The legends of supplemental figures and table

Supplemental Table S1: Manually identified glycoforms of ^{313}pyrQIHATPTPVR^{322} derived from αDG373(T322R)-Fc and their calculated relative abundance

The normalized intensity for each glycopeptide was calculated based on the base peak intensity of its extracted ion chromatogram divided by the sample-dependent normalization factor. The normalization factor for each wild type and mutant sample was calculated based on the average intensity of the three most abundant peptides derived from the Fc region, namely FNWYVDGVEVHNAK (m/z 559.9403, z=3), EPQVYTLPPSR (m/z 643.8424, z=2), TTPPVLDSDGSFFLYSK (m/z 937.4650, z=2), common to all αDG373(T322R)-Fc proteins. This factor was used to correct for different yields of the protein from each sample to allow the abundance of each glycopeptide to be compared across different samples. A representative set of annotated mass labeled HCD/CID spectra for each of the precursors listed here can be found in supplemental Fig. 5.

Supplemental Fig. S1: Entire amino acid sequences of the N-domain (green), mucin domain (black), and Fc region (orange). The possible sites for displaying the laminin-binding glycans are shown in red. T322 was substituted with arginine shown in blue.

Supplemental Fig. S2: Representative HCD MS^2 spectra of tryptic αDG373(T322R)-Fc glycopeptides, ^{323}AIGPPTTAIQEPPSR^{337}, carrying at least a phosphorylated core M3 with and without additional *O*-glycans. The presence of the core M3 is defined by detecting i) a $HexNAc_2$ oxonium ion at *m/z* 407.166 and ii) a phospho-Hex increment from the singly and/or doubly charged peptide core at *m/z* 1534.811 and/or 767.915^{2+}, respectively. Assignment and annotation are similar to those described for Fig. 2.

Supplemental Fig. S3: HCD MS^2 spectra of tryptic αDG373(T322R)-Fc glycopeptides carrying only one phosphorylated core M3 (A), two phosphorylated core M3 moieties with two RboP (B), and two phosphorylated core M3 moieties with one RboP + one GroP (C). As shown in Fig. 2 and listed in Table S1, the presence of 1-2 phosphorylated core M3 along with other mucin-type *O*-glycans, with and without a single RboP or GroP substituent, was common on the ^{313}pyrQIHATPTPVR^{322} peptide derived from αDG373(T322R)-Fc expressed in HEK293T cells. These glycoforms were also commonly found on the same tryptic ^{313}pyrQIHATPTPVR^{322} peptide core derived from αDG373(T322R)-Fc expressed in HCT116 and its various mutants (Table S1, Fig. 7), which also produced a higher proportion of non-pyroglutamylated ^{313}QIHATPTPVR^{322} glycopeptides (peptide core at 560.317^{2+} or 1119.512^{1+}). In general, glycoforms found on the latter could also be identified on the pyroglutamylated peptide counterparts, except for the rare combination of two RboP substituents detected only on non-pyroglutamylated peptide derived from HCT116 *Δ*FKRP mutant (B). No fragment ion could be found to support

their occurrence as tandem RboP. Instead, these are most likely single RboP substituents on each of the phosphorylated core M3 structures. Similarly, the glycoforms with the combination of single RboP and single GroP could only be detected on the pyroglutamylated peptide derived from HCT116 *Δ*FKRP mutant, with no evidence for their occurrence in tandem (C). The assignment and annotation are similar to those described for Fig. 2.

Supplemental Fig. S4: FKTN, FKRP, and TMEM5 are not functionally compensating one another. Wild-type HCT116 and its mutants (*Δ*FKTN, *Δ*FKRP, and *Δ*TMEM5) were transfected with or without FKTN-myc, FKRP-Flag, and TMEM-Flag expression vectors. WGA-enriched cell lysates were subjected to immunoblot analysis using IIH6 and anti-β-DG antibodies. Cell lysates were analyzed for the expression of FKTN-myc, FKRP-Flag, and TMEM-Flag by Western blots using anti-FKTN, anti-Flag, and anti-Flag antibodies.

Supplemental Figure 5: Annotated mass labeled spectra.

In this work, all MS/MS data were manually interpreted along the line described in the Results. After working out the expected and unexpected modifications on the target aDG core peptide with and without N-terminal pyroglutaminylation, all subsequent data assignment was based primarily on i) identifying the peptide backbone by the m/z value, ii) the particular modifications by subtracting the mass of peptide backbone from that of precursor, iii) the diagnostic oxonium ions in HCD, and iv) direct losses from precursor in the case of trap CID. These are appropriately annotated in the mass labeled spectra provided here as supplemental Fig. 5, to comply with the journal requirement, in addition to those shown in Fig. 2, Fig. 3 and supplemental Fig. 3. As the spectra for the same precursors found for each of the wildtype and mutants are very similar, only one representative of each unique precursor as listed in Table S1 is compiled here due to the very laborious process of preparing these annotated spectra. In most cases, the spectra were taken from HEK293, supplemented by those from HCT116 WT, ISPD and FKRP mutants. The occurrence or not of each precursor in different samples and their relative abundance can be inferred from Fig. 7 and Table S1. Raw datasets for each sample have been deposited in public repository. For each precursor, the MS1 profile for the precursor summed over its elution time was shown in the upper panel, its HCD MS2 spectrum in the middle panel and trap CID MS2 spectrum in the bottom panel.