SUPPLEMENTAL DATA

Compromised Mutant EFEMP1 Secretion Associated with Macular Dystrophy Remedied by Proteostasis Network Alteration

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Running head: Manipulating EFEMP1 protein homeostasis

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MATERIALS AND METHODS

Quantitative real-time polymerase chain reaction (qPCR) **analysis** - Cells were harvested by trypsinization and washed once in either Hanks buffered salt solution (HBSS, Gibco, Carlsbad, CA) or Dulbecco's phosphate-buffered saline (DPBS, Gibco). RNA was extracted from the cells using the RNeasy kit (Qiagen) according to the manufacturer's protocol. RNA was eluted in a final volume of 30-50 μL DNase/RNase free water. RNA (500 ng) was reverse transcribed using the Quantitect Reverse Transcription kit (Qiagen). Briefly, the RNA was treated with gDNA wipeout for 2 min at 42°C to remove genomic DNA contamination. Then the RNA was reverse transcribed for 20 min at 42°C

followed by a 5 min 65°C denaturation step. The resulting cDNA was diluted four-fold with DNase/RNase free water before being used for amplification.

qPCR primers were selected using Primer3 software (http://frodo.wi.mit.edu/primer3/) and were optimized for amplification of 130-180 bp amplicons with annealing temperatures centered around 62.5°C. qPCR primers were purchased from Integrated DNA Technologies (IDT, Coralville, Iowa) and were designed as follows: qRibPro (human RPLP2) forward – 5'-CGTCGCCTCCTACCTGCT-3', qRibPro reverse – 5'-CCATTCAGCTCACTGATAACCTTG-3', qEFEMP1 forward – 5'-GGGGATCCTTTGCATGTCAG-3', qEFEMP1 reverse – 5'-TGAAACCAAGGACTGCACTG-3', qGLuc forward – 5'-AGCCCATGGAGCAGTTCATC-3', qGLuc reverse – 5'-CTGGATCCTGCTGGCAAAGG-3'. cDNA was amplified using FastStart Universal Sybr Green Master Mix (Roche). Briefly, 2 µL of diluted cDNA was added to 8 µL of master mix and primer solution, yielding a final primer concentration of 200 nM. cDNA amplification was quantified using an ABI 7900 Fast Real Time PCR machine (ABI, Carlsbad, CA). Amplification was achieved by the following parameters: 50°C for 5 min, 95°C for 2 min, 95°C for 10 sec. followed by 60°C for 30 sec. The reactions were cycled 45 times before proceeding to a thermal melt from 65 to 90°C to confirm the absence of primer dimers.

The resultant data was analyzed using the Relative Quantification (RQ) Manager (ABI) followed by analysis in DataAssist (ABI). Transcripts were normalized to the housekeeping gene with primers targeting human RPLP2.

Sequence analysis of calcium (Ca^{+2})-binding EGF domains in the fibulin family - Information regarding the location and sequence identity of the Ca^{+2}-binding EGF domains (and potential Ca^{+2}-binding domains) in the human fibulin proteins was obtained by analyzing each known fibulin family member using UniProtKB (http://www.uniprot.org/). UniProtKB identification numbers were as follows: Fibulin-1: P23142, Fibulin-2: P98095, Fibulin-3 (EFEMP1): Q12805, Fibulin-4 (EFEMP2): O95967, Fibulin-5 (DANCE): Q9UBX5, and Fibulin-6 (Hemicentin): Q96RW7. Based on homology to other EGF domains, which undergo a 1-3, 2-4, 5-6 native disulfide bond formation, we focused on the 2-4 disulfide bond, or dsb2 because of the proximity of the R345W mutation to the b_n cysteine (see Figure 1B for a diagram of the bonding prediction and terminology). Within each analyzed domain, the second cysteine residue was considered as the b_n cysteine, while the fourth cysteine was considered as its bonding partner, the b_c cysteine. The identity and abundance of the two amino acids on each side of the b_n and b_c cysteines were recorded. Finally, the amino acids were grouped according to side chain characteristics with charge assignment based on physiological pH (7.4).

BNPS-skatole cleavage of secreted EFEMP1 - BNPS-skatole of secreted EFEMP1-GLuc variants was performed essentially as described in the main manuscript. HEK-293T cells were transfected with EFEMP1 constructs for 24 h, after which the cells were replated and allowed to grow for 24 additional hours. Cells were then incubated in serum-free media at 30°C for an additional 24 h. Conditioned media was treated with 20 mM IA in DPBS for 10 min at RT. Total media protein was precipitated with 10% trichloroacetic acid (TCA) for 10 min on ice followed by centrifugation. Protein pellets were washed with 500 μL of cold acetone, followed centrifugation. Protein pellets were dried at RT, followed by resuspension in 150 μL of 8 M urea. Freshly prepared BNPS-skatole (250 μL, 1.6 mg/mL) in glacial acetic acid was added to the concentrated media. Samples were treated overnight at RT in the dark. Proteins were precipitated by adding 1.2 mL cold acetone and incubating at -20°C for ≥2 h followed by centrifugation at ~20,000 x g. Protein pellets were washed with acetone and re-spun at ~20,000 x g for 10 min at 4°C. Acetone was aspirated and the pellet was air dried at RT for 30 min. Samples were then resuspended in 50 μL of 8 M urea in DPBS and heated to 95°C until the protein pellet redissolved. Proteins were further denatured in 1x non-reducing Laemmli buffer for 5 min at 95°C. Samples were then separated by SDS-PAGE and western blotting was performed as in the main text.

Supplemental Table I

Comparison of residues surrounding cysteines involved in dsb2 of typical Ca^{2+}-binding EGF domains of fibulin proteins

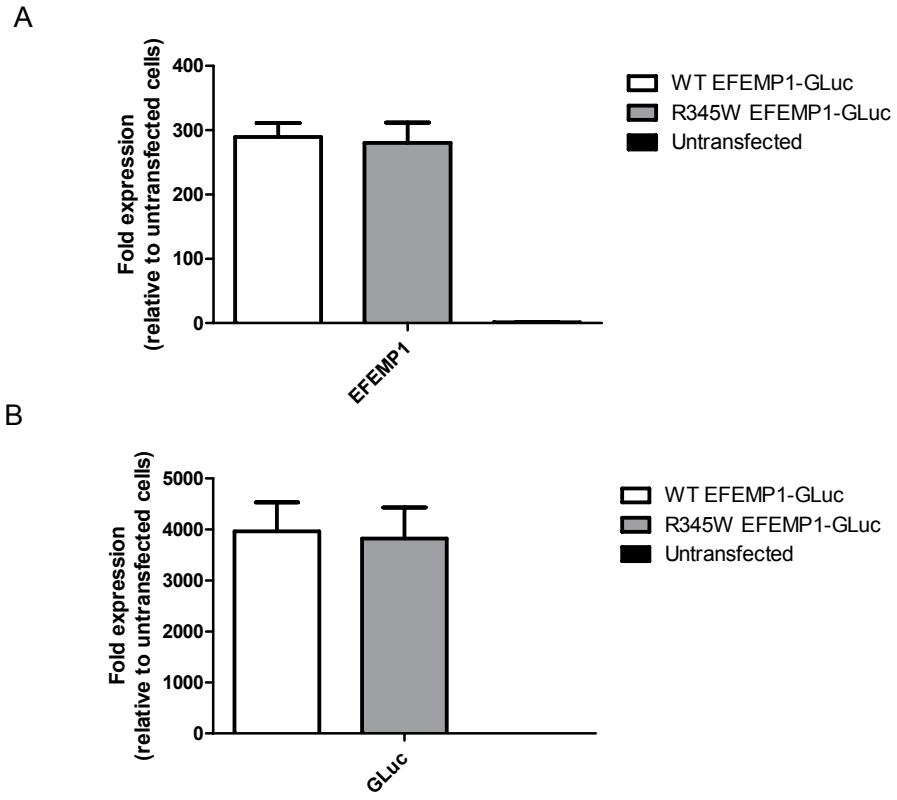
Protein	Typical Ca^{2+} EGF Domain	Residue	b_n surrounding sequence	b_c surrounding sequence
Fibulin-1	Domain 2	216 - 261	HSCRL	FRCQR
	Domain 3	262 - 307	HNCLP	FRCRP
	Domain 4	308 - 355	APCPI	YTCQK
	Domain 5	356 - 398	EPCGK	FRCEC
	Domain 6	399 - 440	RLCGH	YLCSC
	Domain 7	441 - 480	SPCSQ	YQCYC
	Domain 8	481 - 524	HICSY	FQCSC
	Domain 9	525 - 578	HNCSI	FRCLA
Fibulin-2	Domain 1	604 - 645	ELCQH	YHCAC
	Domain 3	719 - 763	HDCSR	FYCVN
	Domain 4	764 - 809	HTCQP	FYCQA
	Domain 5	810 - 857	EPCRP	YTCQR
	Domain 6	858 - 900	HRCGE	YRCDC
	Domain 7	901 - 942	RLCQH	YRCSC
	Domain 8	943 - 981	QRCSQ	YQCYC
	Domain 9	982 - 1024	ILCTF	YQCAC
	Domain 10	1025 - 1069	HNCSE	FRCLR
Fibulin-3 (EFEMP1)	Domain 2	173 - 213	HNCRA	FACQC
	Domain 3	214 - 253	PYCHQ	FYCQC
	Domain 4	254 - 293	NQCAQ	FICQC
	Domain 5	294 - 333	YLCQY	FSCMC
	Domain 6	334 - 378	NECRE	FRCYP
	mut Domain 6	334 - 378	NECWE	FRCYP
Fibulin-4 (EFEMP2)	Domain 2	123 - 163	HDCRP	YQCTC
	Domain 3	164 - 202	RYCQH	FRCQC
	Domain 4	203 - 242	APCEQ	FLCRC
	Domain 5	243 - 282	YLCQY	FSCHC
	Domain 6	283 - 328	HQCSE	YRCVD
Fibulin-5 (DANCE)	Domain 1	42 - 82	EACRG	YLCIP
	Domain 2	127 - 167	HQCNP	YTCSC
	Domain 3	168 - 206	GYCQQ	YSCTC
	Domain 4	207 - 246	NPCVQ	FICRC
	Domain 5	247 - 287	FLCQH	YFCSC
	Domain 6	288 - 333	HTCNL	FKCID
Fibulin-6 (Hemicentin)	Domain 1	5107 - 5146	NPCSH	YYCSC
	Domain 2	5147 - 5191	HTCHA	YRCVV
	Domain 3	5192 - 5229	SPCHQ	FHCGC
	Domain 4	5230 - 5271	NVCRP	YKCID
	Domain 5	5272 - 5307	HQCRY	YRCVC
	Domain 6	5315 - 5355	KPCAH	FKCIC
	Domain 7	5432 - 5471	DACQH	YQCIC

Supplemental Table II

Prevalence of amino acids surrounding cysteines involved in dsb2 of domain 6 EFEMP1

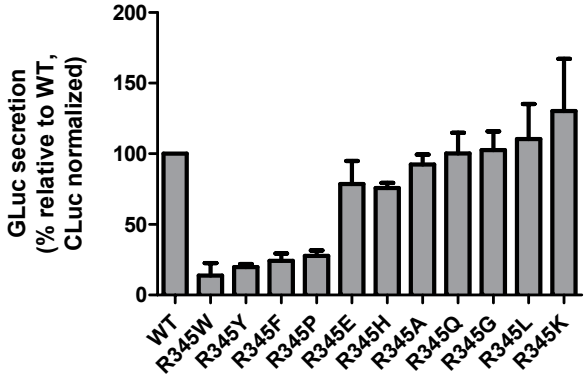
Side chain characteristic	Amino acid	b_n -2	b_n -1	b_n +1	b_n +2	b_c -2	b_c -1	b_c +1	b_c +2
positive	Arginine	3	2	8	1	-	12	3	3
	Histidine	15	-	3	8	-	2	1	-
	Lysine	1	-	-	1	-	3	-	1
negative	Aspartic acid	1	2	-	-	-	-	1	3
	Glutamic acid	4	1	1	4	-	-	1	-
polar, uncharged	Serine	2	1	8	-	-	3	6	-
	Threonine	-	3	1	-	-	3	2	-
	Asparagine	5	4	2	-	-	-	-	1
	Glutamine	1	4	9	8	-	6	8	-
special	Cysteine	-	-	-	-	-	-	-	26
	Glycine	1	-	3	1	-	-	1	-
	Proline	1	9	1	6	-	-	-	3
hydrophobic	Alanine	2	2	2	2	-	1	2	2
	Isoleucine	1	1	-	2	-	2	5	-
	Leucine	-	7	1	2	-	3	2	-
	Methionine	-	-	-	-	-	-	1	-
	Phenylalanine	1	-	-	1	20	1	-	-
	Tyrptophan	-	-	-	-	-	-	-	-
	Tyrosine	2	3	-	4	20	4	3	-
	Valine	-	1	1	-	-	-	4	1

Supplemental Figure 1



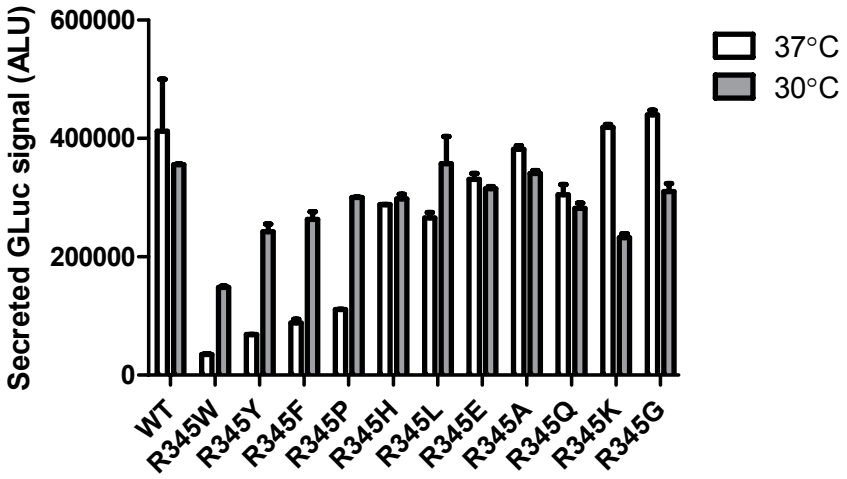
Supplemental Fig 1. EFEMP1 and GLuc transcripts are similar in transfected HEK-293T cells. RNA was extracted 72 h after transfection and qPCR was performed using primers directed against (A) EFEMP1 or (B) GLuc. Transcript levels were normalized to a housekeeping gene, the RPLP2 ribosomal protein. Representative data (mean ± S.D.) of 3 independent experiments are shown.

Supplemental Figure 2



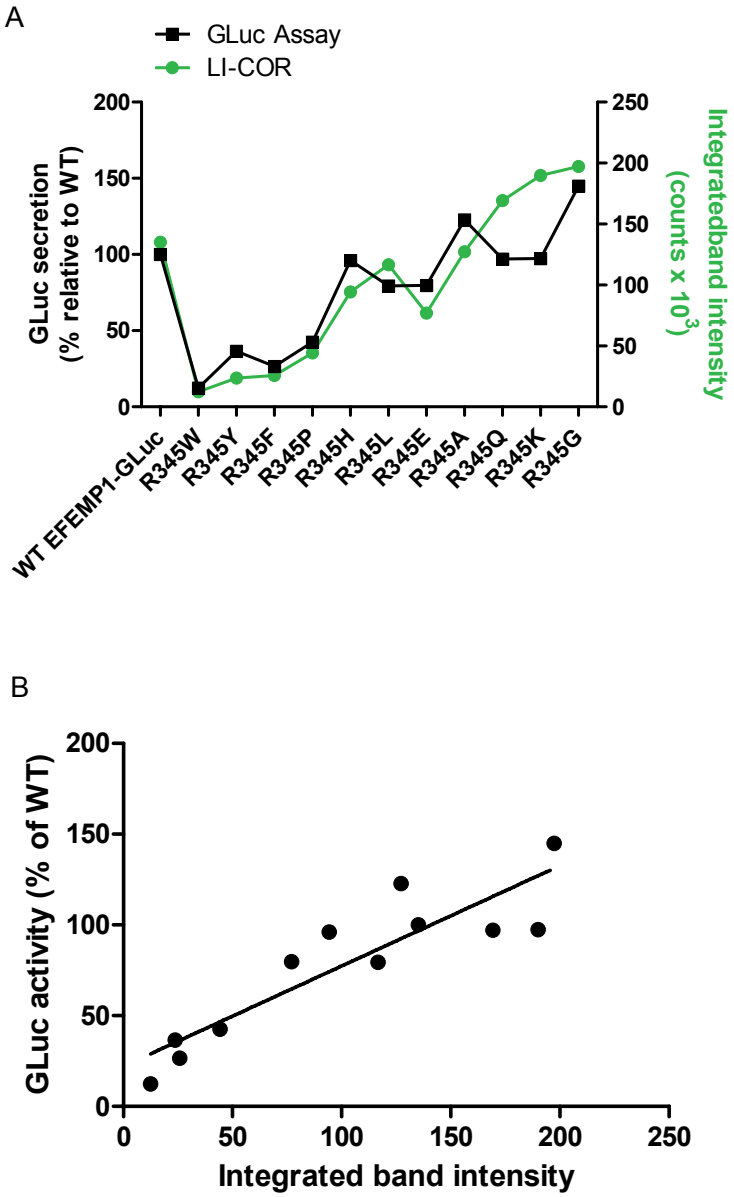
Supplemental Fig 2. Normalization of secreted GLuc assay data to the control luciferase, CLuc does not significantly alter the rank-order of mistrafficked mutants. 45 μL aliquots of conditioned media from co-transfected HEK-293T cells were taken 72 h post transfection and assayed for the presence of the EFEMP1-GLuc fusion protein in an endpoint assay. The GLuc signal was first normalized to differences in the CLuc signal, and then presented as the % relative to WT EFEMP1-GLuc signal ($n \geq 3$, ± S.D.).

Supplemental Figure 3



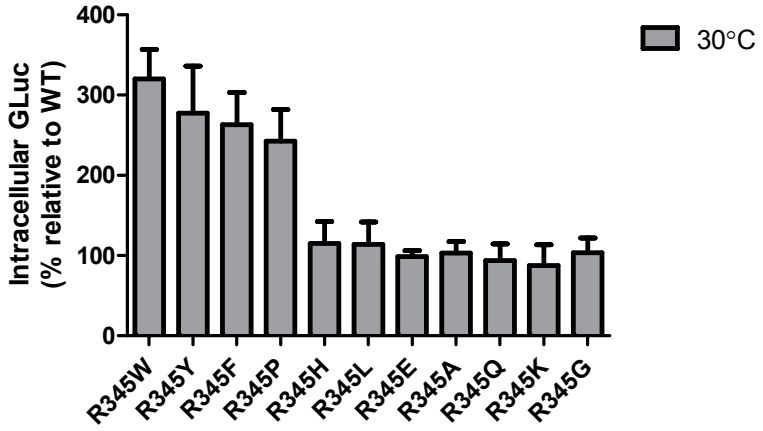
Supplemental Fig 3. Secreted GLuc was assayed from conditioned media from transfected cells incubated at 37 or 30°C for 24 h. Representative data (mean ± S.D. of technical replicates) of ≥ 3 independent experiments are shown.

Supplemental Figure 4



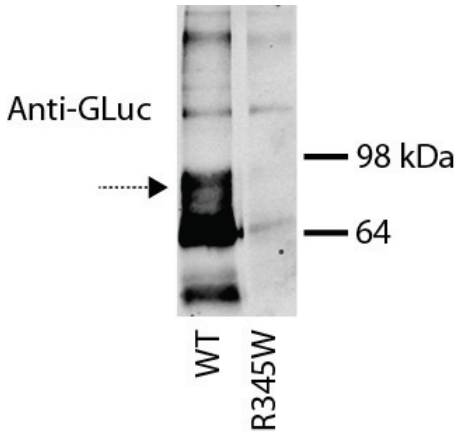
Supplemental Fig 4. Quantification of western blotting band intensities correlate well with the GLuc assay signal. (A) Relative values from one of the GLuc assays summarized in Figure 2A were plotted against the corresponding integrated band intensity of the GLuc protein for that exact sample as determined by LI-COR. (B) Luciferase activity and band intensity (protein concentration) correlate linearly. The coefficient of determination (r^2) following linear regression (black line) is 0.81.

Supplemental Figure 5



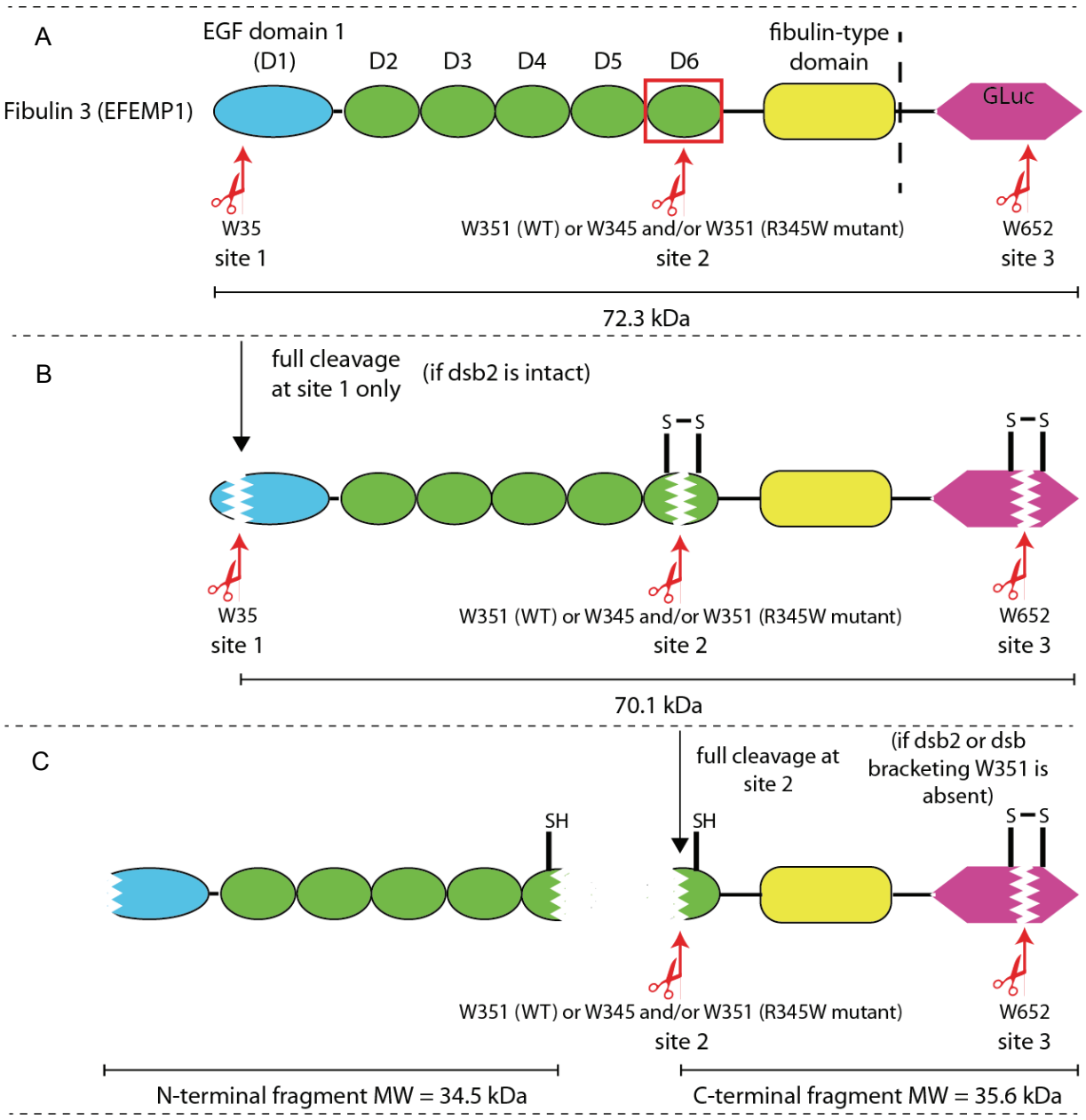
Supplemental Fig 5. The extent of intracellular accumulation of EFEMP1-GLuc variants are inversely proportional to efficiency of secretion. Cells were transfected with the indicated constructs for 48 h followed by temperature shift and media change for 24 h prior to assaying for intracellular GLuc ($n \geq 3$, ± S.D.).

Supplemental Figure 6



Supplemental Fig 6. Secreted R345W EFEMP1 GLuc migrates similarly to WT under non-reducing conditions. Identical samples of WT and R345W EFEMP1 GLuc from the 32 h time point in Figure 1C were boiled under non-reducing conditions and subjected to western blotting detecting for the GLuc epitope as described in Figure 1D. For comparison, the typical migration size of reduced WT and R345W EFEMP1 GLuc is designated by the dotted arrow. Note: the intensity of this image was amplified using LI-COR software to visualize R345W bands. Representative data of 2 independent experiments.

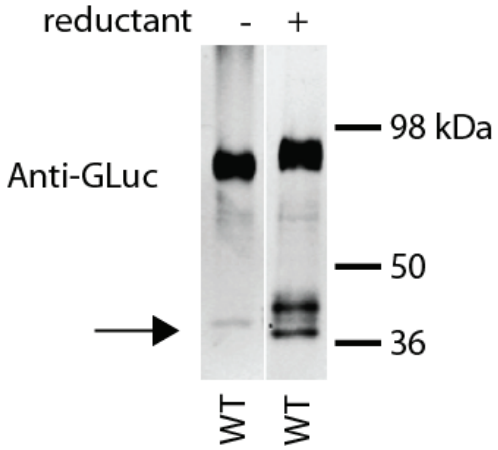
Supplemental Figure 7



Supplemental Fig 7. Schematic of potential BPNS-skatole-generated cleavage products. (A) Full length EFEMP1-GLuc has three possible BNPS-skatole cleavage sites (site 1: the amide bond after W35, site 2: the amide bond after W351 (WT) or W345 and/or W351 (R345W mutant) and site 3: the amide bond after W652). (B) BNPS-skatole cleavage C-terminal to site 1 (W35) results in the generation of a 70.1 kDa isoform containing all but the first 34 amino acids of the fusion protein. (C) If no disulfide bond brackets W351 (WT) or W345 and/or W351 (R345W mutant), such as disulfide bond 2 (dsb2) in domain

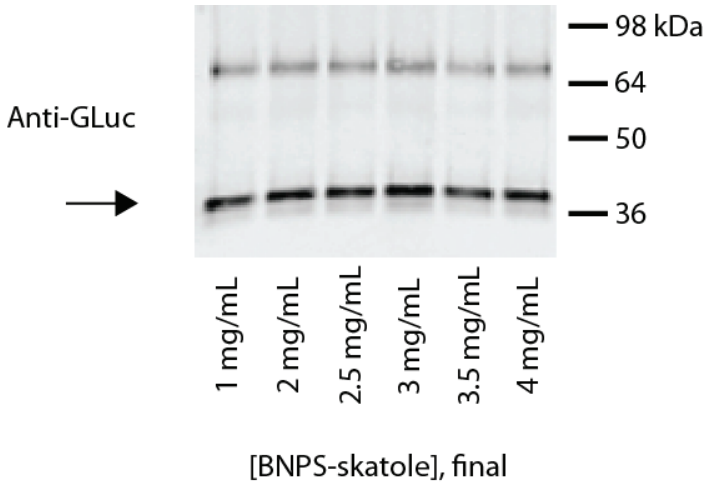
6, BNPS-skatole will cleave C-terminal to site 2 [W351 (WT) or W345 and/or W351 (R345W mutant)], and generate a GLuc-containing isoform of 35.6 kDa. Site 3 (W652) can be cleaved by BNPS-skatole, however a bracketing disulfide bond prevents a MW change upon non-reducing SDS-PAGE.

Supplemental Figure 8



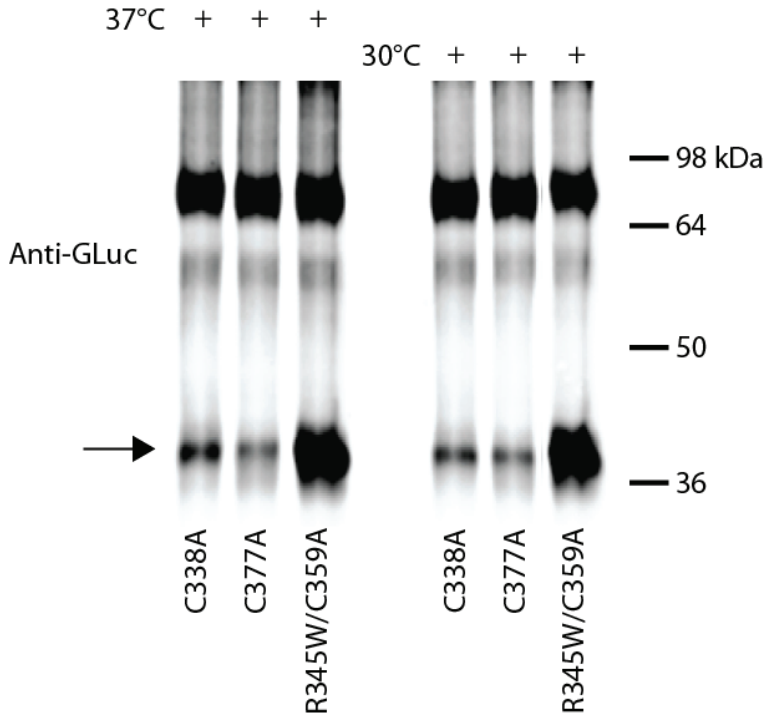
Supplemental Fig 8. BPNS-skatole cleavage is reductant-sensitive. WT EFEMP1-GLuc lysates were cleaved overnight with BNPS-skatole followed boiling in Laemmli buffer with (+) or without (-) reductant (2-mercaptoethanol, 0.83% [v/v], final concentration). Inefficient cleavage by BNPS-skatole may be due in part to tryptophan oxidation. Different sized isoforms are most likely due to an 'opening' up of the EFEMP1 protein after reduction as well as the loss of the C-terminus of GLuc after BNPS-skatole cleavage at W652 followed by reduction of the bracketing disulfide bond. Representative data of 3 independent experiments.

Supplemental Figure 9



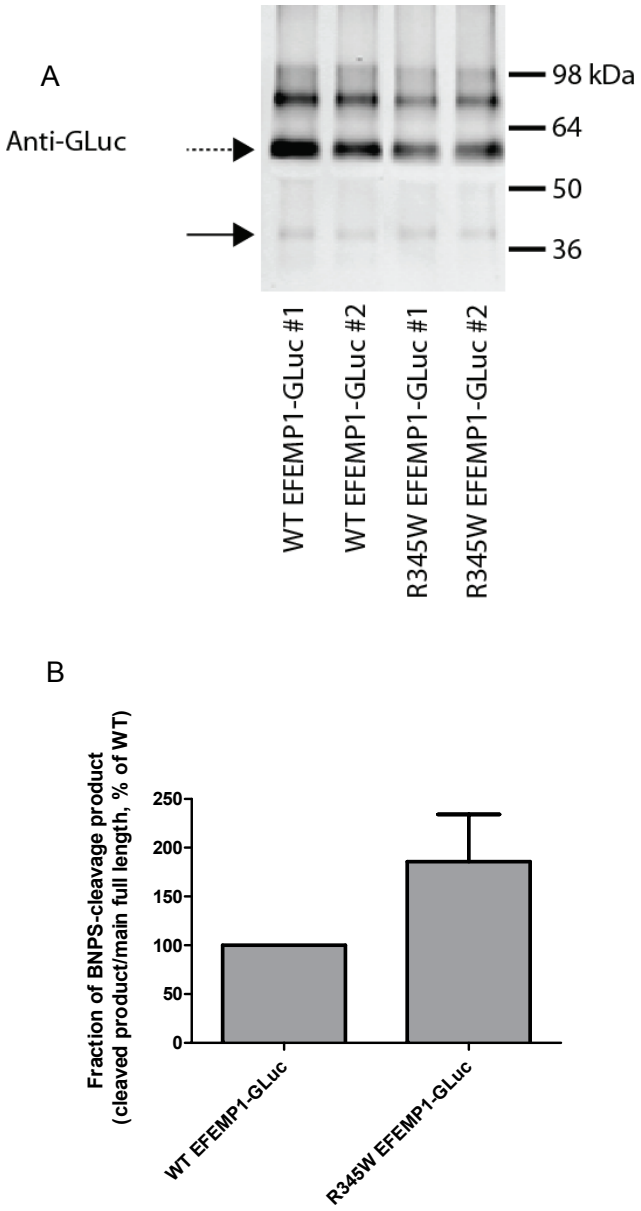
Supplemental Fig 9. Increasing BNPS-skatole concentration does not increase cleavage product. Lysates from C359A EFEMP1 GLuc-expressing cells were treated with increasing concentrations of BNPS-skatole prior to analysis by non-reducing SDS-PAGE and western blotting. The cleavage product (solid arrow) resulting from inadequate dsb2 is designated.

Supplemental Figure 10



Supplemental Fig 10. BNPS-skatole cleavage of cysteine variants is unaffected by temperature reduction. Lysates from transfected cells grown at 37 or 30°C for 24 h were treated with BNPS-skatole, separated by non-reducing SDS-PAGE and probed for the GLuc epitope by western blotting. Representative data of 2 independent experiments.

Supplemental Figure 11



Supplemental Fig 11. Secreted EFEMP1-GLuc is relatively insensitive to BNPS-skatole. (A) Concentrated, serum-free conditioned media aliquots from cells grown at reduced temperatures (30°C) were treated with BNPS-skatole followed by analysis by non-reducing SDS-PAGE and western blotting. The cleavage product (solid arrow) resulting from inadequate dsb2 is designated. TCA precipitation seems to alter the apparent molecular weight of a portion of secreted EFEMP1 (dashed arrow). Biological duplicates (#1, #2) of 3 independent experiments are shown. (B) Quantification of fractional BNPS-skatole cleavage product relative to full length EFEMP1 by LI-COR quantification. The integrated signal intensity of the BNPS-cleavage product (solid arrow in 'A') was divided by the main full length EFEMP1

isoform (dashed arrow in ‘A’) to yield the fractional population of BNPS-skatole cleavage product. The fractional population was then presented as % of WT. Representative data of 3 independent experiments.