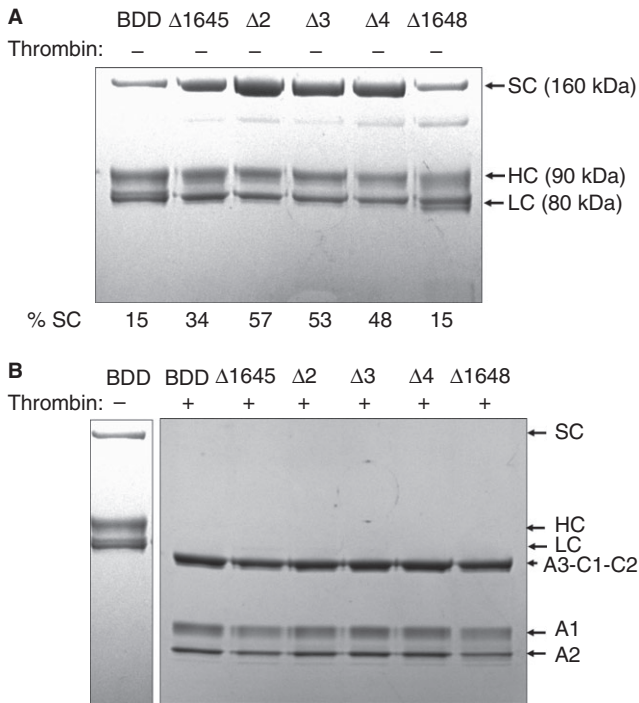


Fig. 2. SDS-PAGE gel analysis of furin deletion variants. (A) Purified protein (3 μ g) was loaded on a reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, followed by staining with Coomassie blue. Optical densitometry assessed by the TotalLab Quant software (Newcastle upon Tyne, UK) was utilized to measure the ratio of the single-chain (SC) to FVIII light chain (LC) and heavy chain (HC). (B) FVIII protein was activated with thrombin (10 nM) prior to loading on the gel.

variants. The Δ 1645 variant was 34% in the SC form, whereas the Δ 1648 variant (15%) was identical to hFVIII-BDD (15%). After complete cleavage by thrombin, the expected species were observed, suggesting that both the SC form and the heterodimer were cleaved by thrombin (Fig. 2B).

Although a significant portion of these variants was a single-chain polypeptide, some of the protein existed as a heterodimer. To determine the alternate site of cleavage, N-terminal sequencing was performed on the light chain fragment. The hFVIII-BDD light chain had a major species that was cleaved at R1648 and a minor species that

was cleaved at S1657 (Table S1). The Δ 2 variant also had both species. For deletion variants Δ 3 and Δ 4, the only species that could be detected was cleaved at S1657. These data suggest that in the absence of the furin site, cleavage occurs only at the downstream position at S1657.

Furin deletion variants have higher procoagulant activity

One-stage and two-stage APTT assays were used to determine the clotting activity of the recombinant proteins. In the one-stage APTT, no difference was detected between hFVIII-BDD and the variants (Fig. 3A). However, in the two-stage assay in which the protein was pre-activated with thrombin before the activity measurement, the specific activities of Δ 2, Δ 3 and Δ 4 were 1.5- to 2-fold higher than hFVIII-BDD, whereas Δ 1645 was 1.2-fold higher (Fig. 3B). Δ 1648 had activity similar to hFVIII-BDD. Western blot confirmed that the FVIII was completely activated under these conditions (data not shown). The increase in specific activity was confirmed in a purified system using an intrinsic Xase assay (Figure S1).

Because the increased specific activity may be related to the stability of the protein upon activation by thrombin, we measured the stability of the furin variants compared with hFVIII-BDD in an A2 domain dissociation study. Following thrombin activation, the A2 domain of FVIIIa rapidly dissociates, followed by a loss in cofactor activity. Initial data about the stability of the variant proteins were obtained using an APTT-based assay. In this assay, the half-life of activated hFVIII (1.0 min) was modestly increased for the variants [Δ 2 (1.4 min), Δ 3 (1.5 min), Δ 4 (1.5 min), Δ 1648 (1.2 min)]; however, Δ 1645 (1.1 min) was similar to hFVIII-BDD (Table 1). A2 domain dissociation studies were also performed in a purified system using a chromogenic Xase assay and similar findings were observed (Figure S2).

To determine whether differential activation by thrombin or FXa may explain the differences in the specific activity, kinetic studies of FVIII activation were performed. For these studies, the Δ 3 variant that had the highest activity in the two-stage APTT was compared with hFVIII-BDD protein that was purified under the same conditions. No

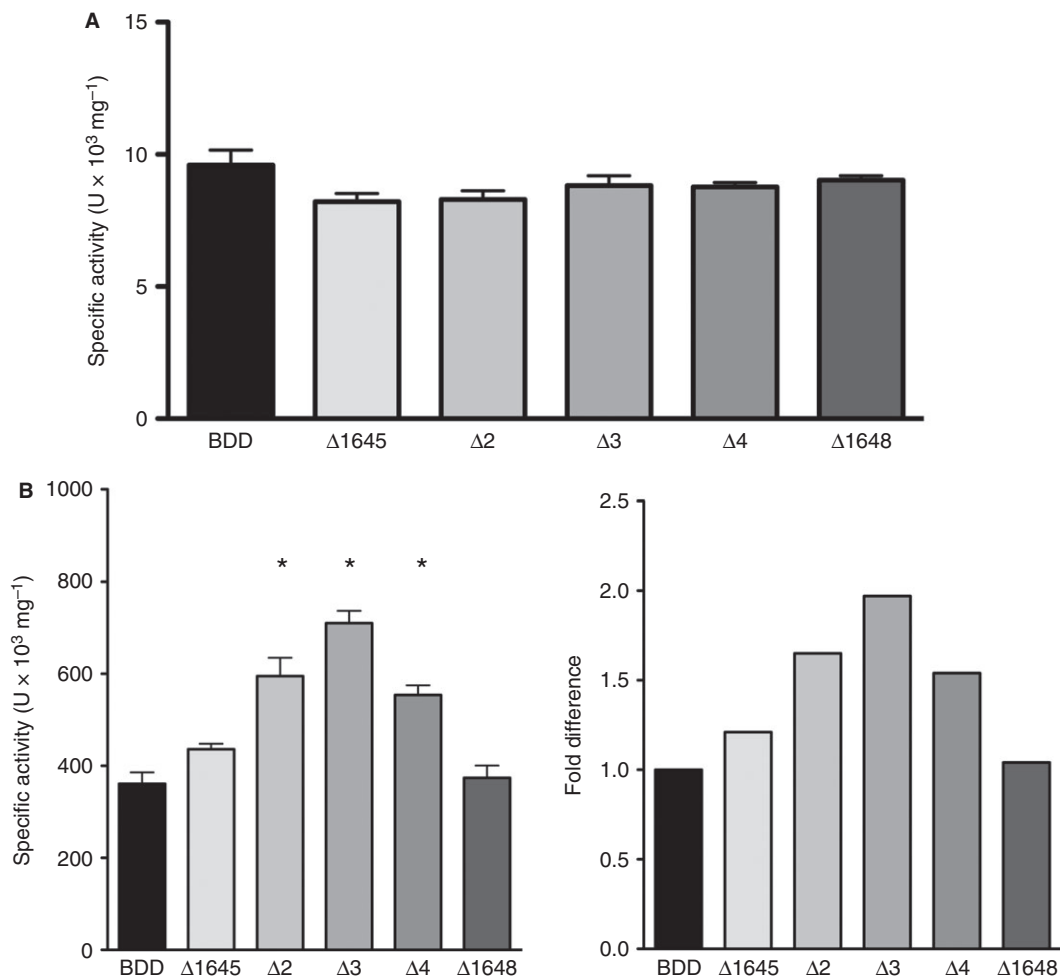


Fig. 3. Comparison of the activity of human FVIII (hFVIII) variants. (A) One-stage activated partial thromboplastin time (APTT). (B) Two-stage APTT. In the two-stage APTT, hFVIII (20 nM) was pre-activated with α -thrombin (40 nM) (Haematologic Technologies, Essex Junction, VT, USA) for 20 s. Hirudin (60 nM) (Sigma-Aldrich, St Louis, MO, USA) was used to quench the reaction and FVIIIa activity was measured (left panel). The fold difference in activity was compared with hFVIII-BDD (B-domain-deleted, BDD) (right panel). Statistically significant difference at $*P < 0.05$ using hFVIII-BDD values as the reference, as determined by one-way ANOVA with Tukey's post-tests. Data presented as mean \pm SEM.

difference was observed between hFVIII-BDD and $\Delta 3$ when activated by various concentrations of thrombin, or by the same concentration of thrombin over time, by Western blot (Figure S3). However, the interpretation of the Western blot studies is confounded by the different ratios of distinctly sized species between variants, which possess different blotting efficiencies. In addition, hFVIII and $\Delta 3$ were activated by FXa at a similar rate when studied using the intrinsic Xase assay (Figure S4). Thus, FVIII-BDD and $\Delta 3$ activation by thrombin proceeds at a similar rate, suggesting that the differences observed in the two-stage APTT are not likely to be due to differential activation by thrombin and FXa.

Assessment of the affinity of furin variants for VWF

VWF stabilizes the FVIII protein in the circulation, which is critical for protecting it from proteolysis [21].

Whereas these proteins were expressed and purified in the absence of VWF, the VWF in the plasma-based assays can influence the activity. To further understand if VWF may affect the proteolytic processing of the variants, we used surface plasmon resonance (SPR) to determine if there was any difference in affinity for VWF between the furin deletion variants and hFVIII-BDD (Figure S5). The K_D was 0.7 nM for hFVIII-BDD, 1.4 nM for $\Delta 1645$, 1.2 nM for $\Delta 2$, 1.0 nM for $\Delta 3$, 2.2 nM for $\Delta 4$ and 0.7 nM for $\Delta 1648$, demonstrating that the variants may have a similar affinity for VWF.

In vivo hemostatic challenges

In vivo assessment of the hemostatic function of the furin deletion variants was carried out using a tail clip bleeding assay. A pilot experiment was performed in HA mice at a range of doses (2–20 $\mu\text{g kg}^{-1}$) to determine an

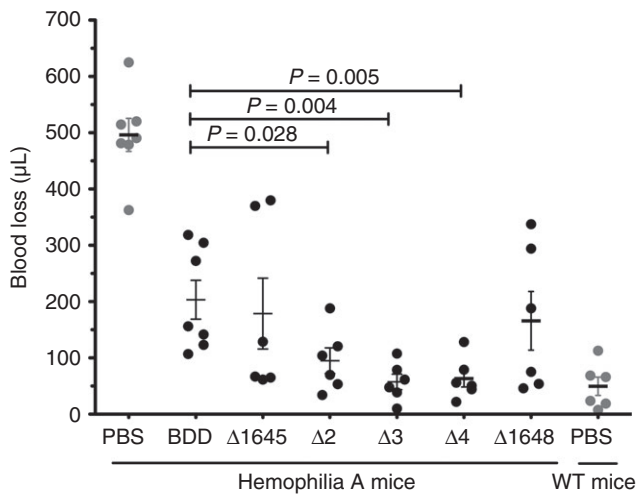


Fig. 4. *In vivo* hemostatic challenge using tail clip bleeding assay. Hemophilia A (HA) mice were infused in the tail vein with hFVIII-BDD ($10 \mu\text{g kg}^{-1}$) or a variant ($\Delta 1645$, $\Delta 2$, $\Delta 3$, $\Delta 4$, $\Delta 1648$) ($10 \mu\text{g kg}^{-1}$) or phosphate buffered saline (PBS) and compared with wild-type mice (PBS). Ten minutes after infusion of the protein, the tail was transected at 3 mm diameter and blood was collected from the tail for 10 min in warm saline. Blood loss was quantitated by lysing the red cell pellet and measuring the absorbance at 575 nm [40]. hFVIII, human FVIII; BDD, B-domain-deleted.

hFVIII-BDD dose that resulted in a partial correction of the bleeding phenotype based on blood loss compared with wild-type mice. Based on these initial studies, HA mice were infused with $10 \mu\text{g kg}^{-1}$ of protein followed by a complete tail transection 10 min post-injection. The blood loss after infusion of $\Delta 3$ ($57 \mu\text{L} \pm 14$) and $\Delta 4$ ($63 \mu\text{L} \pm 15$) was similar to wild-type mice expressing endogenous FVIII ($50 \mu\text{L} \pm 16$) and was significantly reduced compared with hFVIII-BDD ($203 \mu\text{L} \pm 35$) or phosphate buffered saline (PBS) ($496 \mu\text{L} \pm 29$) (Fig. 4). The $\Delta 2$ ($95 \mu\text{L} \pm 23$), $\Delta 1645$ ($179 \mu\text{L} \pm 63$) and $\Delta 1648$ ($166 \mu\text{L} \pm 52$) variants had reduced blood loss compared with HA mice. These data suggest that $\Delta 2$, $\Delta 3$ and $\Delta 4$ have enhanced efficacy compared with hFVIII-BDD upon hemostatic challenge in the macro-circulation.

Expression of furin deletion variants in the setting of AAV delivery

The hFVIII deletion variants were introduced into a liver-specific AAV vector expression cassette that we previously used to deliver hFVIII-BDD to HA mice (Fig. 5A) [3]. HA/CD4 knockout mice were delivered AAV8-hAAT-hFVIII (5×10^{11} vg per mouse) of each variant and peripheral blood was collected for analysis of hFVIII antigen and activity levels. The best performing variants in the AAV setting were $\Delta 3$, $\Delta 4$ and $\Delta 1645$, which expressed 2- to 3-fold higher than hFVIII-BDD (Fig. 5B). The FVIII activity was in agreement with the antigen results (Fig. 5C). DNA copy number analysis of

the variants showed that the animals received similar transgene copies per cell. The copy numbers for each group of mice were: 60.5 ± 8.2 per diploid genome for hFVIII-BDD, 51.5 ± 13.9 for $\Delta 1645$, 39.7 ± 9.6 for $\Delta 2$, 87.5 ± 14.1 for $\Delta 3$, 58.4 ± 14.0 for $\Delta 4$ and 44.0 ± 6.7 for $\Delta 1648$. Together these data demonstrate that the high levels of hFVIII expression after AAV delivery were due to the introduction of the furin deletion variants into the transgene.

Comparison of furin deletion variants with R1645H

To determine if the furin site deletion variants had increased activity and secretion compared with the hFVIII-R1645H variant that we previously described [11], the deletion variants with the highest activity and secretion were compared with R1645H. In the one-stage assay, the specific activity was similar for BDD, R1645H and $\Delta 4$ (Fig. 6A). However, in the two-stage APTT assay (Fig. 6B) both R1645H and $\Delta 4$ had higher activity than hFVIII-BDD. On SDS-PAGE gel, the proportion of the single chain was 34% for R1645H and 33% for $\Delta 1645$, compared with 15% for hFVIII-BDD (Fig. 6C). The $\Delta 3$ and $\Delta 4$ furin deletion variants had a higher proportion of the single chain, 45% and 44%, respectively. After AAV-hFVIII delivery in HA/CD4 KO mice, the deletion variants ($\Delta 3$, $\Delta 4$ and $\Delta 1645$) were secreted at 1.5- to 2-fold higher levels than R1645H, further demonstrating their advantage in the setting of gene therapy (Fig. 6D).

Potential immunogenicity of factor VIII variants

To determine if deletions of the furin cleavage site may change the immunogenicity of the molecule compared with wild-type hFVIII, we tested if the variants break tolerance in a mouse model that is tolerant to hFVIII-BDD. In these studies, we used an immunocompetent HA mouse model that is transgenic for ectopic expression of hFVIII-BDD in platelets (HA/hF8) but has no detectable circulating FVIII antigen or activity [19]. When HA/hF8 mice were challenged with recombinant hFVIII-BDD protein (Xyntha) alongside non-transgenic HA mice, only the HA mice developed an anti-hFVIII antibody response. After eight weekly ($1 \mu\text{g}$) protein challenges, the anti-hFVIII IgG antibody titers were $50.9 \mu\text{g mL}^{-1} \pm 16.7 \mu\text{g mL}^{-1}$ ($n = 5$) for HA mice, whereas the HA/pF8 mice ($1.0 \mu\text{g mL}^{-1} \pm 0.2$) ($n = 5$) had IgG titers similar to baseline levels. In contrast, both the HA and HA/pF8 mice challenged with cFVIII protein developed anti-cFVIII antibody titers between 52 and $57 \mu\text{g mL}^{-1}$, demonstrating that these mice are immunocompetent and that the tolerance is specific to hFVIII, as expected.

Transgenic HA/hF8 mice were challenged using a stringent strategy in which each recombinant FVIII protein variant ($5 \mu\text{g}$) in Complete Freund's adjuvant (CFA) followed by analysis of FVIII-specific IgG at 4 and 8 weeks

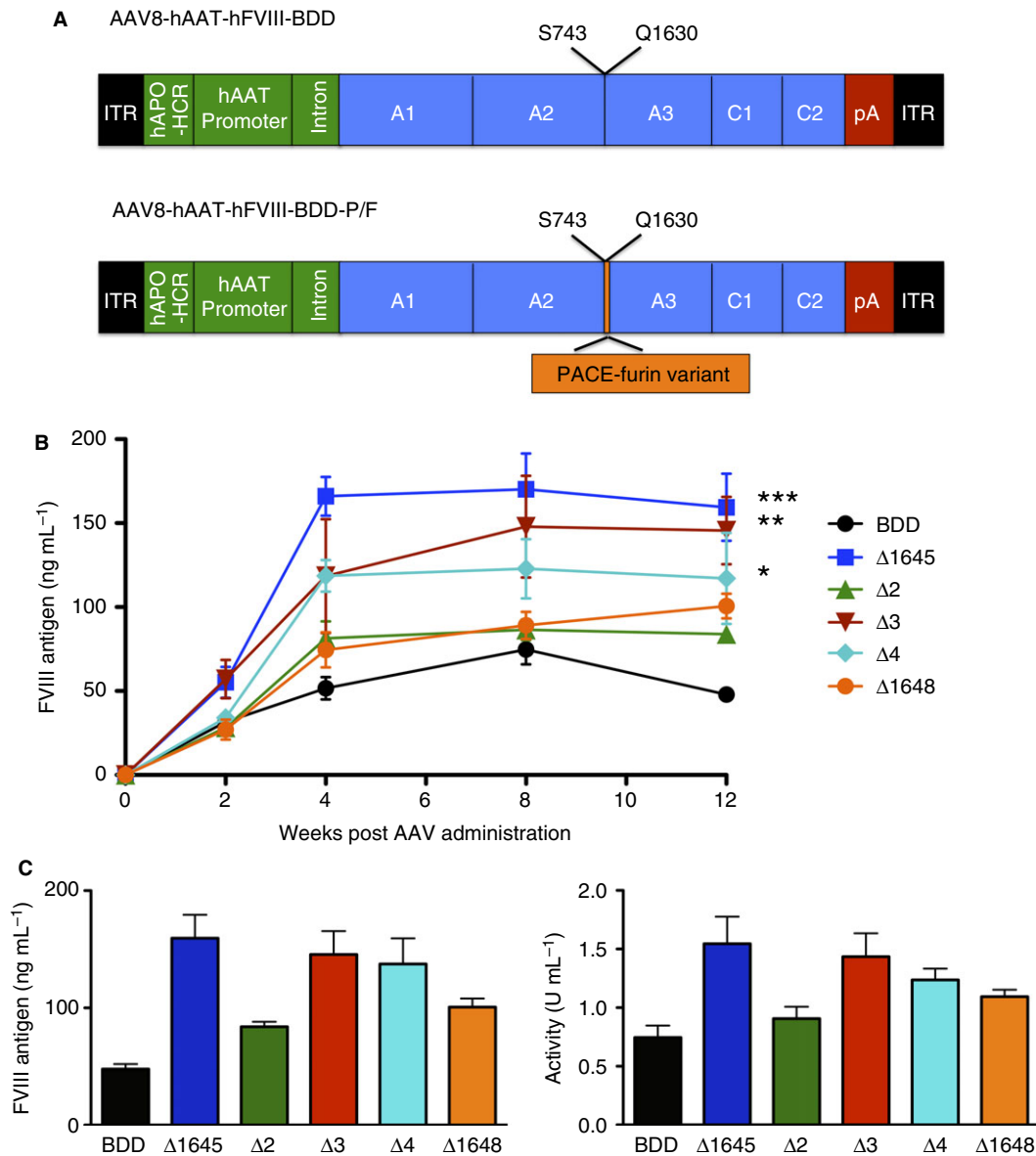


Fig. 5. Adeno-associated viral (AAV) administration of human FVIII (hFVIII) and hFVIII furin deletion variants to hemophilia A/CD4 knockout mice. (A) AAV expression cassettes. (B) AAV8 (5×10^{11} vg per mouse) was delivered intravenously to HA/CD4 KO mice ($n = 4-6$ per variant) at 8–10 weeks of age. Peripheral blood was collected at 2, 4, 8 and 12 weeks post-vector administration. ELISA was used to determine the levels of hFVIII antigen in the circulation at the different time-points after AAV administration. Two-way ANOVA with a repeated measures factor showed that there was a significant difference between the variants ($P < 0.0001$). Bonferroni post-tests suggest a significant difference between hFVIII-BDD and $\Delta 1645$ ($***P < 0.001$), hFVIII-BDD and $\Delta 3$ ($**P < 0.01$) and hFVIII-BDD and $\Delta 4$ ($*P < 0.05$). Data presented as mean \pm SEM. (C) hFVIII antigen and activity correlate after AAV delivery of the furin variants. Antigen was determined by ELISA and activity was determined by Coatest assay. Shown are data from the 12-week time-point. BDD, B-domain-deleted.

post-challenge. All of the mice that were challenged with hFVIII-BDD or a variant did not have an increase in the anti-FVIII-specific IgG after challenge compared with baseline levels (Fig. 7A). HA/hF8 mice challenged with canine FVIII in CFA had anti-cFVIII IgG titers of $1 \mu\text{g mL}^{-1}$ at baseline that increased to $55 \mu\text{g mL}^{-1}$ at 4 weeks and $65 \mu\text{g mL}^{-1}$ at 8 weeks, further demonstrating the specificity of the tolerance to hFVIII.

Another cohort of HA/hF8 mice was administered AAV8-hFVIII-BDD or AAV8-hFVIII-BDD-furin variant (5×10^{11} vg per mouse). The levels of FVIII expression in the AAV-treated mice were similar to the antigen levels observed in the HA/CD4 mice (data not shown). After AAV delivery, the anti-FVIII IgG titers did not increase in animals treated with the hFVIII-BDD-furin proteins, suggesting that the variants did not break tolerance to

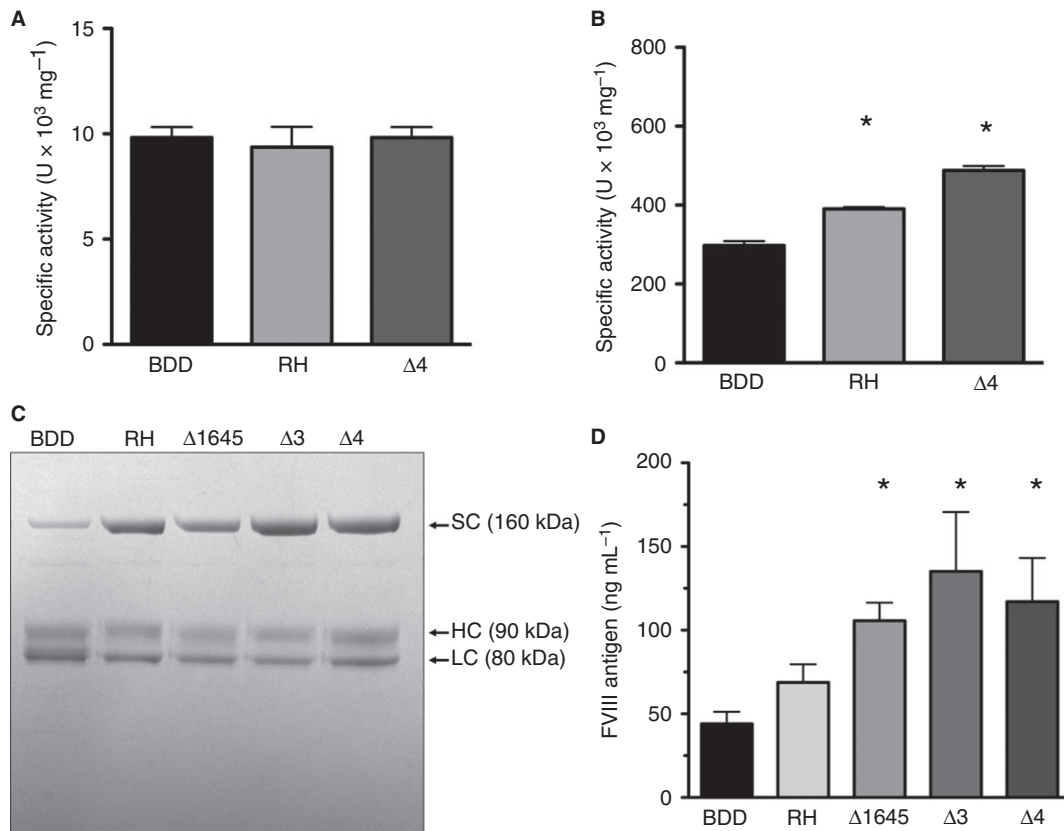


Fig. 6. Comparison of R1645H with the furin deletion variants. (A) One-stage activated partial thromboplastin time (APTT). (B) Two-stage APTT. (C) Purified protein (3 μ g) was loaded on the SDS-PAGE gel under reducing conditions. (D) AAV8 (5×10^{11} vg per mouse) was delivered intravenously to HA/CD4 KO mice ($n = 4$ per variant) at 8–10 weeks of age. At 4 weeks post-vector administration the hFVIII antigen was detected by ELISA. Statistically significant difference at $*P < 0.05$ taking hFVIII-BDD values as the reference, as determined by two-tailed Student's *t*-test. hFVIII, human FVIII; BDD, B-domain-deleted.

hFVIII-BDD (Fig. 7B). Upon stringent challenge with the respective protein in CFA at 24 weeks post-vector administration, no HA/hF8 mice developed an anti-hFVIII immune response. These studies suggest that in animals that are tolerant to hFVIII-BDD, the furin variants are not more immunogenic than hFVIII-BDD.

Discussion

The major hurdle for gene-based therapeutics for hemophilia is the challenge of expressing therapeutic levels of clotting factor at a clinically relevant safe vector dose. Progress in the development of gene therapy for HA has been considerably slower than for hemophilia B because of the challenges of expressing FVIII. Even modest increases in expression may be critical for ensuring that gene therapy for hemophilia is ultimately realized. The development of FVIII variants with enhanced function in the setting of gene-based therapeutics is an important strategy for reducing the vector dose. Other variants of FVIII that confer increased expression have been described; however, they rely on inserting a non-native amino acid sequence into the B-domain [22] or replacing up to 10% of the human amino acid sequence with a

porcine FVIII sequence [23]. In contrast, the furin variants described here result in improved FVIII expression with a minimal modification (one to four amino acid deletion) that results in a new B-domain deleted form.

The furin deletion variants introduced into FVIII-BDD demonstrate that elimination of this cleavage site results in additional material in the uncleaved single-chain polypeptide form. This increase in the amount of protein in the single-chain form depended in part on the extent of the deletion. While the hFVIII-BDD and $\Delta 1648$ had the smallest proportions of the single-chain form (14%), the deletion of two to four residues ($\Delta 2$, $\Delta 3$, $\Delta 4$) resulted in the highest percentage of single chain (57%, 53% and 48%, respectively). Deletion of only the first arginine residue at 1645 resulted in an increase in the single-chain form that was similar to what we observed for R1645H. hFVIII-BDD had a predominant cleavage of the light chain at R1648; however, a minor species was also detected that was cleaved nine residues downstream from the furin site between S1657 and D1658 as previously observed [24]. Interestingly, elimination of the furin site ($\Delta 4$) did not completely prevent cleavage because there remained $\sim 50\%$ that was still processed into the heterodimer form. Although cleavage of $\Delta 2$ occurs at both sites, $\Delta 3$ and $\Delta 4$ were

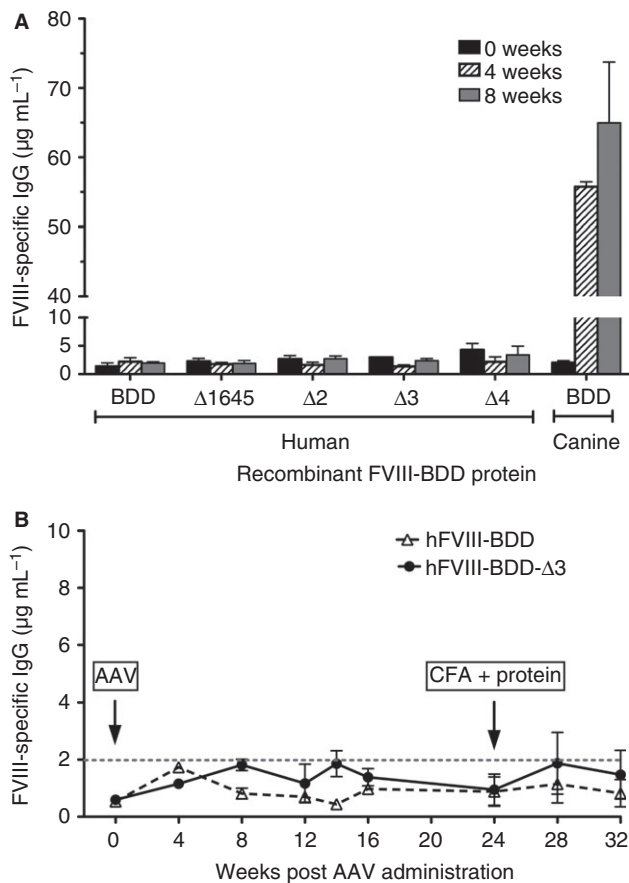


Fig. 7. Immunological studies of the furin deletion variants. (A) HA/pF8 mice ($n = 3/\text{group}$) were challenged subcutaneously with recombinant hFVIII-BDD or hFVIII-furin variant or cFVIII-BDD ($5 \mu\text{g}$) in Complete Freund's adjuvant (CFA). cFVIII-BDD served as a positive control for immune responses. Plasma samples were analyzed for anti-hFVIII-specific total IgG by ELISA at baseline, 4 weeks and 8 weeks post-challenge as previously described using recombinant hFVIII-BDD (Xyntha), specific hFVIII-BDD variant protein or cFVIII-BDD [3]. (B) Anti-FVIII IgG titers after AAV8 delivery of FVIII. Mice were treated with AAV8-hFVIII-BDD (hFVIII-BDD) or AAV8-hFVIII-BDD-furin variant. The $\Delta 3$ variant shown is representative of all of the variants (hFVIII-BDD- $\Delta 3$). The anti-FVIII-specific total IgG was determined by ELISA. At 24 weeks post-vector administration each mouse was challenged with the respective variant protein ($5 \mu\text{g}$) in CFA and anti-FVIII-specific IgG titers were determined at 4 and 8 weeks after CFA challenge. The dotted line represents the upper limit of IgG observed in untreated animals in this assay. hFVIII, human FVIII; BDD, B-domain-deleted; cFVIII, canine FVIII; AAV, adeno-associated viral.

exclusively cleaved at the downstream position, suggesting that the elimination of an arginine and the two internal residues of the consensus sequence is the minimal deletion needed to prevent cleavage at R1648. This downstream site does not contain the canonical R-X-X-R furin recognition sequence, suggesting that a different protease is responsible for cleavage at this position [25]. In the absence of the furin site, cleavage is not completely abolished but only occurs at the downstream site at position S1657. This leads to the

generation of a new heterodimer species. Thus, these $\Delta 3$ and $\Delta 4$ variants have two unique species, a single-chain form and a heterodimer form consisting of a light chain with an N-terminus at $\Delta 1658$, both lacking the furin site.

Importantly, the single-chain FVIII form is chemically identical to the two-chain form upon activation by thrombin. Single-chain forms of FVIII have been described in plasma-derived FVIII [26] and recombinant B-domain-deleted FVIII preparations [7,8,27–29]. These studies also observed that the protein is fully activated by thrombin. Recently, novel single-chain forms of FVIII that have increased affinity for VWF have been developed as a strategy for increasing the half-life of the molecule; however, this results in only a modest half-life extension [30–34]. It is important to consider that each of these single-chain forms is generated in a unique way (i.e. FVIII-Fc fusion protein or covalent bond between the heavy and light chains). The single-chain form that we describe lacks the furin region, becoming a slightly further deleted version of hFVIII-BDD.

The elimination of the furin site impedes proteolytic cleavage at R1648 yet confers higher biological activity both *in vitro* and *in vivo*. This increase in activity may be because of the modest increase in stability upon activation by thrombin. However, this difference in A2 stability alone is not likely to account for the 2-fold increase in specific activity observed in the two-stage APTT. Because the variants are chemically identical to hFVIII-BDD once fully activated, we hypothesized that the gain of activity may be influenced by the difference in activation kinetics. However, these studies are not conclusive and may not be sensitive enough to detect 2- to 3-fold differences. Because other single-chain molecules do not have higher biological activity [30,32], this suggests that it is not the increase in single-chain polypeptide but the absence of the furin site in the single-chain form and/or the heterodimer that confers higher biological activity. Further studies of the alternate cleavage site at S1657 may provide insight into whether the unique heterodimer form that results from cleavage at this site or the cleaved peptide contributes to the enhanced activity. These findings suggest that the proteolysis at this site may have a more complex role in the processing and function of FVIII.

The use of variants of FVIII for therapeutics raises concern over the potential immune response to the neoantigen. Interestingly, hemophilia patients are currently exposed to some single-chain FVIII as well as the heterodimer form in the current protein products, suggesting that the single-chain form will not be a neoantigen [7,8,27–29]. However, the furin deletion variants do represent a modified B-domain-deleted form with minimal differences and may potentially introduce new epitopes. HA mice that are tolerant of hFVIII-BDD provide an opportunity to test if the variants may be more immunogenic than hFVIII-BDD. After a stringent challenge with the recombinant protein in an adjuvant or after AAV delivery in this model, no antibodies to hFVIII were detected. In

contrast, challenge of these mice with canine FVIII-BDD resulted in high-titer anti-canine FVIII antibodies, demonstrating that these mice are immune competent and that the tolerance is transgene specific. Thus, these data suggest that the furin deletion variants are not more immunogenic than hFVIII-BDD.

The furin variants have increased expression compared with hFVIII-BDD after AAV delivery, suggesting that this site may be involved in the intracellular trafficking of hFVIII. Because furin cleavage occurs in the trans-Golgi network, this may create a bottleneck for trafficking out of the cell or may affect the engagement with chaperone proteins involved in secretion [35]. These studies demonstrate that the $\Delta 1645$, $\Delta 3$ and $\Delta 4$ deletions result in increased FVIII expression compared with R1645H and maximize the functional advantage of the furin site. These modifications can be amenable to other therapeutic approaches that target FVIII expression in other cell types, including platelets [36,37] and endothelial cells [38,39]. Combining the furin variants with other approaches such as codon-optimization, enhanced promoter elements or novel AAV vectors can yield an additive effect (D. Sabatino, unpublished data), providing strategies to further reduce the vector dose for AAV-mediated gene transfer. Together these data support the efficacy and safety of the furin variants for the development of protein or gene-based therapeutics.

Addendum

G. Nguyen purified the proteins, carried out the biochemical and *in vivo* experiments, performed data analysis and assisted with the manuscript preparation. L. George performed the immunology studies. J. Siner assisted with biochemical studies. R. Davidson assisted with the *in vivo* experiments. C. Zander performed the VWF studies and X. L. Zheng directed the experimental design and data interpretation of the VWF binding studies. V. Arruda and R. Camire assisted with the study design and editing and provided an intellectual contribution to the manuscript. D. Sabatino planned the studies, performed data analysis and interpretation, and prepared the manuscript.

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Disclosure of Conflict of Interests

D. Sabatino receives research funding from Pfizer and Spark Therapeutics, LLC. R. Camire receives licensing fees from Pfizer and research funding from Pfizer and Novo Nordisk. X. L. Zheng receives funding from Lee's Pharmaceuticals and is a member of the speakers' bureau for Alexion Biotechnological. V. Arruda and R. Camire have a patent, US8,816,054 B2, licensed to Spark Therapeutics. D. Sabatino and G. Nguyen have a patent, US2016/62/278,767, pending.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

- Table S1.** N-terminal sequencing of the hFVIII light chain.
Fig. S1. Specific activity of hFVIII-BDD and $\Delta 3$ in an intrinsic Xase assay.
Fig. S2. A2 domain dissociation of hFVIII-BDD and $\Delta 3$ in an intrinsic Xase assay.
Fig. S3. Western blot of factor VIII activated by thrombin.
Fig. S4. Factor (F) Xa activation of FVIII.
Fig. S5. Binding curves of the factor VIII variants to von Willebrand factor (VWF).

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