Supplementary Figure 1. Full length or C-terminal truncated Syx are equally potent in terms of stabilizing Rnd3.
293T cells expressing streptavidin binding peptide (SBP)-Flag-Rnd3 and full-length (FL) HA-Syx, HA-Syx (1-760) or HA-ROCKα were treated with cyclohexamide (CHX) for the indicated times. Rnd3 was stabilized by both full length and C-terminal truncated Syx but not by co-expression of ROCKα. '*' denotes non-specific band.

Supplementary Figure 2. Syx binding to Rnd3 are blocked by classical loss of function effector mutants.
293T cells were transfected with expression plasmids as indicated and after 16h cells were lysed and streptavidin pulldown was performed. Associated proteins were detected by immunoblotting. No interaction between Rnd3 effector mutant (T55A) and Syx was observed.

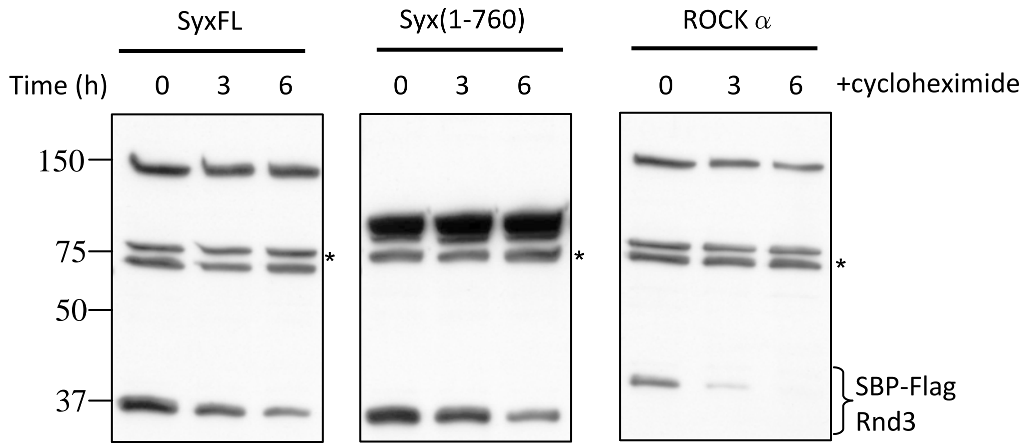
Supplementary Figure 3. Rnd3 is highly expressed in COS 7 cells.
(A) The specificity of anti-Rnd3 used in this study was examined by immunoblotting against HeLa cell lysates containing equal amounts of SBP-tagged Rnd1, Rnd2 and Rnd3. Only weak cross-reaction with Rnd1 was detected (B) Equal amount of protein (50 μg) from MDCK, COS 7, HeLa and NIE-115 lysates were immunoblotted with the anti-Rnd3 to examine endogenous Rnd3 levels.

Supplementary Figure 4. Evidence that Rnd3 stability is independent of Ser11 phosphorylation.
293T cells were transfected with plasmids encoding Rnd3(S11A) or Rnd3(S11D) mutants to assess the effect of preventing ROCK phosphorylation or the effect of a phospho-mimetic mutant on protein stability.

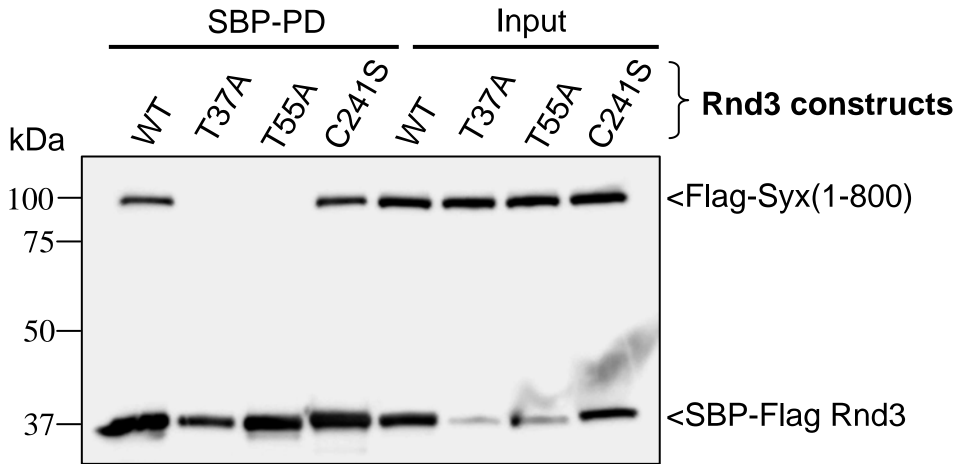
Supplementary Figure 5. Modified Rnd3 was not detected.
293T cells overexpressing Rnd3 were treated for the indicated times with cyclohexamide (CHX) in the presence of 10 μM MG132 or DMSO, then lysed and immunoblotted. To investigate whether Rnd3 modification, as indicated by an upshift, occurs upon MG132 treatment, chemiluminescent detection was performed with Supersignal West Dura (Pierce) and the membrane was exposed to X-ray film for 15 minutes. There was essentially no signal at mobilities corresponding to larger forms of Rnd3.

Supplementary Figure 6. K45 is important for Rnd3 turnover.
293T cells were transfected with plasmids encoding the proteins as indicated. At 16h the cells were treated with CHX for the indicated times, lysed and immunoblottted.

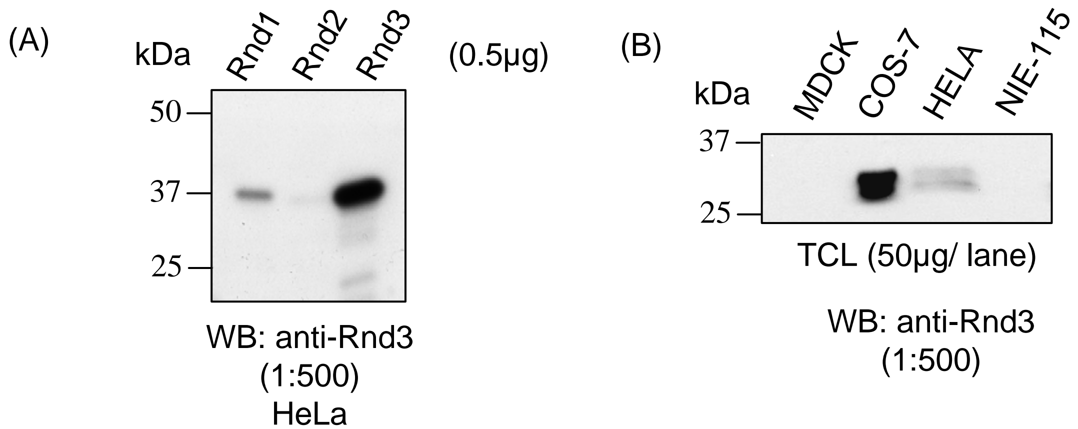
Supplementary Figure 7. Rnd3(K45R) exhibits higher activity in cells.
(A) HeLa cells were transfected with 2μg pXJ-GFP (reporter) and 1μg of the Flag-Rnd3 plasmid as indicated. Shown are representative cell images of GFP-positive and neighboring controls with filamentous actin stained by phalloidin 546. (B) Graphical representation showing percentage of GFP positive cells with disrupted stress fibers. 50 cells were counted for each sample.



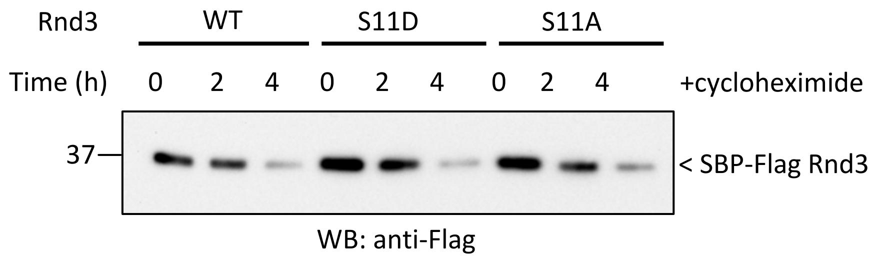
Supplementary Figure 1



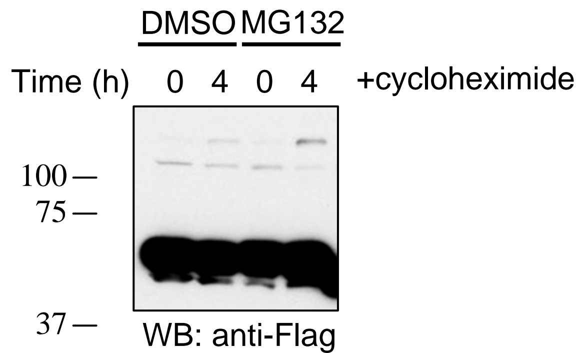
Supplementary Figure 2



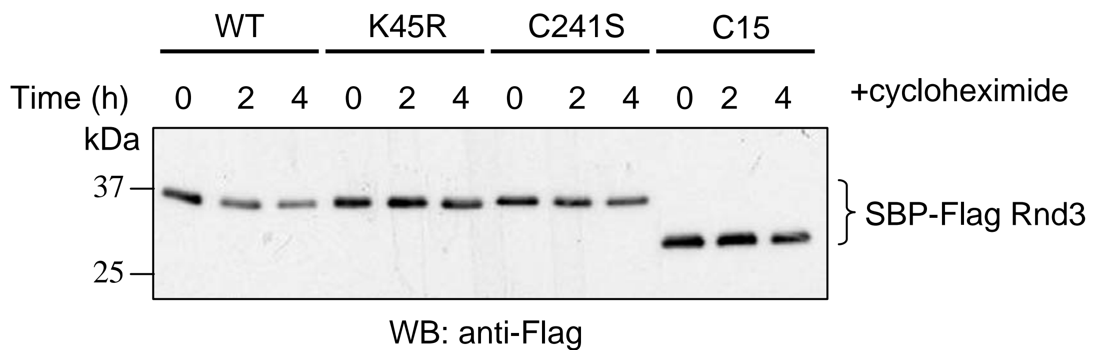
Supplementary Figure 3



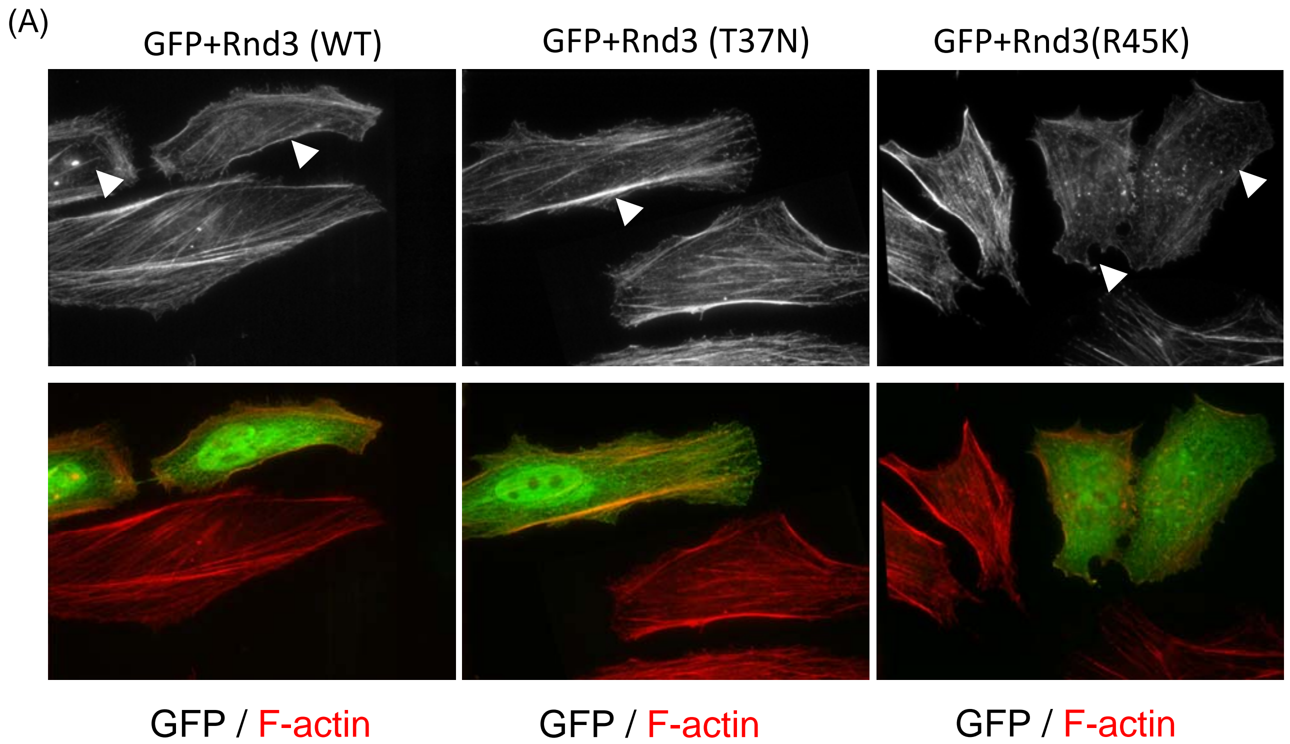
Supplementary Figure 4

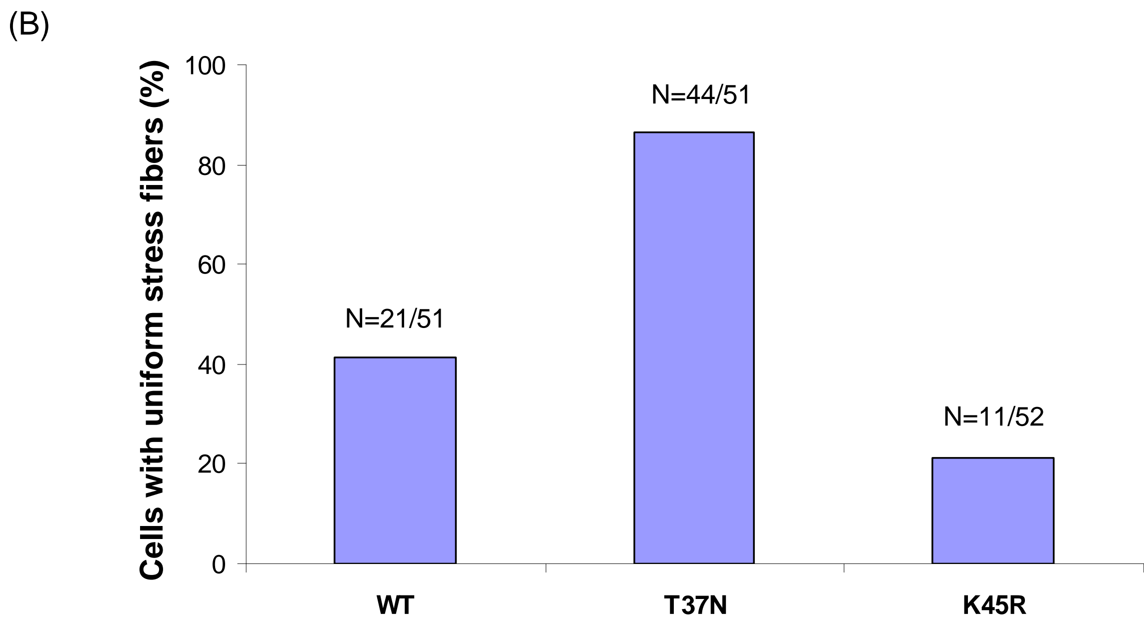


Supplementary Figure 5



Supplementary Figure 6





Supplementary Figure 7