Supplemental Materials
Molecular Biology of the Cell

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SUPPLEMENTAL INFORMATION

Supplemental Figure 1

(A) Full-length (FL) Aslphospho-mutants localize to centrioles. S2 cells were depleted of endogenous Asl for 7 days. On day 4, cells were transfected with wild-type or phospho-mutant
Asl-FL-GFP. The next day, expression was induced for 72 hours. Cells were immunostained for PLP to mark centrioles. Each Asl construct co-localizes with PLP. Scale, 5 μm.

(B) Expression of FLAsl phospho-mutants induces centriole amplification independent of their phosphorylation state. S2 cells were treated as described in A, and the number of centrioles per cell were measured. Nlp-GFP was used as a control. n = 3 experiments per construct (total 300 cells/construct). In all figures, asterisks indicate significant differences and error bars show SEM. ns, not statistically significant.

(C) Plk4 binds FLAsl independently of Asl’s phosphorylation state. A non-degradable (ND) Plk4-myc was co-transfected with the indicated Asl-FL-GFP construct into S2 cells, and the next day expression was induced for 24 hours. Samples were prepared by anti-GFP immunoprecipitation from lysates, and immunoblots were probed for GFP, myc and α-tubulin (loading control).

(D) Asl-A phospho-mutants weakly localize to centrioles. S2 cells were depleted of endogenous Asl for 7 days. On day 4, cells were transfected with wild-type or phospho-mutant Asl-A-GFP and the following day were induced to express for 72 hours. Cells were immunostained for PLP to mark centrioles. Expression of each Asl-A-GFP construct was diffuse throughout the cytoplasm and only weakly localized to centrioles. Scale, 5 μm.

(E) Expression of Asl-A phospho-mutants has no effect on the protein levels of kinase-dead (KD) Plk4. S2 cells were treated with control or Asl RNAi for 6 days. On day 4, cells were transfected with inducible Plk4-KD-GFP either alone or with the indicated Asl-A-V5 construct, and then induced to express the next day for 24 hours. Immunoblots of lysates were probed with anti-GFP, V5, and Asl. Co-transfected Nlp-GFP was used as a loading control.
**SUPPLEMENTAL FIGURE 2:** The phosphorylation state of Asl-A does not influence cellular aggregate formation and has little effect on its binding to Plk4 PB1-PB2.

(A) Co-expression of Plk4 and Asl-A does not induce the formation of intracellular aggregates. S2 cells were co-transfected with Plk4-GFP and the indicated Asl-A-V5 construct, and induced
to express the next day for 24 hours. Since aggregates were rarely observed, images shown are representative images. Scale, 5 μm.

(B) A constant amount of purified Asl-A-His6 (WT, 13A, or 13PM) was incubated with a varying amount of GST-PB1-PB2 bound to glutathione resin. Each assay included an appropriate amount of purified GST on glutathione resin to ensure that the total molar quantity of GST and resin volume were similar in each binding assay. The post-incubation supernatant (S) and twice-washed resin (P) were analyzed by SDS-PAGE, and the Coomassie-stained gels are shown (left). The molar ratios of Asl-A to GST-PB1-PB2 are indicated (top) as well as the positions of the Asl-A-His6 and GST-PB1-PB2 proteins (left and right-side arrowheads, respectively). (Below) The average percent of resin-bound Asl-A is graphed for the four different molar ratios of Asl-A:GST-PB1-PB2. n = 3 experiments. Error bars, SEM. Generally, the pseudo-phosphorylation state of Asl-A does not have much impact on the binding of Asl-A to the tandem PB1-PB2 domains of Plk4.

(C) The phospho-Asl-A mutants associate with Plk4 PB1-PB2 by immunoprecipitation. S2 cells were depleted of endogenous Asl for 6 days. On day 4, cells were co-transfected with inducible PB1-PB2-GFP and the indicated Asl-A-V5 construct, and then induced to express the next day for 24 hours. Samples were prepared by anti-GFP immunoprecipitation from cell lysates. Immunoblots were probed for GFP, V5 and α-tubulin (loading control).

(D) Neither Asl-A-13A nor 13PM mutant associates with Plk4 1-381 (which lacks PB1-PB2; see Figure 1A) by immunoprecipitation. Plk4 1-381-GFP and the indicated Asl-A-V5 constructs were co-transfected into S2 cells. Samples were prepared by anti-GFP immunoprecipitation of cell lysates. Immunoblots were probed for GFP, V5 and α-tubulin.
(E) Validation of the anti-Ana2 phospho-specific antibody. Bacterially expressed and purified MBP-Ana2 was incubated with ATP and +/- purified active Plk4. The anti-pS318 antibodies detect only Ana2 incubated with Plk4. Immunoblots probed with 2 different affinity-purified anti-pS318 polyclonal antibodies are shown.
SUPPLEMENTAL FIGURE 3: Plk4 S228 phosphomutants are catalytically active but do not bind Asl when lacking PB1-PB2.

(A) Comparison of expressed Asl-A-V5 and endogenous Asl levels. Plk4-GFP/Asl-A-V5 dual expression plasmids were transfected into S2 cells. The next day, expression was induced for 72 hrs with the same concentration of CuSO$_4$ (2mM) used in all centriole counting experiments. Blots were probed using Asl-A antibody to compare expressed Asl-A-V5 and endogenous Asl levels.

(B) Plk4 S228 phospho-mutants do not inhibit Plk4 self-destruction. S2 cells were transfected with indicated Plk4-GFP constructs and the next day were induced to express for 24 hours. Lysates were immunoblotted for GFP. Kinase-dead (KD) Plk4 was used as negative control. Nlp-GFP was used as loading control. Note that the levels of WT, S228A, and S228D Plk4 constructs are similar and clearly less than KD Plk4, indicating the S228A and S228D mutants retain the ability to autophosphorylate and thereby stimulate Slimb-mediated proteolysis.

(C) Plk4 S228 phospho-mutants promote centriole amplification (>2 centrioles) in S2 cells when overexpressed. The indicated Plk4-GFP constructs were transfected into S2 cells and induced to express the next day for 72 hours. Centrioles were counted after immunostaining fixed cells to visualize PLP, a centriolar marker. This result further confirms that the S228 mutants are active kinases.

(D) S228 phospho-mutants of a Plk4 construct (amino acids 1-381) truncated to remove PB1-PB2 do not stably associate with Asl-A WT or phospho-mutants. Plk4-GFP and Asl-A constructs were co-transfected into S2 cells and the next day were induced to express for 24 hours.
Samples were prepared by anti-GFP immunoprecipitation of cell lysates. Immunoblots were probed for GFP, V5 and α-tubulin.

(E) Asl-A-13PM does not exhibit increased binding to full length Plk4 S228D mutant. Plk4-GFP and Asl-A constructs were co-transfected into S2 cells and the next day were induced to express for 24 hours. Samples were prepared by anti-GFP immunoprecipitation of cell lysates. Immunoblots were probed for GFP, V5 and α-tubulin. Graph shows the average Asl-A-13D-V5 (normalized to the quantity of Plk4-GFP in the IP) that immunoprecipitates with Plk4-S228D-GFP relative to control (Plk4-WT-GFP). $n = 2$ experiments. Error bar, SEM.
### Supplemental Table 1. Phosphorylation sites of Asl-A.

| Phosphorylated Residue | PeptideSequence | Scaffold Peptide Identification Probability (%) | Phosphate Localization Probability (%) | Mascot Ion Score | Mascot Identity Score | Ascore | Charge | ΔPPM |
|------------------------|----------------|-----------------------------------------------|-------------------------------------|------------------|-----------------------|--------|--------|-------|
| **Phosphorylation Modifications: In Vitro Experimental** | | | | | | | | |
| S7                     | NTPGISLFQGADALNINSTLDRQEEEALQDQKR | 100 | 100 | 86.3 | 53.47 | 39.35 | 2 | 6.61 |
| T337                   | LNETTELDLISVIQHQADESPTSR | 99  | 89  | 61.39 | 53.18 | 10.32 | 3 | -3.89 |
| T338                   | LNETTELDLISVIQHQADESPTSR | 100 | 92  | 74.53 | 52.96 | 12.04 | 3 | 2.69  |
| T339                   | LNETTELDLISVIQHQADESPTSR | 100 | 33  | 97.63 | 41.47 | 0.0    | 3 | -1.66 |
| S346                   | LNETTELDLISVIQHQADESPTSR | 100 | 100 | 116.79 | 53.16 | 48.02 | 3 | -3.06 |
| S359                   | LNETTELDLISVIQHQADESPTSR | 100 | 14  | 69.29 | 41.26 | 0.0    | 3 | 5.15  |
| S362                   | DLIDSVIQHQADEPSRTL$\text{S}$MQGSRL | 94  | 47  | 52.97 | 52.18 | 6.43 | 3 | 4.64  |
| **Phosphorylation Modifications: In Vivo Experimental** | | | | | | | | |
| T3                     | MNTPGISLFQGADALNINSTLDRQEEEALQDQKR | 62  | 100 | 40.76 | 54.34 | 36.93 | 4 | 3.88  |
| T3                     | MNTPGISLFQGADALNINSTLDRQEEEALQDQKR | 92  | 83  | 46.65 | 53.21 | 7.89 | 3 | 16.5  |
| S7                     | MNTPGISLFQGADALNINSTLDRQEEEALQDQKR | 100 | 98  | 64.21 | 54.46 | 20.57 | 3 | -4.62 |
| S19                    | MNTPGISLFQGADALNINSTLDRQEEEALQDQKR | 66  | 90  | 40.52 | 54.55 | 9.14 | 3 | 8.45  |
| S19                    | MNTPGISLFQGADALNINSTLDRQEEEALQDQKR | 99  | 48  | 61.41 | 54.3  | 0.0  | 3 | -2.69 |
| T20                    | MNTPGISLFQGADALNINSTLDRQEEEALQDQKR | 100 | 97  | 76.17 | 54.2  | 15.42 | 3 | -0.338 |
| S134                   | HLSIMQAELDR | 99  | 100 | 57.52 | 46.97 | 1000.0 | 2 | -4.43 |
| S266                   | YNALO$\text{S}$GHETMLVDK | 99  | 100 | 76.22 | 48.99 | 68.55 | 2 | -2.05 |
To identify \textit{in vitro} phosphorylated residues of Asl-A, bacterially-expressed and purified Asl-A was incubated with purified Plk4 kinase domain and MgATP, resolved by SDS-PAGE, prepared for mass spectrometry (MS) (i.e., reduced, alkylated, and proteolyzed), and then analyzed by LC-MS/MS (see Methods). Phosphorylated Ser or Thr residues of recovered peptides are underlined and bold font. To identify \textit{in vivo} phosphorylated residues, transgenic GFP-tagged full-length Asl was co-transfected with either mutant kinase-dead mutant Plk4 (control) or active Plk4 (experimental). Asl was immunoprecipitated from lysates of transfected S2 cells (see MATERIALS AND METHODS), prepared for MS as mentioned above, and then analyzed by LC-MS/MS. No phosphorylated peptides were recovered for the Asl-A regions of the \textit{in vitro} and \textit{in vivo} control samples. Coverages of the Asl-A regions of samples were: \textit{in vitro} control, 94%; \textit{in vitro} experimental, 98%; \textit{in vivo} control, 53%; \textit{in vivo} experimental, 84%. All of the residues listed in the table for ‘\textit{in vitro} experimental’ were present in the recovered peptides of ‘\textit{in vitro} control’, with the exception of S7. All of the listed residues for ‘\textit{in vivo} experimental’ were present in the recovered peptides of ‘\textit{in vivo} control’.

|   | Sequence                  | In vitro | In vitro | In vivo | In vivo | In vitro | In vivo | In vitro | In vivo | In vitro | In vivo |
|---|--------------------------|----------|----------|---------|---------|----------|---------|----------|---------|----------|---------|
| T338 | LNETTELDLIDSVIQQHQADESPTSR | 97       | 4        | 53.6    | 52.92   | 0.0      | 3       | 3.62     |         |          |         |
| T339 | LNETTTELDLIDSVIQQHQADESPTSR | 99       | 97       | 62.39   | 53.12   | 21.83    | 3       | 3.74     |         |          |         |
| S346 | LNETTTELDLIDSVIQQHQADESPTSR | 97       | 98       | 50.09   | 52.63   | 17.78    | 3       | 9.38     |         |          |         |
| S356 | LNETTTELDLIDSVIQQHQADE\textbf{S}PTSR | 100      | 100      | 70.78   | 53.26   | 24.34    | 3       | 3.62     |         |          |         |