SUPPLEMENTARY INFORMATION

Ordering of the N-terminus of Human MDM2 by Small Molecule Inhibitors

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Supplementary information contains:

Figures S1–S7

Supplementary Experimental Section

Supplementary References

Figure S1: Overlay of NMR and X-ray Structure. NMR structure is shown in green and X-ray structure is shown in cyan. Notice the newly formed β-strand in the N-terminus appears longer in the X-ray structure.

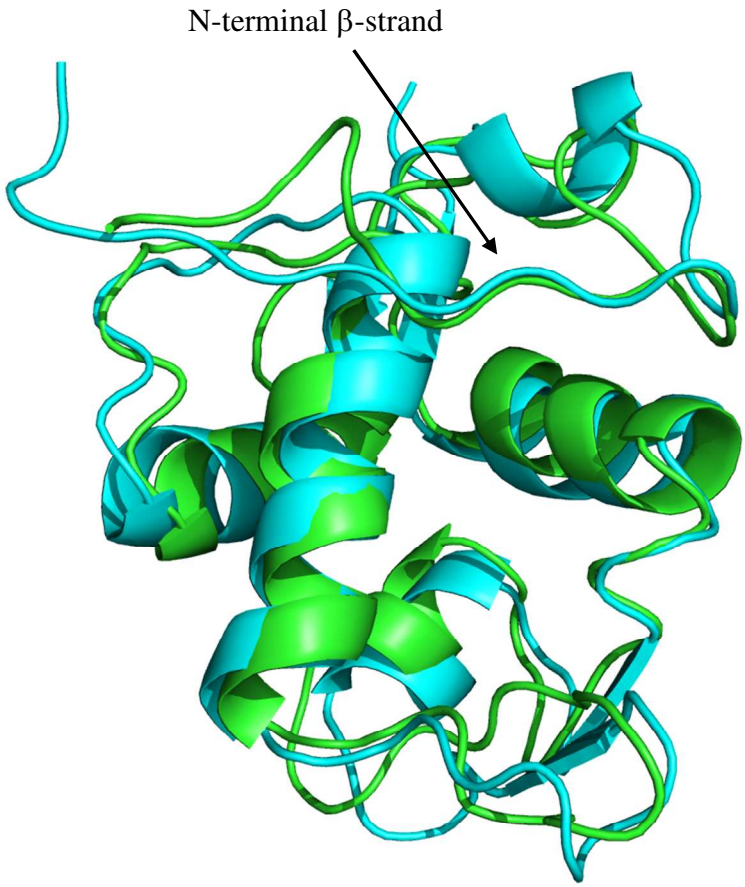

Figure S2: MDM2 (6-125)/Pip-2 dimer observed in the crystal structure. The β-strand that corresponds to the residues 10-16 of one MDM2 (6-125)/Pip-2 complex forms an anti-parallel β-sheet with the β strand from another MDM2(6-125)/Pip-2 complex

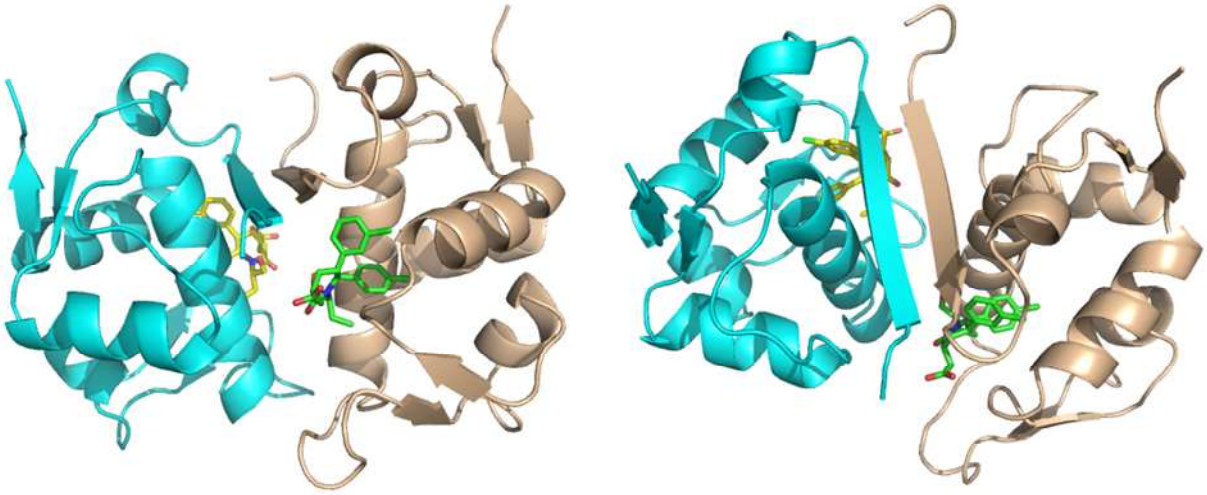

Figure S3: Chemical Shift Indices of Apo- and Holo-MDM2/Pip-1, residues 1-30 are shown. Positive values (blue bars) represent the likelihood of β-sheet secondary structure while negative values (red bars) represent the likelihood of α-helical structure (values of zero correspond to random coil). Predictions are based on HA, CA, CB, and CO chemical shifts as determined using Talos+ [1]. Notice that the chemical shifts of residues 14-16 correspond to β-sheet secondary structure and are in agreement with the NMR structure of MDM2/Pip-1.

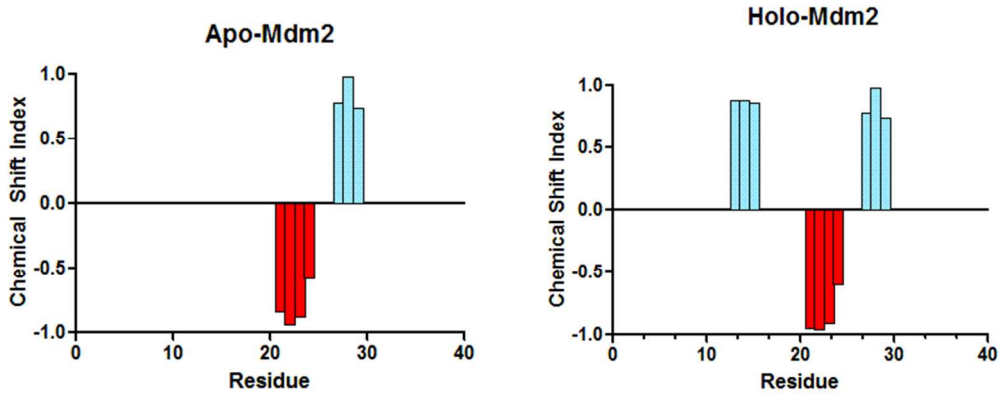

Figure S4: Sequence homology between human, dog, mouse and rat MDM2 (1-188) using ClustalW.[2] Human MDM2 (UniProtKB Q00987), dog MDM2 (UniProtKB P56950), mouse MDM2 (UniProtKB P23804) and rat MDM2 (UniProtKB P3ZQ36).

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                    10        20        30        40        50        60
                     |         |         |         |         |         |
01_Human      MCNTNMSVPTDGAVTTSQIPASEQETLVRPKPLLLKLLKSVGAQKDTYTMKEVLFYLGQY
02_Dog        MCNTNMSVSTGGAVSTSQIPASEQETLVRPKPLLLKLLKSVGAQKDTYTMKEVIFYLGQY
03_Mouse      MCNTNMSVSTEGAASTSQIPASEQETLVRPKPLLLKLLKSVGAQNDTYTMKEIIFYIGQY
04_Rat        MCNTNMSVSTEGAAGTSQIPASEQETLVRPKPLLLKLLKSVGAQKDIYTMKEIIFYIGQY
              ********.* **. ****************************:* *****::**:***
Prim.cons.    MCNTNMSVSTEGA2STSQIPASEQETLVRPKPLLLKLLKSVGAQKDTYTMKE2IFY2GQY

                    70        80        90       100       110       120
                     |         |         |         |         |         |
01_Human      IMTKRLYDEKQQHIVYCSNDLLGDLFGVPSFSVKEHRKIYTMIYRNLVVVNQQESSDSGT
02_Dog        IMTKRLYDEKQQHIVYCSNDLLGDLFGVPSFSVKEHRKIYTMIYRNLVVVNQHEPSDSGT
03_Mouse      IMTKRLYDEKQQHIVYCSNDLLGDVFGVPSFSVKEHRKIYAMIYRNLVAVSQQ---DSGT
04_Rat        IMTKRLYDEKQQHIVYCSNDLLGDVFGVPSFSVKEHRKIYAMIYRNLVVVSQQ---DSGT
              ************************:***************:*******.*.*:   ****
Prim.cons.    IMTKRLYDEKQQHIVYCSNDLLGD2FGVPSFSVKEHRKIY2MIYRNLVVV2QQE2SDSGT

                   130       140       150       160       170       180
                     |         |         |         |         |         |
01_Human      SVSENRCHLEGGSDQKDLVQELQEEKPSSSHLVSRPSTSSRRRAISETEENSDELSGERQ
02_Dog        SVSENSCHREGGSDQKDPVQELQEEKPSSSDLISRPSTSSRRRTISETEEHADDLPGERQ
03_Mouse      SLSESRRQPEGGSDLKDPLQAPPEEKPSSSDLISRLSTSSRRRSISETEENTDELPGERH
04_Rat        SPSESRCQPEGGSDLKDPVQASQEEKPSSSDVVSRPSTSSRRRAISETEENTDELPGERQ
              * **.  : ***** ** :*   *******.:::** *******:******::*:*.***:
Prim.cons.    SVSE2RC2PEGGSD2KDPVQ2LQEEKPSSSDL2SRPSTSSRRRAISETEENTDELPGERQ


01_Human      RKRHKSDS
02_Dog        RKRHKSDS
03_Mouse      RKRRRSLS
04_Rat        RKRHRALS
              ***::: *
Prim.cons.    RKRH2S2S
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MDM2 1-188

	Canine	Mouse	Rat
Identity (*)	107 is 96.40 %	99 is 89.19 %	100 is 90.09 %
Strongly similar (:)	2 is 1.80 %	8 is 7.21 %	6 is 5.41 %
Weakly similar (.)	2 is 1.80 %	4 is 3.60 %	3 is 2.70 %
Different	0 is 0.00 %	0 is 0.00 %	2 is 1.80 %

Figure S5: Sequence homology between human, dog, mouse and rat p53 (1-83) using ClustalW.[2] Human p53 (UniProtKB P04637), dog p53 (UniProtKB Q29537), mouse p53 (UniProtKB P02340) and rat p53 (UniProtKB P10361).

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                    10         20         30         40         50         60
                     |          |          |          |          |          |
01_Human      MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSP---LPSQAMDDLMLSPDDIEQWFTED
02_Dog        MEESQSELNIDPPLSQETFSELWNLLPENNVLSS---ELCPAVDELLL-PESVVNWLDED
03_Mouse      MEESQSDISLELPLSQETFSGLWKLLPPEDILP-----SPHCMDDLLL-PQDVEEFFEGP
04_Rat        MEDSQSDMSIELPLSQETFSCLWKLLPPDDILPTTATGSPNSMEDLFL-PQDVAELLEGP
              **:.**: .:: ******** **:*** :::*.         .:::*:* *:.: : :
Prim.cons.    MEESQSD4SIE2PLSQETFS4LWKLLP2N22L23TAT3SP4AMDDLLLSPQDVEEW2E22

                    70         80
                     |          |
01_Human      PGPDEAPRMPEAAPPVAPAPAAPTPA--
02_Dog        S--DDAPRMPATSAPTAPGPAPSWPL--
03_Mouse      SEALRVSGAPAAQDPVTETPGPVAPAPA
04_Rat        EEALQVS-APAAQEPGTEAPAPVAPASA
                   ..  * :  * :  *..  *
Prim.cons.    SEA2422R2PAAQ4PV22APAPVAPA2A
```

p53 (1-83)

	Canine	Mouse	Rat
Identity (*)	45 is 54.22 %	49 is 55.68 %	42 is 46.67 %
Strongly similar (:)	15 is 18.07 %	13 is 14.77 %	18 is 20.00 %
Weakly similar (.)	11 is 13.25 %	6 is 6.82 %	6 is 6.67 %
Different	12 is 14.46 %	20 is 22.73 %	24 is 26.67 %

p53 (17-26)

	Canine	Mouse	Rat
Identity (*)	8 is 80.00 %	9 is 90.00 %	9 is 90.00 %
Strongly similar (:)	2 is 20.00 %	0 is 0 %	0 is 0 %
Weakly similar (.)	0 is 0 %	1 is 10.00 %	0 is 0 %
Different	0 is 0 %	0 is 0 %	1 is 10.00 %

Figure S6: Secondary structure predictions within N-terminal region of MDM2 (first 30 residues) by DSC (Discrimination of protein Secondary structure Class).[3] C stands for random coil, E for the β-strand and H for α-helix.

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                    10        20        30
                     |         |         |
01_Human      MCNTNMSVPTDGAVTTSQIPASEQETLVRP
02_Dog        MCNTNMSVSTGGAVSTSQIPASEQETLVRP
03_Mouse      MCNTNMSVSTEGAASTSQIPASEQETLVRP
04_Rat        MCNTNMSVSTEGAAGTSQIPASEQETLVRP

DSC_human     CCCCCCCCCCCCCEEEEECCCCHHHHHHCC
DSC_dog       CCCCCCEEECCCCEEEEECCCCHHHHHHCC
DSC_mouse     CCCCCCEEEECCCCCCCCCCCCCHHHHHHCC
DSC_rat       CCCCCCEEEECCCCCCCCCCCCCHHHHHHCC
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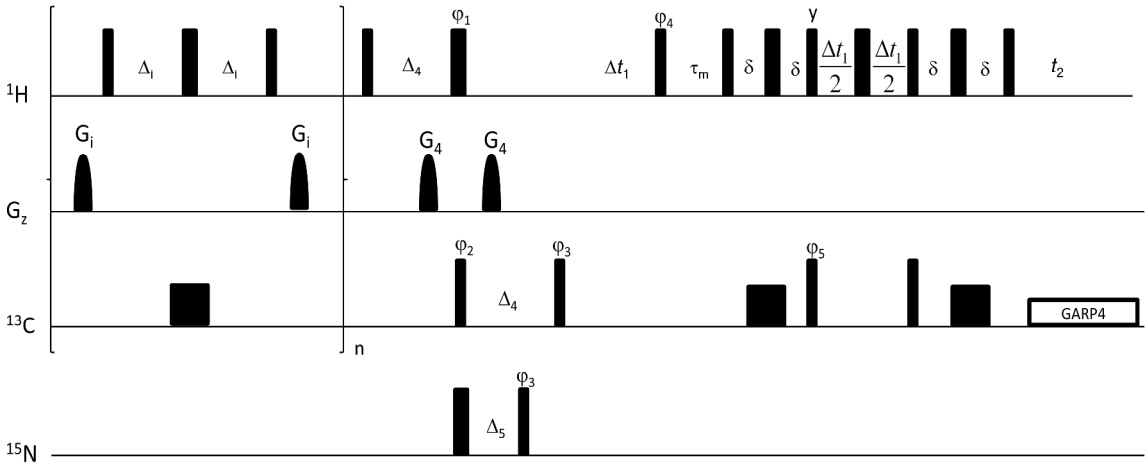
Figure S7: {3,2} ^{13}C,^{15}N filtered NOESY-HSQC pulse sequence for the observation of ligand ^{1}H to protein ^{1}H (^{13}C attached) NOEs in D_2O solutions.[4] The narrow, wide and square bars stand for the hard 90°, hard 180° and adiabatic 180° pulses respectively, with phases set to x unless indicated otherwise above the pulse bar. φ_1=(x,x,y,y,-x,-x,-y,-y), φ_2=16(x),16(-x), φ_2=32(x),32(-x), φ_4=(x,-x) for the cosine and φ_4=(y,-y) for the sine modulation in ω_1 dimension plus TPPI for the quadrature detection [5], φ_5=8(x),8(-x), φ_{rec}=2(x,-x,-x,x),2(-x,x,x,-x). n=3, Δ_1=1.1 ms, Δ_2=1.4 ms, Δ_3=1.8 ms, Δ_4=3.5 ms, Δ_5=1.8 ms, δ=1.8 ms. The z gradients were sine shaped of 500 μs durations and consecutive strength of 6, 15, 24 and 18 G cm^{-1}.



Supplementary Experimental Section

Generation of Human, dog, mouse and rat MDM2 (1-188) for SPR and HTRF.

Cloning: Human, dog, mouse and rat MDM2 (1-188) sequences were cloned into pGEX4t downstream of GST with a thrombin cleavage site. The resulting construct was transformed into competent BL21 (DE3) Star cells (Invitrogen) and selected on LA +Carbenecillin. *Expression:* 12L fermentations were run for each of the above strains at 20°C. The cultures were induced with 0.1M final concentration IPTG. The cells were harvested after 8 hours and frozen at -80°C prior to purification. *Purification: E.coli* cells were resuspended in 25mM Tris pH 7.5, 0.15M NaCl, 0.1% (w/v) CHAPS, 1mM DTT, 10mM imidazole and Complete "Free" protease inhibitor cocktail (Roche) and lysed by a microfluidizer. Lysate was cleared by centrifugation before applying to glutathione Sepharose 4B (GE Biosciences) beads for one hour batch-incubation at 4 ºC. The GST-elution was applied to a Butyl-FF column (GE Biosciences) with 1M $(NH_4)_2SO_4$ and elution peak fractions were applied to Superdex 200 XK 26/60 column (GE Biosciences) equilibrated with; 20 mM Tris pH 7.5, 0.15M NaCl, 10% (v/v) glycerol and 5mM DTT. All proteins identity were confirmed by LC-MS and concentrations were determined according to the calculated molar extinction coefficient at 280nm, and snap frozen in liquid nitrogen and stored at -80ºC

Generation of Human MDM2 (2-118) and (17-125) for SPR and ITC. *Cloning:* Human MMD2 (17-125) and (2-118) sequences either with or without an additional carboxy-terminal sequence GLNDIFEAQKIEWHE (AviTag™) was inserted into pGEX-4t (GE Healthcare) vectors using standard molecular biology techniques. *Expression:* The proteins were expressed in BL21(DE3) *E. coli* by overnight induction using 0.5 mM IPTG at 18C in 2xYT broth. *Purification:* The soluble proteins were purified from cells by glutathione affinity

chromatography as described above. The purified fusion proteins were subjected to thrombin cleavage and purified by size exclusion chromatography. The resulting MDM2 proteins containing the AviTag™ sequence were site-specifically biotinylated using BirA biotin ligase (Avidity, Aurora, CO) according to the manufacturer's instructions and purified by size exclusion chromatography. Biotin occupancy on MDM2 was confirmed to be >99% by mass spectrometric analysis.

Generation of unlabeled/labeled Human MDM2 (6-125) for ITC, X-ray and NMR. *Cloning:* Human MDM2 (6-125) sequence was cloned downstream of a 6X Histidine with a Caspase3 protease cleavage site in the pET30a vector (Novagen). The resulting construct was transformed into competent BL21 (DE3) Star cells (Invitrogen) and selected on LA +Kanamycin. *Expression:* Unlabeled MDM2 (6-125) was generated by incubating pET30-His6-DEVD-huMDM2 (6-125) in Terrific Broth until the culture reached an A600 of 1.0. The culture was then induced with the addition of 0.1M IPTG (final concentration). The culture was incubated at 20°C for 8 hours. The cells were then harvested via centrifugation and frozen at -80°C prior to purification. *Expression and isotopic labeling:* Freshly transformed cells of pET30-His6-DEVD-huMDM2 (6-125) were grown in minimal media containing ^{13}C labeled glucose and ^{15}N ammonium chloride. Cells were grown at 30°C to log phase to an A600 of ~0.8. The culture was then induced with the addition of 0.1M final concentration IPTG. The culture was incubated at 20°C for 8 hours. The cells were then harvested via centrifugation and frozen at -80°C prior to purification. *Purification: E.coli* cell lysate was centrifuged and captured by Ni-NTA. After Caspase3 cleavage and Ni-NTA subtraction, sample was applied to Superdex 200 XK 26/60 column (GE Biosciences) equilibrated with 50mM HEPES pH7, 0.1M NaCl and 1mM TCEP. All proteins identity were confirmed by LC-MS and concentrations were determined

according to the calculated molar extinction coefficient at 280nm, and snap frozen in liquid nitrogen and stored at -80ºC

Generation of Human, dog, mouse and rat p53 (1-83) for SPR and HTRF studies.

Cloning: The human, dog, mouse and rat p53 (1-83) were cloned downstream of a N-terminal tag that includes an Avitag - ecThioredoxinA - 6xHistidines - Thrombin cleavage site - S Tag – Enterokinase cleavage site in the pAMG21 vector (Amgen) using standard molecular biology techniques. The resulting constructs (Avi-TrxA-6His-[thrombin]-S-tag-EK-p53(1-83)) were transformed into BL21 (DE3)Star competent cells and selected on LA+ Kanamycin. *Expression:* The resulting strains were expressed in 15L bioreactor at 30°C. The cultures were induced at an OD600 of ~11 and incubated for an additional 8 hours at 30°C. The cells were harvested via centrifugation and stored at -80°C prior to purification. *Purification: E.coli* cells were resuspended in 25mM Tris pH 7.5, 0.15M NaCl, 0.1% (v/v) CHAPS, 1mM DTT, 10mM imidazole and Complete "Free" protease inhibitor cocktail (Roche) and lysed by a microfluidizer. Lysate was cleared by centrifugation before application to Ni-NTA beads for batch-incubation at 4°C. The Ni-elution was dialyzed in 5mM phosphate buffer pH 6.8 with 2mM DTT and applied to a Hydroxyapatite column (Bio-Red). The p53 protein was collected as Hydroxyapatite flow-through and applied to Superdex 200 XK 26/60 column (GE Biosciences) equilibrated with; 20 mM Tris pH 7.5), 0.15M NaCl, 10% glycerol and 5mM DTT. All proteins identity were confirmed by LC-MS and concentrations were determined according to the calculated molar extinction coefficient at 280nm, and snap frozen in liquid nitrogen and stored at -80ºC.

Generation of Human MDM2 (2-118) WT and mutants (V14A, L57I) for SPR.

Cloning, purification and expression performed as for GST-MDM2 (1-188) constructs.

NMR spectroscopy. NMR spectra were recorded at 15°C on Bruker Avance III 500 MHz and 800 MHz spectrometers (Bruker Biospin, Billerica, MA) equipped with triple-resonance TCI cryoprobes operating at 500.13 and 800.21 MHz, respectively. Assignment of ^{1}H, ^{13}C, and ^{15}N resonances was achieved using a standard set of triple resonance experiments [6] along with an ^{13}C-HCCH-TOCSY[7] spectrum. All experiments were performed in 20 mM sodium phosphate, pH 7.0, 50mM NaCl , 5mM deuterated dithiothreitol (DTT-d10) (Cambridge Isotopes, Cambridge, MA) and 90% H_2O/10% D_2O (except for the HCCH-TOCSY, which was run in 99.99% D_2O). Apo- protein samples were concentrated to approximately 300 µM and were replaced every four to five days until experiments were completed. For the complex structure determination, a dilute sample of the MDM2:Pip-1 complex was concentrated to approximately 500 µM and was stable over the entire time course of the experiments. ^{1}H-^{1}H protein, protein-ligand and interligand NOEs for structure determinations were obtained using ^{15}N- ^{13}C-edited NOESY , ^{13}C,^{15}N-filtered NOESY and {3,2}-^{13}C,^{15}N-filtered NOESY-HSQC spectra, all using 90 ms mixing times). The isotope filtered experiments were performed in 99.996% D_2O.

The ligand proton assignments were based on two-dimensional ^{15}N/^{13}C-filtered TOCSY and NOESY experiments recorded in the ω2-coupled/decoupled mode to discriminate between ligand-ligand and ligand-protein NOEs. The intra-ligand NOEs were used in conjunction with the inter-molecular NOEs obtained from the {3,2}-X-NOESY-HSQC as restraints to dock the ligand into the Holo-MDM2 protein structure. Data for all of these experiments were acquired on the 800 MHz system.

The spectral widths in the ^{13}C-edited NOESY experiment at 800 MHz were 10,416 Hz in F3/F1 and 16,097 Hz in F2, and data were acquired using 1024 x 96 x 128 complex data points.

The spectral widths of the ^{15}N-edited NOESY at 800 MHz were 10,416 Hz in F3/F1 and 2,838 Hz in F2, and data were acquired using 1024 x 20 x 190 complex data points. The HCCH-TOCSY experiment was acquired at 800 MHz using 1024 x 64 x 128 complex data points and using spectral widths of 10,416 Hz in the ^{1}H dimensions and 16,097 Hz in the ^{13}C dimension with the carrier frequency centered at 7,545 Hz. The {3,2}-13C/15N filtered-NOESY-HSQC experiments (described in Supplementary Figure 5) were recorded at 800 MHz with acquisition times of 57 ms and 8.6 ms in t2 and t1 dimensions respectively. Zero-filling in ω2 and linear prediction in ω1 gave 4.4 Hz/pt and 14.6 Hz/pt final resolutions, respectively. The sine and cosine modulated experiments were co-added and superimposed with the non-modulated in ω1 version of the experiment to obtain chemical shifts and assign NOEs to the ligand resonances protons.

All NMR data were processed in Topspin 3.0 or NMRPipe [1] and visualized using Sparky.[8] NMR structures were calculated in a semi-automated fashion using Cyana 3.0.[9] Protein structures were further refined in explicit water using Crystallography and NMR System (CNS).[10] The Ramachandran statistics for the final lowest-energy protein structure were determined using PROCHECK [11] as were as follows: most favored regions (59.9%), additionally allowed regions (37.5%), generously allowed regions (3.6%), and disallowed regions (0%).

Ligand docking was performed using AMBER [12] and was done using 59 protein-ligand and 13 ligand-ligand NOEs. The lowest energy NMR structure from CNS refinement was submitted to 15 ps of simulated annealing with the energy minimized ligand structure at 800 K using both ligand and protein restraints while allowing for ligand and protein-side-chain flexibility in residues for which NOEs were observed in the {3,2}-X-NOESY-HSQC. The

protein backbone was fixed during the docking simulations. The final structure was then submitted to energy minimization with restraints using the SANDER module of AMBER.[12]

Supplementary References

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