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Supplementary Material

Molecular insights into the interaction between the SPRY-domain containing SOCS box protein SPSB2 and peptides based on the binding motif from iNOS

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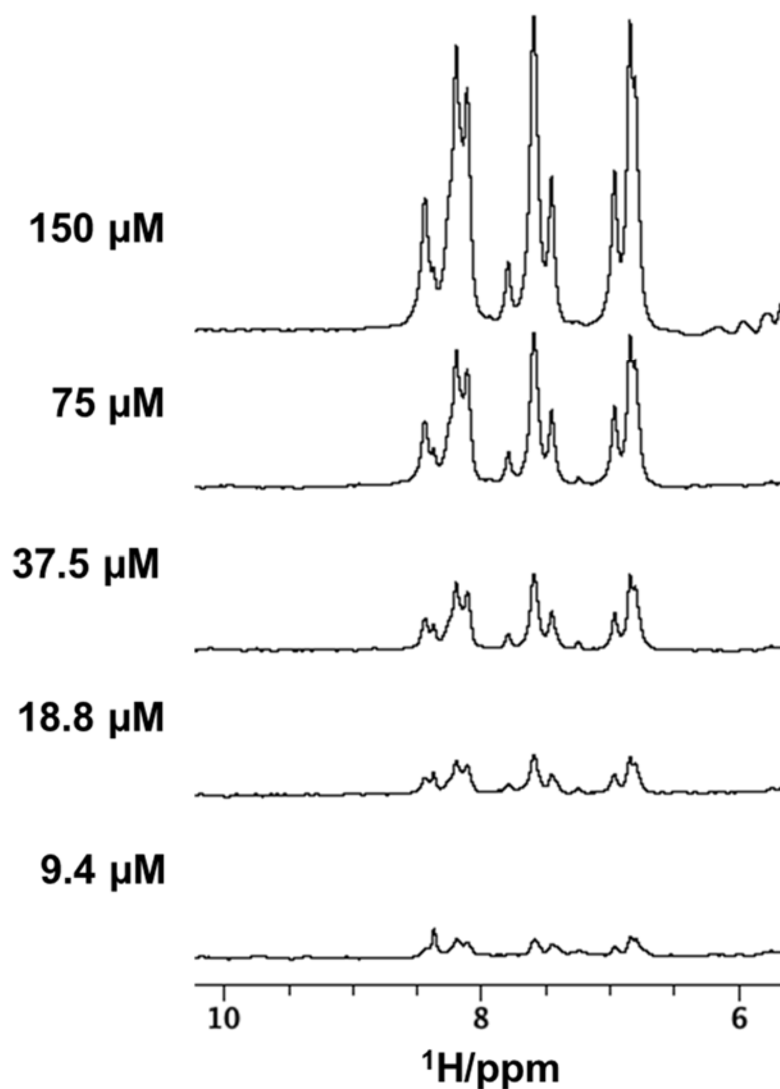

Figure S1. 1D ^{1}H NMR spectra of AINNN with 10% $^{2}H_2O$ recorded at 25ºC and pH 7 at 600 MHz with 512 scans. A series of two-fold dilutions was prepared in 90% H_2O/ 10% $^{2}H_2O$ containing 50 mM phosphate, pH 7.0, 50 mM NaCl, to give a range of concentrations from 9.4 to 150 μM. The expected 2-fold reduction in signal intensities upon 2-fold dilution clearly demonstrates that this peptide is highly soluble in phosphate buffer and not aggregated.

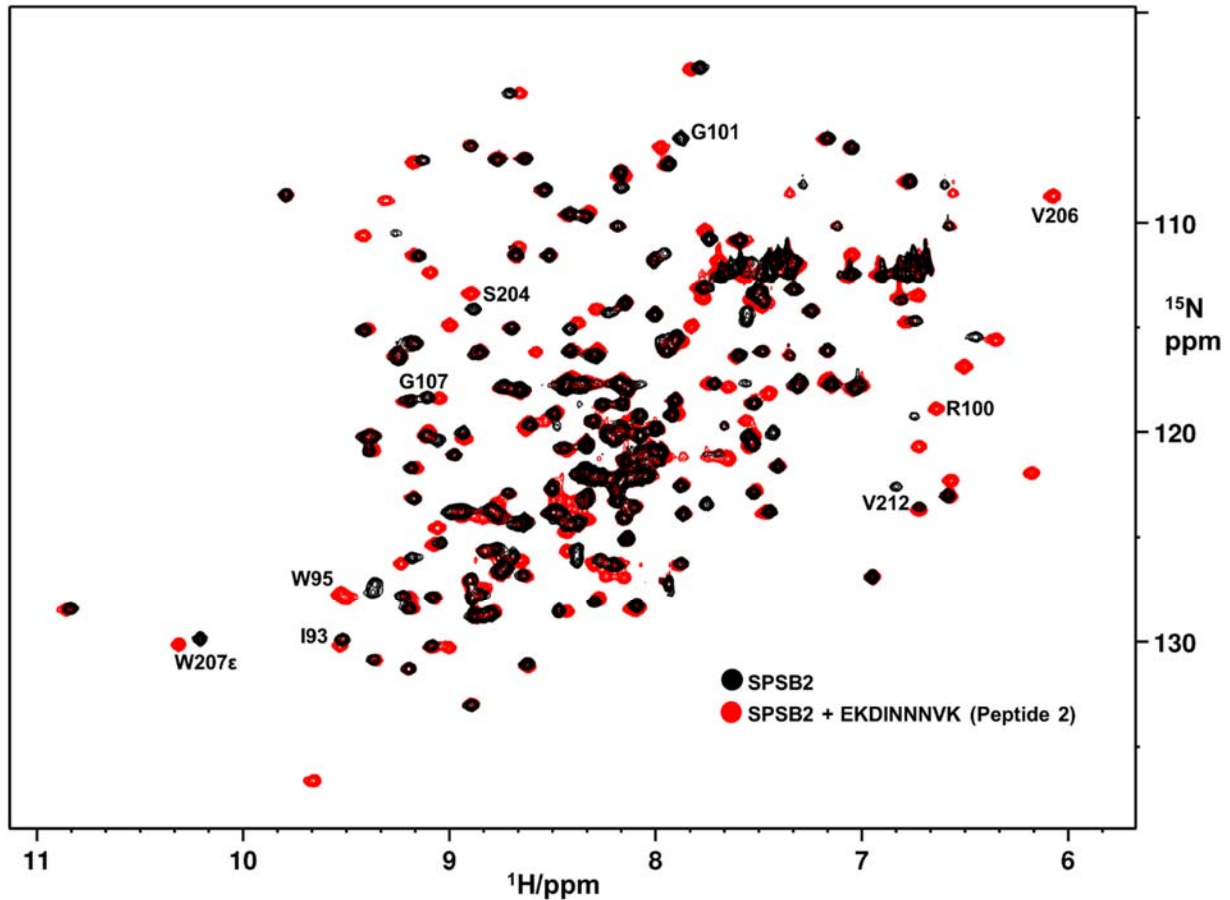

Figure S2. Interaction of 9-residue peptide **2** (EKDINNNVK) with murine SPSB2. Overlay of ^{1}H-^{15}N HSQC of 100 μM ^{15}N-labelled SPSB2 in the absence (black) and presence (red) of unlabelled peptide **2** at a SPSB2/peptide molar ratio of 1:1.5. Folded resonances are shown in grey. Samples were prepared in 90% H_2O/10% $^{2}H_2O$ containing 50 mM phosphate, pH 7.0, 50 mM NaCl, 2 mM EDTA, 2 mM DTT and 0.02% sodium azide. Spectra were acquired at 22ºC and 600 MHz with 64 scans per t_1 increment. Spectra were processed identically and plotted with the same contour levels. Resonances that were significantly perturbed are from amides in close proximity to the iNOS binding site.

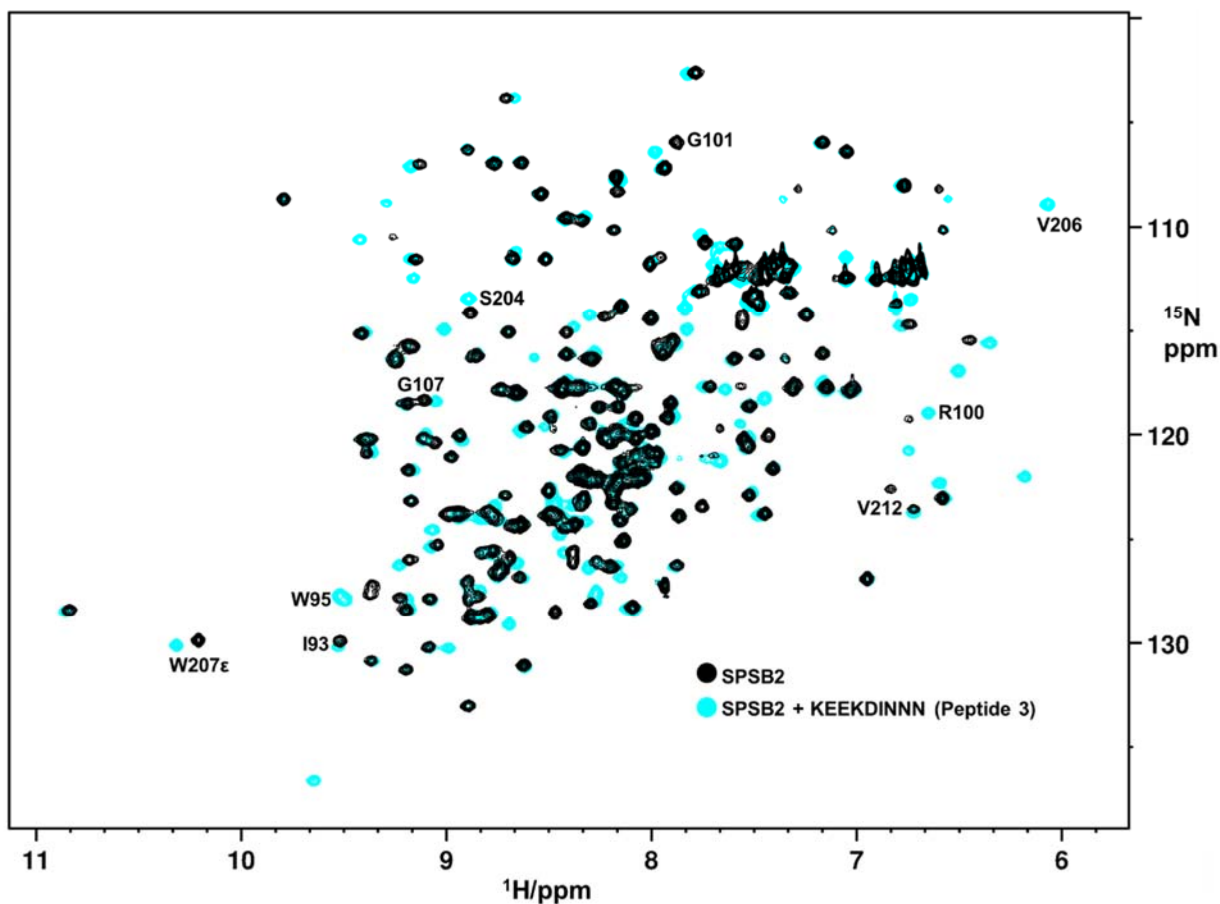

Figure S3. Interaction of 9-residue peptide **3** (KEEKDINNN) with murine SPSB2. Overlay of ^{1}H-^{15}N HSQC of 100 μM ^{15}N labelled SPSB2 in the absence (black) and presence of unlabelled peptide **3** (cyan) at aSPSB2/peptide molar ratio of 1:1.5. Samples were prepared in 90% H_2O/10% $^{2}H_2O$ containing 50 mM phosphate, pH 7.0, 50 mM NaCl, 2 mM EDTA, 2 mM DTT and 0.02% sodium azide. Spectra were acquired at 22ºC and 600 MHz with 64 scans per t_1 increment.

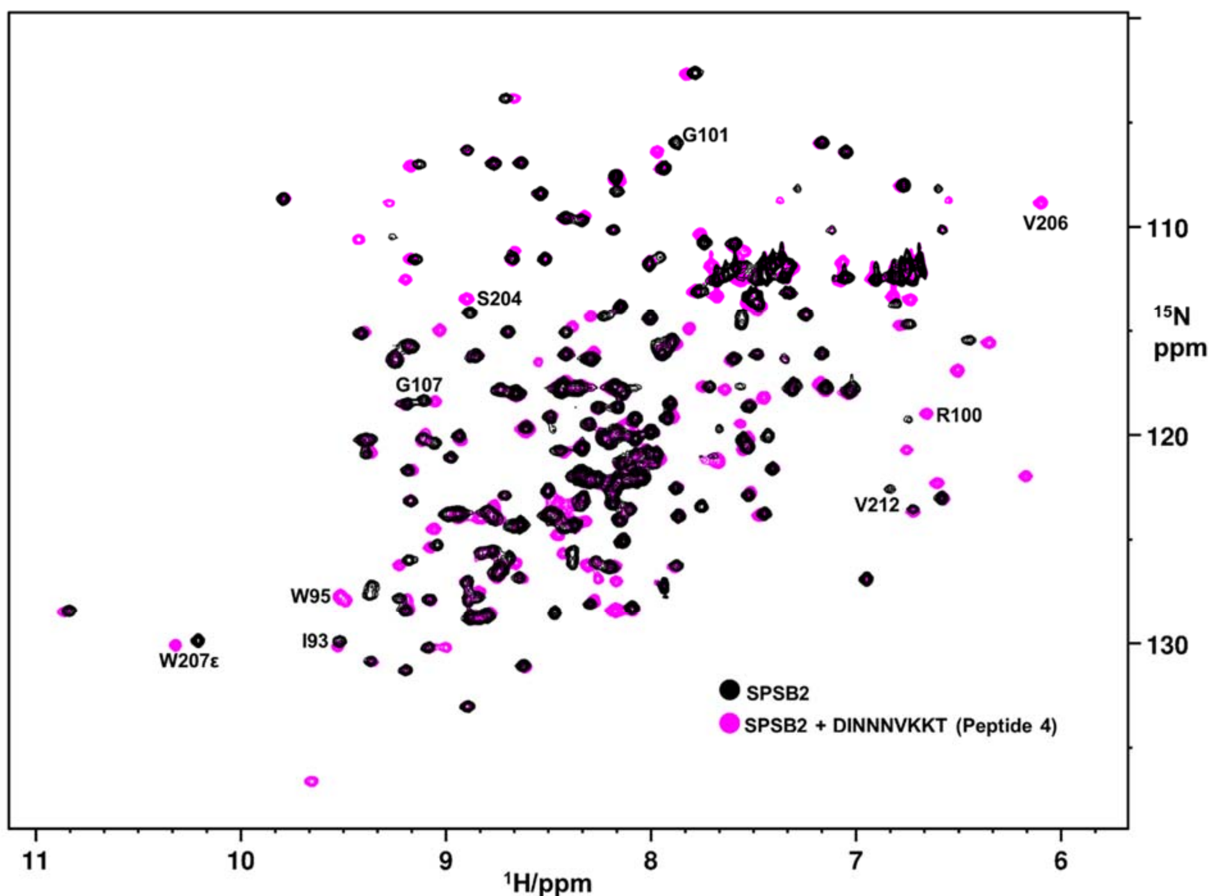

Figure S4. Interaction of 9-residue peptide **4** (DINNNVKKT) with murine SPSB2. Overlay of ^{1}H-^{15}N HSQC of 100 μM ^{15}N labelled SPSB2 in the absence (black) and presence of unlabelled peptide **4** (pink) at a SPSB2/peptide molar ratio of 1:1.5. Folded resonances are shown in grey. Samples were prepared in 90% H_2O/10% $^{2}H_2O$ containing 50 mM phosphate, pH 7.0, 50 mM NaCl, 2 mM EDTA, 2 mM DTT and 0.02% sodium azide. Spectra were acquired at 22ºC and 600 MHz with 64 scans per t_1 increment.

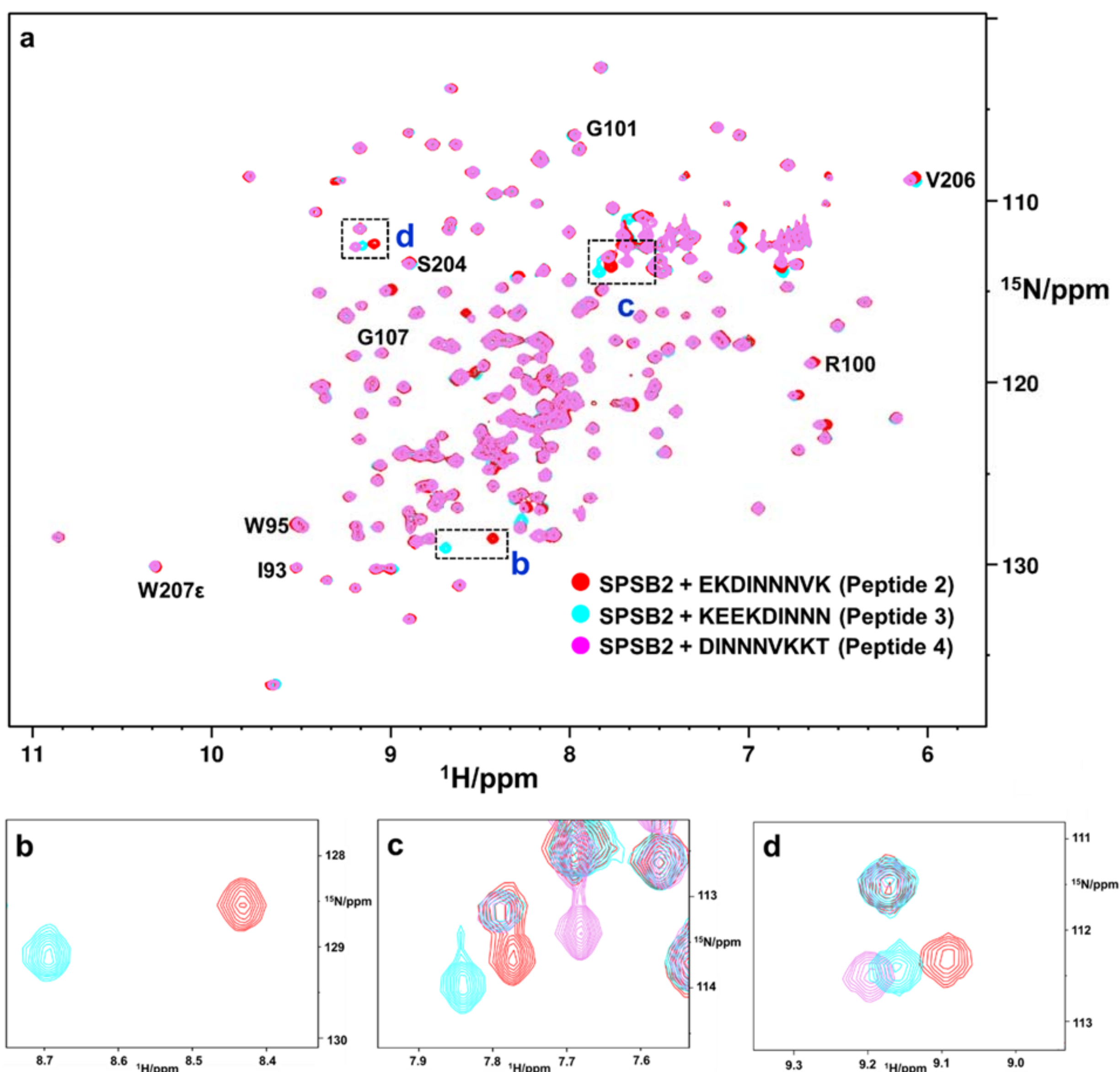

Figure S5. Comparison of ^{1}H-^{15}N HSQC spectra of murine SPSB2 bound to three 9-residue peptides **2-4**. (a) Overlay of ^{1}H-^{15}N HSQC of 100 μM ^{15}N labelled SPSB2 with three 9-residue peptides, **2** (red), **3** (cyan) and **4** (pink). In 2D ^{1}H-^{15}N HSQC spectra of ^{15}N-labelled SPSB2, all three 9-residue peptides resulted in the disappearance of a subset of resonances from the unbound SPSB2 spectrum and simultaneous appearance of a subset of resonances from the bound protein (S2-S4). These observed changes are characteristic of interactions that are in slow exchange on the NMR timescale, which is indicative of tight interactions between the peptides and protein. The slow exchange observed in the spectra precluded transferring the assignments from the unbound protein, so the resonances in each complex were not assigned. Nonetheless, a superimposition of the ^{1}H-^{15}N HSQC spectra of all three complexes showed that the three peptides caused similar spectral perturbations, although several resonances are affected differently by the three peptides. One of the largest perturbations was observed for a peak which, in the presence of a saturating concentration of peptide **2,** appears in the spectrum at ^{1}H/^{15}N chemical shifts of 8.43/128.7 ppm (b). In comparison to peptide **2**, this peak is shifted downfield to 8.69/129.2 ppm in the presence of peptide **3**, whereas it disappears in the presence of **4**. Other differentially-perturbed resonances are depicted in (c) and (d). Shown in (c) is a resonance with ^{1}H/^{15}N chemical shifts of 7.77/113.7 ppm in the presence of peptide **2**. This peak is also observed in peptide **3**, but is shifted downfield, whereas in the presence of peptide

4 it is shifted upfield. Shown in (d) is a resonance at 9.09/112.5 ppm in the presence of peptide **2**. This peak is progressively downfield shifted from peptide **2** to **4**. These data are consistent with the differences in affinity as measured by SPR and suggest that the residues flanking DINNN mediate interactions with the protein that are reflected in differences in the observed chemical shifts of a number of residues of the protein. Samples were prepared in 90% H_2O/10% ^2H_2O containing 50 mM phosphate, pH 7.0, 50 mM NaCl, 2 mM EDTA, 2 mM DTT and 0.02% sodium azide. Spectra were acquired at 22ºC and at 600 MHz with 64 scans per t_1 increment.

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                        19            31
Mouse iNOS                KEEKDINNNVKKT
                   59                     80
Human hPar4         GTPAAAAANELNNNLPGGAPAAP
                      180                        203
Drosophila VASA          RNEDDINNNNNIVEDVERKREFYI
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Figure S6. Amino acid sequence alignments of murine iNOS N-terminal 13-residue peptide sequence (iNOS$_{19\text{-}31}$; Swiss-Prot ID P29477) with human hPar4$_{59\text{-}80}$ and *Drosophila* VASA$_{180\text{-}203}$ (Swiss-Prot ID P09052). Red lettering indicates that amino acid sequence similarity was limited to a consensus motif (D/E)(I/L)NNN. Residues flanking the consensus motif are highly diverse, but are important in improving affinity for the binding proteins.