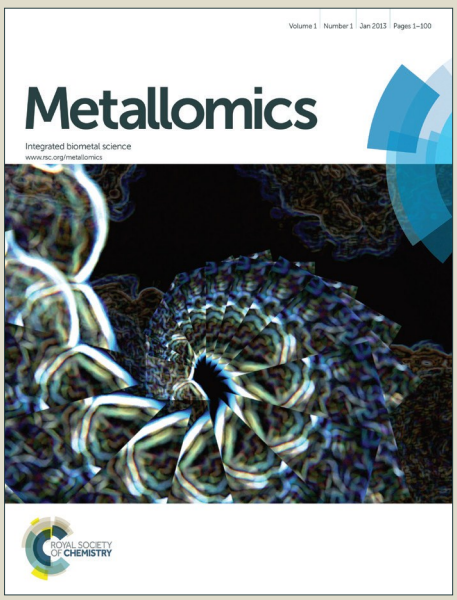
Metallomics

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/metallomics

Journal Name

COMMUNICATION

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

pH-Regulated Metal-Ligand Switching in the HM Loop of ATP7A: A New Paradigm for Metal Transfer Chemistry

Chelsey D. Kline[a], Benjamin F. Gambill[a], Mary Mayfield[a], Svetlana Lutsenko[b], Ninian J. Blackburn[a]*

Abstract Cuproproteins such as PHM and DBM mature in late endosmal vesicles of the mammalian secretory pathway where changes in vesicle pH are employed for sorting and post-translational processing. Colocation with the P1B-type ATPase ATP7A suggests that the latter is the source of copper, and supports a mechanism where selectivity in metal transfer is achieved by spatial colocation of partner proteins in their specific organelles or vesicles. In previous work we have suggested that a lumenal loop of sequence located between trans-membrane helices TM1 and TM2 of the ATPase, and containing five histidines and four methionines acts as an organelle-specific chaperone for metallation of the cuproproteins. The hypothesis posits that the pH of the vesicle regulates copper ligation and loop conformation via a mechanism which involves His to Met ligand switching induced by histidine protonation. Here we report the effect of pH on the HM loop copper coordination via X-ray absorption spectroscopy (XAS), and show via selenium substitution of the Met residues that the HM loop undergoes similar conformational switching to that found earlier for its partner PHM. We hypothesize that in the absent of specific chaperones, HM motifs provide a template for building a flexible, pH-sensitive transfer site whose structure and function can be regulated to accommodate the different active site structural elements and pH environments of its partner proteins.

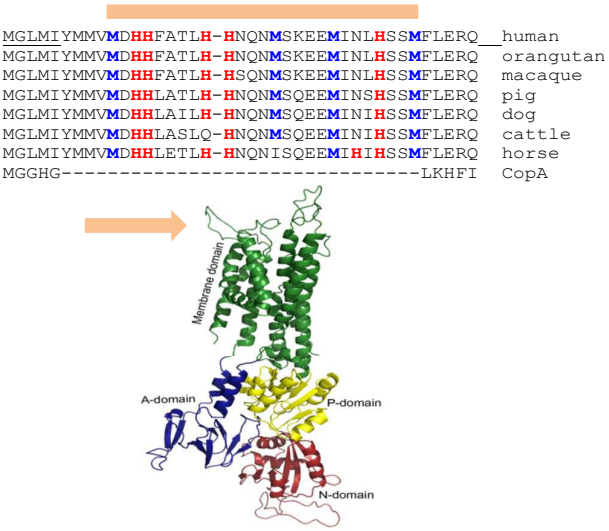


Figure 1. Top, ATP7A lumenal sequences (between TM1 and TM2) for mammalian species compared with CopA from *Legionella pneunmophilia*. The buff bar represents the sequence cloned into the soluble scaffold protein. Bottom, homology model of human ATP7A adapted from reference 9, showing the position of the lumenal loop sequence.

Defects in copper transporting ATPases, ATP7A and ATP7B are responsible for Menkes and Wilson's disease respectively. These transporters are believed to metalate cuproproteins, such as peptidylglycine monooxygenase (PHM),[1-3] dopamine β-monooxygenase (DBM),[4] and tyrosinase (TYR).[5,6] Transfer of copper between donor and acceptor cuproproteins is proposed to occur without the intermediacy of a chaperone,[3] but the precise copper transport mechanism is unknown. These copper enzymes are packaged into vesicles within the transgolgi network (TGN) and are subject to pH environments ranging from 7.2 to 5.2 as maturation in the secretory pathway proceeds.[7,8] ATP7A contains a lumenal loop rich in histidines and methionines[9] important for dephosphorylation and therefore copper release.[10,11] This loop is absent in other eukaryotic P1B-type ATPases (ATP7B, yeast Ccc2), and in bacterial transporters such as CopA[12] (Fig. 1, Table S1), and it has been suggested that its HisMet (HM) sequence elements

[a] *Institute of Environmental Health, Oregon Health and Sciences University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97239, United States*
[b] *Department of Physiology, The Johns Hopkins University, Baltimore, Maryland 21205, USA*

† Electronic Supplementary Information (ESI) available: Materials and Methods, Figures S1 to S6 describing additional XAS data and fits, and Tables S1 to S4 describing location of Met residues and nomenclature, and full parameter lists for fits shown in the main text and in supplementary tables. See DOI: 10.1039/x0xx00000x

may provide a unique role in transferring copper to cuproproteins.[13,14] ATP7A's partners such as PHM, DBM, and TYR exhibit different active site structures and catalytic pH optima, with PHM and DBM being constructed with a pair of uncoupled mononuclear centres with a pH optimum of 5.5,[4,15,16] and TYR having a binuclear dicopper site with pH optimum of 7.[17,18] We hypothesize that in the absent of specific chaperones, HM motifs provide a template for building a flexible, pH-sensitive transfer site whose structure and function can be regulated to accommodate the different active site structural elements and pH environments of its partner proteins.

A feature of chaperone-partner interactions is the tendency for chaperones to mimic the coordination chemistry of their partners. Recently we showed that the HHM motif present at the electron transfer H-centre of PHM is involved in a novel pH and copper-dependent conformational switch.[19-21] The enzyme was found to undergo structural reorganization in the pH range of 8 to 3.5 in which a sulfur ligand (assigned to Met109) bound to copper in place of one of the histidines, abrogating catalytic function. This chemistry provided validation of predictions from model chemistry that His+Met motifs could be important as pH-triggered conformational switches.[22,23] In the present study we asked the question whether its putative donor (the ATP7A lumenal loop) which contains similar (yet more complex) HM elements, undergoes similar pH-dependent copper binding, providing a mechanism whereby ATP7A could tailor its transfer chemistry to the very different conditions required for different cuproprotein metalation.

ATP7A is a membrane protein, which is difficult to express and solubilize. Additionally it contains a number of other metal binding sites in the six sub-domains of the N-terminus, and in the 8-membered trans-membrane helical region (Fig. 1). Therefore, to investigate the copper-binding properties of the ATP7A lumenal loop we generated a construct in which the TM1/TM2 loop sequence was inserted in place of the CXXXC motif of the B. subtilis Sco protein in which all additional His and Met residues had been mutated to Ala.[10] While this loop was shown to bind Cu(I) in a His/Met environment, the details of the Cu(I) binding sites and their pH dependence were elusive. Here we report the effect of pH on copper coordination, and show via selenium substitution of the Met residues that the chimera undergoes similar conformational switching to that found for its partner PHM.

The HM Loop (Fig. 1) contains five histidines and four methionines as potential copper ligands. As shown in Fig. 2, X-ray absorption spectroscopy (XAS) in conjunction with seleno-methionine labelling[24,25] reveals significant pH-induced changes in coordination. Detailed analysis using curve fitting of the EXAFS and FT data (Table S2), show a remarkable degree of plasticity in Cu(I) binding. At pH 8, the simulations of Cu(I) binding to the S(Met) (unlabelled) chimera suggest at least two different environments for Cu(I)-Histidine interactions, one with Cu-N bond lengths diagnostic of bis-histidine coordination, and a second more compatible with a 3-

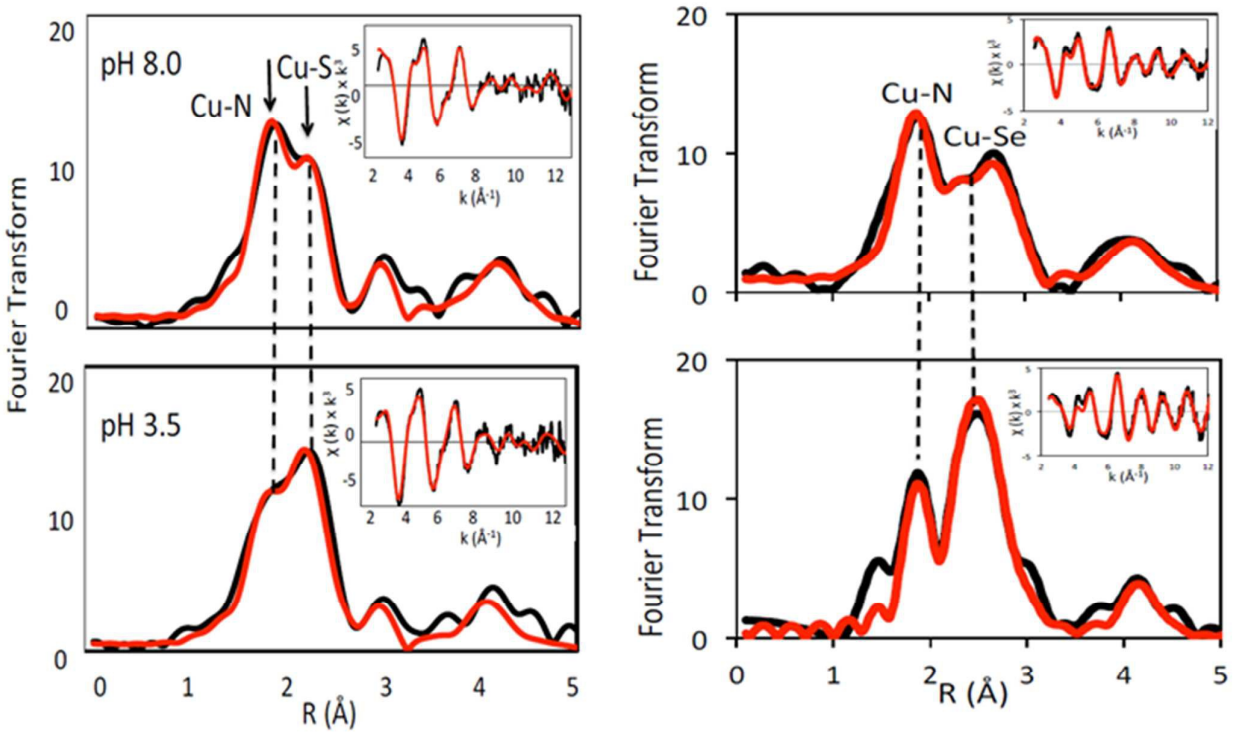


Figure 2 pH dependence of the Cu K EXAFS spectra of S-Met (left) and Se-Met (right) Cu(I) derivatives of WT HM loop at 1:1 metal to protein ratio. Top panels are data collected at pH 8, while bottom panels are for pH 3.5. Parameters used in the simulations shown in red are listed in Table S2 of the Supporting Information.

coordinate structure. In addition, a sub-stoichiometric shell of Cu-S(Met) is found at a distance typical of 3-coordinate Cu(I)-methionine systems. We conclude that the 3-coordinate site consists of a $(His)_2$Met ligand set. As the pH is lowered to 3.5, the coordination changes to a predominately Cu-S environment, with the species trading His ligands for Met as the former protonate. XANES at the Cu edge (Fig. S5) confirmed 3-coordination at both the high and low pHs. Se-labelling of the Met residues allowed the pH transition to be studied with more precision. At pH 8.0 copper adopts a roughly 1:1 mixture of bis-His and His_2SeM coordinated species, while at pH 3.5, the Cu-Se peak dominates the spectrum, accounting for more than 2 SeM ligands per Cu(I). Therefore, as with the unlabelled construct, the HM Loop toggles between various His and Se(Met) ligands, favouring Se(Met) as the pH decreases to an extreme of 3.5. It is likely that each of these different ligand sets represents a different conformation of the loop peptide supporting our hypothesis that His and Met combinations provide conformational switching similar to that observed in PHM, where one or more coordinated histidine in a HM motif protonates inducing coordination of copper to an adjacent Met ligand.[11]

Selenium XAS provides an additional spectroscopic probe[24,25], and allows validation of the conclusions derived from Cu edge data. Figure 3 compares the Se XAS of HM Loop at pH 8.0 and 3.5 where the increase in the Se-Cu peak at 2.4 Å is clearly seen in the data. Corresponding fits are shown in the bottom panels of Figure 3. At pH 8.0 the Se-Cu interaction is weak, and corresponds to a maximum of 0.2 Cu per Se, rising to 0.6 Cu per Se at pH 3.5. Given that the loop sequence contains 4 S(Se)-Met residues, these values suggest ~0.8 and 2.4 Se-Cu bonds respectively. Therefore, Cu(I)-Met coordination increases from a species containing a substoichiometric SeMet component at pH 8.0 to one ligated by at least 2 SeMet ligands at pH 3.5, fully consistent with the Cu edge data.

In order to identify the Met residue(s) that bind copper within the HM Loop, triple met mutants were constructed and analysed at the Cu edge. These mutants are denoted M1 to M4 where M1 retains the first methionine in the sequence, and so on (Table S1). M1 and counterpart, SeM1 (SeMet234 to Ile), were loaded with Cu(I) and subjected to pH 8.0 and 3.5. Interestingly, no signs of Cu-S or Cu-Se were observed at either pH (Fig S1) while the shortening of the Cu-N(His) bond lengths (Table S3) and the increased intensity of the 8983 eV XANES feature (Fig S6) were both consistent with bis-His coordination.[26-28] Triple mutants M2, M3, and M4 also showed no signs of Cu-S(Met) at pH 3.5 (Table S3, Figs S1 and S6). These data provide evidence that constructs containing a single Met experience no pH-dependent switching even in the SeM labelled samples, which imply that multiple methionine residues are required for the pH-triggered transitions. We also constructed double Met mutants containing each of the six possible pairs of Met residues, and studied their Cu-methionine interactions at the Cu and Se K edges. These mutants showed complex behaviour, with no one mutant displaying more than 0.5 S/Se interactions per copper. The data are shown in Figs S2, S3, S4 and S6 with the fit parameters listed in Table S4. Close examination of all the data suggests that M3 and to a lesser extent M1 are most important for copper binding, with the M13 variant (Mets 1 and 3 retained) exhibiting the most prominent Cu-S/Se feature in the FT. However, this variant showed no appreciable pH dependence. Together, the data lead to the unexpected conclusion that at least three methionine residues are required to reproduce the WT coordination properties of the loop, and suggest a highly dynamic environment for copper binding and hand-off to the cuproproteins.

It is interesting that lowering the pH only induces a His to Met switch in the native peptide, while for the mutants no His dissociation occurs. We can speculate that the pK_A for His protonation must be influenced by the availability of a Met residue to coordinate. If the Met is not available or in an unfavourable conformation, then the site remains 2-coordinate, which is known to be a favourable coordination for Cu(I)[27]. The presence of a nearby Met residue in a favourable conformation may induce one of the His residues to protonate and dissociate, since now the methionine can compete successfully for the coordination position on Cu(I). This behaviour has been documented in the low pH (inactive) form of the H-centre of PHM where the presence of M109 in the HHM motif induces a His to Met switch at low pH. However, when the Met is absent (M109A), the enzyme remains active

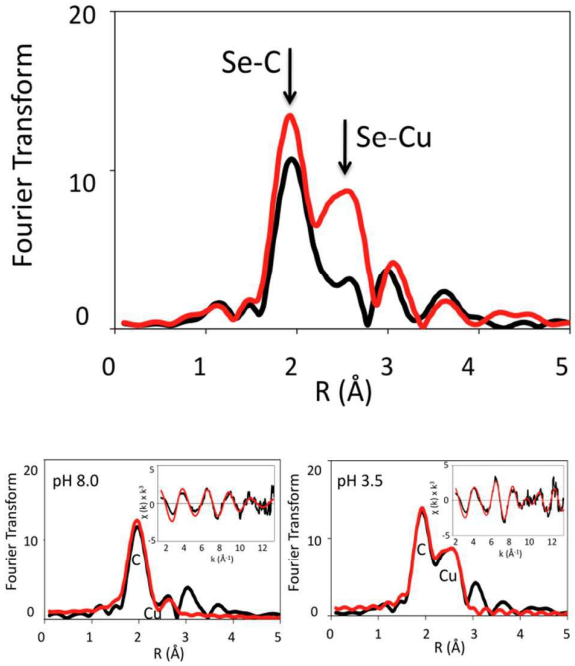


Figure 3. pH-dependent Se EXAFS (insets) and Fourier transforms of SeM labeled HM Loop loaded with Cu(I) at 1:1 metal to protein ratio. Top panel compares the Fourier transforms at pH 8.0 (black) and pH 3.5 (red). Bottom figures are EXAFS (insets) and Fourier transforms at pH 8.0 (bottom left) and pH 3.5 (bottom right). Black traces are experimental and red traces are simulated data.

below pH 4, implying that the nearby Met residue perturbs the pK_A of one of the coordinating His residues, such that it protonates and dissociates.[20]

The lack of specificity, and apparent requirement for the presence of three or four Met residues in the ATP7A lumenal loop is reminiscent of similar multiple Met motifs in the N-terminal ecto-domain of CTR1 where mutation or truncation at the N-terminus has only minor effects on transport efficiency.[29-32] Current models of CTR1 function are suggestive of weak binding to methionine triads in the ecto-domain and entry vestibule, which induce selectivity for Cu(I) without compromising ion lability.[29-32] A similar model applied to the ATP7A lumenal loop would ensure that Cu(I) would be protected by sequestration from solvent interactions during hand-off without being trapped in a thermodynamic sink.

Because metalation intermediates must use metal-binding residues at or close to the catalytic metallocenters, the pH-dependence of metal insertion is likely to mirror that of catalysis, and thus may have profound implications for the way that proteins are sorted and metallated in the secretory pathway. Maturation of the secretory pathway employs sophisticated strategies to sort and process proteins, exploiting vesicular pH for regulation.[7,8,33] PHM, DBM and TYR are all metallated within these vesicles, and evidence from colocation[2,5,6] and other[3] studies implicates the ATPase as the copper donor. However, the environment of the lumen of these vesicles is not the same for all cuproproteins, particularly with respect to pH. For example TYR resides in specialized lysosomes termed melanosomes where the pH is highly variable and current ideas suggest that this may be a factor in determining the differences in pigmentation between different ethnic populations.[34,35] Thus, Caucasian populations are believed to regulate the pH of melanosomes into the acidic range (where the activity of TYR is low) via the action of a H+ importing V-type ATPase[36], whereas African populations maintain neutral pH close to the pH optimum for TYR of 7.4.[35] For PHM, the activity maximum of purified PHMcc (catalytic core) is ~5.5 which corresponds to the pH of its secretory vesicles, but the pH optimum of the intact PAM-1 integral membrane protein containing PHM, PAL and the transmembrane domains together with associated linker peptides is one pH unit lower at 4.5.[37] On the other hand, the presence of the granule associated factor SPAM shifts the pH optimum into the neutral pH range suggesting that protein interactions within the vesicle can perturb the effect of pH on active conformations.[38] It is not yet known under what conditions copper is inserted into the cuproproteins, but one may speculate that transport must likewise be able to respond to the varying conditions of pH. The fluxional metal-binding scaffold of the ATP7A lumenal loop implied by the present findings could likely interact with a variety of different apo-protein environments, and thus be capable of copper transfer to multiple cuproprotein partners under varying pH conditions.

Histidine residues have been implicated as pH conformational switching signals in a number of other systems via their ability to protonate and thus alter H-bonding interactions in the physiological pH range. The H+ importing V-type ATPase has been suggested to sense pH via histidines located on its lumenal loops.[36] PAM itself contains a conserved HisMet rich motif in the linker between PHM and PAL domains which has been shown to alter the trafficking and signalling properties of the protein as it navigates the decreasing pH environment of the secretory pathway.[8] The present work suggests that the role of additional elements such as coupled Met residues and/or metal ions may represent a new paradigm for pH sensing in the maturation of metalloproteins.

Author information

Corresponding Author: blackbni@ohsu.edu
Funding Sources: The authors declare no financial competing interest.

Acknowledgment

This work was funded by grants from the National Institutes of Health GM115214 (NJB) and P01 GM067166 (N.J.B and S.L). We gratefully acknowledge the use of facilities at the Stanford Synchrotron Radiation Lightsource (SSRL), which is supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract No. DE-AC02-76SF00515. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research, and by the National Institutes of Health, National Institute of General Medical Sciences (including P41GM103393).

Significance to metallomics

How mammalian cuproproteins acquire their copper has been a vexing problem since they do not appear to utilize specific metallochaperones, and all appear to be metalated by the ATP7A copper transporter. Recently we proposed a new hypothesis that HisMet (HM) sequence elements in a conserved lumenal loop of the ATP7A copper pump play an important role in copper hand-off to the cuproproteins. In the present communication we show that the pH-dependent His to Met conformational switching predicted by this hypothesis actually occurs in a model system built from a chimera of the lumenal loop sequence and a scaffold protein.

Notes and references

1 De, M.; Ciccotosto, G. D.; Mains, R. E.; Eipper, B. A. J. Biol. Chem. 2007, 282, 23362.
2 El Meskini, R.; Culotta, V. C.; Mains, R. E.; Eipper, B. A. J. Biol. Chem. 2003, 278, 12278.
3 Steveson, T. C.; Ciccotosto, G. D.; Ma, X.-M.; Mueller, G. P.; Mains, R. E.; Eipper, B. A. Endocrinology 2003, 144, 188.
4 Klinman, J. P. J. Biol. Chem. 2006, 281, 3013.
5 Petris, M. J.; Strausak, D.; Mercer, J. F. Hum. Mol. Genet. 2000, 9, 2845.
6 Setty, S. R.; Tenza, D.; Sviderskaya, E. V.; Bennett, D. C.; Raposo, G.; Marks, M. S. Nature 2008, 454, 1142.
7 Paroutis, P.; Touret, N.; Grinstein, S. Physiology 2004, 19, 207.

8 Vishwanatha, K.; Back, N.; Mains, R. E.; Eipper, B. A. J. Biol. Chem. 2014.
9 Schushan, M.; Bhattacharjee, A.; Ben-Tal, N.; Lutsenko, S. Metallomics 2012, 4, 669.
10 Barry, A. N.; Otoikhian, A.; Bhatt, S.; Shinde, U.; Tsivkovskii, R.; Blackburn, N. J.; Lutsenko, S. J. Biol. Chem. 2011, 286, 26585.
11 Otoikhian, A.; Barry, A. N.; Mayfield, M.; Nilges, M.; Huang, Y.; Lutsenko, S.; Blackburn, N. J. J. Am. Chem. Soc. 2012, 134, 10458.
12 Sitsel, O.; Gronberg, C.; Autzen, H. E.; Wang, K.; Meloni, G.; Nissen, P.; Gourdon, P. Biochemistry 2015, 54, 5673.
13 Gourdon, P.; Liu, X. Y.; Skjorringe, T.; Morth, J. P.; Moller, L. B.; Pedersen, B. P.; Nissen, P. Nature 2011, 475, 59.
14 Lutsenko, S.; LeShane, E. S.; Shinde, U. Arch. Biochem. Biophys. 2007, 463, 134.
15 Prigge, S. T.; Mains, R. E.; Eipper, B. A.; Amzel, L. M. Cell. Mol. Life Sci. 2000, 57, 1236.
16 Vendelboe, T. V.; Harris, P.; Zhao, Y.; Walter, T. S.; Harlos, K.; El Omari, K.; Christensen, H. E. M. Science Advances 2016, 2, http://advances.sciencemag.org/content/advances/2/4/e1500980.full.pdf.
17 Matoba, Y.; Kumagai, T.; Yamamoto, A.; Yoshitsu, H.; Sugiyama, M. J. Biol. Chem. 2006, 281, 8981.
18 Sendovski, M.; Kanteev, M.; Ben-Yosef, V. S.; Adir, N.; Fishman, A. J. Mol. Biol. 2011, 405, 227.
19 Bauman, A. T.; Jaron, S.; Yukl, E. T.; Burchfiel, J. R.; Blackburn, N. Biochemistry 2006, 45, 11140.
20 Bauman, A. T.; Broers, B. A.; Kline, C. D.; Blackburn, N. J. Biochemistry 2011, 50, 10819.
21 Kline, C. D.; Mayfield, M.; Blackburn, N. J. Biochemistry 2013, 52, 2586.
22 Haas, K. L.; Putterman, A. B.; White, D. R.; Thiele, D. J.; Franz, K. J. J. Am. Chem. Soc. 2011, 133, 4427.
23 Rubino, J. T.; Chenkin, M. P.; Keller, M.; Riggs-Gelasco, P.; Franz, K. J. Metallomics 2011, 3, 61.
24 Bagai, I.; Rensing, C.; Blackburn, N. J.; McEvoy, M. M. Biochemistry 2008, 47, 11408.
25 Chacon, K. N.; Blackburn, N. J. J. Am. Chem. Soc. 2012, 134, 16401.
26 Kau, L. S.; Spira-Solomon, D. J.; Penner-Hahn, J. E.; Hodgson, K. O.; Solomon, E. I. J. Am. Chem. Soc. 1987, 109, 6433.
27 Park, G. Y.; Lee, J. Y.; Himes, R. A.; Thomas, G. S.; Blackburn, N. J.; Karlin, K. D. J. Am. Chem. Soc. 2014, 136, 12532.
28 Pickering, I. J.; George, G. N.; Dameron, C. T.; Kurz, B.; Winge, D. R.; Dance, I. G. J. Am. Chem. Soc. 1993, 115, 9498.
29 Eisses, J. F.; Kaplan, J. H. J. Biol. Chem. 2005, 280, 37159.
30 Ohrvik, H.; Thiele, D. J. Annals of the New York Academy of Sciences 2014, 1314, 32.
31 Puig, S.; Lee, J.; Lau, M.; Thiele, D. J. J. Biol. Chem. 2002, 277, 26021.
32 Tsigelny, I. F.; Sharikov, Y.; Greenberg, J. P.; Miller, M. A.; Kouznetsova, V. L.; Larson, C. A.; Howell, S. B. Cell Biochem. Biophys. 2012, 63, 223.
33 Williamson, D. M.; Elferich, J.; Ramakrishnan, P.; Thomas, G.; Shinde, U. J. Biol. Chem. 2013, 288, 19154.
34 Ancans, J.; Tobin, D. J.; Hoogduijn, M. J.; Smit, N. P.; Wakamatsu, K.; Thody, A. J. Experimental cell research 2001, 268, 26.
35 Smith, D. R.; Spaulding, D. T.; Glenn, H. M.; Fuller, B. B. Experimental cell research 2004, 298, 521.
36 Marshansky, V. Biochem Soc Trans 2007, 35, 1092.
37 Husten, E. J.; Eipper, B. A. J. Biol. Chem. 1991, 266, 17004.
38 Perkins, S. N.; Husten, E. J.; Mains, R. E.; Eipper, B. A. Endocrinology 1990, 127, 2771.

For Table of Contents