Supporting Information

Metal-Binding Properties of Hpn from *Helicobacter pylori* and Implications for the Therapeutic Activity of Bismuth

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Materials and Methods

Expression and Purification of Hpn-FRET. To construct Hpn-FRET, a synthetic gene encoding Hpn was ordered (Bio Basic), amplified by PCR, and cloned between ECFP and EYFP using *Sph*I and *Sac*I in the plasmid system described in previous reports.[26] The Hpn-FRET plasmid was transformed into BL21star(DE3) and a 10 ml per culture grown from a single colony was diluted into 1 L autoclaved LB medium containing 50 mg ampicilin for protein expression. The cells were grown at 37 ^{o}C, 250 rpm until OD_{600} = 0.6. Then the temperature was lowered to 16 ºC, protein expression was induced with 0.5 mM IPTG, and cells were grown overnight. The cells were harvested by centrifugation and resuspended in 30 ml buffer A (10 mM Tris-HCl [pH 6.5], 300 mM NaCl, 1 mM DTT) with 10 mM EDTA and 10 mM PMSF. The cells were lysed by sonication, and the lysate was cleared and centrifuged at 12,000 rpm for 25 min. The supernatant was incubated with DE23 cellulose resin for 1 hr at room temperature to remove DNA and filtered through a 0.45 μm filter. The supernatant was then dialyzed

against 1.5 L buffer A twice for 2hr each time at 4 ^{o}C. Then, 100 mM Tris [pH 8.8] was added, and the supernatant was applied to a Ni-NTA column pre-equilibrated with buffer B (10 mM Tris [pH 7.4], 300 mM NaCl, 1 mM DTT). The column was washed with 5% buffer C (10 mM Tris-HCl [pH 7.4], 500 mM imidazole, 300 mM NaCl, 1 mM DTT) and eluted with a linear gradient from 5% to 100% buffer C over 40 ml. Peak fractions were pooled, concentrated, and incubated with 20 mM KCN. The sample was then run on a Superdex 200 column with buffer B. The purity of Hpn-FRET was verified by SDS-PAGE gel (Figure S1).

Fluorescent Measurements. Fluorescence measurements were done on a Varian Cary Eclipse Fluorescence Spectrophotometer. The samples were excited at 433 nm, the excitation and emission slit widths were set to 5 nm and the emission spectrum was scanned from 453 nm to 650 nm at 120 nm / min using a moving average smoothing function. For the titration curves, 0 μM to 2 μM of each metal was added to a solution of 250 nM Hpn-FRET in buffer B without DTT and the intensity ratio at the peaks was taken as a measure of FRET change (Ratio = I_{527} / I_{477}). Metal stock solutions (100 μM) were prepared from the following salts with purity > 99 %: $NiCl_2$. $6H_2O$, $ZnCl_2$, $CoCl_2$. $6H_2O$, $CuCl_2$. $6H_2O$, $MnCl_2$, $MgCl_2$, $CaCl_2$, $Cr(NO_3)_3$. $9H_2O$, $Fe(NH_4)_2SO_4$ and $FeCl_3$. A 100 mM $Bi(NO_3)_3$ stock solution in 50% glycerol was used as the Bi^{3+} source. For selectivity measurements the response of 250 nM Hpn-FRET to 1.5 μM of the tested metals in the presence and absence of Ni^{2+}, Zn^{2+}, and Co^{2+} was measured.

CD Measurements. CD measurements were done on an Aviv 202 CD spectrophotometer. The CD spectra of 2 μM Hpn-FRET in 20 mM Tris-HCl [pH 7.4] in the absence and the presence of 12 μM Ni^{2+}, Zn^{2+}, Co^{2+}, and 100 μM Bi^{3+} were scanned

in 1 nm increments in triplicates from 190 nm to 260 nm. The spectra were averaged, smoothed, and subtracted from the buffer before fitting was done using the Olis Global Works fitting program with the CDSSTR algorithm and the SDP 48 (43 soluble and 5 denatured proteins) and SMP56 (43 soluble and 13 membrane proteins) as basis sets (Table S1).

ICP-MS Analysis. 2 ml aliquots of Hpn-FRET (1 μM) in buffer B without DTT were incubated with either 10 μM Ni^{2+}, Zn^{2+} or Co^{2+} or with 20 μM total metal of 1:1 mixtures of Ni^{2+}/Zn^{2+}, Ni^{2+}/Co^{2+} and Zn^{2+}/Co^{2+}. The samples were concentrated 10-fold with a 5000 MW cut-off filter in a centrifugal concentrating device. Each sample was then diluted 3-fold and concentrated again. This was repeated 3 times before the protein concentrations were measured by UV-vis and the samples were analyzed for metal content by ICP-MS to determine the equivalents of metal to protein in each sample (Table S2). ICP-MS measurements were performed on a Thermo Finnigan Element 2 High-Resolution ICP-MS. The samples were diluted with 0.8 N HNO_3 by a factor of 500, analyzed in medium resolution using an external calibration technique. Machine drift was monitored using ^{115}In and ^{75}As as internal standards at ~50 ng/g. Potential interferences were monitored by comparing the measured $^{63}Cu/^{65}Cu$, $^{66}Zn/^{64}Zn$ and $^{112}Cd/^{111}Cd$ rations to those found in nature.

K_d Measurements and Fitting. For the K_d measurement of Hpn-FRET with Ni^{2+} and Zn^{2+}, Ni^{2+}/ citrate, and Zn^{2+}/ CN^- buffers were prepared respectively in 100 mM Tris-HCl (pH 7.4) and 100 mM NaCl. Free Ni^{2+} concentrations were buffered from 1.37×10^{-9} to 6.32×10^{-6} M using 0.3-1.9 mM Ni^{2+} and 1.5-20 mM citrate. Free Zn^{2+} concentrations were buffered to 3.94×10^{-11} to 6.64×10^{-7} M using 0.1-1 mM Zn^{2+} and 5- 20 mM CN^-.

Free metal concentrations were calculated using the program HySS2006[32] and reported complexation constants and K_a values[33] (Supporting Information). The metal loading of Hpn-FRET was measured in each Ni^{2+}/citrate or Zn^{2+}/CN^- buffer. K_d measurements for Hpn-FRET with Bi^{3+} were done by adding increasing amounts of Bi^{3+} (5 μM to 110 μM Bi^{3+} from 1 mM $Bi(NO_3)_3$ stock solution in 50% glycerol) to 250 nM Hpn-FRET in buffer B without DTT. The obtained binding curves were fitted with the Hill 1 equation using Origin 8 (Table S3).

Fluorescent Measurements in *E. coli.* The Hpn-FRET plasmid was transformed into BL21*, cells were pre-cultured overnight in LB medium, harvested by centrifugation, and inoculated into freshly prepared MOPS minimal media[35] with 50 mg/L ampicilin by 1:500 dilution. The cells were grown at 37 ˚C up to OD_{600} = 0.4 - 0.5 and cooled down to room temperature. Protein expression was induced with 0.1 mM IPTG. 1, 2, 5, 10, 20, 50, 100, 200, and 400 μM $NiCl_2$, $ZnCl_2$, bismuth subsalycilate (suspension in water), and Pepto Bismol were added to aliquots of the original culture. Bacterial cells were grown at 16 ˚C overnight. Fluorescent measurements were taken for each culture by diluting cell culture with 600 μM TBS (10 mM Tris-HCl [pH 7.4], 150 mM NaCl) buffer to OD_{600} ~ 0.5. The samples were excited at 420 nm and the emission spectrum was collected from 450 nm to 550 nm four times for each sample and a moving average smoothing function was applied. The signal from TBS was subtracted and the ratio of intensities at 527 nm and 477 nm was used in the calculation of the FRET change.

K_d Measurements of Hpn-FRET for Ni^{2+} and Zn^{2+}.

$H^+ + Cit^{3-} \leftrightarrow HCit^{2-}$ logK= 6.39

$2\ H^+ + Cit^{3-} \leftrightarrow H_2Cit^-$ logK= 11.16

$3\ H^+ + Cit^{3-} \leftrightarrow H_3Cit$ logK= 14.28

$Ni^{2+} + Cit^{3-} \leftrightarrow NiCit^-$ logK= 6.59

$Ni^{2+} + H^+ + Cit^{3-} \leftrightarrow NiHCit$ logK= 10.50

$Ni^{2+} + 2\ H^+ + Cit^{3-} \leftrightarrow NiH_2Cit^+$ logK= 13.30

$Ni^{2+} + 2\ Cit^{3-} \leftrightarrow Ni(Cit)_2^{4-}$ logK= 8.77

$Ni^{2+} + H^+ + 2\ Cit^{3-} \leftrightarrow NiH(Cit)_2^{3-}$ logK= 14.90

$H^+ + CN^- \leftrightarrow HCN$ logK = 9.04

$Zn^{2+} + 2\ CN^- \leftrightarrow Zn(CN)_2$ logK = 11.07

$Zn^{2+} + 3\ CN^- \leftrightarrow Zn(CN)_3^-$ logK = 16.05

$Zn^{2+} + 4\ CN^- \leftrightarrow Zn(CN)_4^{2-}$ logK = 19.62.

For the K_d measurement of Hpn-FRET with Ni^{2+}, Ni^{2+}/citrate buffers were used to obtain free Ni^{2+} concentrations ranging from 1.37×10^{-9} M to 6.32×10^{-6} M using Ni^{2+} binding constant to citrate and the proton association constants for citrate obtained from the *NIST Critical Stability Constants of Metal Complexes* (given above).[1] Free Ni^{2+} concentration were calculated using the program HySS2006[2] for total Ni^{2+} concentrations in the buffer from 3.0×10^{-4} M to 1.9×10^{-3} M and citrate concentrations from 1.5×10^{-3} M to 2.0×10^{-2} M. In the preparation of these buffers, [Tris-HCl] (pH 7.4, 100 mM) > [citrate] (1.5- 20 mM) > [Ni^{2+}] (300 μM- 1.9 mM) >> [Hpn-FRET] (250 nM) are used to maintain buffering capacity at a certain citrate concentration to precisely control free Ni^{2+} concretions. Hpn-FRET concentration used in this measurement is 250 nM in 10 mM Tris-HCl (pH 7.4) and 100 mM NaCl and the Ni^{2+} loading was measured for each Ni^{2+} buffer prepared (Figure 4a). The change in the FRET ratio, which is also equal to the

nickel occupancy of Hpn-FRET, versus the free Ni^{2+} concentration in the buffer was plotted and the binding curve was fitted for the Hill 1 equation[3] using Origin 8 (Table S1)

For the K_d measurement of Hpn-FRET with Zn^{2+}, Zn^{2+}/CN^- buffers were prepared and free Zn^{2+} was computed employing Zn^{2+} complex formation constants with cyanide given above. Free Zn^{2+} concentrations ranging from 3.94×10^{-11} M to 6.64×10^{-7} M were prepared by using total Zn^{2+} concentrations ranging from 1.0×10^{-4} M to 1.0×10^{-3} M and CN^- concentrations ranging from 5.0×10^{-3} M to 2.0×10^{-2} M. The obtained binding curve was fitted with the Hill 1 equation in Origin 8 (Table S1).

Hpn-FRET without His6-tag was cloned, expressed, and purified following the same procedure for Hpn-FRET (Hpn binds Ni-NTA column). It shows the same FRET response to various metal ions as Hpn-FRET with an N-terminal His6-tag (Figure S7).

Reference:

1 A. E. Martell and R. M. Smith, *Standard Reference Database 46,* 2001.
2 L. Alderighi, P. Gans, A. Ienco, D. Peters, A. Sabatini and A. Vacca, *Coordin. Chem. Rev.,* 1999, **184**, 311.
3 J. N.Weiss, *FASEB J.,* 1997, **11**, 835.

Table S1. CD fitting results for Hpn-FRET in the absence and presence of various metal ions using Olis Global Works with CDSSTR algorithm and the SDP 48 and SMP56 basis sets.

	Hpn-FRET	+ 6 eqiv Co^{2+}	+ 6 eqiv Ni^{2+}	+ 6 eqiv Zn^{2+}	+ 100 μM Bi^{3+}
H1:Alpha-helix	0.13	0.11	0.13	0.23	0.23
H2:Disordered alpha-helix	0.1	0.1	0.11	0.16	0.21
S1: Beta-strand	0.23	0.21	0.19	0.16	0.12
S2: Disordered beta-strand	0.18	0.18	0.16	0.16	0.14
T: Turns	0.16	0.19	0.16	0.14	0.13
U: Unordered	0.21	0.2	0.24	0.17	0.15
No.Helix segments per 100mer	2.6	2.6	2.7	3.9	5.3
No.strand segments per 100mer	9	8.9	8.2	7.9	7.2
Ave length of Helix	8.9	8.3	8.9	9.8	8.4
Ave length of strand	4.6	4.4	4.4	4	3.7
Standard Deviation	0.08	0.06	0.07	0.07	0.07

Table S2. ICP-MS results for Hpn-FRET loaded with different metals.

	Concentration (μM)				Metal: Hpn-FRET		
	Hpn-FRET	**Ni**	**Zn**	**Co**	**Ni**	**Zn**	**Co**
Hpn-FRET	10.40	0.32	0.18	--	0.03	0.02	--
Hpn-FRET+ Ni((II)	6.64	36.22	--	--	5.16	--	--
Hpn-FRET+ Zn(II)	6.34	--	28.90	--	--	4.56	--
Hpn-FRET+ Co(II)	5.74	--	--	21.40	--	--	3.73
Hpn-FRET +Ni(II) +Zn(II)	7.84	8.19	33.55	--	1.05	4.28	--
Hpn-FRET +Ni(II) +Co(II)	5.48	20.61	--	3.51	3.76	--	0.64
Hpn-FRET +Zn(II) +Co(II)	6.40	--	29.24	1.49	--	4.57	0.23

Table S3. Fitting results for binding curves of Hpn-FRET to Ni^{2+}, Zn^{2+} and Bi^{3+} with the Hill 1 equation.

	K	**N**	**r^2**
Ni^{2+}	$7.89 \times 10^{-8} \pm 8.8 \times 10^{-9}$	1.21 ± 0.16	0.986
Zn^{2+}	$1.03 \times 10^{-9} \pm 9.3 \times 10^{-11}$	1.42 ± 0.22	0.989
Bi^{3+}	$6.19 \times 10^{-5} \pm 1.0 \times 10^{-6}$	6.23 ± 0.71	0.998

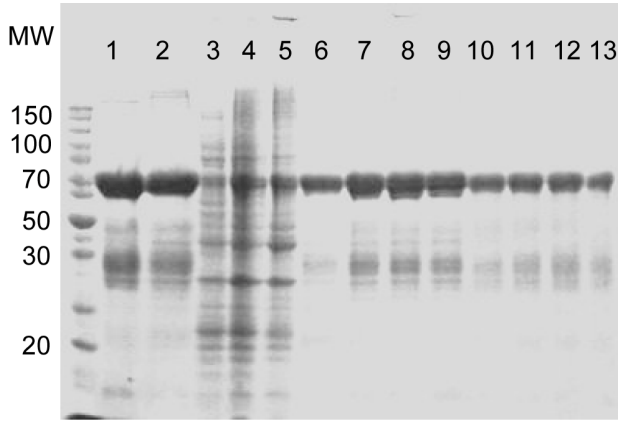

Figure S1. SDS-protein gel of the purified Hpn-FRET. Lane 1 is Hpn-FRET after Ni-NTA column, lane 2 is after gel filtration, lane 3 is the supernatant, lane 4 is the lysate, lane 5 is the insoluble fraction of the cells, lanes 6-9 are different fractions from Ni-NTA column and lanes 10-13 are different fractions from the gel filtration column.

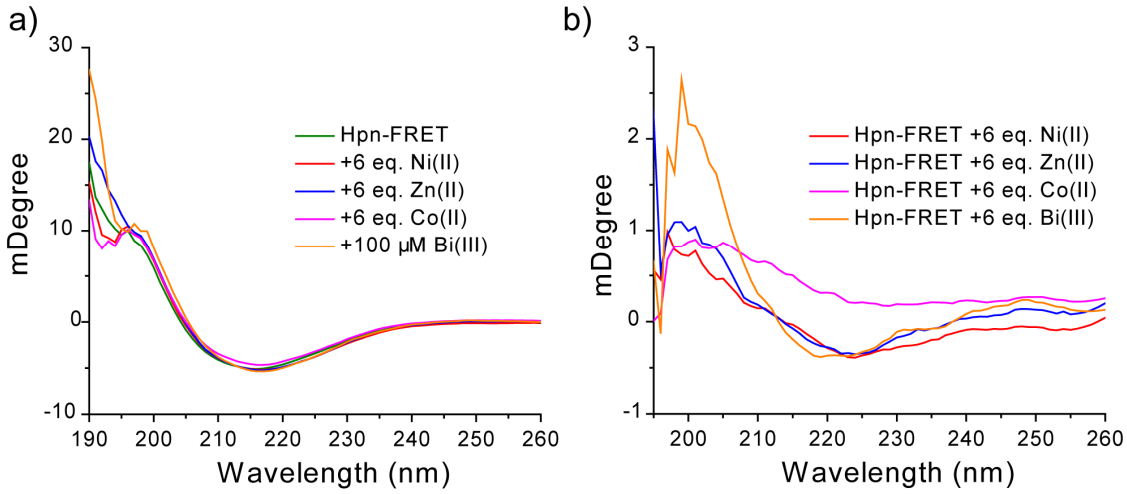

Figure S2. CD spectra for Hpn-FRET in the absence and presence of metals. a) 2 μM Hpn-FRET was used in the absence of metal or with 6 equiv of Ni^{2+}, Zn^{2+}, Co^{2+} or 100 μM Bi^{3+}. b) Difference spectra were generated by subtracting the spectrum of apo-Hpn-FRET from the spectra of Hpn-FRET treated with metals.

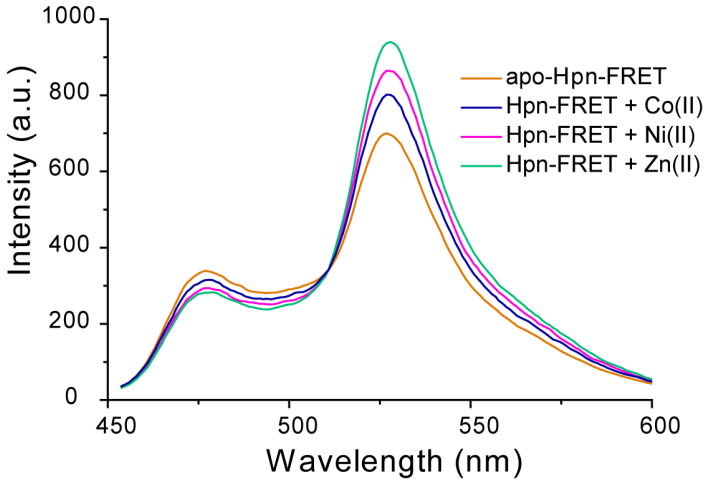

Figure S3. Fluorescence spectra of 250 nM Hpn-FRET without metal (orange), with the addition of 1.5 μM Ni^{2+} (pink), 1.5 μM Zn^{2+} (green), and 1.5 μM Co^{2+} (blue).

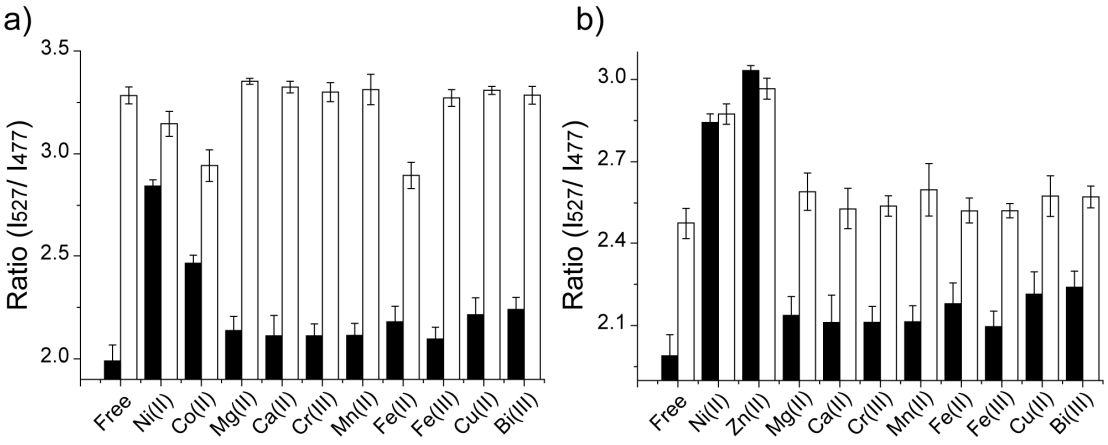

Figure S4. Competition of a) Zn^{2+} and b) Co^{2+} with other metal ions for binding to Hpn-FRET. 250 nM Hpn-FRET with 6 equiv of the respective metal ion is shown in black and the addition of 6 equiv Zn^{2+} or Co^{2+} to the sample is shown in white. All measurements were done in triplicates.

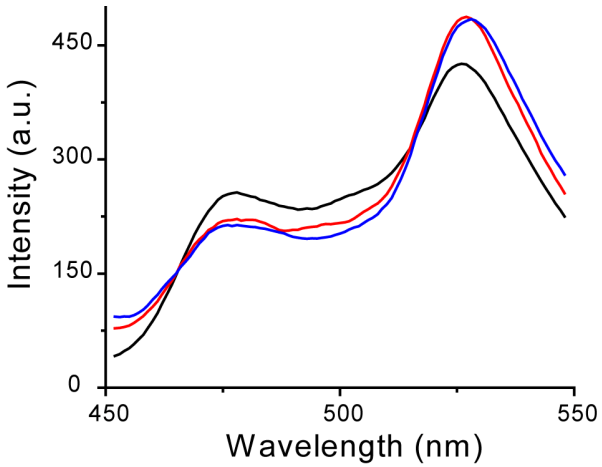

Figure S5. Fluorescence spectra of *E. coli* in the absence of additional metal (black), in the presence of 400 μM bismuth subsalycilate (red) and 400 μM Pepto Bismol (blue).

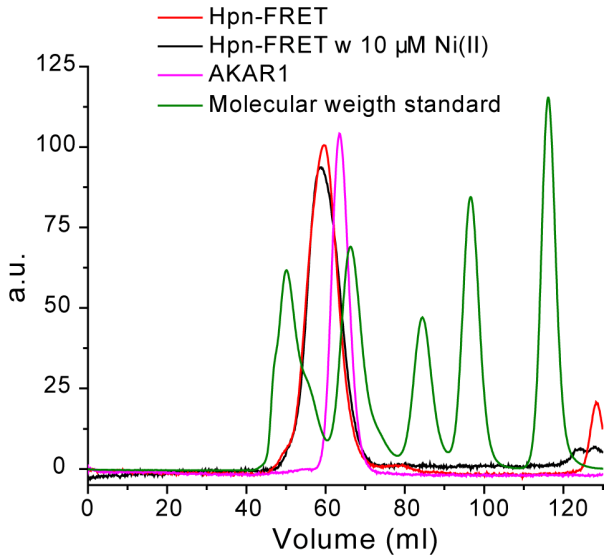

Figure S6. Gel filtration profile of purified Hpn-FRET (MW= 64.0 kDa) in the absence (red) and presence of 10 μM Ni^{2+} (black) on a Superdex 200 column with total column volume of 120 ml. Molecular weight standards (green) (600 kDa, 158 kDa, 44 kDa, 17 kDa and 1.35 kDa) and control protein AKAR1 (pink) (MW= 85.1 kDa) are shown as well. AKAR1 is a protein kinase A activity reporter, which has a sensory domain between YFP and CFP. As both AKAR1 and Hpn-FRET have different domains linked with flexible sequences and are not globular proteins they have large hydrodynamic volumes and elute early on the size exclusion column compared to the molecular weight standards which are globular proteins.

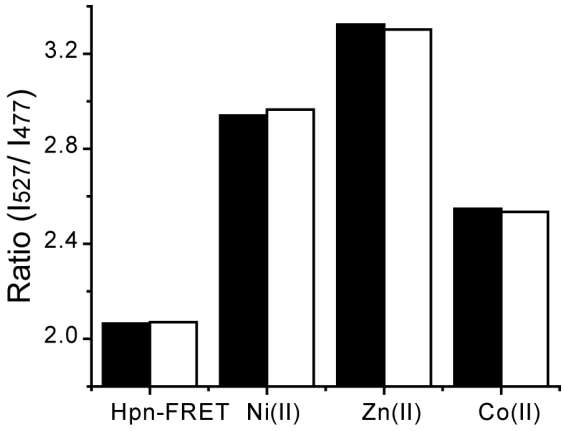

Figure S7. FRET response of 250 nM Hpn-FRET (black) and 250 nM Hpn-FRET without His6-tag (white) in the absence of metal and with the addition of 1.5 μM Ni^{2+}, 1.5 μM Zn^{2+} and 1.5 μM Co^{2+}.