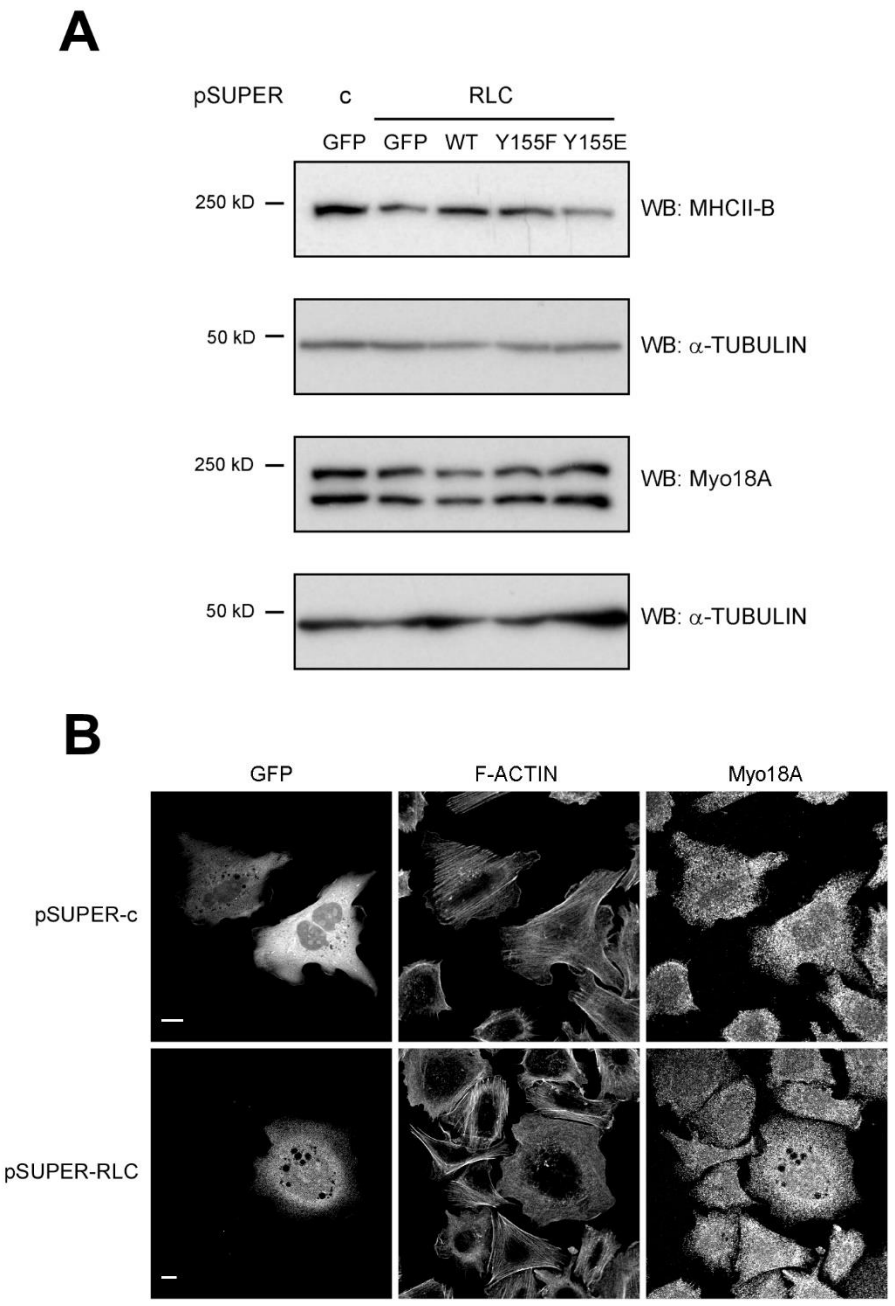

Figure S1. RLC depletion does not affect the protein levels of Myo18A. Related to Figure 3.

(A) CHO.K1 cells were co-transfected with a control sequence (pSUPER-C) or pSUPER-RLC and GFP or the indicated RLC-GFP mutants, cultured for 96h and lysed. Lysates were separated by PAGE/SDS and blotted against MHCII-B or Myo18A. Alpha (α)-tubulin is shown as a loading control. Note the decrease in the levels of MHCII-B in the pSUPER-RLC+GFP and the pSUPER-RLC+Y155E lanes, but the restoration in the wild type and Y155F lanes. Conversely, the levels of Myo18A are not altered significantly. Experiment is representative of three performed.

(B) CHO.K1 co-transfected with GFP and the indicated pSUPER vectors were allowed to spread on fibronectin, fixed and stained for Myo18A and F-actin. Note the effect of RLC depletion in the lower panels (GFP-positive cells round up and do not display large actin filaments), but the lack of depletion of Myo18A. Bars=10 μm. Images are representative of >50 in two independent experiments.

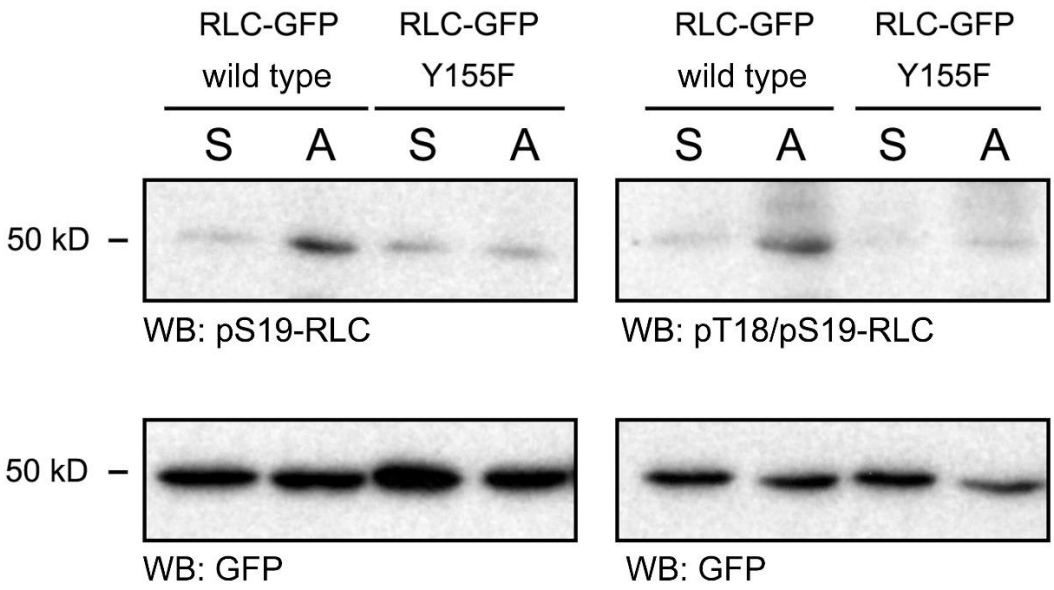

Figure S2. RLC Y155F phosphorylation on S19 and T18/S19 is impaired in response to integrin-mediated adhesion. Related to Figure 3.

CHO.K1 cells expressing GFP-tagged RLC or the Y155F mutant were either kept in suspension (S) or allowed to adhere to fibronectin for 1h (A) and lysed. Lysates were separated by PAGE/SDS, transferred to PVDF membrane and blotted for GFP (loading control, bottom rows), pS19 (left panels) or pT18/pS19 (right panels). Experiment is representative of three performed.

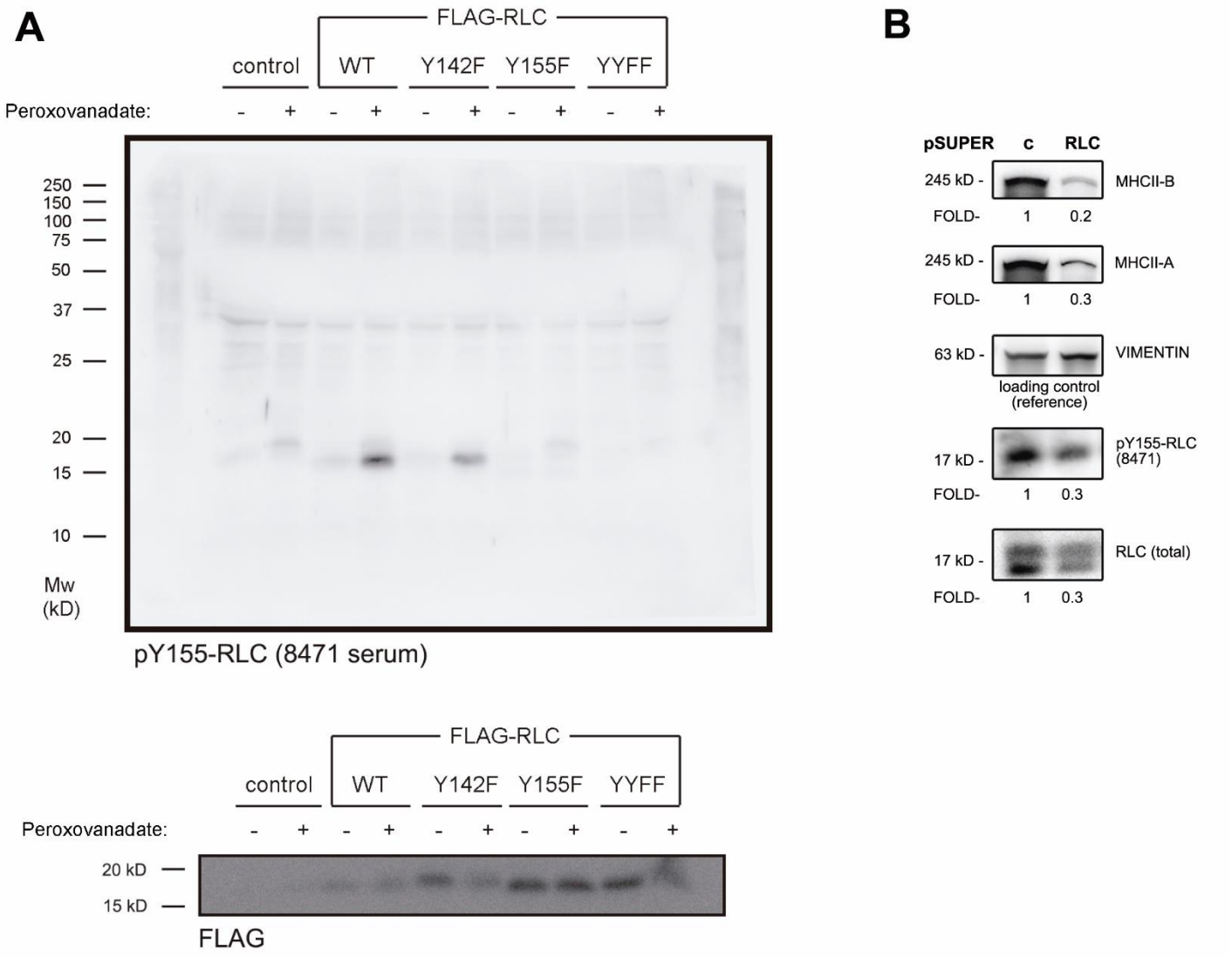

Figure S3. The 8471 antiserum recognizes RLC phosphorylated on Y155 by Western blot. Related to Figure 5.

(A) CHO.K1 cells were transfected, or not (control), with the indicated FLAG-RLC mutant and treated (+) or not (-) with 1µM sodium peroxovanadate for 30 min. Cells were lysed and extracts blotted using the 8471 antiserum (1:1000 dilution, top panel) or anti-FLAG antibody (loading control, bottom panel).

(B) CHO.K1 cells were transfected with pSUPER-RLC or a control sequence (pSUPER-C), cultured for 96h and lysed. Lysates were separated by PAGE/SDS and blotted against pY155-RLC, MHCII-A and MHCII-B. Vimentin is shown as a loading control. Experiment is representative of five performed.

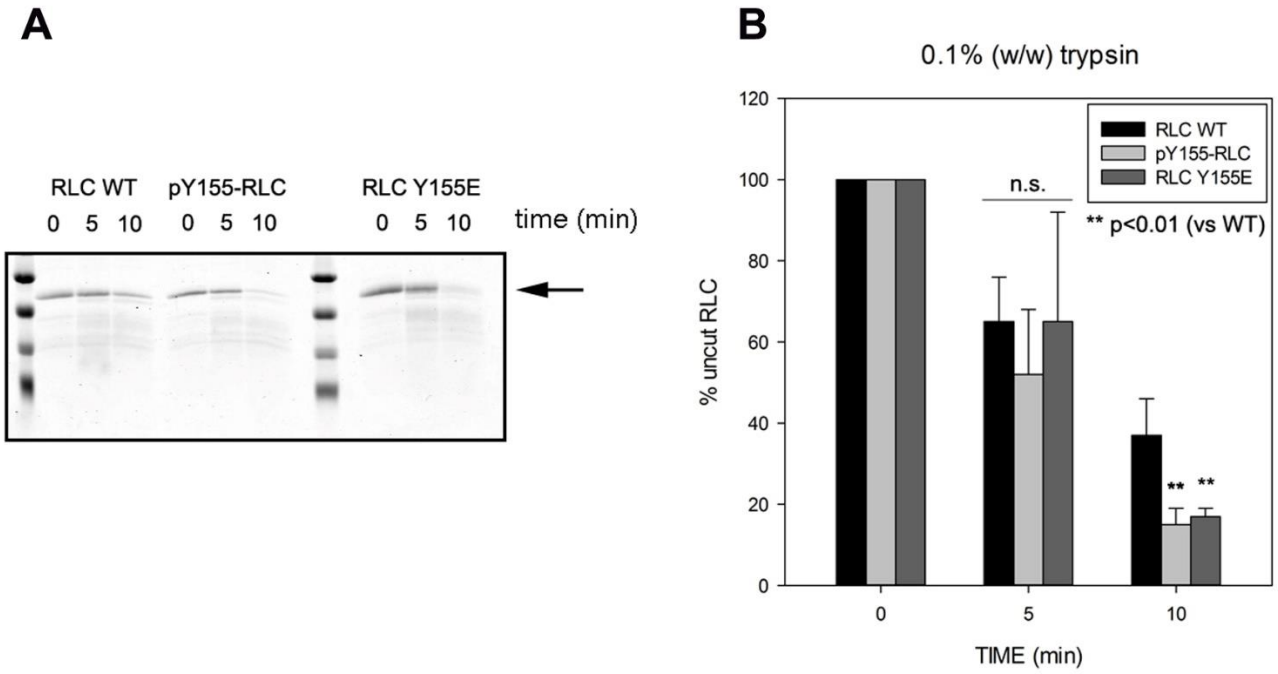

Figure S4. Proteolytic cleavage of RLC by trypsin is increased by phosphorylation of Y155 or its substitution by Glu (E). Related to Figure 5.

(A) Representative Coomassie staining of 1 µg of recombinant, FLAG-tagged wild type RLC, either untreated (RLC WT) or phosphorylated on Y155 (pY155-RLC), and FLAG-RLC Y155E, treated for the indicated times with 1 ng of trypsin. Arrow points to the non-cleaved protein at different time points (0, 5 and 10 min). A representative experiment of three performed is shown.

(B) Densitometric quantification of the experiments shown in (A). A significant difference between pY155-RLC and Y155E-RLC vs. wild type is observed at 10 min incubation.

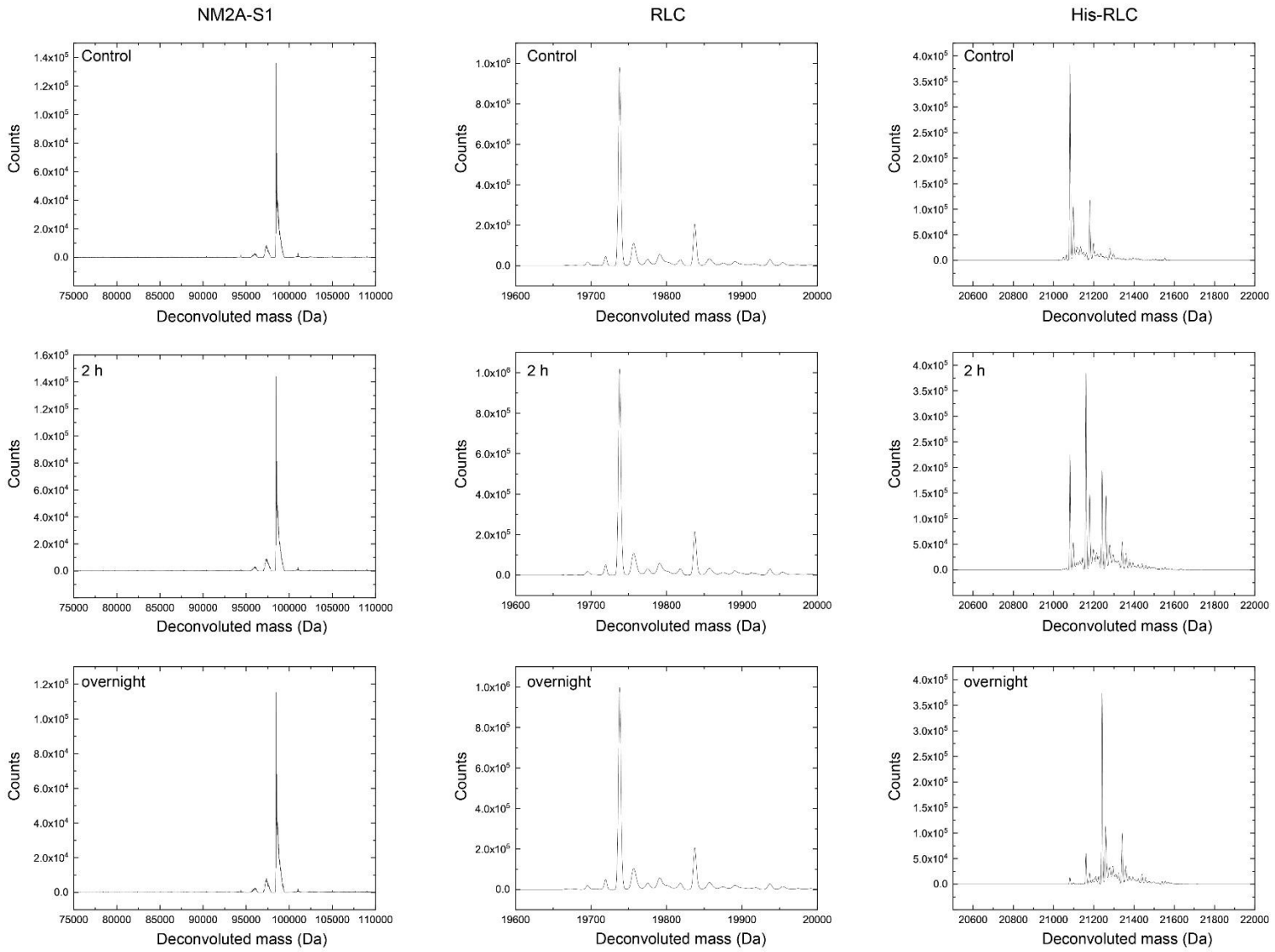

Figure S5. Phosphorylation of free, but not S1-associated RLC determined by intact protein mass spectrometry. Related to Figure 5.

Intact protein mass spectrometry of MHCII-A subfragment-1 and His-RLC after phosphorylation with EGFR. Deconvoluted mass spectra of the MHCII-A motor domain (left), the subfragment-1-bound RLC (center) and His-RLC (right) are shown after 2h and overnight incubation with EGFR as well as non-phosphorylated controls. Note that EGFR neither phosphorylated the MHCII-A motor domain nor the subfragment-1 bound RLC. 55.2% of the His-RLC was single phosphorylated and 23.2% double phosphorylated by EGFR after 2 hrs. Overnight incubation resulted in 11.3% single phosphorylated and 86.5% double phosphorylated His-RLC.

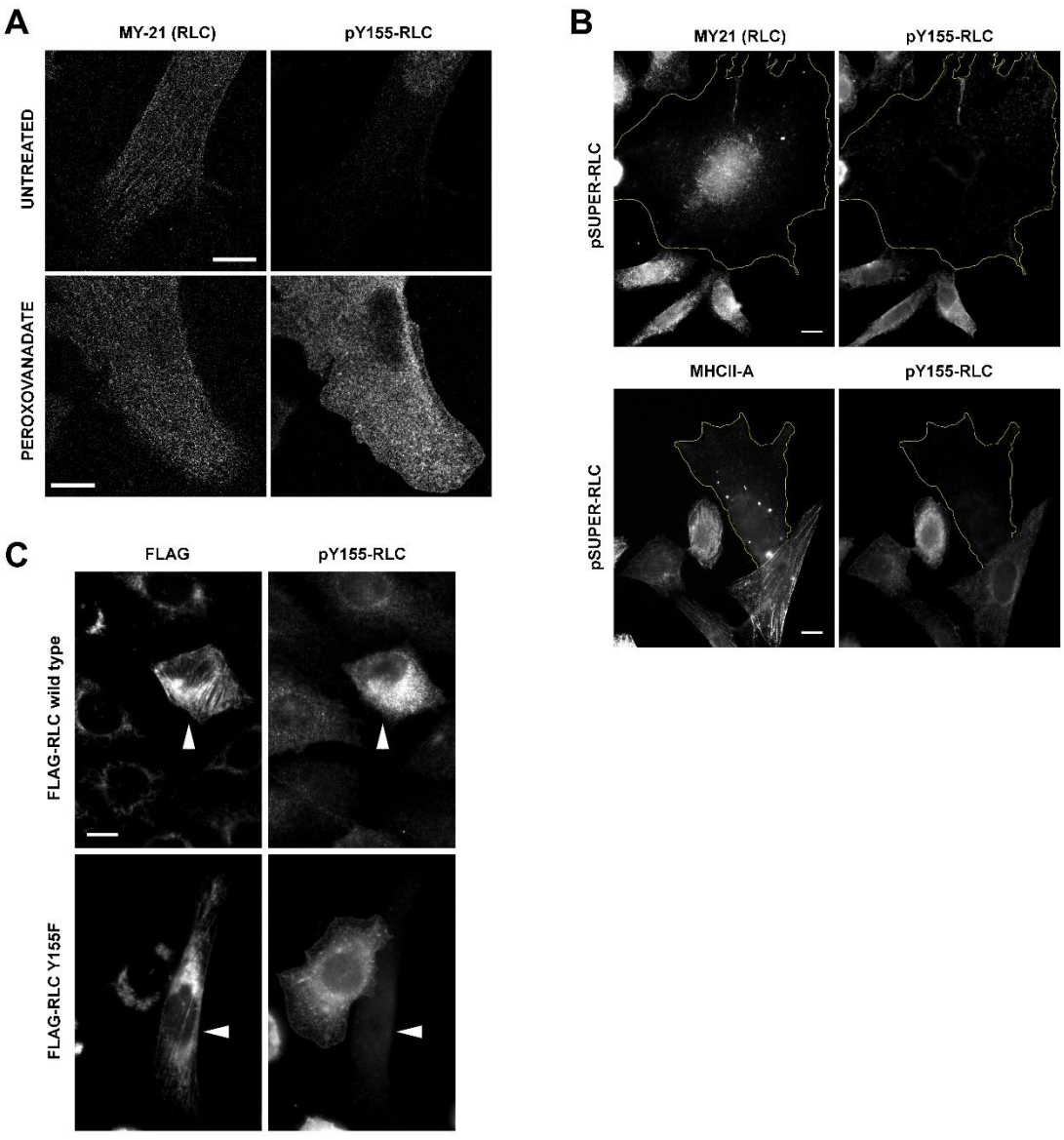

Figure S6. The 8471 antiserum recognizes RLC phosphorylated on Y155 by indirect immunofluorescence. Related to Figure 7.

(A) CHO.K1 cells were allowed to spread on fibronectin, either left untreated (top row) or treated with 1 µM peroxovanadate for 30 min (bottom row). Cells were subsequently fixed and stained for RLC (MY-21, left column) or pY155 RLC (right column). Bars= 10µm. Images were collected and are shown using the same acquisition and representation settings. Note the increase in signal in peroxovanadate-treated cells.

(B) Representative immunofluorescence images of a RLC-depleted cell (outlined in yellow), stained for endogenous, total RLC (MY21, left) and pY155 RLC (right). Note the much bigger size and decreased MY21 signal in the RLC-depleted cell compared to the non-depleted cells at the bottom. Bars=10 µm. Images represent >200 cells examined in three independent experiments.

(C) Representative immunofluorescence images of RLC-depleted cells rescued with FLAG-tagged RLC, wild type (top row) or Y155F (bottom row). Cells were stained for FLAG (left column) and pY155 RLC (right column). Bar=10 µm. Arrowheads point to transfected cells. Note the increased signal in the wild type RLC-transfected cell compared to the Y155F-expressing cell. Images represent >200 cells examined in three independent experiments.

Name	Sequence 5'-3'
pSUPER-RLC RNAi targeting sequence	GGGATGGCTTCATTGACAA
pSUPER-C RNAi targeting sequence (control)	CGTACGCGGAATACTTCGA
RLC Y142E fwd primer (GT)	ACGAGGAGGTGGACGAGATG**GAA**CGGGAGGCGCC
RLC Y142F fwd primer (GT)	ACGAGGAGGTGGACGAGATG**TTC**CGGGAGGCGCC
RLC Y142E/F rev primer (GT)	CATCTCGTCCACCTCCTCGTCAGTGAACCT
RLC Y155E fwd primer (GT)	ACAAGAAGGGCAACTTCAAC**GAA**GTGGAGTTCAC
RLC Y155F fwd primer (GT)	ACAAGAAGGGCAACTTCAAC**TTT**GTGGAGTTCAC
RLC Y155E/F rev primer (GT)	GTTGAAGTTGCCCTTCTTGTCGATGGGCGC
Fwd primer to clone RLC mutants into pCDNA3-FLAG	CGAGCTCGGATCCATGTCCAGCAAACGTGCCAAA
Rev primer to clone RLC mutants into pCDNA3-FLAG	ATATCTGCAGAATTCCTAATCGTCCTTGTCCTTAGCTCC
Fwd primer to clone FLAG-RLC mutants into pGEX-6P1	AAAAAGAATTCATGGACTACAAGGACGACGATGACA
Rev primer to clone FLAG-RLC mutants into pGEX-6P1	TTTTTGCGGCCGCCTAATCGTCCTTGTCCTTAGCTCCGTGC

Table S1. Primers. Related to STAR Methods.