**Supporting Information:**

**A High-Affinity Metal-Binding Peptide from *Escherichia coli* HypB**

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***Materials***. The ubiquitin-pET28 vector was a generous gift of Prof. S. Dhe-Paganon (Structural Genomics Consortium, Toronto). Competent cells were purchased from Novagen. Restriction enzymes and calf intestinal phosphatase (CIP) were obtained from New England Biolabs. T4 DNA ligase was obtained from Fermentas. Kanamycin, tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and isopropyl ß-D-thiogalactoside (IPTG), and phenylmethyl sulfonyl fluoride (PMSF) were purchased from BioShop (Toronto, ON). Ethylenediaminetetraacetic acid (EDTA), 4-(2-pyridylazo)resorcinol (PAR), and *p*-hydroxymercuribenzoic acid (PMB) were purchased from Sigma-Aldrich. Nickel salts were 99.9% pure and purchased from Aldrich except in preparation of XAS samples for which Ni(II) atomic absorption standard solutions (AAS) were used. Primers (Table S1) were purchased from Sigma Genosys. Buffer A (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.5, 100 mM KCl) for metal assays was treated with Chelex-100 (Bio-Rad) to minimize contamination with trace metals. 9-fluorenylmethyl chloroformate (Fmoc) protected amino acids and activating agent O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) were purchased from GL Biochem (Shanghai) Ltd. Novapeg Rink Amide resin was purchased from Novabiochem. All other reagents were analytical grade from Sigma. All samples were prepared with Milli-Q water, 18.2 MΩ-cm resistance (Millipore).

***Cloning and protein expression***. The gene for the fusion of the first 9 amino acids of HypB and ubiquitin was prepared by amplifying the ubiquitin coding sequence from ubiquitin-pET28 by using the B9 primers (Table S1). The PCR product was purified by using the

QIAquick PCR purification kit (Qiagen), followed by digestion with *Nde*I and *Hind*III. The digested PCR product and pET24b digested with same restriction enzymes and treated with CIP were gel extracted by using the QIAquick gel extraction kit, ligated by using T4 DNA ligase, and transformed into XL2-Blue *E. coli* cells that were then plated on LB-kanamycin plates. Plasmids were isolated from an overnight culture of one colony by using the Qiagen plasmid mini-prep kit, and sequenced. The B9E8A mutant of B9ubiquitin was prepared by using the QuickChange method (Stratagene) and the primers listed in Table S1. The unmodified ubiquitin gene was also generated by using the QuickChange method to loop-out the HypB codons in the B9ubiquitin-pET24b vector by using the forward and reverse Ubiquitin primers (Table S1). This was done to avoid the histidine tag encoded in the ubiquitin-pET28 construct.

***Table S1.*** PCR Primers

| Name | Sequence[a] |
|---|---|
| B9 forward | 5'-CATTAT**CATatg**tgtacaacatgcggttgcggtgaaggcCAGATCTTCGTGAAG-3' |
| B9 reverse | 5'-GCAGCCG**AAGCTT**TCACCCACCTCTGAGACGGAG-3' |
| B9E8A forward | 5'-catgcggttgcggtgccggcCAGATCTTCGTG-3' |
| B9E8A reverse | 5'-CACGAAGATCTGgccggcaccgcaaccgcatg |
| Ubiquitin forward | 5'-GAAGGAGATATACATATG/CAGATCTTCGTGAAGACCC-3' |
| Ubiquitin reverse | 5'-GGGTCTTCACGAAGATCTG/CATATGTATATCTCCTTC-3' |

[a]Restriction enzyme sites are shown in bold. HypB-containing sequences are shown in lower case. The alanine codon is underlined in B9E8A primers, and the slash in the Ubiquitin primers indicates the location of the sequence removed to generate the unmodified gene for ubiquitin in the pET24b vector.

For expression of B9ubiquitin, B9E8A, and ubiquitin, the plasmids were transformed into BL21(DE3) cells. A culture of 1.5 L of LB medium supplemented with 50 μg/mL kanamycin and 1 mM $NiSO_4$ was inoculated with 20 mL of an overnight culture and grown at 37˚C until $A_{600}$ reached 0.6. At this point the culture was induced with 0.25 mM IPTG. After 1-2 hours at

15˚C, the cells were harvested by centrifugation and resuspended in 25 mL of 20 mM Tris (pH, 7.5), 100 mM NaCl, 5 mM TCEP and 1 mM PMSF and stored at -80˚C.

***Protein purification***. Following the addition of DNAse, the cells were sonicated on ice and then centrifuged at 18,000 rpm in an SS34 rotor for 40 min at 4˚C. The supernatant was heated for 15 minutes at 86˚C followed by the addition of 1.25 mM more TCEP and centrifugation at 4000 rpm for 40 min at 4˚C. The supernatant was concentrated to ~2 ml by using an Amicon Ultra Centrifugal Filter Device (Millipore, MWCO = 5000 Da) and then diluted to 10 mL with buffer B (20 mM Tris (pH 7.5), 1 mM TCEP). This solution was loaded onto a MonoQ anion-exchange column equilibrated with buffer B and eluted with a NaCl gradient at approximately 20 mM NaCl. The fractions were checked for the presence of protein by using 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), pooled, and concentrated to ~2.5 mL. Proteins were rendered apo by incubation with 10 mM EDTA and 1 mM TCEP for 3 days at 4˚C in an anaerobic glovebox followed by the removal of EDTA and metal by gel filtration. The proteins were quantified by using the calculated molar extinction coefficient of 1615 $M^{-1}cm^{-1}$ (B9ubiquitin and B9E8A) or 1490 $M^{-1}cm^{-1}$ (ubiquitin).[2] B9ubiquitin had an expected mass of 9376.7 Da but was observed to have a mass of 9244.3 Da indicating removal of the N-terminal methionine as observed for HypB.[3] The reduced states of the apo proteins were confirmed by reactions with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and a comparison with a freshly-prepared standard curve of ß-mercaptoethanol (BME).

***Preparation of acetylated B9ubiquitin (AcB9)***. A concentration of 200 μM apo-B9ubiquitin was treated with 500 μM S-(4-nitrophenyl) thioacetate and 10 mM thiophenol and incubated at 4˚C overnight in an anaerobic glovebox.[4,5] ESI-MS (Department of Chemistry, University of Toronto) was used to verify an increase in mass of 42 Da (9286.0 Da). Gel

filtration was performed in the glovebox to remove excess thiophenol and unreacted S-(4-nitrophenyl) thioacetate. A second modification agent, aminothioacetic acid S-benzyl ester was used in an identical way to introduce an amino acetyl group selectively to the N-terminus of B9ubiquitin. This modified protein (NAcB9) yielded similar results to that of AcB9. For both AcB9 and NAcB9, the free thiol content was quantified by a reaction with 2 mM iodoacetamide followed by ESI-MS, revealing the addition of 58 Da for each of the three cysteines.

***Verification of N-terminal modification on AcB9.*** Apo-B9ubquitin and AcB9 were digested with trypsin for 1.5 hours at room temperature and the resulting peptide fragments were analyzed by using MALDI-MS (Department of Chemistry, University of Toronto). Difficulty in observing peptides terminating with lysine was alleviated by the use of a ProteoMass Guanidination Kit (Sigma) to guanidinate the lysine-containing peptides.[6] The guanidination adds 42.02 Da for each lysine modified to an arginine. Guanidination of the N-terminal fragment generated by trypsin (residues Cys2-Lys14, unmodified MW = 1445.6 Da) produces a molecular weight of 1487.6 Da and the addition of acyl group (AcB9) results in a further increase by 42.04 Da (Table S2).

***Table S2.*** N-terminal fragments observed by MALDI-MS.

| | Expected Fragment | Observed Fragment[a] |
|---|---|---|
| B9ubquitin | 1487.6 Da | 1488.4 Da |
| AcB9 | 1529.6 Da | 1529.7 Da |

[a]The protein was digested with trypsin for 1.5 h followed by guanidination of the fragments.

***B9peptide synthesis and purification***. B9peptide (CTTCGCGEGW) was synthesized from the C-terminus to the N-terminus by using Fmoc-based blocking strategies on an Applied Biosystems 431A peptide synthesizer. A tryptophan residue was added at the C-terminus to facilitate quantification of the peptide. A Novapeg rink amide resin with loading capacity 0.44

mmol/g was used. The peptide was cleaved off the resin with a cleavage cocktail of 94% trifluoroacetic acid (TFA), 2.5% MilliQ water, 2.5% ethanedithiol, and 1% triisopropyl silane. For a small-scale cleavage reaction, 2 mL of cleavage cocktail was added to a spatula tip of resin and incubated at room temperature for 3 hours, followed by filtration by using a Biospin disposable chromatography column (Bio-Rad). The TFA was evaporated with nitrogen gas and 1 mL of diethyl ether was added to precipitate the peptide. The solution was centrifuged and the ether was removed. The ether wash was repeated three times and the peptide was finally dissolved in 200 μL of MilliQ water and 1 mM TCEP.

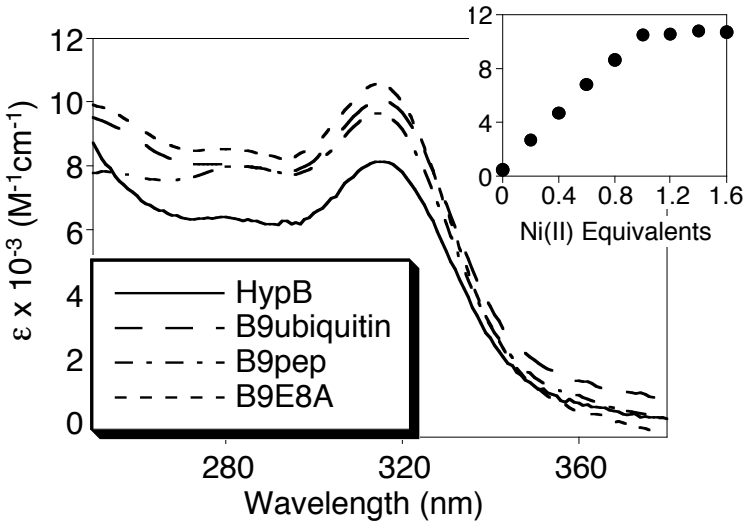
The crude peptide was purified by using analytical reversed-phase HPLC on an Agilent C18 column. A gradient of 5-50% acetonitrile, 0.1% TFA in water at a flow rate of 1 mL/min over 60 minutes was used to elute the peptide. Appropriate fractions were analyzed by MALDI-MS ($MW_{calc}$ = 1016.1 Da, $MW_{obs}$ = 1014.2 Da and 1036.2 Da) and lyophilized. The lyophilized peptide was reconstituted in buffer A and 2 mM immobilized TCEP gel (Pierce). The TCEP gel was removed in an anaerobic glovebox and the full reduction of the peptide was verified by using a DTNB assay. B9peptide was quantitated by using the $A_{280}$ of apo-B9peptide and the calculated molar extinction coefficient of 5625 $M^{-1}cm^{-1}$.[2]

***Metal analysis (HPLC-PAR).*** Triplicate samples of 50 μg B9ubiquitin and ubiquitin, first supplemented with 2 equivalents Ni(II) and then dialyzed overnight in buffer A treated with Chelex-100, were dried by centrifugation under vacuum, reconstituted with metal-free concentrated HCl, hydrolyzed by incubation overnight at 100˚C, dried by vacuum to remove HCl and reconstituted with 80 μL of MilliQ water.[7] Samples were injected onto an IonPak CS5A column attached to a metal-free Dionex AS50 HPLC system followed by postcolumn mixing

with PAR and detected at 500 nm. Several holo-B9ubiquitin samples yielded an average of 107 ± 5 % Ni(II) and metal was not detected in the ubiquitin samples.

***Nickel titration and competition assays.*** Increasing concentrations of nickel were added to 5 μM, 10 μM, or 20 μM apo-B9ubiquitin, apo-B9peptide or apo-B9E8A in buffer A and incubated in an anaerobic glovebox overnight at 4˚C. Nickel binding was monitored at 315 nm for B9ubiquitin, B9peptide, and B9E8A, and 262 nm for AcB9 and NAcB9. Stoichiometric nickel binding to the B9peptide, B98A, and the modified proteins was verified by adding two molar equivalents of nickel folled by gel filtration or dialysis to remove excess nickel, and a subsequent assay with 4-(2-pyridylazo)resorcinol (PAR) in the presence of *p*-hydroxymercuribenzoic acid (PMB).[8] The difference spectra for HypB, B9ubiquitin, the B9 peptide, and the E8A mutation of B9ubiquitin are shown in Figure S1(a). The difference spectra of AcB9 and NAcB9 are presented in Figure S1(b). Nickel titrations in the presence of 10 mM glycine revealed that glycine was not able to compete for nickel with B9ubiquitin or B9E8A but was able to compete for nickel with AcB9 and NAcB9 (data not shown). For competition studies, 1 mM (for B9peptide), 5 mM, 10 mM or 20 mM ethylene glycol bis(2-aminoethylether)N,N,N',N'-tetraacetic acid (EGTA) was also included and samples were incubated in an anaerobic glovebox overnight at room temperature. The data were analyzed as described[3] and by using extinction coefficients for the nickel complexes calculated from a direct titration in the absence of any competitor.

(a)




(b)

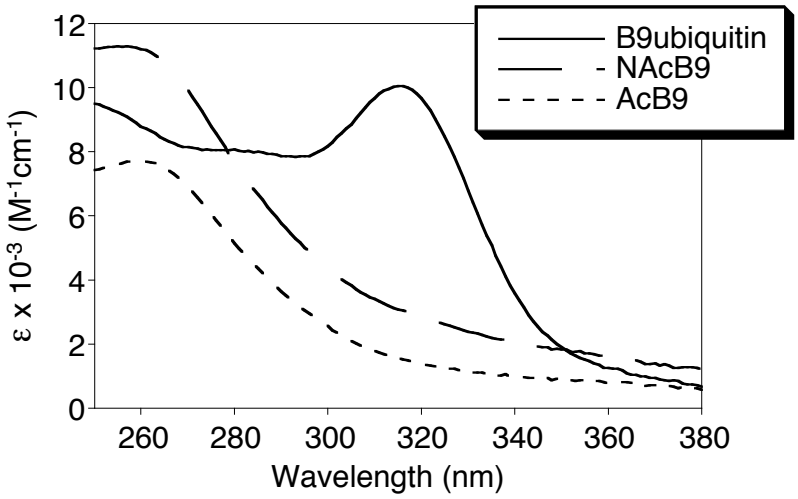



***Figure S1.*** (a) Comparison of the difference spectra of HypB, B9ubiquitin, B9E8A and B9peptide obtained by subtraction of the electronic absorption spectra of apo-protein from that of the corresponding protein with stoichiometric nickel bound. Inset: nickel titration of B9ubiqutin. (b) Difference spectra of AcB9 and NAcB9 with stoichiometric nickel bound.

***PMB titration and nickel release to PAR***. The participation of one or more of the three cysteine residues in the HypB9 residue sequence, CTTCGCGEG, as nickel ligands was verified through the use of the thiol-reactive reagent PMB and the metallochromic indicator PAR.[8] Protein samples with stoichiometric nickel bound were prepared by incubating the protein with nickel followed by gel filtration. The proteins were diluted to 10 μM in buffer A containing 50 μM PAR. Increasing concentrations of PMB were added (0-60 μM PMB). Metal release from the protein and the formation of 2:1 PAR-metal complex in solution was monitored at 500 nm by using electronic absorption spectroscopy (Figure S2),[8] which revealed that approximately three equivalents of PMB were required to produce complete metal release from B9ubiquitin. Identical experiments were performed with ubiquitin, which does not bind nickel. The concentration of Ni(II)-$PAR_2$ complex was calculated by using an $\varepsilon_{500}$ of 66,000 $M^{-1}cm^{-1}$ that was determined from nickel standards.

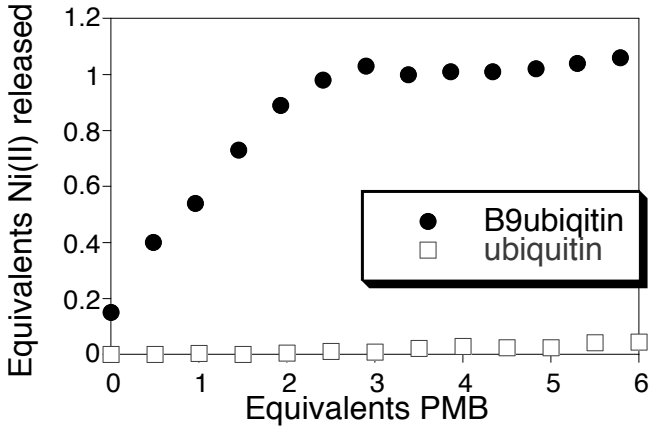



***Figure S2.*** PMB titration of 10 μM B9ubiquitin (circles) or 10 uM ubiquitin (squares) in the presence of 50 μM PAR. Note that ubiquitin does not contain any cysteine residues.

***Metal release assay***. To evaluate the rate that nickel could be released from holo-protein to PAR, a time course experiment was used (Figure S3).[3] For B9ubiquitin and B9E8A the as-purified, nickel-loaded protein was used. For AcB9 the apo-protein was incubated with excess nickel and run through a gel filtration column to remove unbound nickel. The protein was diluted to a final concentration of 5 μM in buffer A containing 100 μM PAR. The increase in absorbance at 500 nm, resulting from formation of Ni(II)-$PAR_2$ complex, was monitored every five minutes for 2 hours. Total metal in the sample was determined by treating the sample protein sample with 100 μM PMB ($A_{max}$). The % metal bound was calculated for each time point by conversion of absorbance through the equation (100*[1-A/$A_{max}$)].

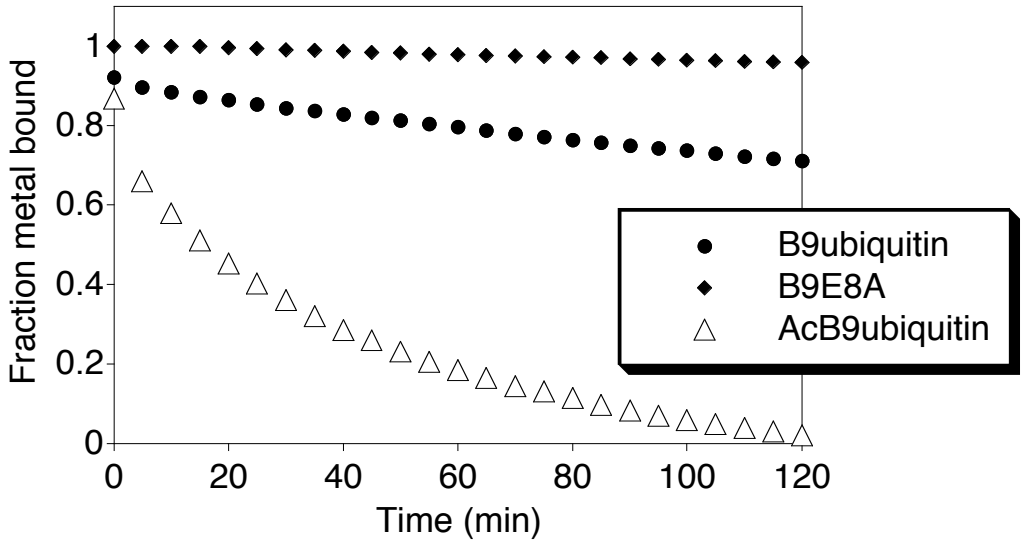



***Figure S3.*** Kinetics of Ni(II) release to PAR from B9ubquitin, B9E8A, and AcB9 as monitored by an increase in absorbance at 500 nm.

***XAS sample preparation***. The Ni(II)-HypB XAS sample was prepared as previously described.[1] For the Ni(II)-B9peptide sample 0.8 equivalents of Ni(II) and 1 mM TCEP was added to a 234 μM solution of the peptide in an anaerobic glovebox and incubated for one hour at room temperature. The sample was subsequently removed from the glovebox and frozen with liquid nitrogen and lyophilized overnight. The sample was then resuspended in 20% glycerol (in water) to a final concentration of 2.2 mM. Stoichiometric Ni(II) binding was confirmed by electronic absorption spectroscopy at 315 nm with a calculated extinction coefficient of 9900 $M^{-1}cm^{-1}$. The Ni(II)-B9ubiquitin XAS sample was prepared by adding 2 equivalents of Ni(II) to a 788 μM solution of protein in an anaerobic glovebox and incubating at room temperature overnight, followed by dialysis against buffer A treated with Chelex-100 to remove excess nickel. The sample was then removed from the glovebox and concentrated. Following concentration, 20% glycerol (v/v) and 1 mM TCEP was added. Stoichiometric Ni(II) binding was again confirmed by electronic absorption spectroscopy at 315 nm with a calculated extinction coefficient of 10,000 $M^{-1}cm^{-1}$. Both samples were subsequently frozen with liquid nitrogen and stored at -80$^{o}$C before being transferred and frozen with liquid nitrogen in (2 x 10 x 10 mm) Lucite sample cuvettes for data acquisition.

***X-ray absorption spectroscopy***. For Ni(II)-HypB and Ni(II)-B9ubiquitin XAS measurements were carried out at the Stanford Synchrotron Radiation Laboratory (SSRL) with the SPEAR 3 storage ring containing between 85 and 100 mA at 3.0 GeV. Nickel K-edge data were collected on the structural molecular biology beamline 9-3 that was operated with a wiggler filed of 2 T and employed a Si(220) double-crystal monochromator. The beamline was also equipped with a rhodium-coated vertical collimating mirror upstream of the monochromator and a downstream bent-cylindrical focusing mirror (rhodium coated). Harmonic rejection was

accomplished by setting the cut-off angle of the mirrors to 13 keV. Incident and transmitted X-ray intensities were monitored using argon-filled ionization chambers, and X-ray absorption was measured as the Ni Kα fluorescence excitation spectrum using a 30 element germanium detector.[9] Samples were maintained at a temperature of approximately 10 K using an Oxford instruments liquid helium flow cryostat. For each sample 3-6 scans were accumulated, and the energy was calibrated by reference to the absorption of a nickel metal foil measured simultaneously with each scan, assuming a lowest-energy inflection point of 9665 eV for Ni(II).

For the Ni(II)-B9peptide sample XAS measurements were carried out at the CLS (Canadian Light Source, 9 scans on the HXMA beamline) by using a similar optical set-up as at SSRL except that harmonic rejection was accomplished by detuning the monochrometer 50% off the intensity peak.

***XAS data analysis***. The EXAFS oscillations, $\chi(k)$, were quantitatively analyzed by curve-fitting using the EXAFSPAK suite of computer programs as described by George et al.,[10] using ab initio theoretical phase and amplitude functions calculated using FEFF, version 7.2.[11] The calculated fitting parameters are displayed in Table S3, and the best fit models are overlayed in Figure S4. No smoothing, filtering or related operations were performed on any of the data. The Ni-S distances fall into the range for planar Ni(II) thiolate complexes,[12] and while the Ni-N distances of the peptide and HypB fall in the observed range for Ni(II)-N(amidate) bonds, they are a bit shorter than expected for a terminal $NH_2$ ligand (1.93 Å).[13,14] In contrast, the Ni-N bond length for the B9ubiquitin is slightly longer than expected, indicating that there is some flexibility with this terminal ligand.

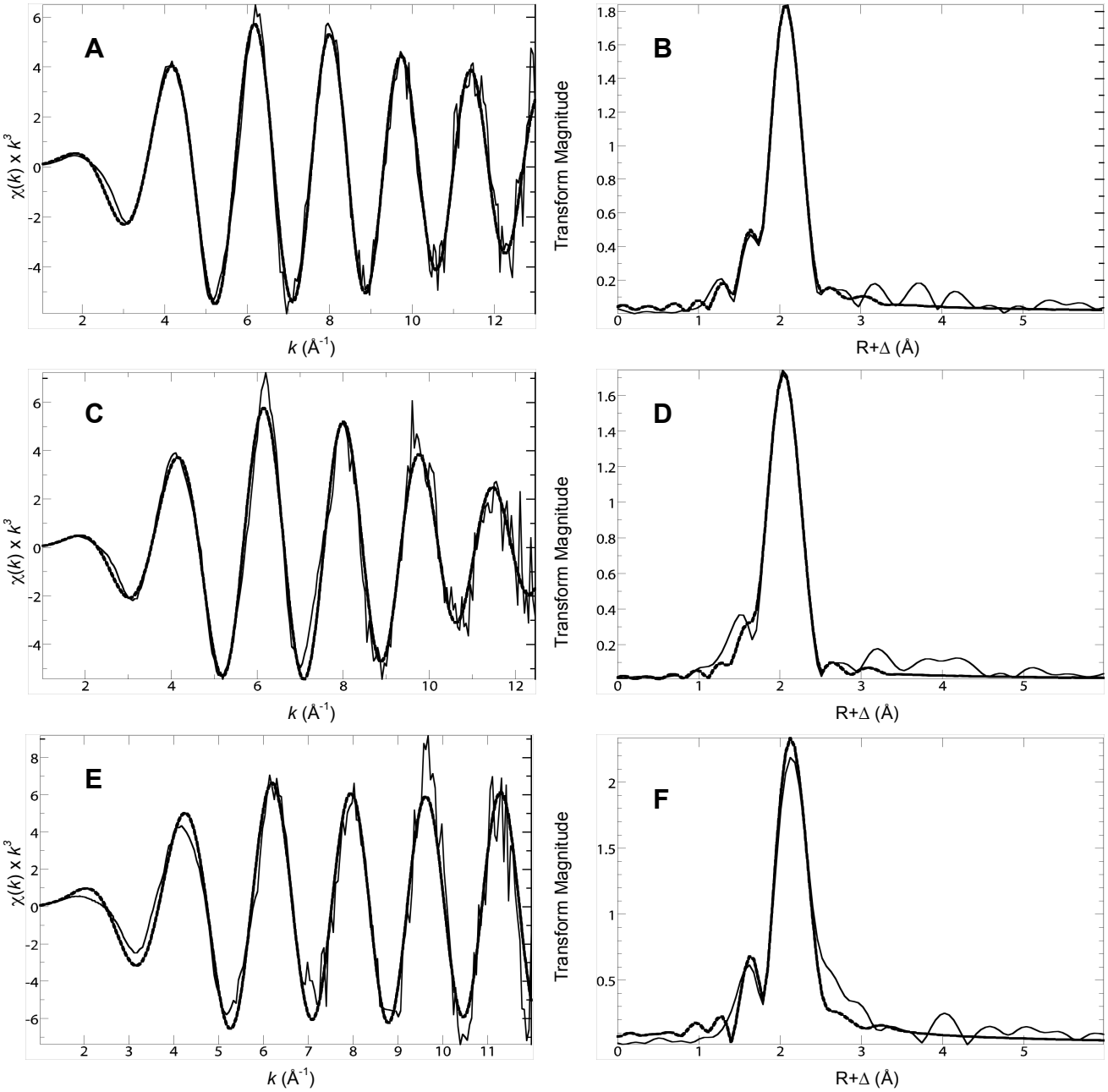



***Figure S4.*** $k^3$-weighted Ni(II) EXAFS data (left) and Fourier Transform data (right). (A & B) HypB loaded with 1 equivalent of Ni(II). (C & D) B9Ubiquitin loaded with 1 equivalent of Ni(II). (E & F) B9Peptide loaded with 0.8 equivalents of Ni(II). Traces: Solid black, raw data for all samples; dashed black line, best-fit model according to Table S3. EXAFS and FT data fits for HypB (A & B) are taken from a previous study.[1]

***Table S3.*** Ni(II) EXAFS Curve-Fitting Results[a,b,c,d]

| Sample Description | A-Bs | N | R(Å) | $\sigma^2$(Å$^2$) | $\Delta E_0$(eV) | *F*-factor |
|---|---|---|---|---|---|---|
| **Ni(II)-HypB** | | | | | | |
| **Model AB1** | **Ni-S** | **3** | **2.168(7)** | **0.00357(1)** | **-17.954(2)** | ***0.198*** |
| | **Ni-N** | **1** | **1.867(34)** | **0.00693(4)** | | |
| **Ni(II)-B9ubiquitin** | | | | | | |
| **Model CD1** | **Ni-S** | **3** | **2.168(11)** | **0.00616(1)** | **-16.264(2)** | ***0.254*** |
| | **Ni-N** | **1** | **2.02(38)** | **0.00440(1)** | | |
| **Model CD2** | Ni-S | 2 | 2.206(17) | 0.00615(1) | -9.706(2) | 0.241 |
| | Ni-N | 2 | 2.024(12) | 0.00143(1) | | |
| **Model CD3** | Ni-S | 4 | 2.16(8) | 0.00707(1) | -18.922(2) | 0.272 |
| **Model CD4** | Ni-S | 1 | 2.234(16) | 0.00319(1) | -6.18(2) | 0.243 |
| | Ni-N | 3 | 2.031(10) | 0.00188(1) | | |
| **Ni(II)-B9peptide** | | | | | | |
| **Model EF1** | **Ni-S** | **3** | **2.194(7)** | **0.00260(1)** | **-13.114(2)** | ***0.328*** |
| | **Ni-N** | **1** | **1.859(24)** | **0.00208(2)** | | |
| **Model EF2** | Ni-S | 2 | 2.201(11) | -0.00033(1) | -13.460(3) | 0.39 |
| | Ni-N | 2 | 1.922(19) | -0.00022(1) | | |
| **Model EF3** | Ni-S | 4 | 2.204(9) | 0.00488(1) | -10.442(2) | 0.41 |
| **Model EF4** | Ni-S | 1 | 2.189(58) | 0.00225(2) | -17.309(9) | 0.88 |
| | Ni-N | 3 | 1.902(44) | 0.00169(2) | | |

[a]A-Bs denotes absorber and backscatterer interaction; N denotes coordination number; R is given in Å and represents interatomic distances; $\sigma^2$ given in Å$^2$, are the Debye-Waller factors (mean-square deviations in interatomic distance); the threshold energy shifts, $\Delta E_0$ are given in eV. The values in parentheses are the estimated standard deviations obtained from the diagonal elements of the covariance matrix. The *F*-factor or fit-error function is defined as $\sqrt{\sum k^6(\chi(k)_{calcd} - \chi(k)_{exptl})^2 / \sum k^6 \chi(k)_{exptl}^2}$; The summation is over all data points included in the refinement.

[b]The best fit models overlayed in Figure S4 are listed in bold and their accompanying *F*-values are in italics. Oxygen donors were not modeled separately from nitrogens due to similar phase and amplitude functions.

[c]Although the *F*-factor for Models CD2 and CD4 were lower, the corresponding Debye Waller factors for the nitrogen donors were extremely low and not considered to be in an acceptable range.

[d]EXAFS fitting parameter data for Ni(II)-HypB (Models AB1) are abstracted from a previously study.[1]

***Molecular Modeling.*** Density Functional Theory (DFT) molecular modeling was performed by using the program Dmol3 Materials Studio Version 4.1.[15,16] We expect bond-length accuracies of around 0.05 Å, and good estimates of energetic trends between postulated molecular entities. The Becke exchange[17] and Perdew correlation[18] functionals were used to calculate both the potential during the self-consistent field procedure, and the energy. Double numerical basis sets included polarization functions for all atoms. Calculations were spin-unrestricted and all electron relativistic core potentials were used. No symmetry constraints were applied, and optimized geometries used energy tolerances of $2.0 \times 10^{-5}$ Hartree. The effects of water solvation were simulated by using the Conductor-like Screening Model (COSMO). [19]

The generated structure (Figure 3) revealed nickel in a square-planar geometry as observed experimentally. Given that examination of structurally characterized small-molecule Ni(II) species using DFT indicates that the energy-minimized DFT structure generally shows an over-estimate bond-lengths by about 0.05 A, there is good agreement between the calculated bond-lengths and those measured by XAS of the Ni(II)-B9peptide complex.

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