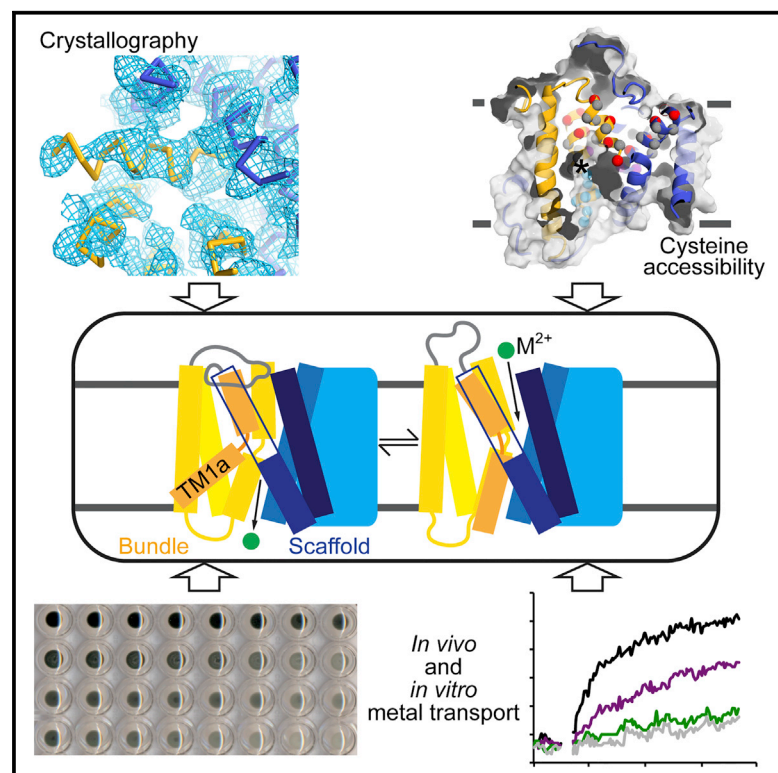


Structure

Crystal Structure and Conformational Change Mechanism of a Bacterial Nramp-Family Divalent Metal Transporter

Graphical Abstract



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In Brief

Bozzi et al. determined the inward-open structure of a bacterial Nramp transition metal transporter with a LeuT fold. Using biochemical experiments, the authors provide mechanistic explanations for how two anemia-causing mutations impede function through altering the conformational landscape of the protein in unique ways.

Highlights

- *Deinococcus radiodurans* Nramp structure reveals TM1a location in inward-open state
- Unfettered movement of TM1a is essential to the metal-transport cycle
- G153R disease-mutant mimic alters selectivity of conserved metal-binding site
- G45R disease-mutant mimic sterically locks protein in inward-open state

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Crystal Structure and Conformational Change Mechanism of a Bacterial Nramp-Family Divalent Metal Transporter

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SUMMARY

The widely conserved natural resistance-associated macrophage protein (Nramp) family of divalent metal transporters enables manganese import in bacteria and dietary iron uptake in mammals. We determined the crystal structure of the *Deinococcus radiodurans* Nramp homolog (DraNramp) in an inward-facing apo state, including the complete transmembrane (TM) segment 1a (absent from a previous Nramp structure). Mapping our cysteine accessibility scanning results onto this structure, we identified the metal-permeation pathway in the alternate outward-open conformation. We investigated the functional impact of two natural anemia-causing glycine-to-arginine mutations that impaired transition metal transport in both human Nramp2 and DraNramp. The TM4 G153R mutation perturbs the closing of the outward metal-permeation pathway and alters the selectivity of the conserved metal-binding site. In contrast, the TM1a G45R mutation prevents conformational change by sterically blocking the essential movement of that helix, thus locking the transporter in an inward-facing state.

INTRODUCTION

Nearly all organisms require iron to survive. The oxidation state cycle of iron is ideal both to catalyze essential redox reactions as a co-factor in numerous enzymes and to provide a pathway for electron transport across membranes. In addition, heme iron is used for oxygen transport and storage, enabling aerobic respiration. Organisms have thus evolved mechanisms to acquire, traffic, and safely store this crucial transition metal (Aisen

et al., 2001; Andrews, 2008). The natural resistance-associated macrophage protein (Nramp) family of divalent metal transporters plays a vital role in mammalian iron homeostasis (Mackenzie and Hediger, 2004). Expressed in phagosomal membranes, Nramp1 both helps macrophages kill engulfed pathogens by extracting iron and other essential transition metals (Cellier et al., 2007), and enables iron recycling from dying erythrocytes (Soe-Lin et al., 2009). Mammals abundantly express a second homolog, Nramp2 (also known as divalent metal transporter 1) in intestinal enterocytes to enable absorption of dietary iron (Gunshin et al., 1997). Nramp2 is also expressed at lower levels in the endosomal membranes of most somatic cells (Mackenzie et al., 2007), where it enables extraction of transferrin-bound iron from vesicles, an especially important process in erythropoiesis (Canonne-Hergaux et al., 2001; Gunshin et al., 2005).

To maintain homeostasis, mammals tightly regulate iron uptake and transport (Lieu et al., 2001), primarily through translation and localization of Nramps (Gunshin et al., 2001; Hubert and Hentze, 2002). An overabundance of free iron generates free radicals that cause tissue damage and increase susceptibility to infection (Ganz, 2009). In contrast, iron deficiency causes anemia (Abbaspour et al., 2014). Accordingly, mutations in Nramp2 are implicated in anemia in humans and rodents (Iolascon and De Falco, 2009; Shawki et al., 2012). The same glycine-to-arginine mutation (G185R) causes microcytic anemia in both mice (Fleming et al., 1997) and Belgrade rats (Fleming et al., 1998; Veuthey and Wessling-Resnick, 2014), and altered protein localization in enterocytes (Canonne-Hergaux et al., 2000). This mutation reduces iron transport when expressed in mammalian cell lines (Su et al., 1998; Touret et al., 2004; Xu et al., 2004), with a concomitant increase in permeability to calcium (typically a poor Nramp substrate) in G185R-transfected cells compared with wild-type (WT) counterparts (Xu et al., 2004). A glycine-to-arginine mutation (G75R) in human anemia patients may abrogate iron transport function (Barrios et al., 2012; Blanco et al., 2009; Shawki et al., 2012). However, the molecular mechanisms

Table 1. Data Collection and Refinement Statistics

	Fab	DraNrapm
PDB	NA	5KTE
SBGid Databank	335	332, 333, 334
Data Collection		
Wavelength (Å)	0.97917	1.139
Resolution range (Å)	30.00–3.00 (3.11–3.00)	46.47–3.94 (4.08–3.94)
Space group	P2 ₁ 2 ₁ 2 ₁	I222
Unit cell (a, b, c)	116.17, 183.66, 299.88	113.13, 132.08, 221.0
No. of crystals	1	3
Total reflections	558,353	245,867
Unique reflections	119,718	11,791 (462)
Redundancy	4.7 (4.8)	16.6 (11.4)
Completeness (%)	99.5 (100.0)	95 (96)
Mean I/σ(I)	8.4 (2.2)	6 (.61)
R _{merge}	0.151	0.171
R _{meas}		0.177
R _{pim}		0.047
CC _{1/2}		0.99 (0.189)
Refinement		
Resolution range (Å)		46.47–3.94 (4.12–3.94)
R _{work}		0.2666 (0.2672)
R _{free}		0.3128 (0.3656)
No. of atoms		5,625
Protein		5,622
Ions (Os)		3
Protein residues		762
Ramachandran plot (%)		
Favored		692 (90.8)
Allowed		66 (8.7)
Outliers		4 (0.52)
RMSD (bonds)		0.005
RMSD (angles)		1.12
Average B factor		89.8
Protein		89.8
Ions (Os)		190

NA, not applicable; RMSD, root-mean-square deviation.

by which these mutations perturb Nrapm metal transport and cause anemia remain unknown.

The Nrapm family spans the tree of life, with homologs that perform a range of essential divalent transition metal-transport functions, likely as secondary transporters that harness a proton gradient (Courville et al., 2006). Based on sequence analyses, Nrapm homologs form four major phylogenetic clades: the eukaryotic Nrapms and the prokaryotic A, B, and C clades (Cellier et al., 2001). The crystal structure of *Staphylococcus capitis* Nrapm (ScaNrapm), belonging to clade C, confirmed a LeuT fold (named for the bacterial sodium/amino acid symporter [Yamashita et al., 2005]) for Nrapms, as predicted previously (Cellier, 2012; Ehrnstorfer et al., 2014).

Because of the alternating access model for membrane transport proteins (Jardetzky, 1966), Nrapms should cycle between at least two stable conformations: outward-facing to bind its metal substrate, and inward-facing to release its cargo into the cytosol. The ScaNrapm structure, in an inward-facing state, revealed a metal-binding site that consists of conserved aspartate, asparagine, and methionine residues, and a backbone carbonyl from transmembrane segments (TMs) 1 and 6 that coordinate a range of divalent metal substrates (Ehrnstorfer et al., 2014), with the methionine providing a selective preference for transition metals over alkaline earth metals (Bozzi et al., 2016).

Here we present the crystal structure of the *Deinococcus radiodurans* Nrapm homolog (DraNrapm) from prokaryotic clade A. The DraNrapm structure represents an inward-facing apo conformation, with TM1a, absent from the ScaNrapm crystallization construct, swung up to open a large intracellular vestibule. We use this structure along with extensive cysteine accessibility and metal-transport measurements to propose a model for conformational change in this LeuT-fold transporter and explain the mechanistic effects of two anemia-causing mammalian Nrapm2 mutations. In our model, reaching the outward-facing state requires TM1a to approach the protein core to close the intracellular vestibule. This motion is prevented by an N-terminal glycine-to-arginine mutation that mimics an anemia-causing mutation in human Nrapm2. The second disease-mimicking glycine-to-arginine mutation alters the extracellular vestibule and metal-binding site, resulting in reduced transport activity and altered selectivity.

RESULTS

The DraNrapm Inward-Facing Structure Shows a Highly Kinked Transmembrane Helix 1

We determined the crystal structure of detergent-solubilized DraNrapm (38% sequence identity with ScaNrapm) in complex with a monoclonal antibody fragment (Fab) to 3.9-Å resolution (Table 1). Crystallization was facilitated by intracellular surface mutations (Figure 1B; see below). We used a ScaNrapm-based homology model and the Fab fragment crystal structure as molecular replacement (MR) search models, with additional phasing provided by single-wavelength anomalous signals from three osmium ions bound to the Fab or at crystal contacts (Figure S1). Refinement was facilitated by using xMDFF (McGreevy et al., 2014; Singharoy et al., 2015). In particular, we used a combination of xMDFF and steered molecular dynamics to optimize our TM1a model, exploring different TM1a positional registries corresponding to a screw axis rotation of approximately one helical turn. The assigned TM1a registry (Figure 1D) yielded both the lowest R factor and the best agreement with cysteine accessibility data (see Figures 6 and 7; see below). The final asymmetric unit comprises one DraNrapm transporter, one Fab bound to the DraNrapm periplasmic face, and three osmium ions, with crystal-packing interactions between the Fab and the DraNrapm cytoplasmic face (Figure S1). All 11 DraNrapm TMs are visible in the electron density, including TM1a, which was truncated in ScaNrapm (Ehrnstorfer et al., 2014).

Like ScaNrapm, DraNrapm has a LeuT fold with two pseudosymmetry-related structural repeats comprising TMs 1–5

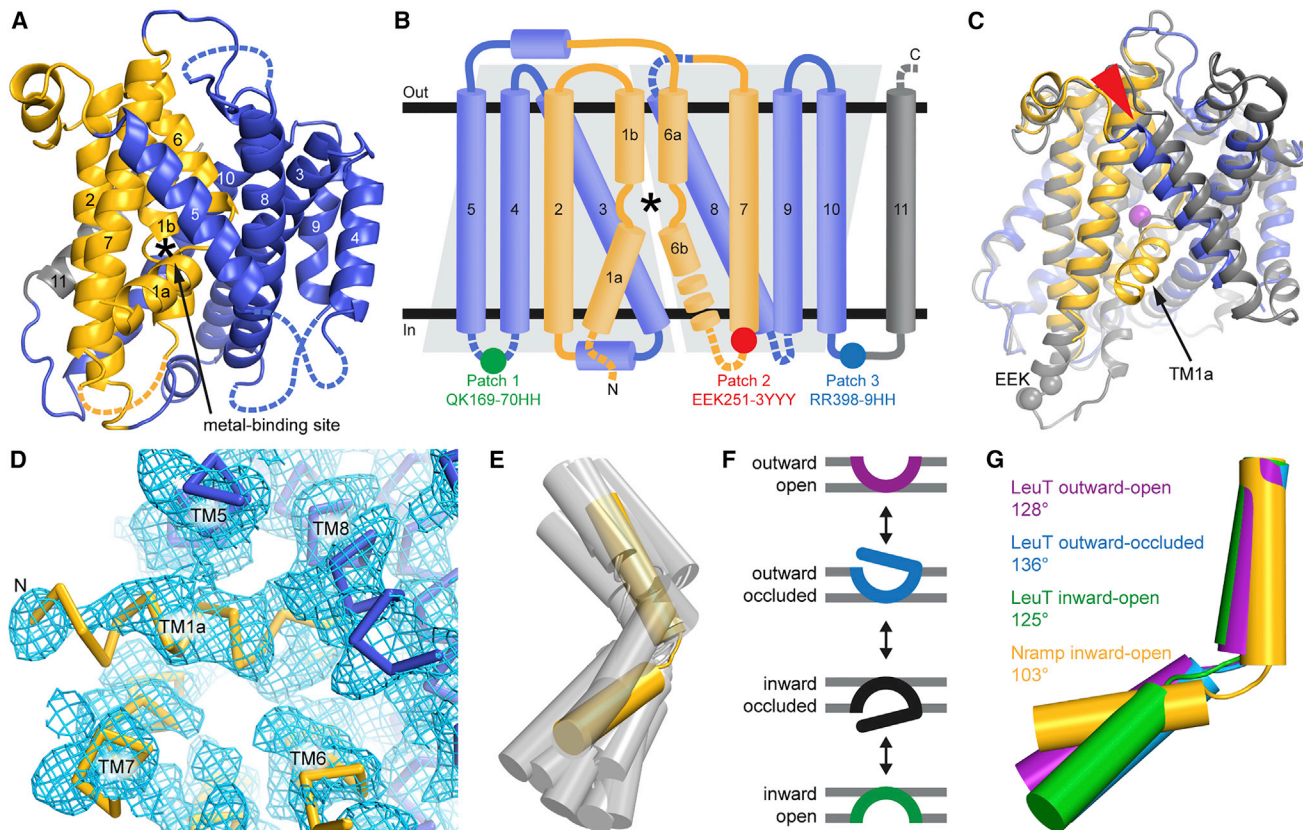


Figure 1. DraNrap Structure in the Inward-Facing State Shows a Highly Kinked TM1

(A) Cartoon representation of DraNrap with TM helices labeled; the bundle (TMs 1, 2, 6, and 7) is gold, scaffold (TMs 3, 4, 5, 8, 9, and 10) blue, and TM11 gray. Dashed loops are disordered in the structure. (B) DraNrap topology diagram, with helices as cylinders, and gray trapezoids highlighting the inverted structural repeats (TMs 1–5 and TMs 6–10). Intracellular loop mutations patch 1 to 3 in the crystallized construct are indicated. (C) Superposition of DraNrap (blue and gold) and ScaNrap (PDB: 4WGW; gray and Mn²⁺ magenta) indicates a similar overall fold. The main differences are the position of TM5 (red arrowhead) and the presence of TM1a in DraNrap. Gray spheres mark the ScaNrap EEK motif corresponding to patch 2, disordered in DraNrap.

(D) Final 2F_o – F_c electron density map at 0.8 σ showing density for TM1a. DraNrap is represented as a C α trace.

(E) Comparison of TM1 kink angle of DraNrap (yellow) with published LeuT-fold structures (gray; LeuT PDB: 2A65, 3TT1, 3TT3, 5JAE; Mhp1 PDB: 2JLN, 4D1B, 2X79; vSGLT PDB: 2XQ2, 3DH4; BetP PDB: 4LLH, 4AIN, 4DOJ, 4C7R). The scaffolds of the corresponding structures were superimposed and oriented as in (A). (F) Conformational states in a transport cycle, color coded as in (G).

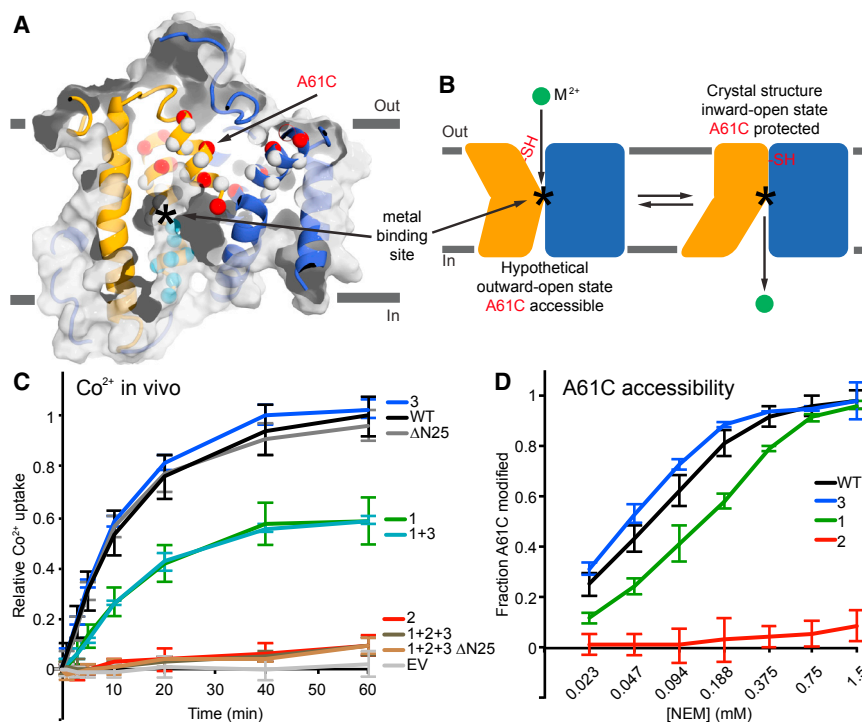
(G) The TM1b helices were aligned for DraNrap and three distinct LeuT conformations, highlighting the common kink at the unwound substrate-binding region in the middle of TM1. The kink is even more pronounced in inward-open DraNrap than any LeuT structure.

See also Figure S1.

and TMs 6–10, respectively (Figures 1A and 1B) (Yamashita et al., 2005). As in other LeuT folds, TMs 1, 2, 6, and 7 form a “rocking bundle” whose putative movements relative to the “scaffold,” made up of the remaining TMs, likely effects the switch in active site accessibility from extracellular to intracellular (Forrest and Rudnick, 2009). TM1 and 6 are unwound in the center of the membrane plane, providing substrate-binding residues as observed in ScaNrap (Ehrnstorfer et al., 2014). In DraNrap, the metal-binding site is unoccupied and exposed to the intracellular side (see Figure 2A). Thus the structure represents a substrate-free inward-facing conformation. Accordingly, DraNrap and ScaNrap superimpose well with a root-mean-square distance of 1.54 Å over 279 C α atoms. Clade A members such as DraNrap have a four-residue deletion near the TM9 N terminus in comparison with clade C members such as ScaNrap, resulting in a shorter he-

lix (Figure S1D) (Cellier, 2012). The main difference between the two structures is the position of scaffold helix TM5 (Figure 1C), more angled relative to the membrane plane in DraNrap, perhaps influenced by the presence of TM1a in the structure.

As in other LeuT-fold proteins (Figure 1E), DraNrap TM1a is bent outward and lies nearly parallel to the membrane between the pillars of scaffold helix TM5 and bundle helix TM7, creating a large aqueous vestibule on the cytoplasmic side of the transporter. The angle between TM1a and 1b is 103°, smaller than that observed in the inward-facing LeuT structure (125°; Krishnamurthy and Gouaux, 2012), and outward-occluded LeuT (136°; Yamashita et al., 2005) (Figures 1F and 1G). Correspondingly this deep bend in TM1 yields a larger vestibule than in LeuT, which could facilitate water coordination of transported metal ions.



(D) While the transport-competent patch 1 and 3 mutants retained A61C accessibility (patch 1 at a reduced level), the transport-dead patch 2 mutant eliminated A61C accessibility, suggesting it locks the protein in the inward-open state. All data are averages \pm SD ($n \geq 3$). For reference, WT and empty vector/vesicle (EV) Co $^{2+}$ uptake time course and WT A61C accessibility data are repeated in subsequent figures. See also Figure S2 and Table S1.

Cysteine Accessibility Scanning Reveals an Outward-Facing Metal-Permeation Pathway

To identify a metal-permeation pathway, we created a panel of sequential single-cysteine mutants spanning DraNrap TM1, 3, and 6, which typically line the inward- and/or outward-facing permeation pathway in LeuT-fold transporters (Shi, 2013). The mutations were introduced in the C382S background, which removed the lone endogenous cysteine while retaining full activity (Figure S2E). We measured in vivo accessibility to thiol-specific modifier N-ethylmaleimide (NEM) or inner membrane-impermeable 2-(trimethylammonium)ethyl methane thiosulfonate bromide (MTSET) (Figures S2A and S2B), which both specifically react with aqueous-exposed cysteines (Kaback et al., 2007; Karlin and Akabas, 1998). We classified the cysteine positions in three groups: (1) NEM- and MTSET-reactive residues were deemed as extracellularly exposed in at least one DraNrap conformation; (2) NEM-reactive but MTSET-protected residues as intracellularly accessible; and (3) NEM-protected as buried (Figures S2C and S2D and Table S1). We observed high NEM accessibility all along TM1a and 6b, consistent with the large aqueous vestibule below the metal-binding residues in our structure. Between the metal-binding site and the extracellular face, we observed a helical pattern of accessible positions on TM1b, 3, and 6a that line the bundle-scaffold interface (Figure S2D). As many of these NEM-reactive positions are buried in the inward-facing structure (Figure 2A), we conclude that they face an aqueous pathway for periplasmic metal ions to reach the binding site in an alternate outward-open conformation. Thus, our cysteine accessibility

measurements, rather than describing a single state, instead provide a composite picture of multiple conformations that DraNrap cycles through in the native membrane.

A Crystallization Construct Mutation Locks the Transporter in an Inward-Open State

We exploited this knowledge of the extracellular metal-permeation pathway to select A61C as a reporter to assess the conformational preferences of various DraNrap mutants (Figures 2A and 2B). A61C was accessible to inner membrane-impermeable modifier MTSET (Figure S2B), indicating that it faces a periplasmic aqueous environment, and A61C/C382S showed WT-level Co $^{2+}$ transport (Figure S2E). In contrast, our crystallization construct, which contained entropy-reducing mutation patches in three intracellular loops: (1) QK169-70HH, (2) EEK251-3YYY, and (3) RR398-9HH, as well as an N-terminal 25-residue deletion (Figure S2F), did not transport Co $^{2+}$ (Figure 2C). Patch 2 on its own completely eliminated Co $^{2+}$ uptake (Figure 2C). This mutation is in intracellular loop 6-7, far from the metal-binding site; thus we tested A61C accessibility over a range of NEM concentrations to probe for conformational preference. Unlike the concentration-dependent increase in NEM modification of A61C for WT DraNrap, A61C was essentially not modified in the patch 2 background (Figures 2D and S2G). Therefore, the patch 2 mutant protein cannot switch to the outward-open state, which explains its loss-of-function phenotype, as substrate ions cannot reach the binding site (below A61C) from the outside. The patch 3 mutant had WT-level Co $^{2+}$ transport and A61C modification, while the patch 1 mutant had

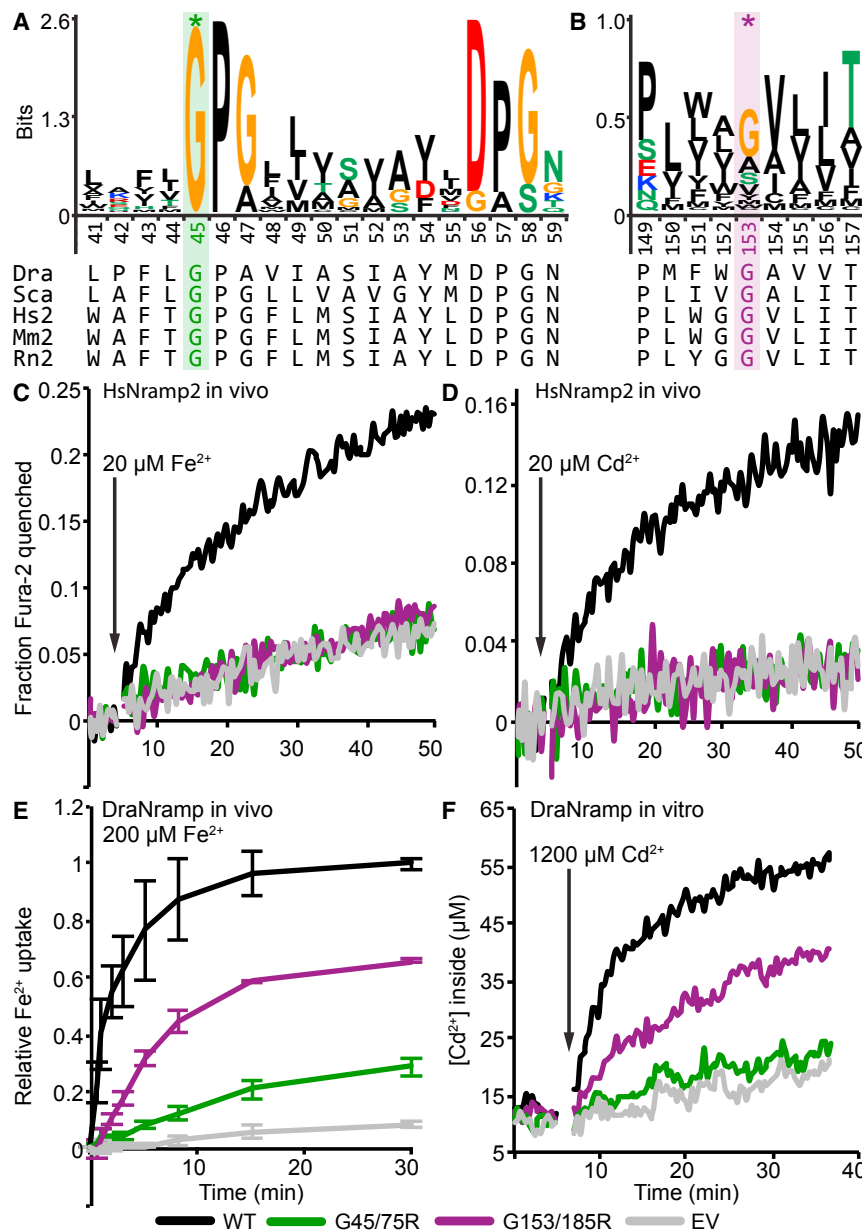


Figure 3. Glycine-to-Arginine Mutations Impair Transition Metal Transport in Both Human Nramp2 and DraNramp

(A and B) Sequence logos of the TM1a region (A) showing that G45 (shaded green with *, G75 in human Nramp2) on TM1a is absolutely conserved in Nramps, and a TM4 segment (B) showing that G153 (shaded purple with *, G185 in human/mouse/rat Nramp2) is generally a small amino acid. Logos were generated from an HMMER alignment of 2,691 sequences using DraNramp to search the UniProt database, with an E value cutoff of 1×10^{-9} . Dra, *D. radiodurans* MntH; Sca, *S. capitis* MntH; Hs2, *Homo sapiens* Nramp2; Mm2, *Mus musculus* Nramp2; and Rn2, *Rattus norvegicus* Nramp2.

(C and D) Fura-2 fluorescence quenching traces showing severe loss of function (no transport activity above baseline) for both G-to-R mutants compared with WT human Nramp2 for transport of the transition metals Fe^{2+} (C) and Cd^{2+} (D) in transfected HEK cells. Traces are representative of at least three independent transfection experiments.

(E) Relative Fe^{2+} uptake of *E. coli* expressing the analogous G-to-R DraNramp mutants also showed significantly decreased transport activity compared with WT. Plotted are averages \pm SD ($n = 3$).

(F) Both G-to-R DraNramp mutants had decreased Cd^{2+} transport when reconstituted into proteoliposomes. Traces are representative of three experiments.

See also Figure S3.

both slightly reduced Co^{2+} transport and A61C accessibility (Figures 2C and 2D). This clear correlation of impaired metal uptake with loss of A61C accessibility further implicates the opening of the interface between the bundle and scaffold as an essential conformational change within the Nramp transport cycle.

Anemia-Causing Mutations Impair Metal Transport in Human Nramp2 and DraNramp

Naturally occurring glycine-to-arginine mutations G75R in TM1a in human Nramp2 and G185R in TM4 in mouse and rat Nramp2 all cause anemia (Blanco et al., 2009; Fleming et al., 1997, 1998). The TM1a glycine is absolutely conserved; the TM4 glycine is generally conserved as a small residue (Figures 3A and 3B). When introduced in human Nramp2, both G-to-R mutations abrogated transport of the physiological substrate Fe^{2+} (Figure 3C), as

well as Cd^{2+} , Co^{2+} , and Mn^{2+} (Figures 3D, S3A, and S3B), as detected in transfected HEK cells using the metal-binding fluorescent dye Fura-2. Similarly, the analogous disease-mutant mimics in DraNramp significantly impaired Fe^{2+} transport as detected colorimetrically in DraNramp-expressing *Escherichia coli* (Figure 3E), and reduced Cd^{2+} (Figure 3F) and Mn^{2+} transport (Figure S3C) as detected with Fura-2 using purified DraNramp reconstituted into proteoliposomes. In both homologs, the TM1a G-to-R mutant expressed similarly to WT, while TM4 G-to-R mutant expression was reduced (Figures S3D and S3E), which could contribute to the loss-of-function phenotype in the in vivo assays. However, the DraNramp G153R mutation clearly impaired both Cd^{2+} and Mn^{2+} transport in proteoliposomes with normalized protein concentrations (Figure S3F). Notably, the G185R mutation in mouse Nramp2 similarly impaired Fe^{2+} transport (Su et al., 1998). These consistent loss-of-function data for the analogous mutants indicate that DraNramp is a useful model to further investigate how these disease-causing mutations inhibit transport.

The G153R Mutation Alters the Conformational Equilibrium and Metal Selectivity

G153 is far (~ 20 Å) from the metal-binding site, near the top of TM4 within the scaffold (Figure 4A). We tested the in vivo Co^{2+}

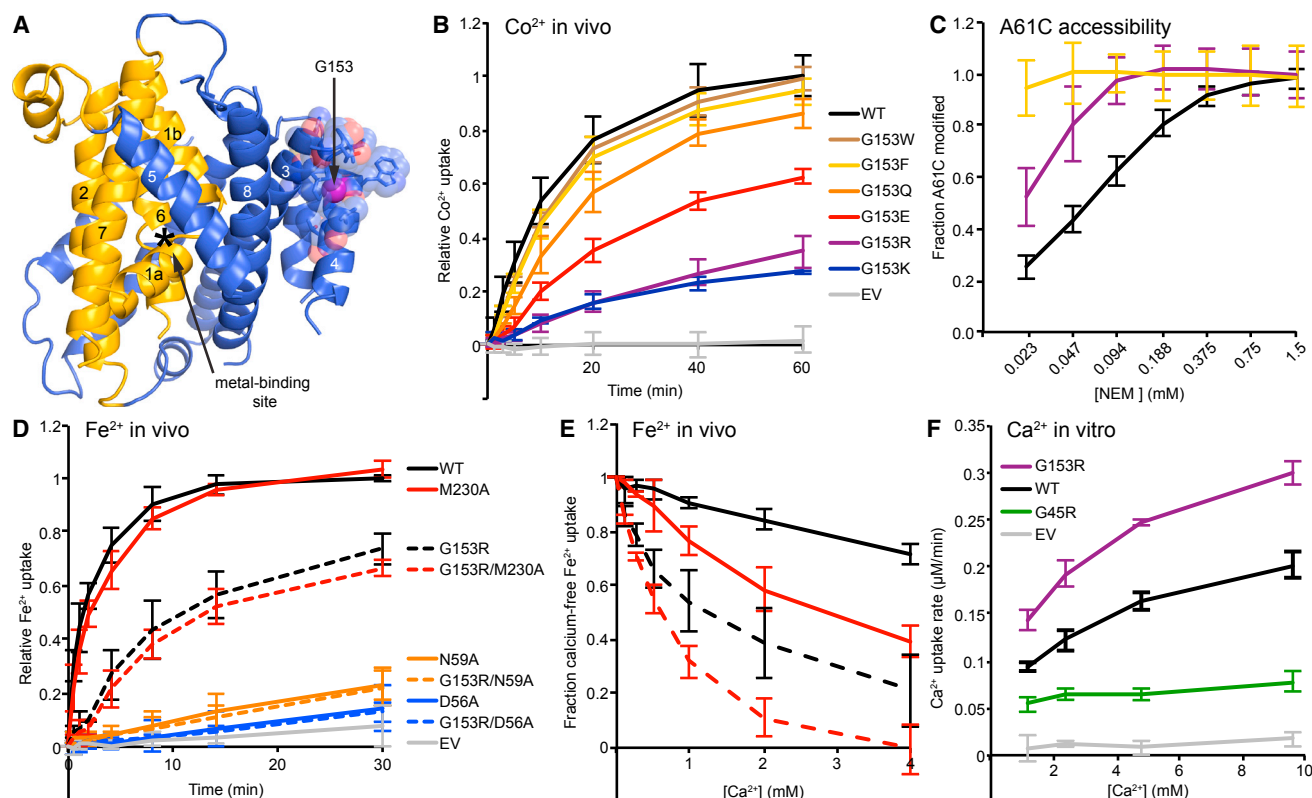


Figure 4. G153R Mutation Perturbs Outward-Facing State and Alters Metal Selectivity of the Binding Site to Favor Ca^{2+}

(A) In the inward-facing DraNrapm structure, G153 is located on the extracellular end of TM4 tightly packed in the back of the scaffold, far from the conserved metal-binding site. The G153 C α is a magenta sphere; nearby residues are shown as transparent spheres with side chains as sticks.

(B) Co^{2+} uptake data showing charged bulky substitutions at G153 reduced transport, while aromatic substitutions retained WT-level transport.

(C) G153R showed increased accessibility to NEM modification of the A61C outward-open conformational reporter, and G153F showed a further increase.

(D) In in vivo Fe^{2+} uptake, G153R showed little additional effect when combined with metal-binding site mutations, and its residual Fe^{2+} transport still uses the conserved metal-binding site as double mutations G153R/D56A and G153R/N59A both severely reduced transport.

(E) Competing Ca^{2+} reduced in vivo Fe^{2+} uptake more for G153R than WT, and double mutant G153R/M230A was even more susceptible to Ca^{2+} competition. The Ca^{2+} -free Fe^{2+} uptake level (2 min uptake for WT/M230A; 15 min uptake for G153 mutants) was set to 100% for each variant to facilitate direct % inhibition comparison.

(F) G153R transported more Ca^{2+} (as detected by Fura-2) than WT or the G45R mutant in an in vitro proteoliposome assay. All data are averages \pm SD ($n \geq 3$). See also Figure S4.

uptake activity of various substitutions at this position. G153R, and the similarly large and positively charged G153K, were most impaired (Figure 4B). In contrast, replacing G153 with large aromatics (G153F and G153W) did not significantly alter Co^{2+} uptake (although Fe^{2+} uptake by G153F was somewhat reduced; Figure S4B). A negatively charged glutamate (G153E) reduced uptake much more than a polar glutamine (G153Q), although less than G153R. Overall, charged side chains caused the largest reductions in transport activity.

In our inward-open structure, modeling a bulky substitution at position 153 leads to major steric clashes with nearby residues such as I142 on TM3, indicating that G153R or similarly large substitutions likely alters DraNrapm conformation at least locally. We therefore compared outward-open reporter A61C accessibility for this G153X panel (Figures 4C and S4C). All mutations resulted in increased NEM modification of A61C, with even more pronounced effects for G153F and G153W than for G153R. These data alone cannot distinguish between two sce-

narios: (1) increased occupancy of the outward-open state with concomitant decreased inward-open state occupancy; or (2) local structural changes that increase A61C accessibility in a protein that can still undergo conformational cycling. We further investigated this effect in Figure 6 (below).

Previous work showed that in mouse Nrapm2 the G185R mutation decreased Fe^{2+} transport, but surprisingly enabled Ca^{2+} transport (Xu et al., 2004). This could occur through either distortion of the conserved metal-binding site or opening of an alternative metal-permeation pathway. We first tested whether the residual Fe^{2+} transport by G153R DraNrapm relied on the canonical metal-binding site, introducing an alanine substitution for each binding-site side chain into the WT or G153R background. Either a D56A or N59A substitution abolished Fe^{2+} transport in both backgrounds, while M230A had negligible effects in both cases (Figure 4D), consistent with our previous results (Bozzi et al., 2016). Thus, G153R still relies on the conserved metal-binding residues for Fe^{2+} transport. We next tested whether

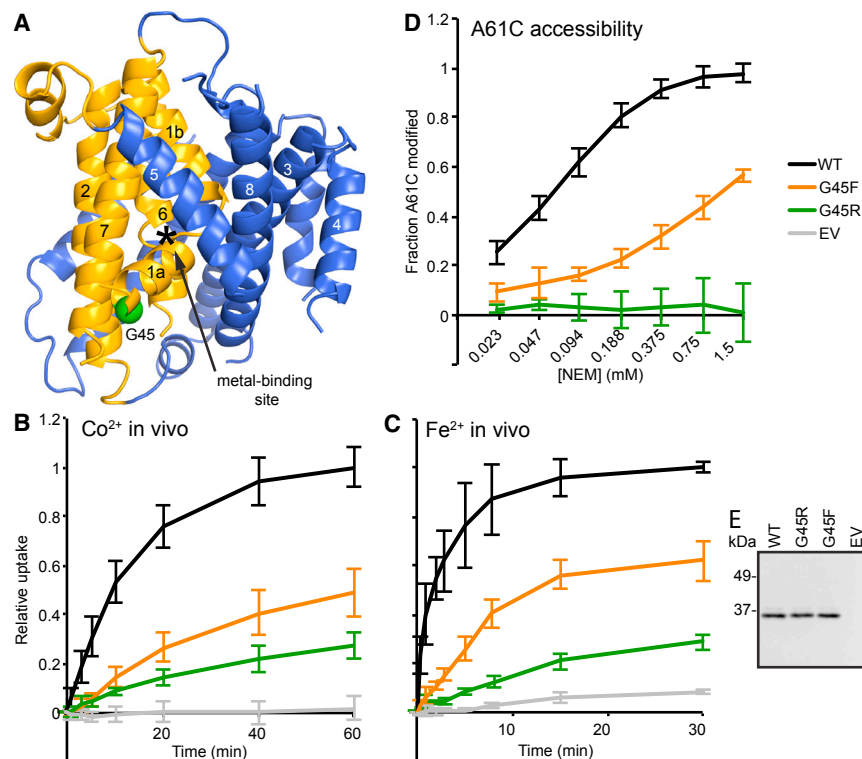


Figure 5. G45R Mutation Inhibits Transport by Locking Nrapm in an Outward-Closed Conformation

(A) G45 (green sphere) is on TM1a on the intracellular side of the bundle, 11 residues below the metal-binding D56. (B) G45R and G45F were significantly impaired in *in vivo* Co²⁺ transport. (C) G45F also impaired *in vivo* Fe²⁺ transport (WT, G45R, and EV data reproduced from Figure 3E for comparison). (D) G45R and G45F drastically decreased accessibility of outside-open conformational reporter A61C, indicating a strong preference for the inward-open state. (E) Western blot showing G45R and G45F expressed similarly to WT DraNrapm. All data are averages \pm SD ($n \geq 3$).

site in a manner that both improves Ca²⁺ transport and impairs transition metal transport, analogously to mouse Nrapm2.

The G45R Mutation Wedges the Transporter in an Inward-Facing Conformation

Like G153, the completely conserved G45 is ~ 20 Å away from the metal-binding

G153R perturbed the selectivity of this binding site by measuring Fe²⁺ transport in the presence of competing Ca²⁺ for WT, G153R, M230A, and G153R/M230A (Figure 4E), as well as G153F (Figure S4D). As expected, M230A, lacking the methionine “selectivity filter” that excludes alkaline earth metals (Bozzi et al., 2016), was more susceptible to Ca²⁺ competition than WT. Interestingly, G153R was even more susceptible to Ca²⁺, and the G153R/M230A double mutant was most susceptible, as 4 mM Ca²⁺ effectively abolished Fe²⁺ uptake. This suggests that G153R indeed perturbs the metal-binding site in favor of Ca²⁺ in DraNrapm in a manner that is complementary rather than redundant with removing the conserved methionine. We observed a similar pattern using a plate-based toxicity assay in which we assess the growth of *E. coli* expressing DraNrapm or its variants on various metal concentrations: G153R or M230A increased Ca²⁺ susceptibility, and G153R/M230A again had an additive effect (Figure S4F). This synergy was not observed for the similar alkaline earth metal Mg²⁺, as G153R/M230A had higher Mg²⁺ tolerance than M230A alone (Figure S4G), while G153R and WT grew similarly, suggesting that the G153R perturbation may be somewhat specific for Ca²⁺. G153R also had a reduced susceptibility to Cd²⁺ compared with WT (Figure S4H), but was more susceptible than M230A. To directly demonstrate the improved Ca²⁺ transport of the G153R mutant, we purified and reconstituted it into proteoliposomes, and observed decreased Cd²⁺ transport (Figure S4E) but increased Ca²⁺ transport compared with WT (Figure 4F). Of note, the other disease-mutant mimic, G45R, impaired both Ca²⁺ and Cd²⁺ transport (Figure 4F), thus the metal specificity perturbation is unique to G153R. In summary, the G153R mutation in our bacterial DraNrapm model system alters the conserved metal-binding

ing site at the N terminus of TM1a, which juts out at an angle to provide high cytoplasmic solvent access to the metal-binding residues (Figure 5A). Modeling an arginine at this position in our inward-facing structure yields no steric clash. We thus hypothesized that the mutation is not tolerated in the alternate outward-open state: a bulky residue at this position may act as a wedge forcing the protein to remain in the inward-open state, thus explaining the severe metal-transport impairment of G45R (Figure 3). Consistently, the bulky uncharged G45F substitution impaired *in vivo* uptake of Co²⁺ (Figure 5B) and Fe²⁺ (Figure 5C) nearly as much as G45R. Furthermore, single-cysteine reporter A61C was essentially fully protected in G45R, indicating that the outward-open state was rarely if ever sampled, while A61C accessibility in G45F suggested a shifted conformational preference, strongly favoring the inward-open state relative to WT (Figure 5D). Thus, decreased A61C accessibility of G45 substitutions correlated with decreased metal transport. This is similar to the patch mutant phenotypes (Figures 2C and 2D), especially patch 2, which just like G45 is located in the intracellular half of the bundle, indicating the importance of proper conformational cycling for protein function.

Mutations at Glycine Positions Illustrate the Conformational Change Process in DraNrapm

To further explore the DraNrapm conformational cycle, we investigated how G45R and G153F altered solvent accessibility for a panel of inward- or outward-accessible single-cysteine substitutions that retained significant transport activity (Figures S5A and S5B). Accessibility of outward reporters A61C and L140C was correlated; G45R greatly decreased while G153F greatly

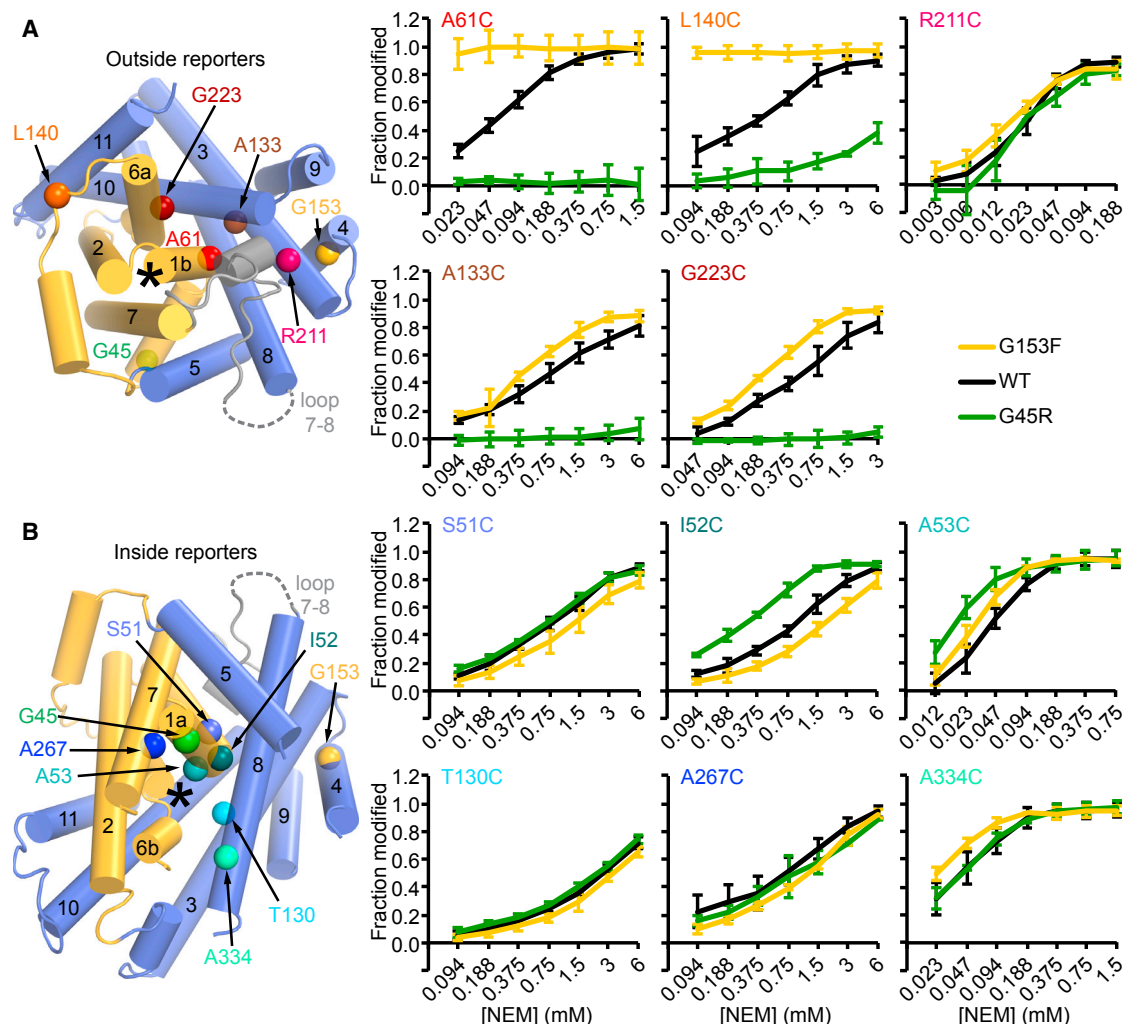


Figure 6. Mutations of Conserved Glycines Shift the Conformational Landscape of DraNrapm

(A) Cysteine modification as a function of NEM concentration for five extracellularly accessible reporters, mapped on a top view of the structure (left). A61C data are repeated from [Figures 3C and 4D](#) for comparison.

(B) NEM modification for five intracellularly accessible cysteine reporters, as well as T130C, which could not clearly be assigned as intracellularly or extracellularly accessible. Reporter positions are indicated on the structure, viewed down the cytoplasmic vestibule.

* indicates the metal-binding site. All data are averages \pm SD ($n \geq 4$). See also [Figure S5](#).

increased accessibility ([Figure 6A](#)). This suggests that G153F favors a conformational state in which part of the bundle-scaffold interface above the metal-binding site is highly exposed (perhaps by dislodging loop 7–8 that blocks solvent access to A61 and L140 in the inward-open structure). Closer to the metal-binding site within the bundle-scaffold interface, outward-accessible G223C and A133C were also fully protected in G45R, further supporting our inward-locked model for G45R ([Figure 6A](#)). However, at those cysteine positions G153F only had a modest effect, slightly increasing accessibility. Finally, neither G45R nor G153F altered accessibility of R211C on extracellular loop 5–6.

Accessibility was also unaltered at T130C, on TM3 adjacent to the unwound regions of TM1 and 6, indicating that the metal-binding site remains solvent-accessible in both G153F and G45R ([Figure 6B](#)).

To probe for water-excluding structural changes in the cytoplasmic vestibule, we compared NEM accessibility of five cysteines located below the metal-binding site in the WT, G45R, and G153F backgrounds ([Figure 6B](#)). Accessibility was the same in all backgrounds for S51C on TM1a, A267C on TM7, and A334C on TM8. For I52C and A53C on TM1a just below the metal-binding D56, accessibility was higher in G45R, suggesting that these positions form part of the cytoplasmic gate regulating access to the metal-binding site. We observed only minor perturbations at all inward-reporter positions for G153F. Thus, this mutation likely does not lock the protein in the outward-facing state with a closed intracellular gate, consistent with its high metal-transport activity ([Figures 4B and S4B](#)). In summary, the intracellular gate likely includes the TM1a C terminus, although a more complete picture awaits a truly outward-locked mutant. On the extracellular side, our crystal structure

and cysteine accessibility results clearly demonstrate a ~ 15 Å thick, solvent-excluding gate that remains firmly closed in the G45R mutant.

TM1a Movement Is Essential to the DraNrap Transport Cycle

To determine the functional importance of the DraNrap N terminus, we designed a truncation series that eliminated 25–49 residues (Figures S6A–S6C). Co^{2+} uptake activity was at WT levels up through the $\Delta\text{N}34$ construct, and slight reductions in transport accompanied each further truncation for $\Delta\text{N}37$, $\Delta\text{N}40$, and $\Delta\text{N}43$ (Figure S6). In contrast, $\Delta\text{N}46$ and $\Delta\text{N}49$, which both truncate beyond the TM1a N terminus at the invariant G45, showed pronounced drops in Co^{2+} uptake activity.

To further assess the importance of TM1a movement for metal transport, we compared Co^{2+} uptake for single-cysteine mutants, either unmodified or pre-modified with NEM (Figure 7A). For cysteine-less mutant C382S, WT (with the inaccessible C382), and extracellular loop control R211C, NEM treatment did not impair transport. However, for six of seven TM1a positions where the cysteine mutant retained transport activity, NEM modification greatly impaired transport. Thus, while those cysteines were highly accessible in at least one conformation, they must move into a more congested environment as part of the metal-transport cycle, which NEM modification sterically blocks. This phenomenon was not observed for positions preceding TM1a (residues 40–44, disordered in the structure). Cysteine substitution alone impaired transport at four positions: G45C, consistent with the importance of this conserved glycine and loss of function of G45R and G45F (Figures 3 and 5); D56C and G58C, which disrupt the metal-binding site; and A50C, a surprising result given the subtle size increase, which may reflect how close this face of TM1a must approach TM7 and/or the scaffold (TM8) as the inside gate closes in the outward-open state. Surprisingly, we did not observe NEM-dependent impairment for the two accessible TM1b positions A61C and E65C, the first of which clearly lines the outward metal-permeation pathway and is buried in the inward-open state. An A61W mutant similarly retained WT-level Co^{2+} transport, showing that DraNrap can indeed tolerate added bulk at this position (Figure S6D). However, pre-modifying A61C with the positively charged MTSET selectively inhibited Co^{2+} uptake (Figure S6G), thus some perturbation of the outward metal-permeation pathway at position A61 can indeed disrupt transport.

Finally, to further test the functional importance of TM1a movement, we substituted tryptophan (an alternative way of adding steric bulk) at each of the six TM1a positions where NEM modification of cysteine mutants drastically impaired transport. Analogously to G45R, these tryptophan mutations greatly impaired Co^{2+} transport and fully protected the A61C outward-open reporter (Figures S6D–S6F), indicating that bulky modifications on TM1a lock the protein in an inward-facing conformation. In summary, these results indicate that TM1a movement is an integral part of Nrapm conformational rearrangement and is thus required for metal transport. From all of our results, we propose a model of Nrapm conformational change in which significant inward movement of TM1a toward the scaffold is crucial to opening the outside interface between the bundle (TM1b and 6a) and

scaffold (TM3, 8, and 10) to allow metal ions (and bulk water) to access the binding site from the outside (Figure 7B).

DISCUSSION

Using X-ray crystallography, cysteine accessibility, and metal-transport measurements, we developed DraNrap as a model to understand the conformational change process for the Nrapm family of divalent metal transporters. The DraNrap structure, while also a LeuT fold in an inward-facing conformation, complements the ScaNrap structure (Ehrnstorfer et al., 2014) by identifying the location of the functionally important TM1a and (with cysteine-scanning mutagenesis) the external metal-permeation pathway. It also provides a structural example of a second distinct evolutionary clade within the Nrapm family. Furthermore, our structure showed the locations of two highly conserved glycines where mutations to arginine cause anemia in either rodents (Fleming et al., 1997, 1998) or humans (Blanco et al., 2009) and impair transport in both human Nrapm2 and DraNrap (Figure 3).

The G153R mutation on DraNrap TM4 mimics the phenotype first observed in mouse Nrapm2 (Xu et al., 2004), altering metal-transport selectivity, despite these two homologs having only 29% sequence identity. G153R perturbs the conserved metal-binding site, thus reducing transport of the typical transition metal substrates of Nrapm and increasing Ca^{2+} transport (Figure 4). A bulky residue cannot be accommodated at position 153 in our inward-facing structure. Our data are consistent with the G153R mutation distorting the scaffold in a manner that interferes with closing of the extracellular metal-permeation pathway (Figure 6). Precisely how a mutation 20 Å from the binding site increases Ca^{2+} permeability remains undetermined, but interestingly G153R and M230A, which remove the sulfur ligand responsible for excluding alkaline earth metals (Bozzi et al., 2016), have additive effects (Figures 4 and S4). Other residues besides the four previously identified may be important to metal binding and/or selectivity and, in at least one other Nrapm homolog, mutations distant from the metal-binding site affect substrate preference (Pottier et al., 2015).

G45R on TM1a forms a steric wedge that prevents the protein from reaching the outward-open state (Figure 5). Moreover, our results imply that the unfettered movement of TM1a is essential to the conformational rearrangement that must occur to allow metal transport (Figures 7 and S6).

Our study adds to a wealth of structural and functional information available for LeuT-fold secondary transporters (Penmatsa and Gouaux, 2014; Shi, 2013) that expanded on the original “rocking bundle” model of alternating access in which the bundle (TM1, 2, 6, and 7) moves relative to the scaffold (Forrest and Rudnick, 2009; Forrest et al., 2008). Comparison of outward- and inward-open LeuT structures (Krishnamurthy and Gouaux, 2012) showed TM1a swinging up by 45° and a smaller TM6b motion exposing the substrate-binding site to the cytosol. However, the physiological relevance of this large TM1a movement in LeuT is disputed: while some movement is essential to substrate release (Zhao et al., 2011), the Y268A mutation on TM6b in the crystallized construct disrupts a hydrogen-bonding network in the inward-facing state and stabilizes a conformation not highly sampled by the WT protein (Kazmier et al., 2014b),

EXPERIMENTAL PROCEDURES

Cloning of Nramp Constructs

The DraNramp sequence was inserted into the NdeI and NotI sites of pET21-N8H and human Nramp2 into pCDNA3 vectors as described previously (Bozzi et al., 2016). Mutagenesis was performed using QuikChange protocols (Stratagene), and confirmed by sequencing.

DraNramp Protein Purification

DraNramp C41(DE3) cells were grown at 37°C in 12 L of terrific broth with 10% (w/v) glycerol and 100 mg/L ampicillin, inoculating with 1:50 overnight culture to an optical density at 600 nm of 1.0, induced with 100 μ M isopropyl- β -D-thiogalactoside for 4 hr. Proteins were purified at 4°C. Cells were lysed by sonication in three volumes of lysis buffer (20 mM NaPO₄ [pH 7.0], 75 mM imidazole-HCl [pH 7.0], 500 mM NaCl, 10% glycerol) plus 1 mM PMSF, 1 mM benzamidine, 0.3 mg/mL each DNase I and lysozyme. Debris was removed by 20 min centrifugation at 27,000 \times g, membranes were pelleted in 70 min at 230,000 \times g, solubilized in 35 mL lysis buffer + 1% (w/v) β -dodecylmaltoside (DDM) for 1 hr, and clarified by 35 min centrifugation at 140,000 \times g, filtered through a 0.45 μ m filter, loaded onto 5 mL Ni-Sepharose (GE Healthcare) pre-equilibrated with lysis buffer + 0.03% β -DDM, and washed thrice with 50 mL lysis + 0.03% β -DDM. Protein was eluted in 25 mL increasing imidazole to 450 mM, and concentrated to \sim 1 mL in a 50 kDa cutoff centrifugal concentrator. Following buffer exchange on a Superdex S200 (GE Healthcare) to 20 mM HEPES (pH 7.5), 150 mM NaCl, and 0.1% (w/v) β -decylmaltoside (DM), protein-containing fractions were identified using SDS-PAGE, pooled, and concentrated to \sim 5 mg/mL.

Fab Production

BALB/c mice were immunized four times with DraNramp reconstituted into proteoliposomes using *E. coli* lipids (Avanti Polar Lipids). All procedures were approved by the Massachusetts Institute of Technology Committee on Animal Care. Hybridomas were generated and cultured using established protocols (Yokoyama, 2008), and secreted monoclonal antibodies (mAbs) were tested in ELISA by being captured on anti-mouse IgG-coated 96-well plates. Binding mAbs were identified using detergent-solubilized biotinylated DraNramp coupled to streptavidin-horseradish peroxidase. Cells in ELISA-positive wells underwent four rounds of recloning by limiting dilution to ensure monoclonality and stable mAb expression. Conformation-specific mAb clones were selected as non-binders in slot blot assays for binding to SDS-denatured DraNramp. The cocrystallized mAb was purified by capture from filtered hybridoma supernatant on protein A-agarose pre-equilibrated with binding buffer (BB; 20 mM NaPO₄ [pH 8.0], 150 mM NaCl). After a ten-column-volume BB wash, mAbs were eluted in seven column volumes of 100 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5) and 3.6 M MgCl₂, dialyzed against BB, and concentrated using a 50 kDa cutoff concentrator. Purified mAbs (15 mg at 1.5 mg/mL) in 20 mM NaPO₄, 20 mM EDTA (pH 6.0) were digested with 5 units of ficin suspension (Sigma-Aldrich) at room temperature for 5 hr. After adding 50 mg iodacetamide and 50 mg N-ethyl maleimide, the digest was concentrated and run on a Superdex S200 16/600 (GE Healthcare) in BB. Fab-containing fractions were passed through 5 mL protein A-agarose to remove any Fc fragment or uncleaved mAb, and concentrated to \sim 10 mg/mL. The Fab sequence was obtained using standard protocols (Verma, 2000).

Fab Crystallization and Structure Determination

Fab crystals were grown by sitting drop-vapor diffusion, mixing 0.5 μ L 10 mg/mL Fab with 0.5 μ L reservoir (0.1 M 3-(cyclohexylamino)-1-propanesulfonic acid [pH 9.7], 0.1 M MgCl₂, 30% polyethylene glycol [PEG] 3350), cryo-protected in the reservoir plus 25% glycerol, and frozen in liquid nitrogen. Diffraction data to 3.1 Å were collected at the Advanced Photon Source beamline ID24-E (Table 1). An Ig2b Fab structure (PDB: 1KNO) was used as a MR search model, and the Fab structure was refined to $R_{\text{work}}/R_{\text{free}}$ of 0.22/0.30 using REFMAC.

DraNramp-Fab Crystallization and Structure Determination

The DraNramp crystallization construct was truncated by 25 residues at the N terminus, and mutations were introduced in intracellular loops (OK169-170HH, EEK251-3YYY, and RR398-9HH). DraNramp was incubated for 2 hr with Fab in

a 1:1.2 molar ratio, and run on a Superdex S200 10/300 (GE Healthcare) in 10 mM HEPES (pH 7.5), 150 mM NaCl, and 0.1% DM. DraNramp-Fab-containing fractions were pooled and concentrated to \sim 5–10 mg/mL using a 50 or 100 kDa cutoff concentrator. DraNramp-Fab crystals were grown by vapor diffusion against 0.1 M sodium acetate (pH 4.5), 0.05 M magnesium acetate, 24% PEG 400, and 0.4%–1% β -octylglucoside in 1:1 volume ratio of sitting drops at 4°C. Crystals derivatized with 1 mM OsCl₃ for 1 day turned dark brown and were frozen in liquid nitrogen.

Diffraction data were collected from three crystals at the Advanced Photon Source ID24-E and -C beamlines, using vector scanning and full transmission, processed with HKL2000 (Otwinowski and Minor, 1997), and merged to improve completeness. For initial phasing, a ScaNramp-based homology DraNramp model built using SWISS MODEL (Biasini et al., 2014) and the Fab structure were used as MR search models in Phaser (McCoy et al., 2007). Experimental phase information from single-wavelength anomalous diffraction from the osmium-soaked crystals was determined using AutoSol in PHENIX (Adams et al., 2010). The resulting osmium sites were combined with the MR solution and data containing the experimental phase information were used for refinement. Coot (Emsley and Cowtan, 2004) was used for model building. Initial rounds of refinement used xMDFF, with input files prepared in VMD (Humphrey et al., 1996) using the MDFF-GUI (McGreevy et al., 2016) with default molecular dynamics parameters (McGreevy et al., 2014), improving $R_{\text{work}}/R_{\text{free}}$ from 0.50/0.54 to 0.41/0.45. The model was further refined using PHENIX with additional local refinements using xMDFF. TM1a was placed using a combination of xMDFF and steered molecular dynamics (SMD) simulations. As with the CiVSP voltage sensor protein (Li et al., 2014), a helix-screw motion was induced in SMD simulations using the orientation quaternion (Jiang et al., 2014) of the helix as a collective variable, while simultaneously using electron density restraints. The TM1a helix position featuring the lowest R value was identified, resulting in a 2% increase in the local cross-correlation. It is also consistent with cysteine accessibility data (Figure 6). After final rounds of refinement in PHENIX, the final model includes residues 43–165, 176–236, 259–304, 310–341, 353–428 for DraNramp, 1–129 + 132–213 and 1–213 for the Fab heavy and light chains, respectively, and three osmium ions. Figures were made in PyMOL (Schrödinger).

Cysteine Accessibility Measurements

Adapting the cysteine-labeling protocol from Tetsch et al., (2011), *E. coli* expressing single-cysteine DraNramp variants were exposed to thiol modifiers in 100 mM Tris (pH 7), 60 mM NaCl, 10 mM KCl, 0.5 mM MgCl₂, and 0.75 mM CaCl₂. All incubations were at 37°C. To determine overall accessibility, 3 mM NEM was applied for 60 min. To determine inside versus outside accessibility, 3 mM MTSEA or MTSET was applied for 30 min followed by 1.5 mM NEM for 30 min. For all NEM-gradient experiments, the indicated NEM concentration was applied for 15 min at room temperature. Excess cysteine was added to quench reactions. Cells were washed twice, resuspended and incubated 1 hr in 100 mM Tris (pH 7), 6 M urea, 0.5% SDS, and 0.5 mM DTT, incubated with a 2-fold excess of 5 kDa PEG maleimide (Creative PEGWorks), then quenched with sample buffer containing β -mercaptoethanol. Protein was detected in western blots using an Alexa 647-conjugated anti-His-tag antibody (QIAGEN) and a Typhoon Imager (GE Healthcare), and background-subtracted band intensities (I) were measured using ImageJ64. The upper (5-kDa PEG maleimide modified) to lower band (NEM modified) ratio was determined and compared with the no-NEM sample ratio (defined as maximal upper-to-lower band ratio = 0% NEM modified) to calculate the NEM-modified cysteine fraction using: modified-cysteine fraction = $1 - [I_{\text{upper}}/(I_{\text{lower}} + I_{\text{upper}})]/[I_{\text{upper-no-NEM}}/(I_{\text{lower-no-NEM}} + I_{\text{upper-no-NEM}})]$.

Metal Uptake Assays

Metal-transport experiments in proteoliposomes, *E. coli*, and HEK293T cells were performed as described previously (Bozzi et al., 2016). See the [Supplemental Information](#) for additional description.

ACCESSION NUMBERS

The accession number for the DraNramp crystal structure reported in this paper is PDB: 5KTE. The unprocessed diffraction images used to determine the Fab and DraNramp structures were deposited in the Structural Biology Data

Grid (<https://data.sbggrid.org/>) with accession numbers SBDG: 332, 333, 334, 335.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.str.2016.09.017>.

AUTHOR CONTRIBUTIONS

R.G. oversaw and designed the research with W.A.W., L.B.B., and A.T.B.; W.A.W. and E.R.G. generated mAbs, which H.L.P. supervised; W.A.W. obtained DraNrapm-Fab crystals and all diffraction data; L.B.B. purified DraNrapm mutants and determined the DraNrapm-Fab structure; L.B.B. and A.T.B. developed in vitro metal-transport assays; A.T.B. made most Nrapm mutants, performed all cysteine accessibility and metal-transport experiments, and analyzed the resulting data; A.S. performed the xMDFF and SMD simulations, which K.S. supervised; A.T.B., L.B.B., and R.G. wrote the manuscript, with input from all authors.

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REFERENCES

- Abbaspour, N., Hurrell, R., and Kelishadi, R. (2014). Review on iron and its importance for human health. *J. Res. Med. Sci.* **19**, 164–174.
- Adams, P.D., Afonine, P.V., Bunkoczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.W., Kapral, G.J., Grosse-Kunstleve, R.W., et al. (2010). PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 213–221.
- Aisen, P., Enns, C., and Wessling-Resnick, M. (2001). Chemistry and biology of eukaryotic iron metabolism. *Int. J. Biochem. Cell Biol.* **33**, 940–959.
- Andrews, N.C. (2008). Forging a field: the golden age of iron biology. *Blood* **112**, 219–230.
- Barrios, M., Moreno-Carralero, M.I., Cuadrado-Grande, N., Baro, M., Vivanco, J.L., and Moran-Jimenez, M.J. (2012). The homozygous mutation G75R in the human SLC11A2 gene leads to microcytic anaemia and iron overload. *Br. J. Haematol.* **157**, 514–516.
- Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Gallo Cassarino, T., Bertoni, M., Bordoli, L., et al. (2014). SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res.* **42**, W252–W258.
- Blanco, E., Kannengiesser, C., Grandchamp, B., Tasso, M., and Beaumont, C. (2009). Not all DMT1 mutations lead to iron overload. *Blood Cells Mol. Dis.* **43**, 199–201.
- Bozzi, A.T., Bane, L.B., Weihofen, W.A., McCabe, A.L., Singharoy, A., Chipot, C.J., Schulten, K., and Gaudet, R. (2016). Conserved methionine dictates substrate preference in Nrapm-family divalent metal transporters. *Proc. Natl. Acad. Sci. USA* **113**, 10310–10315.
- Canonne-Hergaux, F., Fleming, M.D., Levy, J.E., Gauthier, S., Ralph, T., Picard, V., Andrews, N.C., and Gros, P. (2000). The Nrapm2/DMT1 iron transporter is induced in the duodenum of microcytic anemia mk mice but is not properly targeted to the intestinal brush border. *Blood* **96**, 3964–3970.
- Canonne-Hergaux, F., Zhang, A.S., Ponka, P., and Gros, P. (2001). Characterization of the iron transporter DMT1 (NRAMP2/DCT1) in red blood cells of normal and anemic mk/mk mice. *Blood* **98**, 3823–3830.
- Cellier, M.F. (2012). Nrapm: from sequence to structure and mechanism of divalent metal import. *Curr. Top. Membr.* **69**, 249–293.
- Cellier, M.F., Bergevin, I., Boyer, E., and Richer, E. (2001). Polyphyletic origins of bacterial Nrapm transporters. *Trends Genet.* **17**, 365–370.
- Cellier, M.F., Courville, P., and Campion, C. (2007). Nrapm1 phagocyte intracellular metal withdrawal defense. *Microbes Infect.* **9**, 1662–1670.
- Claxton, D.P., Quick, M., Shi, L., de Carvalho, F.D., Weinstein, H., Javitch, J.A., and McHaourab, H.S. (2010). Ion/substrate-dependent conformational dynamics of a bacterial homolog of neurotransmitter:sodium symporters. *Nat. Struct. Mol. Biol.* **17**, 822–829.
- Courville, P., Chaloupka, R., and Cellier, M.F. (2006). Recent progress in structure-function analyses of Nrapm proton-dependent metal-ion transporters. *Biochem. Cell Biol.* **84**, 960–978.
- Ehrnstorfer, I.A., Geertsma, E.R., Pardon, E., Steyaert, J., and Dutzler, R. (2014). Crystal structure of a SLC11 (NRAMP) transporter reveals the basis for transition-metal ion transport. *Nat. Struct. Mol. Biol.* **21**, 990–996.
- Emsley, P., and Cowtan, K. (2004). Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2126–2132.
- Fleming, M.D., Trenor, C.C., 3rd, Su, M.A., Foernzler, D., Beier, D.R., Dietrich, W.F., and Andrews, N.C. (1997). Microcytic anaemia mice have a mutation in Nrapm2, a candidate iron transporter gene. *Nat. Genet.* **16**, 383–386.
- Fleming, M.D., Romano, M.A., Su, M.A., Garrick, L.M., Garrick, M.D., and Andrews, N.C. (1998). Nrapm2 is mutated in the anemic Belgrade (b) rat: evidence of a role for Nrapm2 in endosomal iron transport. *Proc. Natl. Acad. Sci. USA* **95**, 1148–1153.
- Forrest, L.R., and Rudnick, G. (2009). The rocking bundle: a mechanism for ion-coupled solute flux by symmetrical transporters. *Physiology (Bethesda)* **24**, 377–386.
- Forrest, L.R., Zhang, Y.W., Jacobs, M.T., Gesmonde, J., Xie, L., Honig, B.H., and Rudnick, G. (2008). Mechanism for alternating access in neurotransmitter transporters. *Proc. Natl. Acad. Sci. USA* **105**, 10338–10343.
- Ganz, T. (2009). Iron in innate immunity: starve the invaders. *Curr. Opin. Immunol.* **21**, 63–67.
- Grouleff, J., Sondergaard, S., Koldso, H., and Schiott, B. (2015). Properties of an inward-facing state of LeuT: conformational stability and substrate release. *Biophys. J.* **108**, 1390–1399.
- Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L., and Hediger, M.A. (1997). Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* **388**, 482–488.
- Gunshin, H., Allerson, C.R., Polycarpou-Schwarz, M., Rofts, A., Rogers, J.T., Kishi, F., Hentze, M.W., Rouault, T.A., Andrews, N.C., and Hediger, M.A. (2001). Iron-dependent regulation of the divalent metal ion transporter. *FEBS Lett.* **509**, 309–316.
- Gunshin, H., Fujiwara, Y., Custodio, A.O., Drenzo, C., Robine, S., and Andrews, N.C. (2005). Slc11a2 is required for intestinal iron absorption and erythropoiesis but dispensable in placenta and liver. *J. Clin. Invest.* **115**, 1258–1266.
- Hubert, N., and Hentze, M.W. (2002). Previously uncharacterized isoforms of divalent metal transporter (DMT)-1: implications for regulation and cellular function. *Proc. Natl. Acad. Sci. USA* **99**, 12345–12350.
- Humphrey, W., Dalke, A., and Schulten, K. (1996). VMD: visual molecular dynamics. *J. Mol. Graph.* **14**, 33–38, 27–38.

- Iolascon, A., and De Falco, L. (2009). Mutations in the gene encoding DMT1: clinical presentation and treatment. *Semin. Hematol.* **46**, 358–370.
- Jardetzky, O. (1966). Simple allosteric model for membrane pumps. *Nature* **211**, 969–970.
- Jiang, W., Phillips, J.C., Huang, L., Fajer, M., Meng, Y., Gumbart, J.C., Luo, Y., Schulten, K., and Roux, B. (2014). Generalized scalable multiple copy algorithms for molecular dynamics simulations in NAMD. *Comput. Phys. Commun.* **185**, 908–916.
- Kaback, H.R., Dunten, R., Frillingos, S., Venkatesan, P., Kwaw, I., Zhang, W., and Ermolova, N. (2007). Site-directed alkylation and the alternating access model for LacY. *Proc. Natl. Acad. Sci. USA* **104**, 491–494.
- Karlin, A., and Akabas, M.H. (1998). Substituted-cysteine accessibility method. *Methods Enzymol.* **293**, 123–145.
- Kazmier, K., Sharma, S., Islam, S.M., Roux, B., and McHaourab, H.S. (2014a). Conformational cycle and ion-coupling mechanism of the Na⁺/hydantoin transporter Mhp1. *Proc. Natl. Acad. Sci. USA* **111**, 14752–14757.
- Kazmier, K., Sharma, S., Quick, M., Islam, S.M., Roux, B., Weinstein, H., Javitch, J.A., and McHaourab, H.S. (2014b). Conformational dynamics of ligand-dependent alternating access in LeuT. *Nat. Struct. Mol. Biol.* **21**, 472–479.
- Krishnamurthy, H., and Gouaux, E. (2012). X-ray structures of LeuT in substrate-free outward-open and apo inward-open states. *Nature* **481**, 469–474.
- Li, Q., Wanderling, S., Paduch, M., Medovoy, D., Singharoy, A., McGreevy, R., Villalba-Galea, C.A., Hulse, R.E., Roux, B., Schulten, K., et al. (2014). Structural mechanism of voltage-dependent gating in an isolated voltage-sensing domain. *Nat. Struct. Mol. Biol.* **21**, 244–252.
- Lieu, P.T., Heiskala, M., Peterson, P.A., and Yang, Y. (2001). The roles of iron in health and disease. *Mol. Aspects Med.* **22**, 1–87.
- Mackenzie, B., and Hediger, M.A. (2004). SLC11 family of H⁺-coupled metal-ion transporters NRAMP1 and DMT1. *Pflugers Arch.* **447**, 571–579.
- Mackenzie, B., Takanaga, H., Hubert, N., Rolfs, A., and Hediger, M.A. (2007). Functional properties of multiple isoforms of human divalent metal-ion transporter 1 (DMT1). *Biochem. J.* **403**, 59–69.
- Malinauskaitė, L., Quick, M., Reinhard, L., Lyons, J.A., Yano, H., Javitch, J.A., and Nissen, P. (2014). A mechanism for intracellular release of Na⁺ by neurotransmitter/sodium symporters. *Nat. Struct. Mol. Biol.* **21**, 1006–1012.
- McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C., and Read, R.J. (2007). Phaser crystallographic software. *J. Appl. Crystallogr.* **40**, 658–674.
- McGreevy, R., Singharoy, A., Li, Q., Zhang, J., Xu, D., Perozo, E., and Schulten, K. (2014). xMDFF: molecular dynamics flexible fitting of low-resolution X-ray structures. *Acta Crystallogr. D Biol. Crystallogr.* **70**, 2344–2355.
- McGreevy, R., Teo, I., Singharoy, A., and Schulten, K. (2016). Advances in the molecular dynamics flexible fitting method for cryo-EM modeling. *Methods* **100**, 50–60.
- Otwinowski, Z., and Minor, W. (1997). Processing of X-Ray Diffraction Data Collected in Oscillation Mode. (Elsevier), pp. 307–326.
- Penmatsa, A., and Gouaux, E. (2014). How LeuT shapes our understanding of the mechanisms of sodium-coupled neurotransmitter transporters. *J. Physiol.* **592**, 863–869.
- Perez, C., Koshy, C., Yildiz, O., and Ziegler, C. (2012). Alternating-access mechanism in conformationally asymmetric trimers of the betaine transporter BetP. *Nature* **490**, 126–130.
- Pottier, M., Oomen, R., Picco, C., Giraudat, J., Scholz-Starke, J., Richaud, P., Carpaneto, A., and Thomine, S. (2015). Identification of mutations allowing natural resistance associated macrophage proteins (NRAMP) to discriminate against cadmium. *Plant J.* **83**, 625–637.
- Shawki, A., Knight, P.B., Maliken, B.D., Niespodzany, E.J., and Mackenzie, B. (2012). H⁺-coupled divalent metal-ion transporter-1: functional properties, physiological roles and therapeutics. *Curr. Top. Membr.* **70**, 169–214.
- Shi, Y. (2013). Common folds and transport mechanisms of secondary active transporters. *Annu. Rev. Biophys.* **42**, 51–72.
- Shimamura, T., Weyand, S., Beckstein, O., Rutherford, N.G., Hadden, J.M., Sharples, D., Sansom, M.S., Iwata, S., Henderson, P.J., and Cameron, A.D. (2010). Molecular basis of alternating access membrane transport by the sodium-hydantoin transporter Mhp1. *Science* **328**, 470–473.
- Singharoy, A., Venkatakrishnan, B., Liu, Y., Mayne, C.G., Lee, S., Chen, C.H., Zlotnick, A., Schulten, K., and Flood, A.H. (2015). Macromolecular crystallography for synthetic abiological molecules: combining xMDFF and PHENIX for structure determination of cyanostar macrocycles. *J. Am. Chem. Soc.* **137**, 8810–8818.
- Soe-Lin, S., Apte, S.S., Andriopoulos, B., Jr., Andrews, M.C., Schranzhofer, M., Kahawita, T., Garcia-Santos, D., and Ponka, P. (2009). Nramp1 promotes efficient macrophage recycling of iron following erythrophagocytosis in vivo. *Proc. Natl. Acad. Sci. USA* **106**, 5960–5965.
- Su, M.A., Trenor, C.C., Fleming, J.C., Fleming, M.D., and Andrews, N.C. (1998). The G185R mutation disrupts function of the iron transporter Nramp2. *Blood* **92**, 2157–2163.
- Tavoulari, S., Margheritis, E., Nagarajan, A., DeWitt, D.C., Zhang, Y.W., Rosado, J., Ravera, S., Rhoades, E., Forrest, L.R., and Rudnick, G. (2016). Two Na⁺ sites control conformational change in a neurotransmitter transporter homolog. *J. Biol. Chem.* **291**, 1456–1471.
- Tetsch, L., Koller, C., Donhofer, A., and Jung, K. (2011). Detection and function of an intramolecular disulfide bond in the pH-responsive CadC of *Escherichia coli*. *BMC Microbiol.* **11**, 74.
- Touret, N., Martin-Orozco, N., Paroutis, P., Furuya, W., Lam-Yuk-Tseung, S., Forbes, J., Gros, P., and Grinstein, S. (2004). Molecular and cellular mechanisms underlying iron transport deficiency in microcytic anemia. *Blood* **104**, 1526–1533.
- Verma, R. (2000). PCR of the v region. *Methods Mol. Med.* **40**, 453–459.
- Veuthey, T., and Wessling-Resnick, M. (2014). Pathophysiology of the Belgrade rat. *Front Pharmacol.* **5**, 82.
- Watanabe, A., Choe, S., Chaptal, V., Rosenberg, J.M., Wright, E.M., Grabe, M., and Abramson, J. (2010). The mechanism of sodium and substrate release from the binding pocket of vSGLT. *Nature* **468**, 988–991.
- Xu, H., Jin, J., DeFelice, L.J., Andrews, N.C., and Clapham, D.E. (2004). A spontaneous, recurrent mutation in divalent metal transporter-1 exposes a calcium entry pathway. *PLoS Biol.* **2**, E50.
- Yamashita, A., Singh, S.K., Kawate, T., Jin, Y., and Gouaux, E. (2005). Crystal structure of a bacterial homologue of Na⁺/Cl⁻-dependent neurotransmitter transporters. *Nature* **437**, 215–223.
- Yokoyama, W.M. (2008). Production of monoclonal antibody supernatant and ascites fluid. *Curr. Protoc. Mol. Biol.* *Chapter 11*, Unit 11.10.
- Zhao, Y., Terry, D.S., Shi, L., Quick, M., Weinstein, H., Blanchard, S.C., and Javitch, J.A. (2011). Substrate-modulated gating dynamics in a Na⁺-coupled neurotransmitter transporter homologue. *Nature* **474**, 109–113.