Supplemental Information for

KNL1 binding to PP1 and microtubules is mutually exclusive

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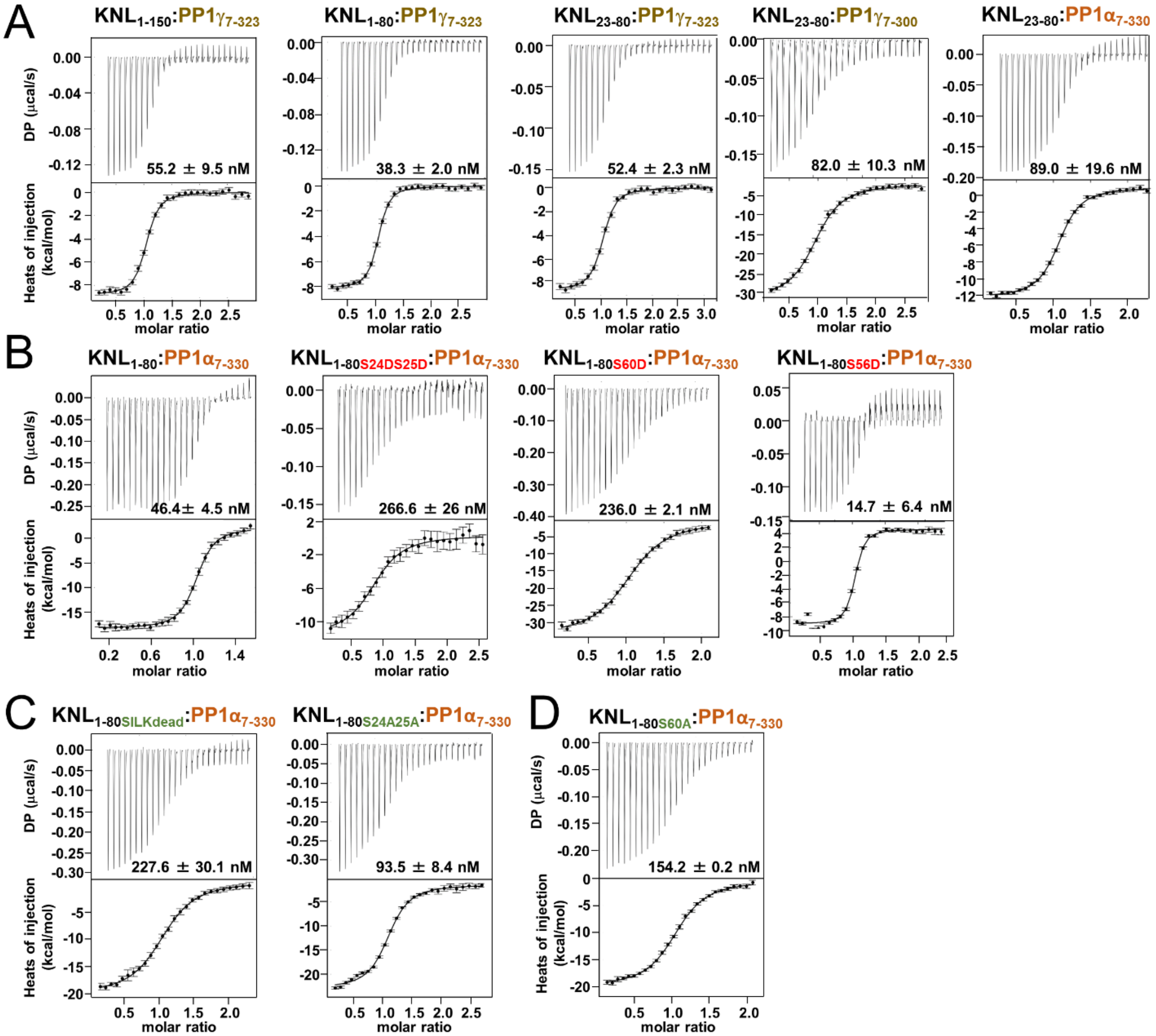

Figure S1. ***Isothermal titration calorimetry of KNL1 with PP1.***

Related to Figure 1.

(**A**) KNL1$_{1-150}$:PP1γ$_{7-323}$, KNL1$_{1-80}$:PP1γ$_{7-323}$, KNL1$_{23-80}$:PP1γ$_{7-323}$, KNL1$_{23-80}$:PP1γ$_{7-300}$, KNL1$_{23-80}$:PP1α$_{7-330}$.

(**B**) WT and phosphomimetic mutants of KNL1 with PP1: KNL1$_{1-80}$:PP1α$_{7-330}$, KNL1$_{1-80S24DS25D}$:PP1α$_{7-330}$, KNL1$_{1-80S60D}$:PP1α$_{7-330}$, KNL1$_{1-80S56D}$:PP1α$_{7-330}$.

(**C**) SILK mutants of KNL1 with PP1: KNL1$_{1-80SILKdead}$:PP1α$_{7-3300}$, KNL1$_{1-80S24AS25A}$:PP1α$_{7-330}$.

(**D**) RVxF mutant of KNL1 with PP1: KNL1$_{1-80S60A}$:PP1α$_{7-330}$

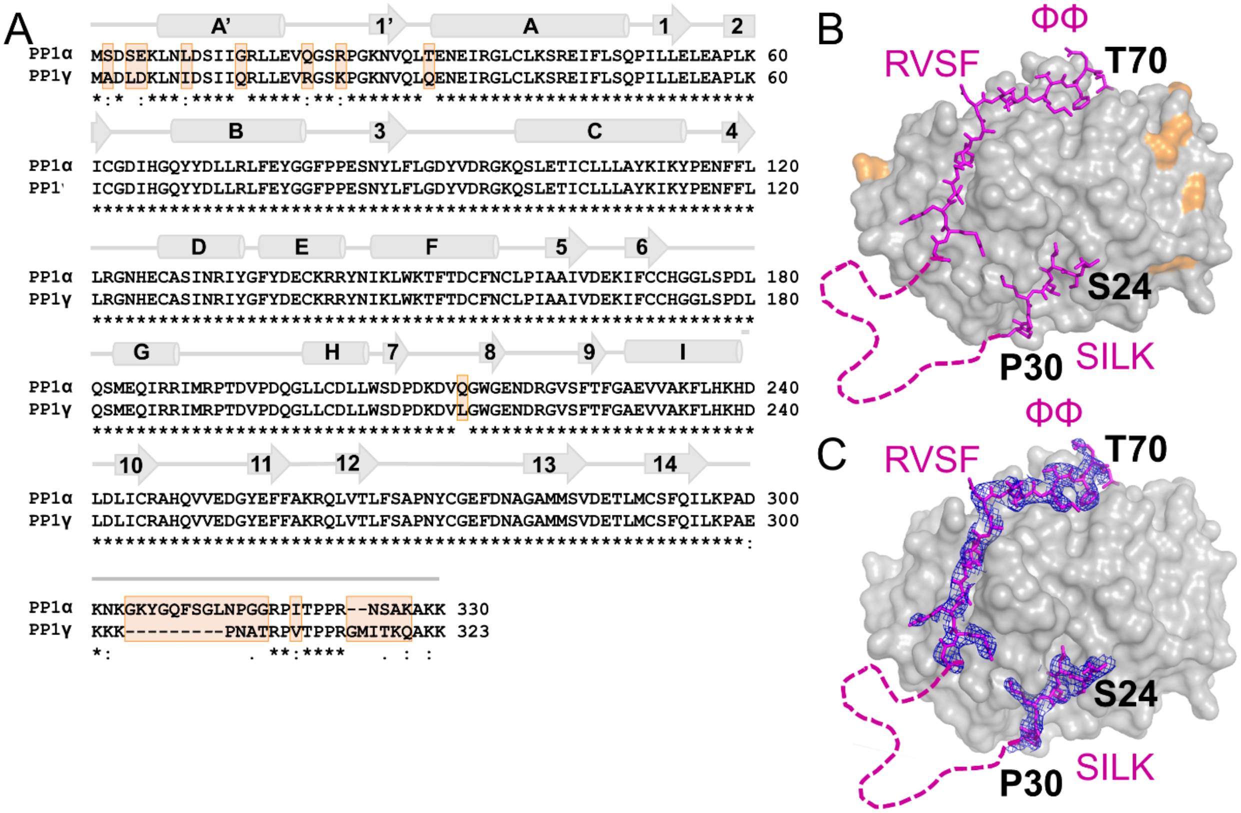

Figure S2. ***KNL1 is not isoform specific***.

Related to Figure 1.

(**A**) Sequence alignment of PP1α and PP1γ, with sequence difference shaded in orange. PP1 secondary structure elements are shown as cylinders (helices) and arrows (strands) above the sequence.

(**B**) The structure of the KNL1:PP1 holoenzyme with KNL1 in pink and PP1 shown as a grey surface. Residues that differ between PP1α and PP1γ are shaded grey. KNL1 does not interact with any residues that differ between the two PP1 isoforms.

(**C**) F_o-F_c omit map contoured at 1σ of bound KNL1.

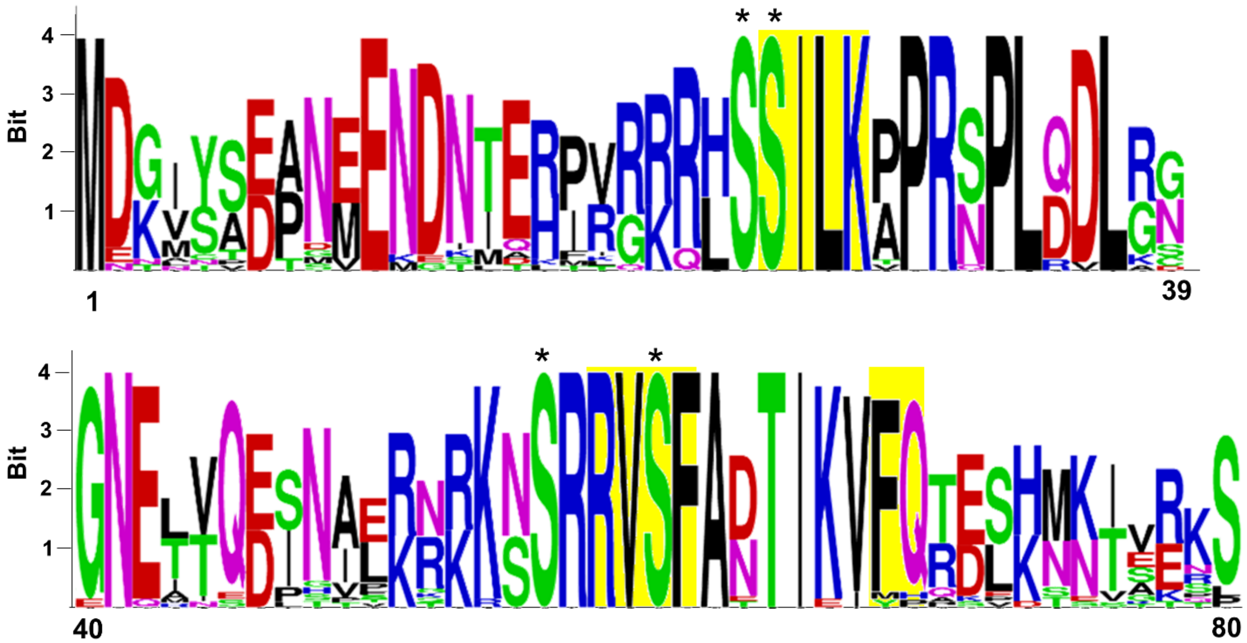

Figure S3. ***Sequence logo of an alignment of KNL1 PP1 interaction domain (residues 1-80) from 27 organisms from T. adhaerens to H. sapiens.***

Related to Figure 1.

PP1 interaction motifs, which are highly conserved, are highlighted in yellow. Serine residues phosphorylated by Aurora B Kinase are starred.

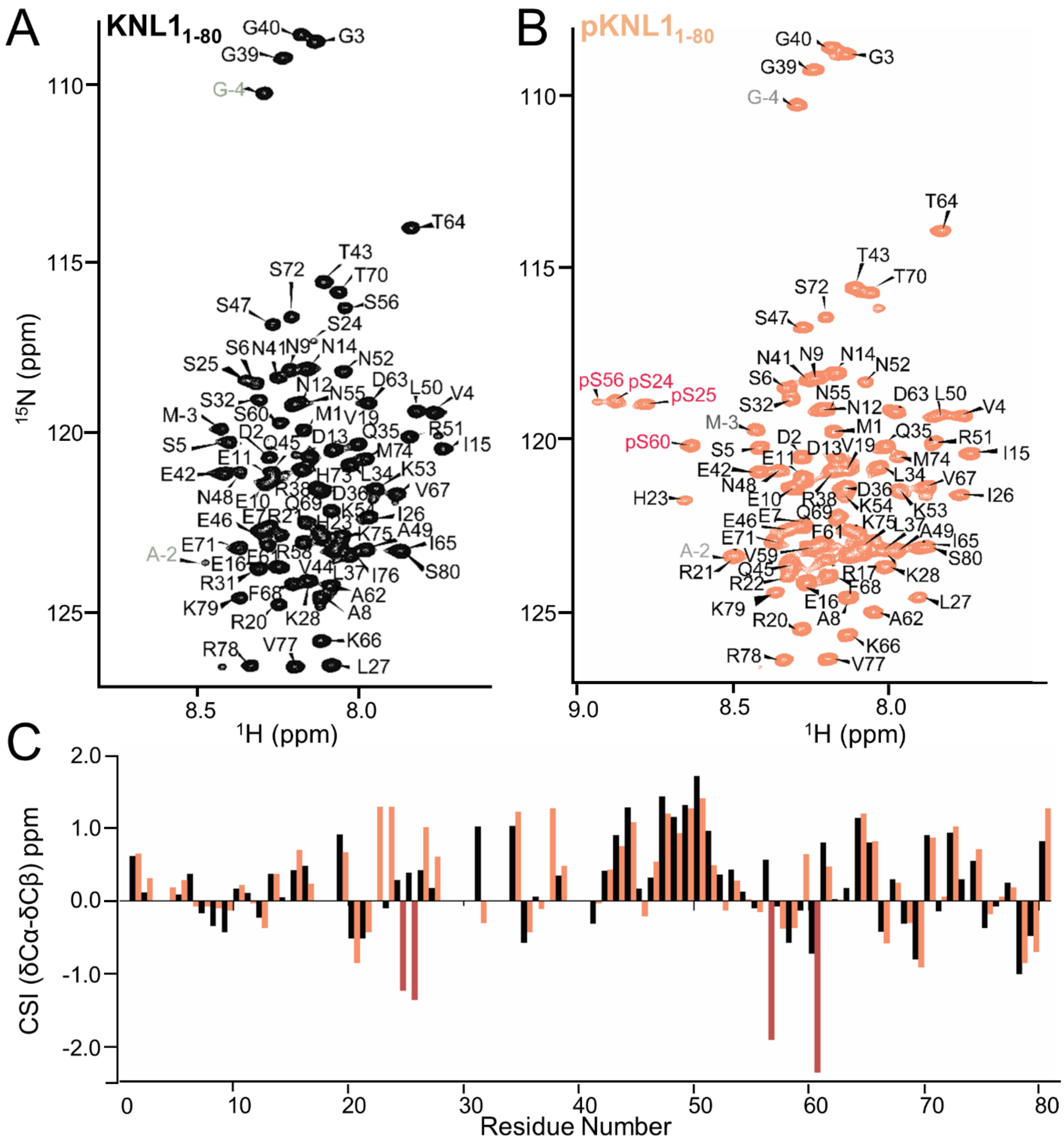

Figure S4. ***KNL1 is phosphorylated by Aurora Kinase at four residues.***

Related to Figure 3.

2D [^{1}H,^{15}N] HSQC of **(A)** KNL1$_{1-80}$ (black) and (**B**) pKNL1$_{1-80}$ (phosphorylated by Aurora Kinase; orange). Aurora B phosphorylates KNL1 at residues S24, S25, S56 and S60.

(**C**) Chemical shift index (CSI) plotted against residue number for KNL1 (black) and pKNL1 (orange); the phosphorylated serine residues are indicated (red). Both un-phosphorylated and phosphorylated KNL1 lack preformed secondary structure elements.

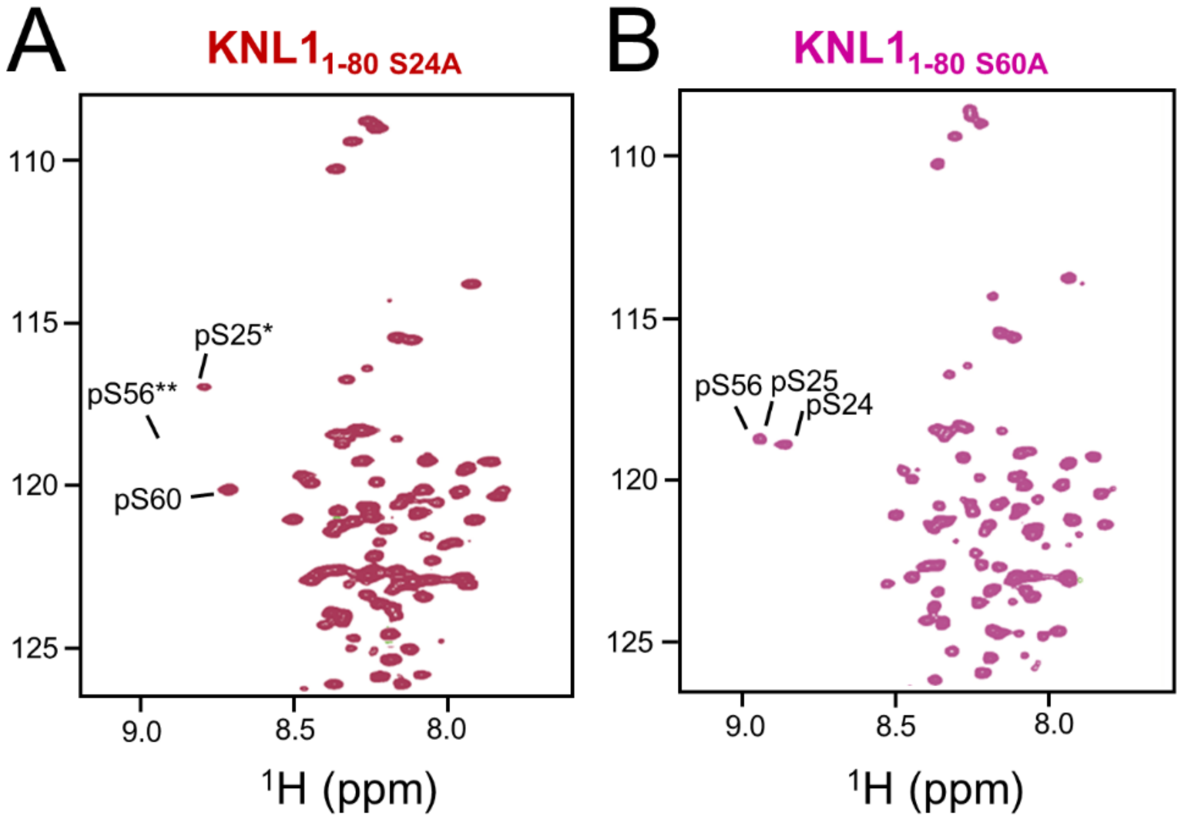

Figure S5. ***KNL1 residues are independently phosphorylated by Aurora B Kinase*.** Related to Figure 3.

2D [^{1}H,^{15}N] HSQCs of Aurora B kinase phosphorylated KNL1 variants: (**A**) pKNL1$_{1\text{-}80}$S24A and (**B**) pKNL1$_{1\text{-}80}$S60A. Each single point mutation does not alter the ability of Aurora B kinase to phosphorylate the remaining three Ser residues. *, N-H^N cross-peak of pS25 in KNL-1$_{1\text{-}80}$ S24A. **pS56 peak is visible at lower contours.

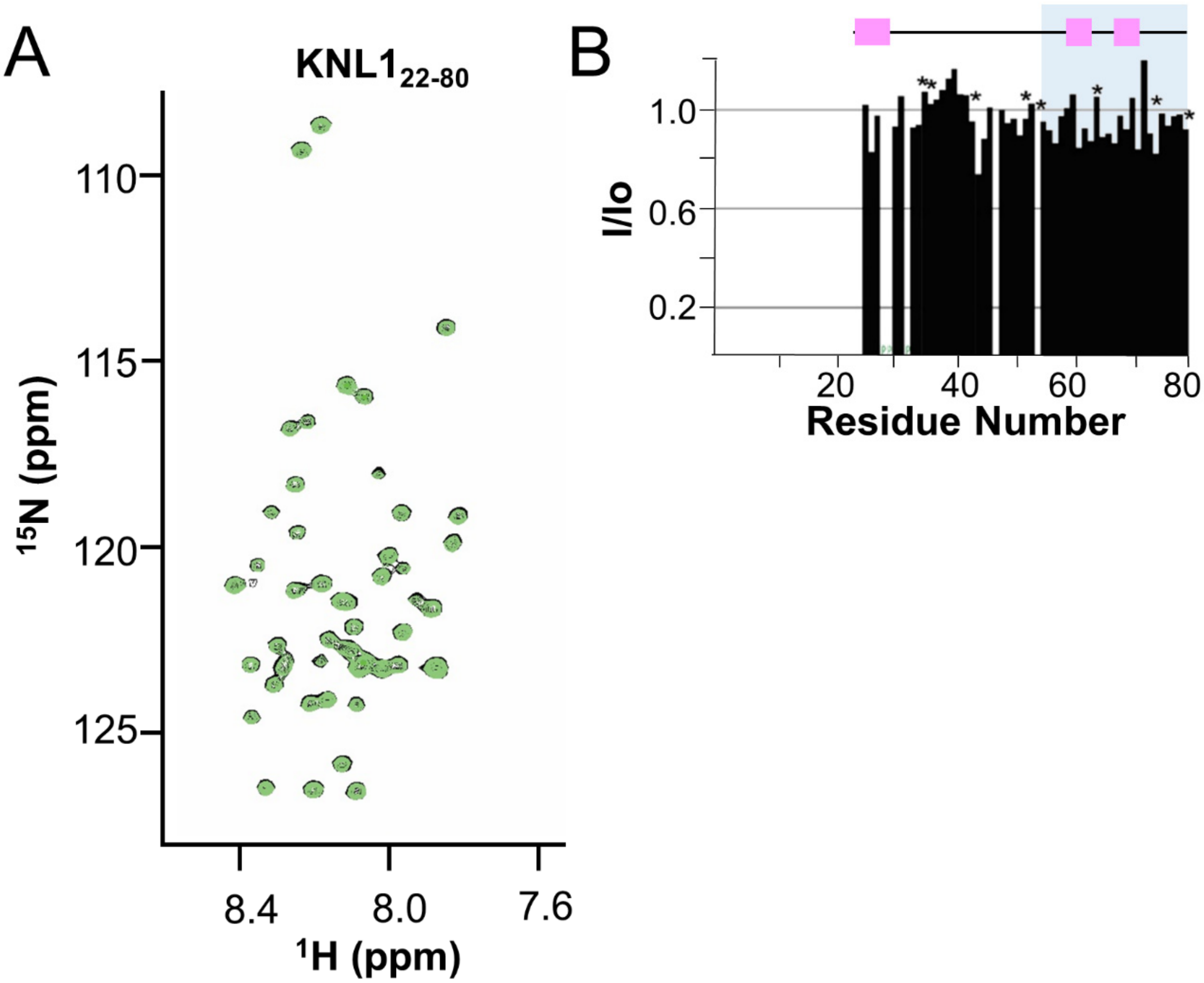

Figure S6. ***MTBS1 is the primary MT binding site in KNL1.***

Related to Figure 4

(A) An overlay of 2D [^{1}H,^{15}N] HSQC spectrum of free (black) and MTB-bound KNL1$_{22-80}$ (green). (**B**) Normalized Intensity ratios of KNL1$_{\text{MTbound}}$(I)/ KNL1$_{\text{free}}$ (I$_0$) upon addition of microtubules plotted vs amino acid sequence of KNL1$_{1-80}$; MTB binding site 2 is shaded in light blue. PP1 binding sites (SILK, RVxF and ΦΦ) are highlighted in pink.