Supporting Information

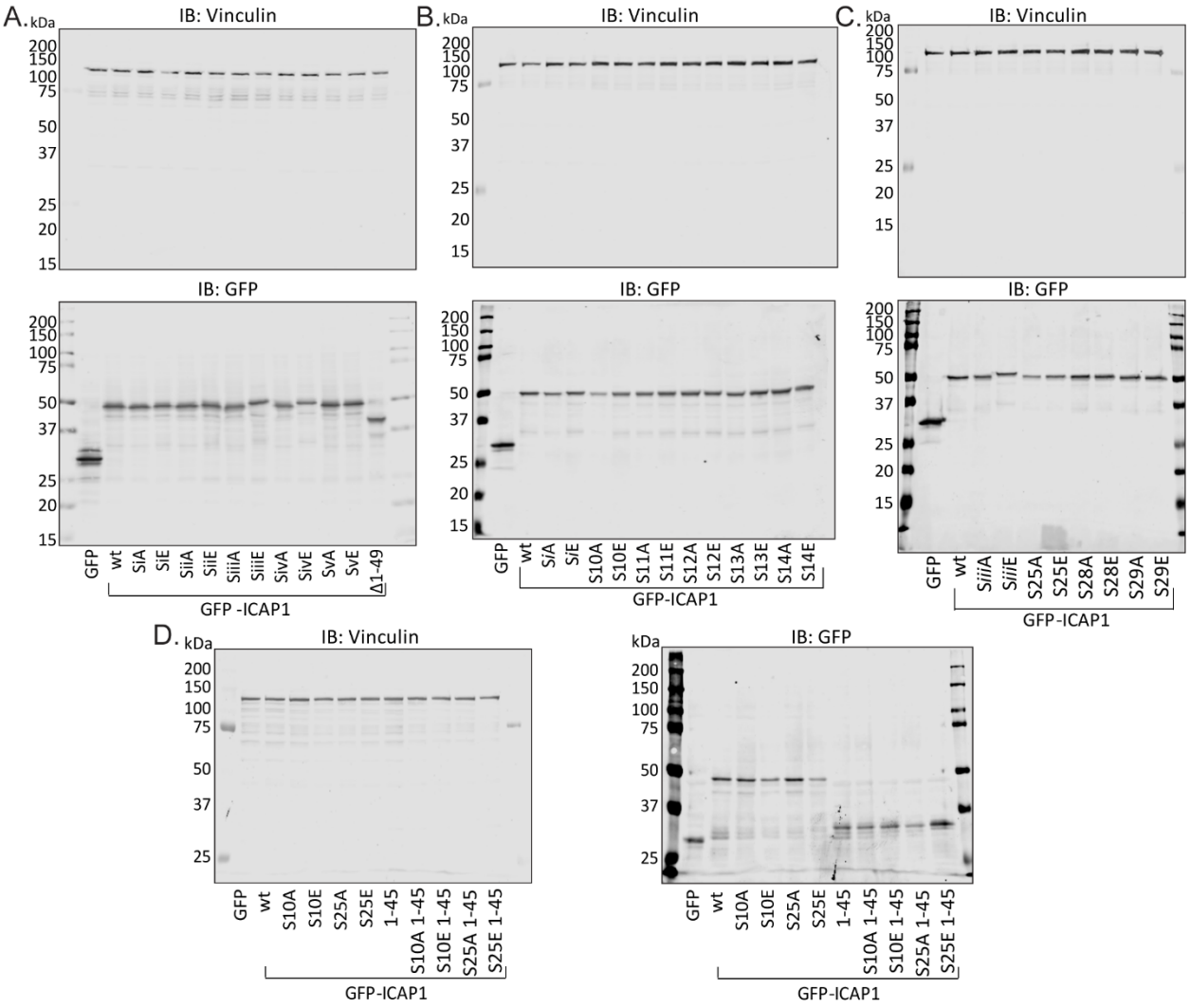

Figure S1: Representative immunoblots indicating construct expression for experiments shown in ***Fig 2D, 3C, 3F, and 6C****.* Lysates of CHO cells expressing GFP, GFP-ICAP1, or GFP-ICAP1 grouped *i-v* (A), individual group *i* (B), individual group *iii* (C), and truncation 1-45 (D) phosphorylation site mutants were immunoblotted using anti-GFP antibody. Vinculin was used as a loading control.

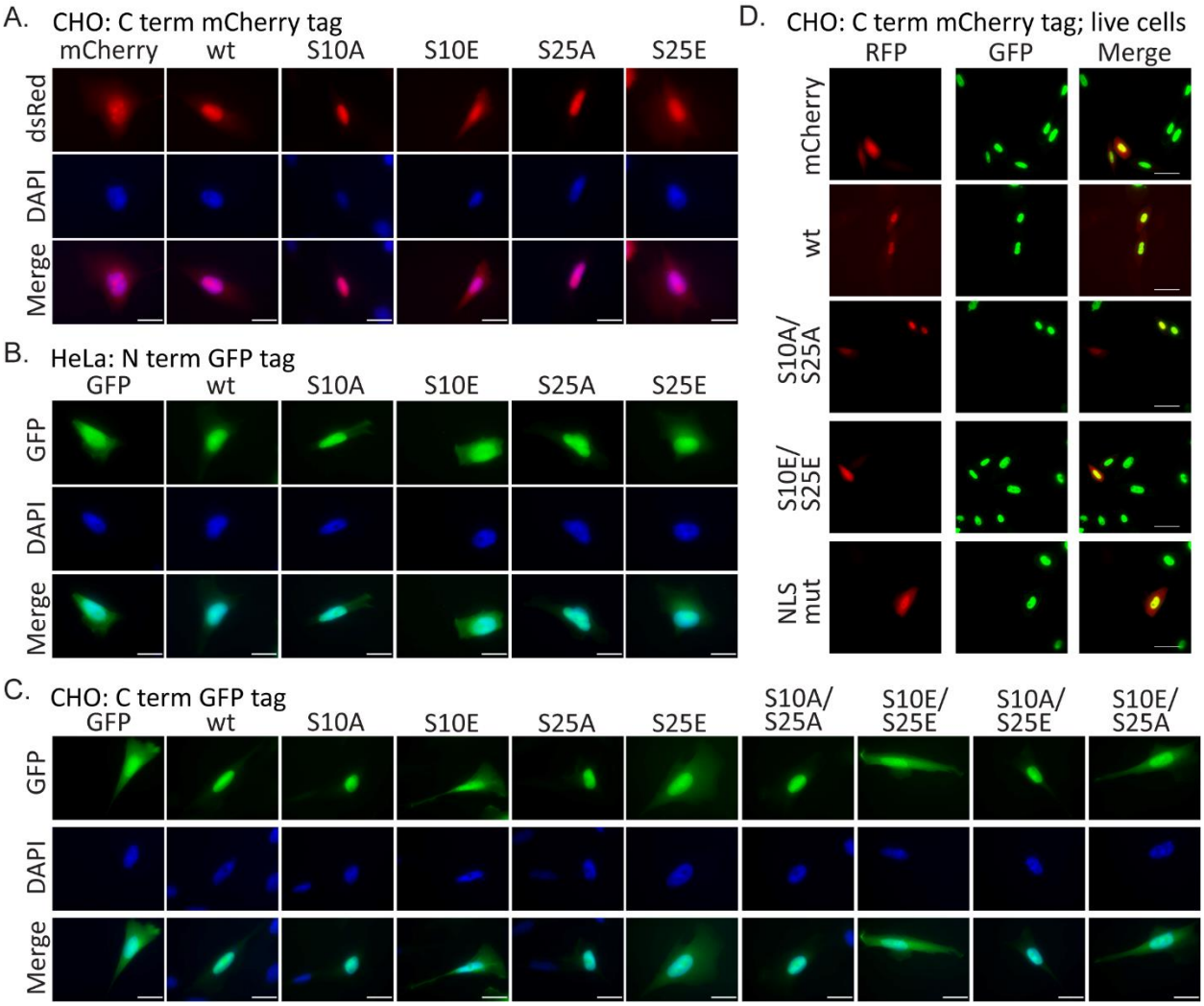

Figure S2: Representative images for quantitative microscopy experiments shown in ***Fig 4***. CHO cells (A, C) or HeLa cells (B) were transfected with constructs encoding mCherry, C-terminally mCherry-tagged ICAP1 and phosphomutants (A), or GFP, N-terminally GFP-tagged ICAP1 and phosphomutants (B), or GFP, C-terminally tagged ICAP1-GFP and phosphomutants (C). Cells were plated on fibronectin, fixed 24 hours later and stained with DAPI (to identify nuclei). (D) CHO cells stably expressing GFP-Histone 2B (to identify nuclei) and transiently transfected with constructs expressing mCherry or C-terminal mCherry-tagged ICAP1, ICAP1 phosphomutants, or ICAP1 containing mutations in the NLS (ICAP1 KK6,7AA), were plated on fibronectin-coated glass bottom dishes (MatTek), stained with HCS CellMask Deep Red Stain (to identify cell boundaries), and imaged live. Representative images, bar represents 10 μm (A-C) or 50 μm (D).

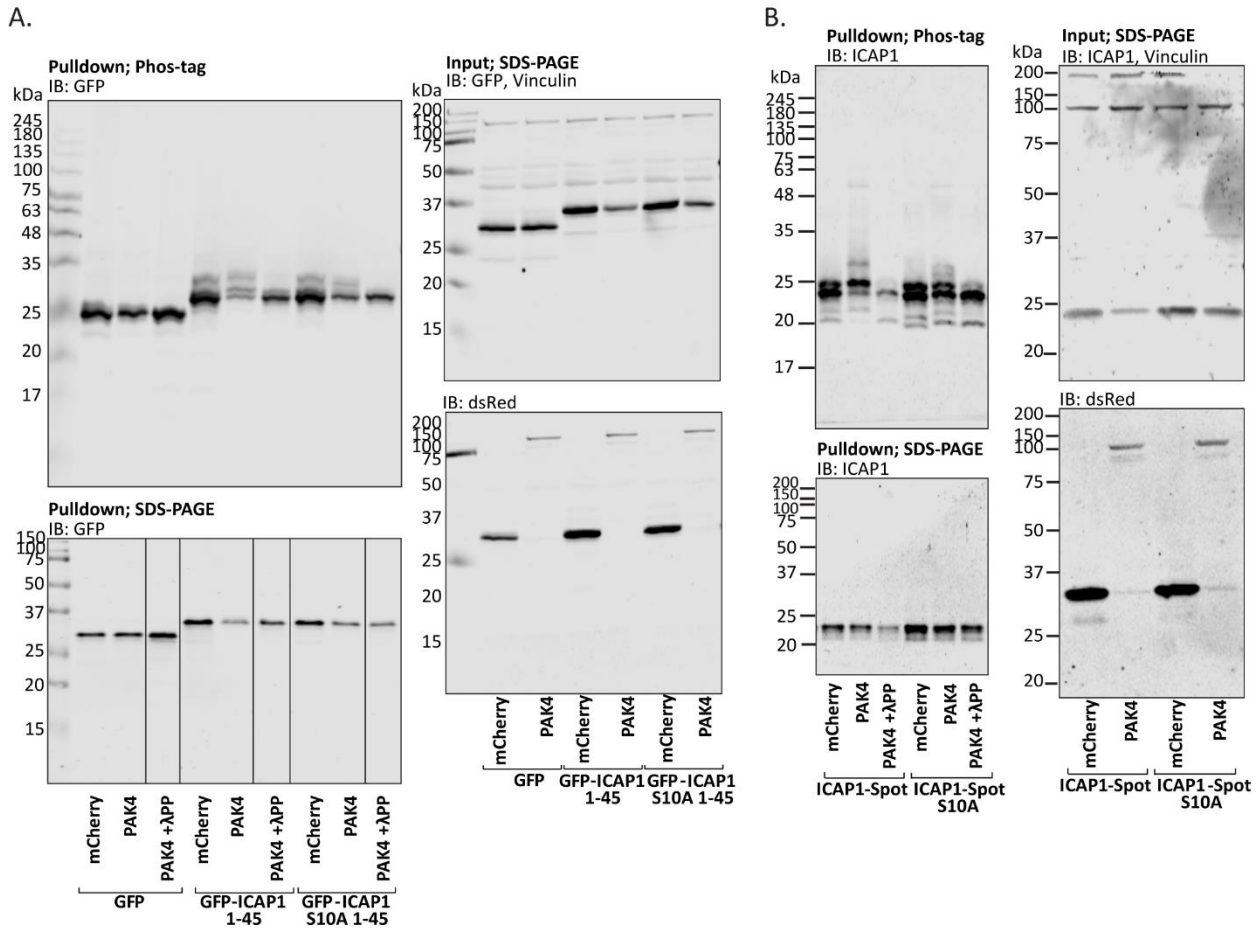

Figure S3: Representative immunoblots indicating construct expression for experiments shown in ***Fig. 8A,D***. Phos-tag™ gel mobility shift analyses were performed in CHO cells co-expressing mCherry, mCherry-PAK4 S445N and either GFP, GFP-ICAP1 1-45, or GFP-ICAP1 1-45 S10A (A) or ICAP1-Spot or ICAP1-Spot S10A (B) in the presence and absence of lambda protein phosphatase (λPP). (A) GFP-nanotrap pulldowns were resolved by Phos-tag™ PAGE and by standard SDS-PAGE (as indicated) and analyzed by immunoblotting against GFP. Input samples were also assessed by standard SDS-PAGE to evaluate expression levels of all constructs. (B) Spot-Trap® agarose pulldowns, were resolved by Phos-tag™ PAGE and by standard SDS-PAGE and analyzed by immunoblotting against ICAP1. Input samples were also assessed by standard SDS-PAGE to evaluate expression levels of all constructs.

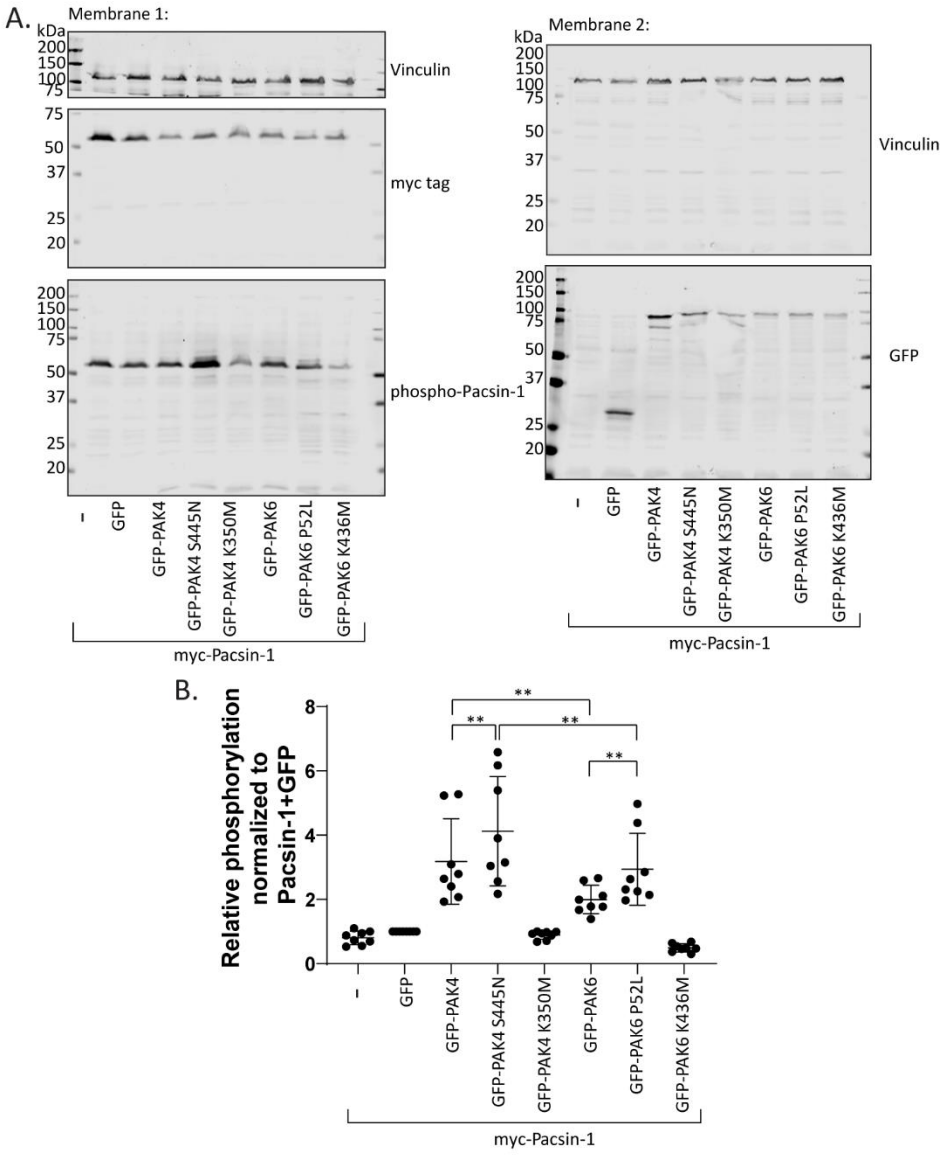

Figure S4: PAK4 and PAK6 phosphorylate Pacsin-1 to levels dependent on their kinase activity. (A,B). CHO cells expressing myc-tagged Pacsin-1 and GFP, GFP-PAK4 or PAK6 constructs were lysed, resolved by SDS-PAGE and immunoblotted against phospho-pacsin-1, myc tag, GFP, and Vinculin. The relative phosphorylation of Pacsin-1 in all conditions was normalized to Pacsin-1+GFP for 8 independent experiments. Bars show mean with standard deviation. Statistical analysis was performed using one-tailed paired t test. ** indicates $p \leq 0.01$.

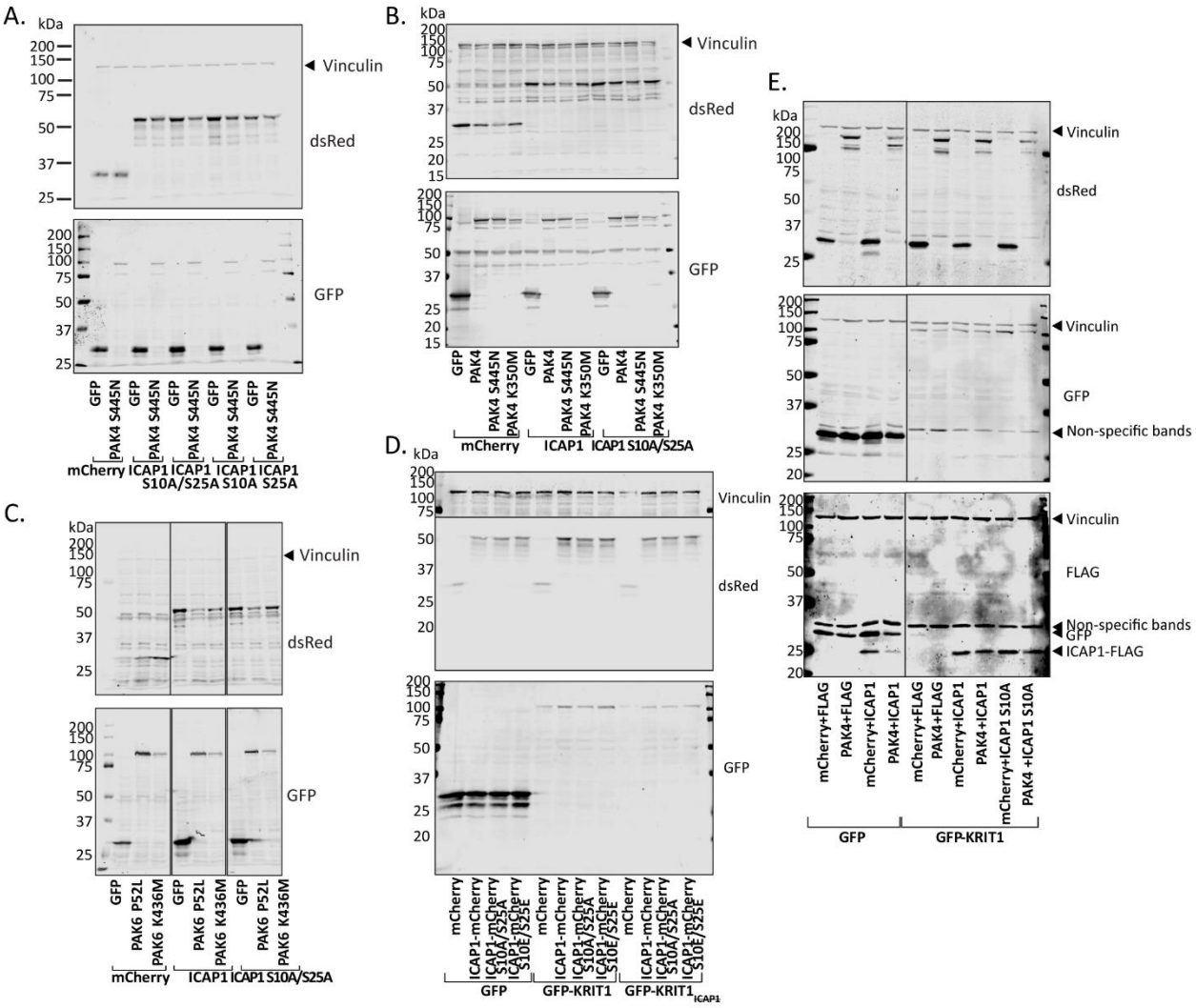

Figure S5: Representative immunoblots indicating construct expression for experiments shown in ***Fig. 9*** *and* ***10***. Lysates of CHO cells expressing mCherry, ICAP1-mCherry, or ICAP1-mCherry phospho-blockers and GFP or GFP-PAK4 (A,B) or PAK6 (C) were immunoblotted using anti-GFP or anti-DsRed antibodies as indicated. Vinculin was used as a loading control. Lysates of CHO cells expressing mCherry, ICAP1-mCherry, or ICAP1-mCherry phosphomutants and GFP, GFP-tagged KRIT1 or a KRIT1 mutant defective in binding ICAP1 (KRIT1$_{\text{ICAP1}}$;GFP-KRIT1 R179A/R185A/N192A/Y195A) (D) or co-expressing FLAG, C-terminally triple FLAG tagged ICAP1 (ICAP1-FLAG) or ICAP1-FLAG S10A, with mCherry or mCherry-PAK4 S445N, and GFP or GFP-KRIT1 (E) were immunoblotted with the indicated antibodies.