Figure S1. Live-cell, confocal FRET acceptor photobleaching scans of hBest1-CFP and mutant (R218C, W93C, and V9M) hBest1-YFP in MDCK II cells. Live-cell acceptor photobleaching demonstrates that hBest1-CFP and hBest1^{V9M}-YFP exhibit FRET in intracellular compartments. In contrast, hBest1-CFP and either hBest1^{W93C}-YFP or hBest1^{R218C}-YFP exhibited FRET at the periphery of the cell, consistent with localization at the plasma membrane. FRET efficiency was highest for the positive control, a CFP-YFP fusion protein, and lowest for the negative control, which was hBest1-CFP co-expressed with YFP. Scale bar: 10 μm.

Figure S2. Reciprocal co-immunoprecipitation of mBest1 and hBest1 or hBest1^{V9M} in MDCK II cells. MDCK II cells were co-transfected with mBest1-GFP and either hBest1 or hBest1^{V9M}. Lysates of co-transfected cells were immunoprecipitated using an anti-mBest1 or anti-hBest1 antibody and western blotted using a separate GFP- or hBest1-specific antibody. Control lanes were loaded with immunoprecipitates prepared from untransfected MDCK II cells**.** Lysate lanes were loaded using lysates from co-transfected cells.

Figure S3. Live-cell, confocal FRET acceptor photobleaching of mBest1-CFP and hBest1-YFP or hBest1^{V9M}-YFP in MDCK II cells. (A) Representative X-Y scan of mBest1-CFP (*blue*, donor) and hBest1-YFP (*yellow*, acceptor) co-expressed in MDCK II cells via transfection. Live-cell acceptor photobleaching was performed by bleaching the acceptor, generating the resultant image in **(B)**, which highlights regions in the plasma membrane where donor intensity increased. **(C)** Both hBest1-YFP (n=24) and hBest1^{V9M}-YFP (n=24) exhibited a similar %E that differed significantly ($p < 0.001$) from both the negative (n=23) and positive control (n=26). Scale bar: 10 μm. Error bars indicate ± SD.

Figure S4. Western blotting of hBest1-YFP and untagged hBest1^{W93C} in MDCK II cells. Polarized monolayers of MDCK II cells were made to co-express hBest1-YFP, untagged hBest1^{W93C}, or hBest1-YFP and hBest1^{W93C} via adenovirus mediated gene transfer. Western blotting for YFP identified hBest1-YFP at ~95 kDa in MDCK II cells, but did not detect untagged hBest1. Western blotting for hBest1 detected hBest1-YFP as well as untagged hBest1^{W93C} at ~68 kDa. Lanes loaded with lysates from co-expressing MDCK II cells displayed both hBest1 bands (tagged and untagged) when blotted for hBest1, while lanes loaded with lysates from cells expressing either hBest1-YFP or hBest1^{W93C} showed only one hBest1 band.