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# **Molecular characterization of two novel cases of complete complement inhibitor Factor I deficiency.**

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*Abbreviations:* C3, third complement factor; C4BP, C4b-binding protein; CR1, complement receptor 1; FB, Factor B; FH, Factor H; FI, Factor I; FIMAC, Factor I membrane attack complex; JIA, juvenile idiopathic arthritis; LDLr, low density lipoprotein receptor; MCP, membrane cofactor protein; SP, serine protease.

*Keywords:* Factor I, Complement system, primary immunodeficiency

## **Abstract**

Factor I (FI) is the major complement inhibitor that degrades activated complement components C3b and C4b in the presence of specific cofactors. Complete FI deficiency results in secondary complement deficiency due to uncontrolled spontaneous alternative pathway activation. In this study we describe two unrelated patients with complete FI deficiency and undetectable alternative complement pathway activity. Both patients were suffering from recurrent infections and arthralgia/arthritis. In one patient, analysis of genomic DNA revealed deletion of two adenine nucleotides in exon 2 of the *CFI* gene (c.133-134delAA), causing a frame shift and premature STOP codon/termination in the FIMAC (FI-membrane attack complex) domain (p.K45SfsX11). The other patient carried an A>T substitution in exon 6 (c.866A>T) encoding the LDLr2 (low density lipoprotein receptor) domain (p.D289V), resulting in an aspartic acid to valine change. Both patients were homozygous for the mutations while their healthy parents were heterozygous carriers. The mutations were introduced into recombinant FI, causing lack of FI expression and secretion upon transient transfection. Mutation p.K45SfsX11 theoretically allows expression of a 55 amino acid fragment of FI that lacks the serine protease domain, preventing proteolytic activity. In contrast, aspartic acid D289 is crucial for folding of FI. This report describes the molecular and functional consequences of two novel mutations of FI, providing a unique insight into the pathogenesis of complete FI deficiency in these patients.

## 1. Introduction

The complement system plays a major role in defense against pathogens. It also identifies dying cells (Ricklin et al., 2009) and guides adaptive immunity (Markiewski and Lambris, 2007). The physiological relevance of complement is demonstrated by illnesses affecting complement deficient patients such as recurrent infections, autoimmune diseases and kidney diseases (Pettigrew et al., 2009; Welch and Blystone, 2009). Invading pathogens activate complement either spontaneously, due to differences in surface composition compared to host cells (alternative and lectin pathways), or through antibody binding (classical pathway) (Lambris et al., 2008). This leads to initiation of a proteolytic cascade, release of pro-inflammatory anaphylatoxins (C5a, C3a) that attract white blood cells, opsonisation of the target with C3b and finally formation of the membrane attack complex (MAC). The destructive potential of complement against the host is tightly controlled by inhibitors. Some of these inhibitors circulate in blood while others are expressed on the cell surface. C4b-binding protein (C4BP) is the major soluble inhibitor of the classical and lectin pathways whereas factor H (FH) inhibits the alternative pathway. Membrane bound complement inhibitors include membrane cofactor protein (MCP, CD46) and complement receptor 1 (CR1) (Kim and Song, 2006; Zipfel and Skerka, 2009). These inhibitors promote degradation of activated complement factors C3b or C4b by the serine protease factor I (FI). The inhibitors regulate complement in such a way that complement is only active when required, to prevent tissue damage and systemic depletion. Activation and inhibition are finely balanced and a slight imbalance may result in tissue damage and autoimmunity, or recurrent infection (Sjoberg et al., 2009).

FI is an 88 kDa glycoprotein that circulates in blood and inhibits all pathways of complement. It degrades activated complement factors C4b and C3b, but only when they are bound to a cofactor such as FH, C4BP, CR1 or MCP (Sim et al., 1993). Structurally, FI is a heterodimer of a heavy chain consisting of one FI-membrane attack complex (FIMAC) domain, one CD5 domain and two low-density lipoprotein receptor domains (LDLR), and a light chain containing the serine protease, linked together by a disulfide bond. Although the three dimensional structure of FI has not been determined, we have constructed models of all domains of FI (Nilsson et al., 2009). The FI gene is located on chromosome 4q25 and spans 63 kb. It comprises 13 exons with exons 1-8 encoding the heavy chain and exons 9-13 the serine protease region (Goldberger et al., 1987). Complete FI deficiency is associated with recurrent infections and in some cases also with glomerulonephritis and autoimmune diseases such as systemic lupus erythematosus (Nilsson et al., 2007; Vyse et al., 1996). Heterozygous deficiency of FI predisposes to atypical haemolytic uremic syndrome (Bienaime et al., 2010; Nilsson et al., 2010a; Richards et al., 2001). In the current study we characterize the molecular defects of FI in two patients with complete FI deficiency carrying novel mutations in the *CFI* gene.

## 2. Materials and methods

### 2.1. Patients and their families

Patients from two unrelated families from Australia and Turkey were the focus of this evaluation. Patient 1 was a 14 years old Caucasian Australian boy from nonconsanguineous parents presenting with a history of recurrent infections with *S. pneumoniae* and *S. pyogenes* (Table 1). Infections included septicaemia, joint infections and pneumonias. He

also had a history of juvenile idiopathic arthritis (JIA) from ages 2 to 3.5 years. There was no history of meningitis or renal disease. Family history included ankylosing spondylitis and psoriasis in the maternal grandfather and a distant maternal relative who died at 4 years from JIA. He had absent alternative pathway activity, reduced classical pathway activity, low C3 but normal C4 levels, undetectable Factor B (FB) and a low concentration of FH, confirmed in two separate evaluations (Table 2). The patient's 10 years old brother, who had a history of recurrent otitis media but was otherwise well, also had absent alternative pathway activity, reduced classical pathway activity, low C3 with normal C4, undetectable FB and low FH. FI was undetectable in the index case and his younger brother, low in his sister and mother, and normal in his older brother.

Patient 2 was an 18 years old Turkish girl of consanguineous parents (first cousins), with a history of recurrent upper and lower respiratory tract infections and recurrent vasculitic eruptions and arthralgias (Table 3). One of her sisters suffered from recurrent upper respiratory tract infections and recurrent pyogenic infections during childhood, without autoimmune manifestations. Two other sisters were healthy. She had absent alternative pathway activity, low classical pathway activity, low C3 but normal C4 concentrations, low levels of FB, FH and properdin, and undetectable FI (Table 3). Her parents, who were well, had normal FI concentrations.

Blood samples from both patients and their family members were obtained with informed consent and the study was approved by the ethics committee of Lund University. Unfortunately samples from the father of Patient 1 and the three sisters of Patient 2 were not available for analysis.

## *2.2. Sequencing of genomic DNA*

Direct sequencing of all 13 *CFI* exons was performed as described (Fremaux-Bacchi et al., 2004). In brief, the DNA was extracted from the whole blood using QIAmp DNA Blood mini kit (Qiagen) and PCR products were sequenced with the Big Dye® Terminator Cycle Sequencing Kit (Applied Biosystems), followed by purification with the DyeEx 2.0 Spin kit (Qiagen) and analysis on the ABI 31 30xl Genetic Analyzer (Applied Biosystems). None of the described mutations were found in one hundred healthy controls. The numbering of the amino acid includes the 18-residue signal peptide commencing with the ATG initiation codon.

## *2.3. CFI cDNA clones for recombinant proteins*

Full-length cDNA encoding the human *CFI* was cloned into the eukaryotic expression vector pcDNA3 (Invitrogen) (Nilsson et al., 2007). The mutations found in Patients 1 and 2 (c.133-134delAA and c.866 A>T) were introduced using the QuikChange site-directed mutagenesis kit (Stratagene) using primers: c.133-134delAA fwd 5'-GCT CTT TTC CTT CAC TTA CTC ATC TTC-3'; and c.866 A>T fwd 5'-CTT ATG TTT GCA CAG CAT GCC AAG G-3' as well as their reverse counterparts (Eurofins MWG Operon). These mutations were confirmed by sequencing.

## *2.4. Transient transfection*

Transient transfection of HEK 293 cells (ATCC no 1573-CRL) with the construct for wild type (WT) FI as a positive control, the empty vector (pcDNA3) as a negative control and the two mutant FI constructs, was accomplished using lipofectin (Invitrogen). After collecting

the supernatants the cells were lysed in ice-cold solubilization buffer (1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% aprotinin and 2 mM phenylmethylsulfonyl fluoride; PMSF). FI concentrations were measured in the cell lysates and supernatants, by ELISA.

### 3. Results

#### 3.1. Genetic analysis of patients

Sequencing of genomic DNA of Patient 1 and his family revealed a deletion of two adenine nucleotides in exon 2 (c.133-134delAA) coding for the FIMAC domain, in the patient and his affected brother (Fig. 1B). This deletion caused a frame shift and premature STOP codon termination (p.K45SfsX11) in the FIMAC domain (Fig. 1A). His healthy brother did not carry the mutation (Fig. 1A) while his sister and mother were heterozygous carriers of the mutation (Fig. 1C).

Patient 2 carried a homozygous missense mutation (adenine to thymidine in exon 6; c.866A>T), changing D289 to V in the LDLr2 domain (Fig. 1A and 1B). Her parents were heterozygous carriers of this substitution (Fig. 1B and 1C).

#### 3.2. Expression and characterization of recombinant FI mutants

Since both mutations were associated with a complete lack of FI in plasma, we hypothesized that the mutations would affect the secretion of recombinant FI from cells. WT FI and the recombinant mutant constructs, as well as the empty pcDNA3 vector (mock), were transfected in a transient manner into HEK293 cells. When FI concentrations were measured in the cell supernatants and lysates, only WT FI could be detected in both supernatants and lysates. There was no increase in signal in the cells transfected with either mutant compared to the mock cells (Fig. 2A and 2B). This indicates that mutated FI was not secreted from the cells and most probably efficiently degraded intracellularly or not even translated.

### 4. Discussion

Both patients in this evaluation had a type I deficiency of FI in which no production or secretion of the protein was detected. In Patient 1, the mutation occurred in exon 2 by deletion of two adenine nucleotides at position 133-134, which causes a frame shift, a premature stop codon (TGA at positions 165-166-167) and termination of elongation. As a result of this mutation, the protein is severely truncated and probably not secreted. We did not detect any FI in cell lysates and supernatants that were transfected with FI carrying this mutation. However, even though we used two polyclonal antibodies in our ELISA method, it is possible that a severely truncated fragment of FI cannot be detected by this antibody combination. Importantly, it is certain that such a truncated FI completely lacks the serine protease domain and cannot act as a protease.

In Patient 2, the mutation occurred in exon 6 in the LDLr2 domain, changing D289 to V. This is a large change of amino acid sequence from a large negatively charged D to a small hydrophobic V. D289 is poorly exposed on the surface of the LDLr2 domain and our homology-based model predicts that it coordinates  $\text{Ca}^{2+}$  buried in the core of the domain (Fig 3A and 3B). Change of this amino acid probably causes a serious folding problem and lack of secretion of the protein. We did not detect any FI carrying this mutation in cell media

or lysates of transfected cells, which also implies that the protein was rapidly degraded intracellularly or poorly translated. We described a similar mutation – G170V (G188V when numbering includes signal peptide) - in another FI deficient patient (Nilsson et al., 2009). It is possible that the mutated FI mRNA transcripts in the patients in the current study have greater turnover to reduce the rate of synthesis of faulty protein and consequently decrease the accumulation of truncated or misfolded products that may be toxic. This phenomenon, known as nonsense mediated decay, was observed previously in a FI deficient patient (Baracho et al., 2003). Unfortunately we could not gain access to patient fibroblasts to verify if this occurs in the currently studied patients.

In a previous report of structure-function relationships of FI, we constructed mutant K249Q/Q259R/E270Q (or K267Q/Q277R/E288Q including signal sequence), which caused misfolding and lack of secretion of this recombinant protein (Nilsson et al., 2010b). In Patient 2, E288 lies next to mutated D289. To assess the importance of the region of negatively charged amino acids on the surface of the LDLr2 domain, we constructed mutant E288Q/D289N/E290Q. However, the protein was not expressed by HEK293 cells (data not shown). Taken together, these results imply that the cluster of negatively charged amino acids in the LDLr2 domain, which includes mutated D289 in our patient, is important for folding and may indeed be involved in binding of  $\text{Ca}^{2+}$  (Fig. 3B).

The alternative pathway of complement appears to be more compromised in FI deficient patients than the classical pathway, as shown in this and previous studies. This could be due to the fact that the classical pathway operates at very low concentrations of complement components. *In vitro* assays can detect classical pathway activation at serum concentrations of less than 0.1% whereas at least 5% is required for alternative pathway activation. Thus, depletion of complement components may inactivate the alternative pathway while the classical pathway may retain some function.

FI is a proteolytic enzyme that regulates several physiological activities of the C3b molecule. Deficiency of FI causes continuous activation of the alternative pathway and a subsequent depletion of C3, FB, properdin and FH (Naked et al., 2000; Rasmussen et al., 1990), as observed in the patients in this evaluation. Furthermore, in the absence of FI, no iC3b and C3dg can be generated and these are responsible for efficient phagocytosis as well as B-cell memory generation. This explains the predisposition to pyogenic infections in FI deficient patients (Castillo et al., 2009; Vyse et al., 1996; Wahn et al., 1984).

Individuals with FI deficiency present with recurrent bacterial infections of the upper and lower respiratory tract, septicaemia and/or meningitis (Floret et al., 1991; Leitao et al., 1997). Although complete deficiency of FI is rare, with less than 100 cases reported in the literature, the diagnosis should be considered in patients with recurrent invasive bacterial infections. This report describes the genetic and immunological workup of two FI-deficient patients with novel mutations of the FI gene, contributing to a greater understanding of the molecular mechanisms underlying this rare disorder of complement.

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## Figure legends

Figure 1. FI and FI mutations in Patients 1 and 2 and their families. (A) FI contains a FIMAC domain, a CD5 like domain, LDLr1 and 2 domains, a region of no known homology and a SP domain. Two important inter-domain disulfide bridges are indicated. Mutations found in Patients 1 (p.K45Sfs11) and 2 (p.D289V) are numbered including the signal peptide. (B) Genetic analyses in both families. Electropherograms showing sequences of DNA regions containing mutated nucleotides in Patient 1 (c.133-134delAA) and Patient 2 (c.866A>T). Results obtained for a healthy control and some family members are shown for comparison. (C) The patients analyzed in this study are marked with arrows. Symbols used: rectangle; man, circle; female, white; wt, black; homozygous, black and white; heterozygous; question mark, DNA not available for analysis. In the family of Patient 1, the patient and his younger brother were homozygous carriers of the mutation, while the mother and sister were heterozygous carriers of the mutation. The older brother did not carry the mutation. In the family of Patient 2, a homozygous mutation was identified in the patient while her parents carried the same mutation in the heterozygous form.

Figure 2. Expression of FI wt and mutants in transiently transfected cells. HEK 293 cells were transiently transfected with an empty plasmid (mock), FI wt, p.K45SfsX11 and p.D289V. FI levels in the cell lysates (A) and supernatants (B) were measured by ELISA. Only wt was secreted into the cell medium. FI could be detected only in wt cell lysate. Experiments were conducted in triplicate on three different occasions and results are presented as means  $\pm$  standard deviation (SD). One-way ANOVA tests were performed to test the significance of differences between FI variants and mock transfected cells. \* $P < 0.05$ ; \*\*\* $P < 0.001$ ; ns, not significant.

Figure 3. Structural analysis of the FI mutation identified in Patient 2. The LDLr2 domain is shown as a solid surface (A) and as a 3D image using PyMol (B). The mutation is marked in red, the negatively charged residues that bind to the  $\text{Ca}^{2+}$ -ion are marked in blue and the  $\text{Ca}^{2+}$ -ion is shown in light blue.

## Tables

Table 1. Clinical history of the patients.

	Patient 1	Patient 2
Sex (Gender)	Male	Female
Geographic background	Australia (Caucasian origin)	Turkey
Nucleotide change	c.133-134delAA	c.866A>T
FI mutation	p.K45SfsX11	p.D289V
Genetic status	Homozygous	Homozygous
Age at diagnosis of FI deficiency (years)	14	18
Types of infections	Recurrent infections with <i>S. pneumoniae</i> and <i>S. pyogenes</i> Septicaemia, joint infections and pneumonias	Recurrent upper and lower respiratory system infections (no organisms identified)
Rheumatologic disease	Juvenile idiopathic arthritis	Vasculitis and arthralgia
Renal disease	No	No

Table 2. Complement profiles for Patient 1 and his family. Values outside normal range are indicated in bold.

	Reference interval	Index Case	Younger brother	Older brother	Sister	Mother
C3 <sup>a</sup>	(0.7-2.06 g/L)	<b>0.32</b>	<b>0.22</b>	0.87	0.79	1.09
C4 <sup>a</sup>	(0.11-0.61 g/L)	0.12	0.11	0.12	<b>0.10</b>	0.22
Factor B <sup>b</sup>	(191-382 mg/L)	<b>&lt;38</b>	<b>&lt;38</b>	221	<b>150</b>	284
Factor H <sup>b</sup>	(345-590 mg/L)	<b>189</b>	<b>148</b>	456	398	631
Factor I <sup>b</sup>	(39-100 mg/L)	<b>&lt;2</b>	<b>&lt;2</b>	43	<b>20</b>	<b>34</b>
Classical pathway <sup>c</sup>	(>85%)	<b>41</b>	<b>11</b>	96	<b>84</b>	109
Alternative pathway <sup>c</sup>	(>64%)	<b>0</b>	<b>0</b>	<b>49</b>	<b>55</b>	79
Genetic status: p.K45SfsX11 mutation		Homo-zygous	Homo-zygous	Wild type	Hetero-zygous	Hetero-zygous

<sup>a</sup> Measured by nephelometry on the Immage 800 (Beckman Coulter)

<sup>b</sup> Measured by radial immunodiffusion (Binding Site)

<sup>c</sup> Measured by Wielisa ELISA (Euro-Diagnostica AB)

Table 3. Complement profiles for Patient 2 and her parents. Values outside normal range or statistically different from pooled NHS are indicated in bold.

	<b>Reference interval</b>	<b>Index case</b>	<b>Father</b>	<b>Mother</b>
C3 <sup>a</sup>	0.77-1.38 g/l	<b>0.47</b>	1.20	1.38
C4 <sup>a</sup>	0.12-0.33 g/l	<b>0.44</b>	0.33	0.32
C1q <sup>b</sup>	78-131 %	124	nd	nd
C5 <sup>b</sup>	72-171 %	75	155	160
Factor B <sup>b</sup>	59-154 %	<b>~ 10</b>	65	123
Properdin <sup>b</sup>	54-157 %	<b>40</b>	137	164
Factor H <sup>b</sup>	69-154 %	<b>65</b>	132	134
Factor I <sup>b</sup>	60-152 %	<b>&lt; 6</b>	77	123
Factor I <sup>c</sup>	nd	<b>&lt; 2% of NHS</b>	as pooled NHS	as pooled NHS
C3dg <sup>d</sup>	< 5 mg/l	< 3.5	nd	nd
Classical pathway <sup>e</sup>	nd; %	<b>13</b>	75	100
Alternative pathway <sup>e</sup>	nd; %	<b>0</b>	64	118
Genetic status: <b>p.D289V</b> mutation		Homozygous	Heterozygous	Heterozygous

<sup>a</sup> Measured by immunonephelometry (Heuck et al., 1983)

<sup>b</sup> Measured by electroimmunoassay (Johnson et al., 1983)

<sup>c</sup> Measured by ELISA using two polyclonal antibodies against FI (Nilsson et al., 2009)

<sup>d</sup> Measured by double decker electrophoresis (Brandslund et al., 1981)

<sup>e</sup> Measured using hemolytic assays (Nilsson et al., 2007)

nd: not determined

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Figure 1.

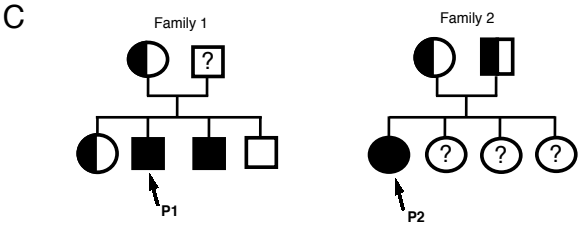
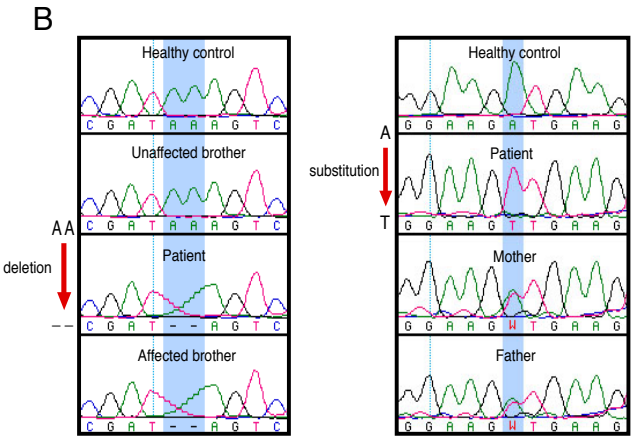
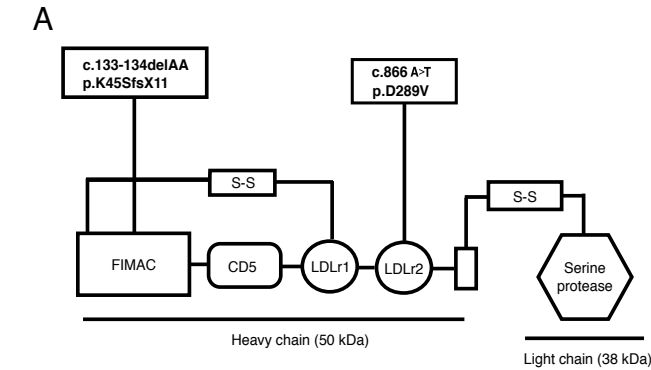


Figure 2.

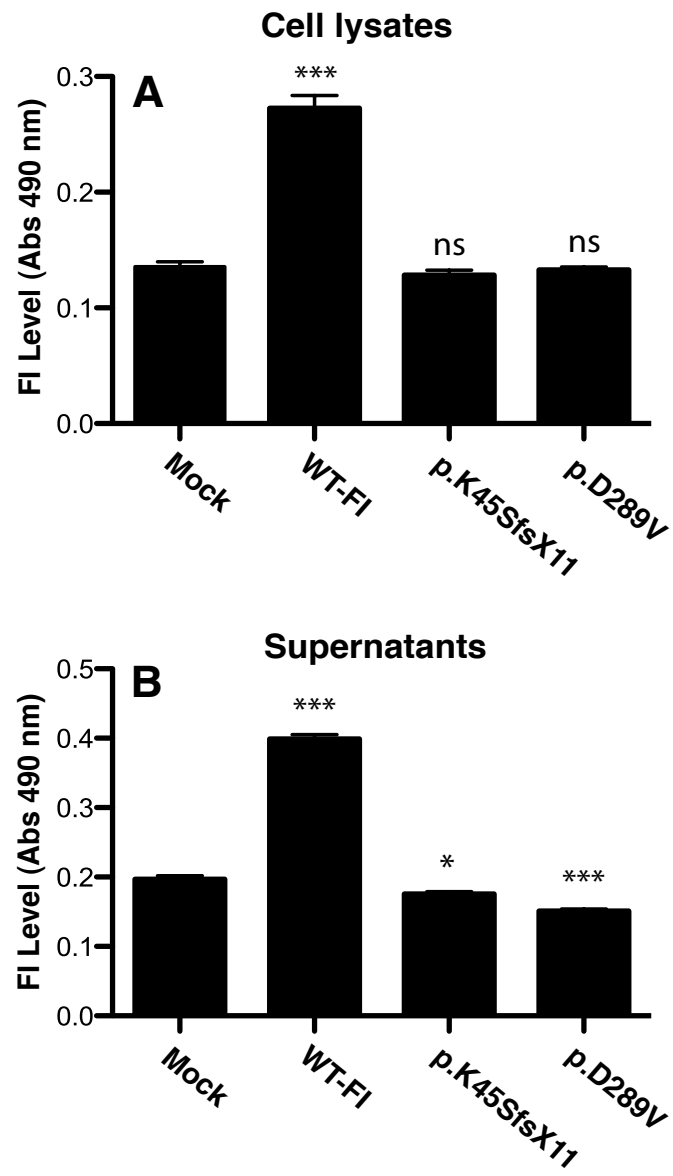




Figure 3.

