

1 **Structure of human ferroportin bound to hepcidin reveals mechanisms of iron**
2 **homeostasis**

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35

36 **Abstract**

37 The serum iron level in humans is tightly controlled by the action of the hormone hepcidin on the
38 iron efflux transporter ferroportin. Hepcidin negatively regulates iron absorption and recycling by
39 inducing ferroportin internalization and degradation. Aberrant ferroportin activity can lead to
40 diseases of iron overload, like hemochromatosis, or iron limitation anemias. Here, we
41 determined cryogenic electron microscopy (cryo-EM) structures of ferroportin in lipid nanodiscs,
42 both in the apo state and in complex with cobalt, an iron mimetic, and hepcidin. These
43 structures and accompanying molecular dynamics simulations identify two divalent metal
44 binding sites within the N- and C-domains of ferroportin. Hepcidin binds ferroportin in an
45 outward-open conformation and completely occludes the iron efflux pathway. The
46 carboxy-terminus of hepcidin directly contacts the divalent metal in the FPN C-domain. We
47 further show that hepcidin binding to ferroportin is coupled to iron binding, with an 80-fold
48 increase in hepcidin affinity in the presence of iron. These results suggest a new model for
49 hepcidin regulation of ferroportin, where only iron loaded ferroportin molecules are targeted for
50 degradation. More broadly, our structural and functional insights are likely to enable more
51 targeted manipulation of the hepcidin-ferroportin axis in disorders of iron homeostasis.

52 Introduction

53 Iron is essential for life. Complexed to heme, iron enables oxygen transport and cellular
54 respiration. As a cofactor for many proteins, iron coordinates redox chemistry by alternating
55 between ferrous (Fe^{2+}) and ferric (Fe^{3+}) oxidation states. Despite this central role in biology, free
56 ferrous iron is toxic. In excess, iron can catalyze the production of free radicals, leading to
57 cellular damage. Iron levels are therefore tightly controlled, both at the cellular and organism
58 level.

59

60 In mammals, iron levels are regulated by the action of hepcidin, a peptide hormone, on
61 ferroportin (FPN), the only known iron efflux transporter¹⁻³ (Fig. 1a). FPN mediates absorbance
62 of dietary iron by transport of ferrous iron across the basolateral surface of intestinal
63 enterocytes. FPN also mediates iron recycling from hepatocytes and macrophages⁴. Iron efflux
64 by FPN is controlled by the amount of transporter located at the cellular surface. FPN synthesis
65 is transcriptionally regulated by cellular hypoxia, iron and heme concentrations, and
66 inflammatory signaling⁵. In settings of elevated serum iron levels, liver-derived hepcidin levels
67 increase and this hepcidin negatively regulates cell surface FPN by acutely blocking iron
68 transport⁶ and inducing FPN ubiquitination, internalization, and degradation⁷⁻¹⁰. Hepcidin
69 activity decreases serum iron levels by suppressing FPN-mediated dietary iron absorption and
70 release of iron from cellular stores.

71

72 Iron disorders in humans can result from dysregulation of hepcidin or FPN, reflecting the central
73 role of the hepcidin-FPN axis in iron homeostasis. Deficits in hepcidin-mediated regulation of
74 FPN, often due to hereditary hemochromatoses, lead to iron overload and widespread tissue
75 damage affecting the liver, pancreas, and joints¹¹⁻¹³. By contrast, inappropriate elevation of
76 hepcidin levels yields iron-restricted anemia^{14,15}. Although several approaches to restore
77 aberrant FPN function have been evaluated in clinical trials¹⁶⁻¹⁸, none have thus far succeeded.

78

79 The molecular mechanism of FPN regulation by hepcidin remains incompletely defined at the
80 atomic level. A confluence of human genetics studies and structure-function evaluations have
81 identified key regions of FPN important in hepcidin regulation^{6,19-22}. A key recent advance was
82 determination of the X-ray crystal structure of a divalent metal transporter from the bacterium
83 *Bdellovibrio bacteriovorus* (bbFPN) with 40% similarity to human FPN^{23,24}, which revealed a
84 unique architecture among the broader major facilitator superfamily (MFS) of membrane

85 transporters. Although bbFPN is predicted to share structural features with human FPN, the
86 precise mechanisms of iron coordination likely differ and bbFPN is not regulated by hepcidin.
87

88 To understand how FPN transports iron, and how this process is regulated by hepcidin, we used
89 a combination of cryogenic electron-microscopy (cryo-EM), molecular dynamics simulations,
90 and *in vitro* biochemical assays. These studies reveal the molecular recognition of iron and
91 hepcidin by FPN and suggest a new regulatory mechanism enabling hepcidin to selectively
92 target actively transporting ferroportin molecules for degradation.

93

94 **Structures of apo- and hepcidin-bound human FPN**

95 We screened the antigen-binding fragments (Fabs) of antibodies previously raised against
96 FPN²⁵ for use as a fiducial mark to guide image alignment of a small membrane protein
97 embedded in a lipid nanodisc for structure determination by single particle cryo-EM, a strategy
98 we proposed many years ago²⁶. Among the many Fabs that bound purified FPN
99 (Supplementary Fig. 1), a single clone, Fab45D8, yielded interpretable class averages in
100 negative stain EM and was selected to facilitate cryo-EM structure determination. Unlike many
101 antibodies and antibody fragments targeting FPN, Fab45D8 was previously determined to be
102 non-competitive with hepcidin and, on its own, did not induce FPN internalization²⁵. Indeed, in
103 nanodisc-reconstituted preparations of FPN, Fab45D8 did not alter the binding properties of
104 hepcidin (Supplementary Fig. 2).

105

106 We determined cryo-EM structures of nanodisc-reconstituted FPN bound to Fab45D8, both in
107 the apo state (3.2 Å, Supplementary Fig. 3) and bound to hepcidin and Co²⁺ (2.5 Å,
108 Supplementary Fig. 4). We independently validated prior reports that FPN transports cobalt²⁷
109 (Supplementary Fig. 1). Unlike Fe²⁺, Co²⁺ is not readily oxidized and therefore provides a
110 tractable surrogate divalent metal for FPN biochemical and structural studies. The cryo-EM
111 density map of FPN enabled building of an atomic model of FPN regions important for iron
112 transport and hepcidin binding^{1,6}, and a portion of the intracellular loop 3 (ICL3) important in
113 hepcidin-induced FPN internalization⁸⁻¹⁰ (Supplementary Fig. 3 and 5a & Supplementary Table
114 1). The entire FPN extracellular loop 5 (ECL5) remains unresolved, likely due to significant
115 conformational flexibility. To enable modeling of Fab45D8, we separately obtained its X-ray
116 crystal structure at 2.1 Å (Supplementary Fig. 6 and Supplementary Table 2)

117

118 Both cryo-EM structures reveal a monomeric FPN bound to a single Fab45D8 molecule, which
119 recognizes a short alpha helical segment in FPN extracellular loop 2 (ECL2) (Fig. 1b and
120 Supplementary Fig. 6). Similar to other MFS transporters, FPN contains twelve transmembrane
121 (TM) helices arranged in two domains (Fig. 2a). Both the N-terminal and C-terminal domains are
122 composed of six helices, with a large central cavity that, in both apo- and hepcidin-bound
123 structures, is open to the extracellular side and closed intracellularly (Fig. 2b). Ferroportin
124 shares significant structural similarity with the bacterial bbFPN transporter, with an overall root
125 mean squared deviation (RMSD) of 2.0 Å when compared to the outward-open conformation of
126 bbFPN (Fig. 2c). The overall backbone conservation is even higher within the isolated
127 C-terminal domain (RMSD 1.4 Å). Unlike most other MFS transporters, the alpha helix of FPN
128 TM7 is interrupted by a short non-helical stretch of six residues. This unique feature, previously
129 posited to be important in iron binding²⁴, is shared between FPN and bbFPN.

130

131 Several interacting residues define an intracellular gate that keeps the N- and C- domains of
132 FPN in an outward open conformation. Similar to a previously observed interaction network in
133 bbFPN²³, R489 in TM11 of the C-domain forms an ionic interaction with D157 in TM4 of the
134 N-domain (Fig. 2d). This interaction is further supported by an extended ionic and
135 hydrogen-bonding network including residues E486 (TM11) and R88 (TM3). In FPN, an
136 additional cluster of ionic and hydrogen bonding interactions between TM5 in the N-domain and
137 TM10 in the C-domain further stabilizes the outward open conformation (Fig. 2e). Mutation of
138 several residues within the intracellular gate leads to FPN loss of function in ferroportin disease,
139 highlighting the importance of the gate in coordinating the conformational steps necessary for
140 iron efflux^{19,28}.

141

142 **Iron binds to the N and C domains of FPN**

143 Two distinct sites capable of binding divalent cations have previously been proposed for bbFPN.
144 Although initial crystallographic studies suggested that iron primarily binds in a cavity within the
145 N domain of the bbFPN transporter²³, further mutagenesis studies found a critical divalent cation
146 binding site within the C domain²⁴. Structural elucidation of the FPN iron binding site, however,
147 remains elusive. Previous studies on bbFPN either used supraphysiological concentrations of
148 iron or found Ni²⁺ bound as an EDTA complex.

149

150 We obtained cryo-EM data for a Co^{2+} -hepcidin-FPN complex in the presence of a 8-fold molar
151 excess of CoCl_2 to FPN ($100 \mu\text{M CoCl}_2$: $12.5 \mu\text{M FPN}$) to minimize artifacts arising from
152 supraphysiological metal concentrations. Comparison of this map with the apo-FPN map
153 revealed two new densities in the central cavity of FPN, corresponding to single metal binding
154 sites in the N and in the C domain respectively (Fig. 3a-c). Within the C domain, Co^{2+} directly
155 interacts with C326 in TM7b and H507 in TM11 while making a water-mediated contact with
156 D325 and the backbone carbonyl of T320 (Fig. 3b). Intriguingly, the tetrahedral coordination
157 geometry for Co^{2+} is fulfilled by the carboxy terminus of hepcidin. Within the N domain, we
158 observe density for Co^{2+} coordinated directly by TM1 residues D39 and H43 (Fig. 3c).

159

160 We captured Co^{2+} bound to FPN in the presence of hepcidin. Hepcidin binding likely influences
161 the structure and dynamics of FPN and it is therefore possible that the metal binding sites are
162 different in FPN in the absence of hepcidin. To better understand divalent metal binding to FPN
163 in the absence of hepcidin, we used all-atom molecular dynamics simulations. We first
164 performed six simulations of apo-FPN in a hydrated lipid bilayer with Fe^{2+} ions initially positioned
165 randomly in bulk solvent. In all six independent simulations, Fe^{2+} ions bound spontaneously to
166 the C domain, localizing to the unwound region of TM7 near residues D325, D504, and H507
167 within hundreds of nanoseconds of simulation time (Fig. 3d and Supplementary Fig. 7). The Fe^{2+}
168 ion also occasionally moves closer to TM1 to interact with D39, consistent with the additional
169 N-domain site observed in the hepcidin-bound structure. In parallel simulations of apo-FPN run
170 without Fe^{2+} ions, we observed mobility of TM7b, with significant fluctuations of D325 (Fig. 3e).
171 This observation is consistent with comparatively weaker cryo-EM density for TM7b as
172 compared to other transmembrane helices in apo-FPN (Supplementary Fig. 7). By contrast,
173 both D325 and TM7b are less mobile in simulations with iron bound at the C domain site (Fig.
174 3e). Divalent metal binding to the C domain may therefore stabilize an otherwise dynamic TM7b
175 in a conformation that favors hepcidin-binding.

176

177 The identification of two metal binding sites within FPN raises the question of whether both sites
178 are required for iron efflux. Several human FPN mutations that lead to hereditary
179 hemochromatosis map to residues that directly coordinate Co^{2+} in the C domain, including
180 C326S/F/Y^{13,29-31} and H507R³². Although these mutations likely disrupt the precise coordination
181 geometry required for metal binding, they are fully competent to transport iron even in the
182 presence of hepcidin; indeed, this lack of hepcidin-responsiveness leads to iron overload.

183 Mutation of D325 leads to decreased iron efflux²², which initially suggested a key site for iron
184 efflux in the C domain of FPN²⁴. However, recent modeling studies of an inward open
185 conformation of FPN based on bbFPN suggest that D325 may interact with the N domain; loss
186 of iron efflux in D325 mutants may therefore be caused by disruption of the extracellular gate³³.
187 By contrast, mutation of D39 to alanine in the N domain metal binding site of FPN completely
188 abolishes iron efflux from HEK293 cells²², suggesting that the N domain may be a primary site
189 for effluxed iron. Because the C domain metal-binding site is important for hepcidin binding, it
190 may primarily serve an important iron-dependent regulatory function in hepcidin control of FPN
191 activity.

192

193 **Hepcidin occludes outward open FPN**

194 Hepcidin binds FPN in a central cavity between the N and C domains, acting as a molecular
195 cork to completely occlude the iron efflux pathway (Fig. 1c and 4a). This structural observation
196 supports recent studies demonstrating acute inhibition of iron transport by hepcidin that is
197 independent of FPN ubiquitination and degradation⁶. Although the conformations of apo- and
198 Co²⁺-hepcidin-bound FPN are highly similar (RMSD 0.73 Å), the Co²⁺-hepcidin-bound structure
199 shows a ~3 Å rigid body separation of the N and C domains on the extracellular side of FPN
200 (Fig. 4b). Within the N domain, hepcidin binding leads to displacement of TM2 from the central
201 cavity, driven in part by a specific contact between I6 of hepcidin and T61 and Y64. Within the C
202 domain, the largest conformational changes occur around the Co²⁺ binding site, leading to
203 changes in the conformation of TM7b and the extracellular side of TM11 (Fig. 4b).

204

205 Hepcidin makes extensive polar and hydrophobic contacts with FPN with a total buried surface
206 area of ~1300 Å². Our structure of hepcidin bound to FPN provides insight into disease-causing
207 mutations associated with FPN gain of function in hereditary hemochromatoses. Several FPN
208 mutations decrease hepcidin binding to FPN, including N144H/D/T^{34–36}, C326S/F/Y^{13,29–31},
209 Y333H³⁷, Y501C^{6,38}, D504N^{6,39} and H507R³². Of these, C326 and H507 directly coordinate the
210 cobalt ion and mutations therefore indirectly affect hepcidin affinity or alter the atomic basis of
211 binding specificity. Other interactions between FPN and hepcidin are either hydrophobic or
212 depend on hepcidin amide backbone atoms, which is consistent with the relatively high
213 tolerance of amino acid substitutions within hepcidin⁴⁰. For example, Y333 fits into a
214 hydrophobic cavity in hepcidin and hydrogen bonds with the backbone carbonyl of hepcidin
215 residue M21 (Fig. 4c). D504 in FPN coordinates the backbone amide of hepcidin H3 while Y501

216 π -stacks with the imidazole side chain of H3 (Fig. 4c). A further hydrogen bond between N144
217 and Y501 further stabilizes this interaction network. In contrast to these mutations, the Y64N/H
218 mutants retain hepcidin binding⁶, but are completely resistant to hepcidin-induced FPN
219 ubiquitination^{41,42}. The outward displacement of TM2 near Y64 induced by hepcidin may
220 therefore be important for FPN ubiquitination (Fig. 4c).

221

222 The structure of hepcidin bound to FPN provides insight into prior efforts to engineer hepcidin
223 mimetics as potential therapeutics for diseases of iron overload^{20,40,43,44}. Although the first two
224 residues of hepcidin (DT) are dispensable for activity, residues 3-8 (HFPICI) are absolutely
225 required for function²⁰. Indeed, the first two residues of hepcidin make minimal interactions with
226 FPN (Fig. 4c). By contrast, alanine scanning mutagenesis suggested an important role for
227 hydrophobic hepcidin residues including F4 and F9, and to a lesser extent H3 and I6^{40,43,44}.
228 Residues F4 and F9 insert between the N and C domains of FPN at opposite ends of the central
229 cavity (Fig. 4c); hydrophobic residues at these positions likely stabilize the FPN outward open
230 state. More unexpected is the extensive set of contacts between hepcidin residues 10-25 and
231 TM7b of FPN. Prior studies have recapitulated hepcidin activity with a linear peptide composed
232 of hepcidin residues 1-9, though with a ~8-20 fold reduction in potency^{40,45}. Whether these
233 minihepcidins fully plug the iron efflux pathway remains unclear. Furthermore, in the absence of
234 structural data, it remains unclear whether minihepcidin variants coordinate divalent metals in
235 the same manner as observed for hepcidin in our structure.

236

237 In addition to blocking the iron transport pathway, hepcidin regulates FPN by causing
238 ubiquitination of lysine residues in intracellular loop 3 (ICL3)^{9,10}. Among these, K240 is critical for
239 hepcidin-induced FPN internalization and degradation. Neither the apo- or Co²⁺-hepcidin-bound
240 structures resolve residues 239-288 of ICL3, precluding a structural understanding of how
241 hepcidin regulates the conformation of K240. In the resolved regions of ICL3, we observe no
242 significant conformational changes between apo- and Co²⁺-hepcidin-bound FPN (Fig. 4d,e).
243 Several caveats may limit our structural analysis of hepcidin-induced conformational changes in
244 FPN, including the lack of a membrane voltage or proton gradient across the lipid nanodisc, the
245 requirement for specific lipids for FPN function, or other cofactors important in hepcidin-induced
246 FPN ubiquitination. However, the structures provide clues into the role of ICL3 in FPN function.
247 The N terminal portion of ICL3 (residues 230-238) forms interactions with the N domain (Fig.
248 4d). Notably, K236 makes an ionic interaction with the intracellular gate residue D157. The C

249 terminal portion of ICL3 (residues 291-304) forms an amphipathic helix that makes a number of
250 contacts with both TM2 in the N domain and TM11 in the C domain, which both undergo
251 conformational changes upon binding hepcidin (Fig. 4e). In both cases, the resolved regions of
252 ICL3 are primed to sense the conformation of the transporter as it shuttles iron and binds
253 hepcidin. These regions may therefore serve as important conduits linking the conformation of
254 hepcidin binding on the extracellular side to K240 conformation on the intracellular side.

255

256 **Hepcidin is coupled to iron binding**

257 The direct interaction between hepcidin and Co^{2+} in our structure is unexpected (Fig. 5a), and
258 suggests that divalent metals may be important for hepcidin binding to FPN. We therefore
259 directly tested the effect of Fe^{2+} and Co^{2+} on hepcidin affinity at FPN. A fluorescently tagged
260 version of hepcidin (Rhodamine green-hepcidin²⁵, RhoG-Hep) bound to nanodisc-reconstituted
261 ferroportin with an apparent K_D of 210 nM ($\text{p}K_D = -6.67 \pm 0.02$) (Fig. 5b). In the presence of 10
262 μM FeCl_2 , we observed a significantly increased affinity of 2.5 nM ($\text{p}K_D = -8.61 \pm 0.21$), an
263 almost 80-fold change in the potency of hepcidin at FPN. Addition of CoCl_2 also increased
264 hepcidin affinity. Consistent with a cooperative effect, the effect of CoCl_2 on hepcidin binding
265 was saturable (Fig. 5c).

266

267 The reference range for hepcidin concentration in healthy adults is $\sim 1\text{-}30 \text{ nM}^{46}$. Our *in vitro*
268 binding experiment with purified FPN indicates minimal hepcidin binding to FPN in the absence
269 of a divalent metal, suggesting that metal binding to FPN may regulate hepcidin activity *in vivo*.
270 Our structure of FPN bound to Co^{2+} and hepcidin revealed conformational changes in TM7b
271 associated with hepcidin binding (Fig. 3e) and a direct contact between hepcidin and Co^{2+} (Fig.
272 3b); both could be important for the observed effect of Fe^{2+} and Co^{2+} on hepcidin binding. We
273 therefore tested whether disruption of the C domain iron binding site influences hepcidin
274 binding. Even in the presence of 50 μM CoCl_2 , the D325N, C326S, and H507R mutants bound
275 hepcidin weakly, titrating in a micromolar range similar to wild-type FPN in the absence of
276 divalent metals (Fig. 5c). These results highlight the critical role of the C domain metal site in
277 potent hepcidin binding to FPN, which is likely important in homeostatic control of iron levels in a
278 physiological setting.

279

280 **Discussion**

281 Ferroportin is a central regulator of iron homeostasis in humans. Both human FPN and the
282 bacterial homologue bbFPN show remarkable similarities in their overall architecture, with a
283 unique conformation of TM7 responsible for molecular recognition of iron within the C domain.
284 This similarity suggests a deep evolutionary history for FPN-like transporters within the broader
285 major facilitator superfamily. Heparin, by contrast, is specific to vertebrates and likely evolved
286 as a new strategy to regulate a critical point in iron absorption. The structures presented here
287 map, at high resolution, metal and hepcidin binding to FPN.

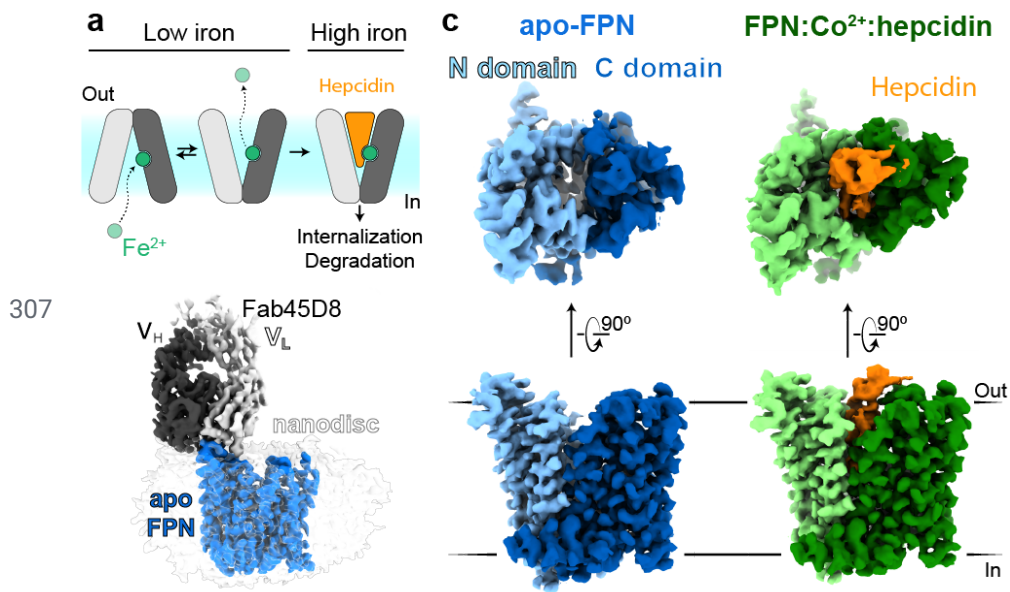
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289 We determined that hepcidin binding to FPN is greatly potentiated by iron itself, potentially due
290 to the stabilizing effect iron has on the hepcidin-binding site of FPN (Fig. 5d). With iron, the
291 binding affinity of hepcidin falls in a range concordant with the concentration of hepcidin
292 observed in healthy human adults. In normal iron homeostasis, this may enable hepcidin to
293 selectively bind and regulate FPN molecules actively transporting iron and loaded with Fe^{2+} ,
294 while sparing FPN molecules located on cells with low transport activity. Heparin binding to
295 FPN would both trap the transporter in an outward open state and limit iron egress; both actions
296 acutely decrease iron efflux, as has been recently reported⁶. Elevated hepcidin levels likely
297 inappropriately overcome this regulatory strategy and degrade FPN even in the absence of
298 active iron efflux. The potentiation of hepcidin activity by iron may therefore have immediate
299 consequences for the development of hepcidin mimetics currently in clinical trials¹⁶.
300 Furthermore, hepcidin antagonism by direct targeting of FPN may require molecules with high
301 potency to overcome the nanomolar effect of the hormone in the presence of iron. The structural
302 and functional insights into FPN function presented here therefore provide critical foundations
303 for the discovery of therapeutics for human disorders of iron homeostasis.

304

305

306 MAIN TEXT FIGURES



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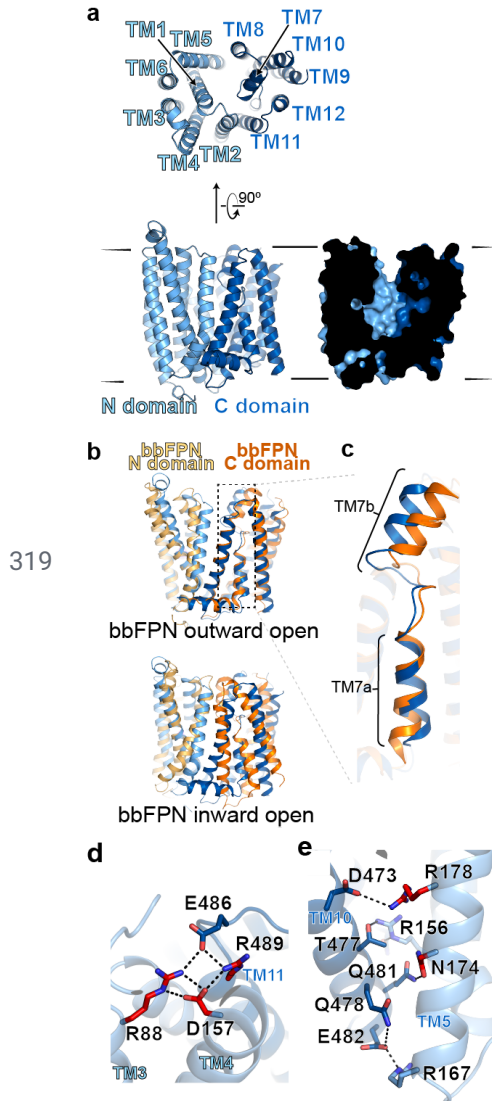
309 **Figure 1. Structures of human ferroportin.** **a**, Ferroportin effluxes cellular iron (Fe²⁺) by an
310 alternating access mechanism. Hepcidin binds to outward open ferroportin and induces
311 ubiquitination and degradation. **b**, Cryo-EM map of apo-FPN-Fab45D8 complex in lipid
312 nanodisc. **c**, Cryo-EM density of apo and Co²⁺/hepcidin bound FPN. The N and C domains are
313 colored in different shades of blue for apo-FPN and green for Co²⁺/hepcidin bound FPN.
314 Hepcidin (orange) binds to an extracellular facing cavity in FPN.

315

316

317

318



320 **Figure 2. Structure of apo-FPN and similarity to bbFPN.** **a**, Ribbon diagram of FPN reveals
321 12 transmembrane helices. The N- and C-domains are colored in different shades of blue.
322 Cutaway surface view (right) shows outward open conformation. **b**, Human FPN aligned to the
323 outward-open (PDB: 5AYN) and inward-open (PDB: 5AYO) conformations of bbFPN. **c**, Unique
324 architecture of TM7 shared between human FPN and bbFPN. **d**, Intracellular gating residues
325 are shown as sticks. Residues in red are known FPN loss-of-function mutations **e**, TM10 and
326 TM5 form an extensive network of interactions, further stabilizing the outward open
327 conformation. Residues highlighted in red are known loss-of-function mutations that lead to
328 ferroportin disease in humans.

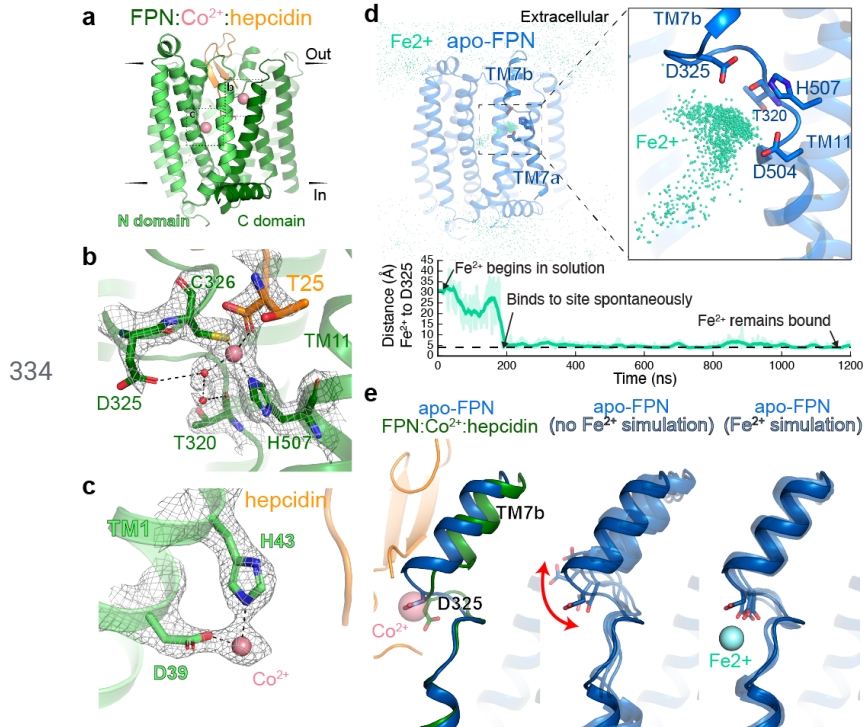
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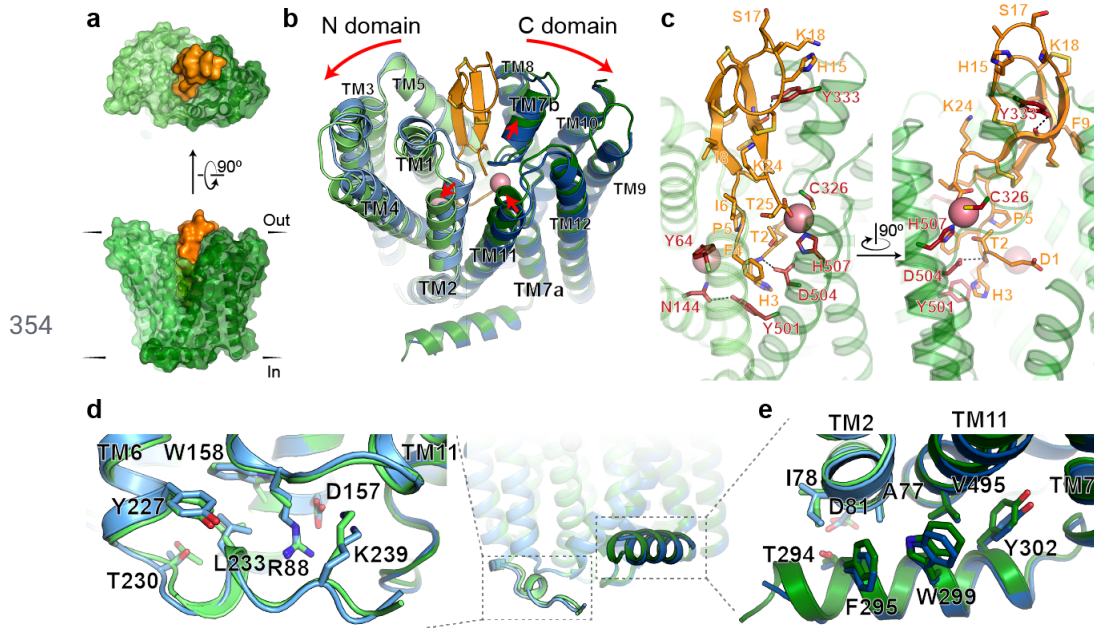
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336 **Figure 3. Iron binds to the N and C domains of FPN.** **a**, Ribbon diagram of
337 FPN-Co²⁺-hepcidin complex. Heparin binds an extracellular-facing pocket in FPN. **b**, Closeup
338 view of cryo-EM density for Co²⁺ ion in the FPN C domain. Co²⁺ binds with tetrahedral
339 coordination to C326, H507, a water molecule, and the hepcidin C-terminus. **c**, Closeup view of
340 cryo-EM density for Co²⁺ ion in the FPN N domain, coordinated by H43 and D39. **d**, In molecular
341 dynamics simulations with Fe²⁺ initially positioned randomly in bulk water surrounding FPN, the
342 Fe²⁺ ions spontaneously bind to a region near H507, D325, and D504. The aggregated position
343 of Fe²⁺ ions from six simulations, each 2 μ s in length, is shown superimposed with apo FPN. **e**,
344 In one representative simulation, an Fe²⁺ ion spontaneously binds within 200 ns and remains
345 localized at this site for more than 1000 ns. Distance shown is from the ion to the nearest
346 oxygen atom of the D325 side chain. Thick trace represents a 15-ns sliding mean and thin
347 traces represent unsmoothed values. **e**, Comparison of TM7b conformation in apo-FPN and
348 FPN bound to Co²⁺ and hepcidin. In simulations without Fe²⁺, TM7b is dynamic, with significant
349 fluctuation of D325. D325 coordinates Fe²⁺ in simulations, and is associated with decreased
350 TM7b motion.

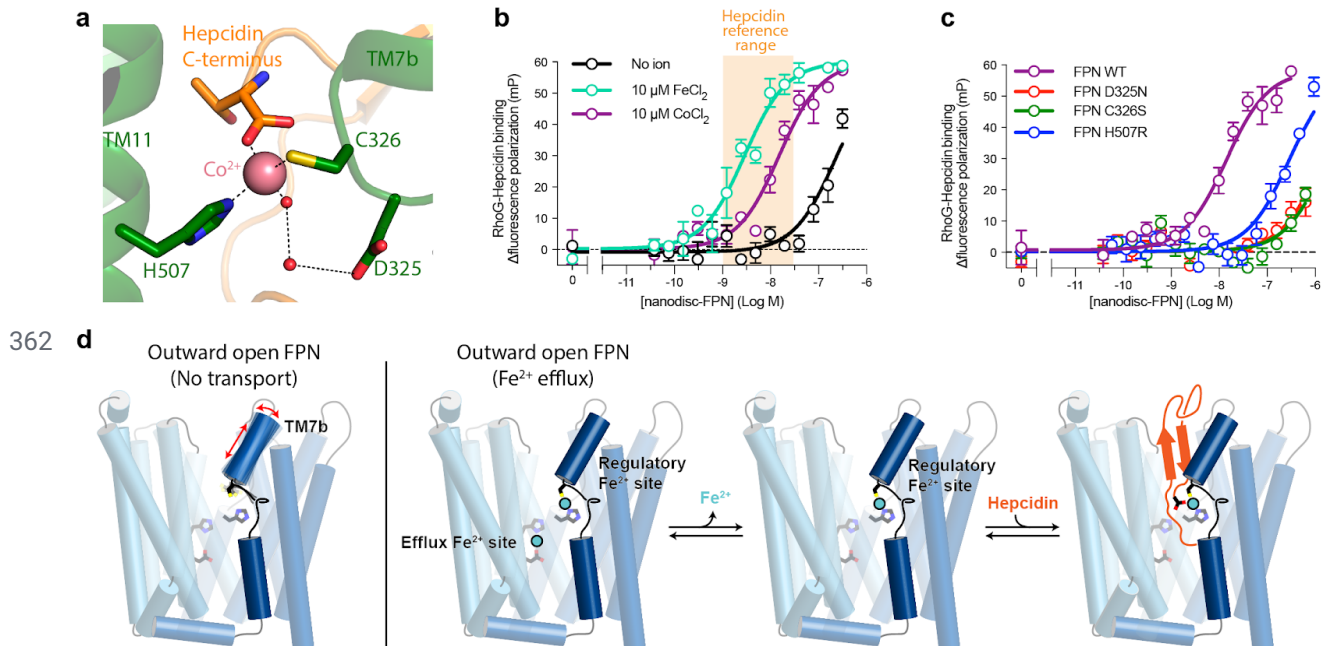
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355 **Figure 4. Hepcidin binding to FPN.** **a**, Surface representation of the FPN-Co²⁺-hepcidin
356 complex viewed from the extracellular side and perpendicular to the membrane plane. **b**,
357 Ribbon diagrams of apo-FPN (blue) aligned to FPN-Co²⁺-hepcidin (green, orange, and pink
358 spheres) showing the overall separation of the N and C domains, and the displacement of TMs
359 containing key residues for hepcidin binding. **c**, Close-up views of the hepcidin binding site.
360 Residues in red are known hepcidin resistance mutations involved in ferroportin disease. **d,e**
361 Close-up of interactions between the transmembrane regions and intracellular loop 3.



363 **Figure 5. Hepcidin binding is potentiated by iron.** **a**, FPN C domain metal binding site.
364 Shown are residues that interact with Co^{2+} , including D325, C326, and H507 in FPN as well as
365 the hepcidin C-terminus. **b**, Fluorescence polarization increase in rhodamine green-labeled
366 hepcidin (RhoG-hepcidin) as nanodisc-reconstituted FPN is titrated with a K_D of 210 nM.
367 Addition of 10 μM FeCl_2 or CoCl_2 increases the affinity of hepcidin to 2.5 nM and 7.7 nM ($\text{pK}_D =$
368 -8.11 ± 0.16), respectively. Hepcidin concentration range in healthy human adults is shown in
369 orange. **c**, Mutation of the C domain metal binding sites decreases RhoG-hepcidin binding
370 affinity at FPN, even in the presence of 50 μM CoCl_2 . All values are reported as mean \pm s.e.m.
371 Error bars represent s.e.m. **d**, Model for iron-coupled hepcidin regulation of FPN function. In
372 settings of iron efflux, TM7b is conformationally stabilized by iron coordination in the C domain
373 regulatory site. High affinity hepcidin binding to outward open FPN depends on the direct
374 coordination of iron in the C domain.

375
376
377

378 **METHODS**

379 No statistical methods were used to predetermine sample size. The experiments were not
380 randomized and the investigators were not blinded to allocation during experiments and
381 outcome assessment.

382

383 **Expression and purification of human ferroportin**

384 The wild-type human FPN gene was cloned into a pVL1392 vector containing an expression
385 cassette comprised of a C terminal human rhinovirus 3C (HRV-3C) protease recognition
386 sequence followed by a human protein C epitope tag (EDQVDPRLIDGK) and an 8x
387 polyhistidine tag. Baculovirus was generated using *Spodoptera frugiperda* Sf9 insect cells
388 (unauthenticated and untested for mycoplasma contamination, Expression Systems 94-001F)
389 and the construct was expressed in *Spodoptera frugiperda* Sf9 insect cells. Cells were collected
390 48 h after transduction and stored at -80°C until further use. Frozen cell pellets were thawed
391 and washed with a hypotonic buffer (20 mM HEPES pH 7.50, 1 mM EDTA, supplemented with
392 20 $\mu\text{g}/\text{mL}$ leupeptin, and 160 $\mu\text{g}/\text{mL}$ benzamidine) before solubilizing with 50 mM HEPES pH
393 7.5, 300 mM NaCl, 1% (w/v) n-dodecyl- β -D-maltopyranoside (DDM, Anatrace), 0.1% (w/v)
394 cholesteryl hemisuccinate (CHS, Steraloids), 1 mM EDTA supplemented with 20 $\mu\text{g}/\text{mL}$
395 leupeptin, and 160 $\mu\text{g}/\text{mL}$ benzamidine for 1 h at 4°C . Following centrifugation, the resulting
396 supernatant was loaded on homemade anti-protein C antibody Sepharose beads and washed
397 extensively in 50 mM HEPES pH 7.50, 300 mM NaCl, 2 mM CaCl_2 , 0.1% (w/v) DDM, 0.01%
398 (w/v) CHS. FPN was eluted with 50 mM HEPES pH 7.50, 300 mM NaCl, 0.1% (w/v) DDM,
399 0.01% (w/v) CHS, 0.2 mg/mL Protein C peptide (Genscript) and 5 mM EDTA. The protein was
400 concentrated with a Vivaspin 100-kDa MWCO concentrator and the monomeric FPN fraction
401 was collected after size-exclusion chromatography (SEC) over a Superdex S200 Increase
402 10/300 GL column (GE Healthcare) equilibrated with 20 mM HEPES pH 7.50, 100 mM NaCl and
403 0.1% (w/v) DDM, and 0.01% (w/v) CHS.

404

405 **Expression and purification of MSP**

406 Constructs encoding MSP-NW9 or MSP-NW11⁴⁷ in the pET28b vector (Addgene #133442) were
407 transformed into BL21(DE3) Rosetta *Escherichia coli*, and grown in terrific broth medium
408 supplemented with 2 mM MgCl_2 and 0.1% (w/v) glucose at 37°C . At OD_{600} of ~ 0.6 , expression
409 was induced by addition of 400 μM isopropyl β -d-1-thiogalactopyranoside (IPTG) and lowering
410 the temperature to 20°C . Cells were harvested after 16 hours and resuspended into 5 mL lysis

411 buffer (200 mM Tris pH 8.0, 500 mM NaCl, 1% (v/v) Triton X-100 (Sigma), 0.02 mg/mL
412 leupeptin, 0.16 mg/mL benzamidine, and benzonase) per gram pellet. After stirring for 30 min at
413 4°C, cells were lysed by pulsed sonication on ice. The lysate was cleared by centrifugation at
414 15,000 x g for 25 min at 4°C and loaded on Ni-NTA Sepharose. Ni-NTA beads were washed
415 with 50 mM Tris pH 8.0, 500 mM NaCl, 1% (v/v) Triton, then 50 mM Tris pH 8.0, 500 mM NaCl,
416 50 mM sodium cholate, then 50 mM Tris pH 8.0, 500 mM NaCl, and finally with 50 mM Tris pH
417 8.0, 500 mM NaCl, 30 mM Imidazole. MSP was eluted with 50 mM Tris-HCl pH 8.0, 500 mM
418 NaCl, 400 mM Imidazole and dialyzed into 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM
419 EDTA, 0.1 mM TCEP at 4°C. The following day, MSP was concentrated on a Vivaspin 10-kDa
420 MWCO concentrator, aliquots were flash frozen in liquid nitrogen and stored at -80°C for
421 reconstitution.

422

423 **Isolation, expression and purification of Fab45D8**

424 The heavy and light chain sequences of mAb45D8²⁵ were separately cloned into pcDNA3.4 and
425 the resulting vectors were transfected into Expi293F Human Embryonic Kidney cells (Life
426 Technologies) using a 1:2 mass ratio of light and heavy chain DNA with the Expifectamine
427 transfection kit (Life Technologies) as per the manufacturer's instructions. Supernatant
428 containing mAb45 was harvested 136 h after transfection and loaded on homemade Protein G
429 Sepharose beads and extensively washed with a buffer comprising 20 mM HEPES pH 7.50,
430 and 100 mM NaCl. mAb45D8 was eluted with 100 mM glycine (pH 3.0) and fractions were
431 immediately neutralized with 200 mM HEPES pH 7.50. To generate the Fab fragment, 10 mg of
432 purified mAb45D8.1 was diluted into 9.5 ml freshly prepared cleavage buffer (20 mM sodium
433 phosphate pH 7.00, 10 mM EDTA, and 10 mM cysteine) and treated with 0.5 ml agarose
434 immobilized papain (Thermo Scientific) at 37°C. After 16 h the cleaved Fab45D8 fragment was
435 purified by reverse Protein A affinity chromatography, followed by SEC into buffer comprising 20
436 mM HEPES pH 7.50 and 100 mM NaCl. Fab45D8 was concentrated on a Vivaspin 10-kDa
437 MWCO concentrator, and aliquots were flash frozen in liquid nitrogen and stored at -80°C for
438 later use.

439

440 **Reconstitution of FPN into lipidic nanodisc**

441 Purified FPN (0.2-0.5 mg) was mixed with purified MSP and a lipid mixture containing a 2:3
442 weight ratio of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC, Avanti) and
443 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG, Avanti). For reconstitution

444 into NW9 nanodiscs, an FPN:MSP:Lipid molar ratio of 1:20:1100 was used. For reconstitution
445 into NW11 nanodiscs, an FPN:MSP:Lipid molar ratio of 1:20:800 was used. The reconstitution
446 sample was nutated for 1 h at 4°C before addition of 0.2 g/mL SM2-BioBeads (BioRad), and the
447 reconstitution sample was further nutated overnight at 4°C before removal of the biobeads. FPN
448 containing nanodiscs were purified by loading the reconstitution sample on anti-protein C
449 antibody Sepharose beads and washing extensively with 20 mM HEPES pH 7.50, 100 mM
450 NaCl, and 1 mM CaCl₂ to remove empty nanodiscs. FPN containing nanodiscs were eluted with
451 20 mM HEPES pH 7.50, 100 mM NaCl, 0.25 mM EDTA, and 0.2 mg/mL Protein C peptide
452 (Genscript), and concentrated on a Vivaspin 50-kDa concentrator.

453

454 **Crystallization and structure determination of Fab45D8**

455 Purified Fab45D8 was diluted to 13.0 mg/mL in 20 mM HEPES pH 7.5, 100 mM NaCl. Fab45D8
456 crystals were obtained in 0.3 M trimethylamine-N-oxide (TMAO), 0.1 M Tris pH 8.5, and 30%
457 (w/v) PEG 2000 MME at 20 °C. Individual crystals were flash frozen in liquid nitrogen after a 30
458 s soak in 0.3 M trimethylamine-N-oxide (TMAO), 0.1 M Tris pH 8.5, and 30% (w/v) PEG 2000,
459 and 20% v/v ethylene glycol. A full diffraction dataset was collected at the Advanced Photon
460 Source GM/CA-CAT beamline 23ID-B, and processed using xia2dials⁴⁸ implementation of
461 XDS⁴⁹. The structure of the Fab was solved by molecular replacement using Phaser⁵⁰, with a
462 search model of a closely related germline mouse monoclonal antibody (PDB ID: 6BZV⁵¹)
463 lacking complementarity determining regions (CDRs). The model was iteratively improved by
464 refinement in Coot⁵² and Phenix⁵³. Data collection and refinement statistics are summarized in
465 Supplementary Table 1. The final model contained 96.77%, 2.23% and 0% in the favored,
466 allowed and outlier regions of the Ramachandran plot, respectively as assessed by
467 MolProbity⁵⁴.

468

469 **Calcein transport assay for divalent cations**

470 FPN was reconstituted into liposomes for divalent cation transport assays. Empty liposomes
471 were prepared as a 3:1 mass ratio of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
472 (POPE, Avanti) to POPG dissolved in chloroform, followed by gentle evaporation of the
473 chloroform under a stream of nitrogen gas, and overnight desiccation. The lipids were dissolved
474 in 20 mM HEPES pH 7.40, 100 mM KCl to a final concentration of 12.5 mg/mL, sonicated until
475 optically clear, subjected to multiple freeze-thaw cycles, and extruded through a 400 nm
476 polycarbonate filter (Avestin) to generate unilamellar vesicles. Subsequently 0.13% (w/v)

477 Triton-X100 (Sigma) was added to destabilize liposomes, corresponding to the concentration
478 yielding 80% of the maximum OD₅₄₀ obtained in a liposome destabilization curve. Purified FPN
479 was added at a 1:50 protein to lipid mass ratio and incubated for 15 min at 4°C. Control
480 liposomes devoid of FPN were prepared in parallel using the same concentration of DDM. To
481 remove excess detergent, 0.05 g/mL of SM2-BioBeads were added to the sample and nutated
482 for 1 hr at 4°C, then 0.05 g/mL SM2-BioBeads were added followed by incubation overnight at
483 4°C, and finally addition of 0.08 g/mL SM2-BioBeads followed by incubation for 2 hr at 4°C.
484 Proteoliposomes were harvested by ultracentrifugation at 300,000 x g for 30 min and
485 resuspended at a concentration of 2.0 mg/mL lipids in internal buffer comprised of 20 mM
486 HEPES pH 7.40 and 100 mM KCl, before flash freezing in liquid nitrogen and storage at -80°C.
487 On the day of the transport assay, proteoliposomes were thawed and incubated with 500 mM
488 calcein (Sigma), then subjected to three freeze-thaw cycles, and extruded through a 400 nm
489 polycarbonate filter. The liposomes were washed four times with external buffer comprising 20
490 mM HEPES pH 7.40 and 100 mM NaCl, by repeated ultracentrifugation and resuspension.
491 Immediately prior to the assay, the calcein containing proteoliposomes were diluted to 0.25
492 mg/mL lipid in external buffer. Time-course fluorescence traces were recorded as 1 s
493 integrations using a FluoroMax-4 (Horiba) with λ_{ex} of 490 nm and λ_{em} of 520 nm. Steady state
494 fluorescence was recorded for at least 5 min, before addition of small aliquots of freshly
495 prepared stocks of either FeCl₂ or CoCl₂. To stabilize the ferrous (Fe²⁺) state, we prepared iron
496 as a 1:10 ratio of sodium ascorbate:FeCl₂ immediately prior to the experiment. To determine the
497 full extent of the calcein quenching response, 10 μM of the divalent cation ionophore calcimycin
498 (Sigma) was added at the end of each experiment. Transport data was normalized to the mean
499 baseline fluorescence intensity prior to addition of ion.

500

501 **Hepcidin binding assays**

502 Fluorescence polarization measurements were performed using rhodamine-green labeled
503 hepcidin (RhoG-hepcidin)²⁵. For FPN saturation binding experiments, samples were prepared in
504 a black 384-well plate (Greiner) containing 0-1 μM of nanodisc reconstituted NW11-FPN and 5
505 nM RhoG-hepcidin in sample buffer comprising 20 mM HEPES pH 7.50, 100 mM NaCl, and
506 supplemented with FeCl₂, CoCl₂ or MnCl₂ as indicated. For ion stimulation experiments, 100 nM
507 NW11-FPN and 5 nM RhoG-Hepcidin was mixed with 0-600 μM of CoCl₂. For Fab binding
508 experiments, 100 nM NW11-FPN and 5 nM RhoG-Hepcidin was mixed with 0 - 3 μM of
509 Fab45D8 in sample buffer containing 10 μM CoCl₂. Binding reactions were equilibrated for 60

510 min at RT, and fluorescence polarization was recorded on a Biotek Synergy H4 (Agilent) in
511 polarization mode using fixed bandpass filters with λ_{ex} of 484 nm and λ_{em} of 520 nm.

512

513 Analytical fluorescence size exclusion chromatography (FSEC) was performed by mixing 25 μg
514 of NW11-FPN with 2 x fold molar excess of RhoG-Hepcidin in sample buffer comprised of 20
515 mM HEPES (pH 7.50), 100 mM NaCl and 10 μM CoCl_2 . Samples were incubated for 20 min on
516 ice and 1.5 x molar excess of Fab45D8, or sample buffer, was added followed by incubation for
517 30 min on ice. For homologous competition, 1 μM NW11-FPN was mixed with 30 μM unlabelled
518 hepcidin (Bachem) in sample buffer comprised of 20 mM HEPES (pH 7.50), 100 mM NaCl and
519 10 μM CoCl_2 , and incubated for 30 min on ice. Then 2 μM RhoG-Hepcidin was added and the
520 sample incubated for 30 min on ice. Samples were injected on a Superdex 200 Increase 10/300
521 GL column (GE Lifesciences) pre-equilibrated in 20 mM HEPES pH 7.50, 100 mM NaCl, and 10
522 μM CoCl_2 . RhoG-Hepcidin fluorescence was recorded using an FP-1520 Intelligent
523 Fluorescence Detector (Jasco) with λ_{ex} of 493 nm and λ_{em} of 524 nm.

524

525 **Cryo-EM Sample Preparation and Data Collection**

526 Nanodisc-reconstituted apo-FPN was mixed with 1.15 molar excess of Fab45D8 and incubated
527 on ice for 30 min. The complex was purified by size-exclusion chromatography over a Superdex
528 S200 Increase 10/300 GL column (GE Healthcare) equilibrated with 20 mM HEPES pH 7.50,
529 100 mM NaCl. For Co^{2+} /hepcidin samples, 600 μM CoCl_2 and 30 μM hepcidin (Bachem) was
530 added to nanodisc-reconstituted FPN and incubated for 20 minutes on ice prior to addition of
531 Fab45D8. The resulting complex was purified over size-exclusion chromatography as for the
532 apo sample but with the addition of 100 μM CoCl_2 in the chromatography buffer. Collected
533 fractions were supplemented with fresh hepcidin to 30 μM . For both preparations, fractions
534 containing the nanodisc-FPN-Fab45D8 complex were concentrated to ~ 3 mg/ml on a Vivaspin
535 50-kDa MWCO concentrator and freshly used for electron microscopy.

536

537 For high-resolution cryo-EM, the apo-FPN-Fab45D8 complex was diluted to 0.0375 mg/mL in 20
538 mM HEPES pH 7.5, 100 mM NaCl directly prior to vitrification, and 2 μL sample was applied to
539 glow-discharged gold holey carbon 1.2/1.3 300-mesh grids (Quantifoil) coated in-house with
540 graphene oxide⁵⁵⁻⁵⁷. Grids were blotted for 2-4 seconds at 0 force and 10 seconds wait time
541 before being plunge vitrified in liquid ethane using a MarkIV Vitrobot (ThermoFisher). The
542 blotting chamber was maintained at 22°C and 100% humidity during freezing.

543

544 Co²⁺/hepcidin samples were diluted to 1.5 mg/mL in gel filtration buffer (20 mM HEPES pH 7.5,
545 100 mM NaCl, 100 μ M CoCl₂) before vitrification. Grids were blotted for 3 seconds at 0 force
546 and 5 seconds wait time before being plunge vitrified in liquid ethane using a MarkIV Vitrobot
547 (ThermoFisher). The blotting chamber was maintained at 22°C and 100% humidity during
548 freezing.

549

550 FPN-Fab45D8 and Co²⁺/hepcidin-FPN-Fab45D8 movies were collected using a Titan Krios
551 (ThermoFisher) outfitted with a K3 camera and Bioquantum energy filter (Gatan). The K3
552 detector was operated in superresolution mode and the energy filter slit width was set to 20 eV.
553 Movies were collected at a nominal magnification of 105,000x, physical pixel size 0.834Å/pix,
554 with a 70 μ m C2 aperture and 100 μ m objective aperture at a dose rate of 8 e⁻/pixel/second. A
555 total dose of 66 e⁻/Å² was collected as a 120-frame movie, resulting in a 6-second movie with
556 0.55 e⁻/frame. Data were collected using semi-automated imaging scripts in SerialEM⁵⁸. For
557 FPN-Fab45D8, 5009 movies were collected using a 3x3 image shift pattern at 0° tilt and 406
558 movies were collected on-axis with a 30° stage tilt in two separate data collection sessions. For
559 Co²⁺/hepcidin-FPN-Fab45D8, 4395 movies were collected at 0° tilt in a single data collection
560 session.

561

562 **Cryo-EM Image Processing**

563 For FPN-Fab45D8, data were motion corrected and 2x binned on-the-fly using MotionCor2⁵⁹ in
564 the SCIPION pipeline⁶⁰. Motion corrected micrographs were imported into cryoSPARC⁶¹ and
565 RELION⁶² and contrast transfer function parameters were calculated using CTFFIND4⁶³. CTF
566 information for tilted images were estimated using patch CTF estimation in cryoSPARC.
567 138,314 particles were selected from 497 micrographs using the Blob picker in cryoSPARC. 2D
568 class averages were generated after extracting the putative particles with a 300-pixel box and
569 binning to 64 pixels. Six of these averages were used as templates for further particle picking.
570 Template picking yielded 4,737,795 particles. These were split into 6 groups to increase speed
571 of processing, extracted in a 300-pixel box, and binned to 64 pixels. 2D classification was run in
572 cryoSPARC with default settings except: number of 2D classes 200, initial classification
573 uncertainty factor 4, number of online-EM iterations 40, batch size per class 300. Objectively
574 “good” (showing clear Fab and receptor density) class averages were selected and exported to
575 RELION format using csparc.py⁶⁴. Class averages that were not classified as “good”, but were

576 not clearly ice contamination or graphene oxide edges, were run through a second round of 2D
577 classification with default settings except: number of 2D classes 200, number of online-EM
578 iterations 40, batch size per class 300. All “saved” class averages from the second rounds of 2D
579 classification in cryoSPARC selected and exported to RELION format using `csparc.py`. Particles
580 were extracted from CTF-corrected images in RELION at a box size of 300 pixels, binned to 128
581 pixels. 1,326,130 particles, in three groups (detailed in Supplementary Fig. 3) were classified in
582 3D with image alignment in RELION using an initial model generated in cryoSPARC from
583 80,000 particles collected on a Talos Arctica filtered to 40 Å, C1 symmetry, a regularization
584 parameter of 4, for 30-35 iterations with no mask. Particles from classes with resolved
585 transmembrane (TM) helices were selected, extracted in a 300-pixel box, and imported back
586 into cryoSPARC. Non-uniform refinement was run with default settings and no resolution limit,
587 resulting in angle and shift assignments for 850,000 particles. These particles were
588 subsequently exported to RELION format using `csparc.py` and run through 3D classification
589 without image alignment in RELION. Four of the 12 classes were selected, imported into
590 RELION, run through non-uniform alignment with an automatically generated mask, and refined
591 to a reported global resolution of 3.2 Å. The resulting map showed clear signs of mild preferred
592 orientation (Supplementary Fig. 3). The particles were exported into RELION format using
593 `csparc.py`, converted into an image stack, and imported into cisTEM⁶⁵ as a refinement package.
594 The particles were reconstructed and half-maps were generated using the “generate 3D”
595 command. These half-maps, as well as the half-maps from cryoSPARC were run through our
596 lab’s directional Fourier shell correlation (dFSC) program⁶⁶ clearly showing a more distributed
597 range of views in the map generated by cisTEM. Maps were sharpened in RELION. Resolutions
598 are reported using the FSC = 0.143 cut-off⁶⁷ and were estimated in cryoSPARC and cisTEM.
599
600 For Co²⁺/hepcidin-FPN-Fab45D8, data were motion corrected and 2x binned on-the-fly using
601 MotionCor2⁵⁹ in the SCIPION pipeline⁶⁰. Motion corrected micrographs were imported into
602 cryoSPARC⁶¹ and contrast transfer function parameters were calculated using CTFFIND4⁶³.
603 3,753,516 particles were selected from 4395 micrographs using the template picker in
604 cryoSPARC with 2D averages from the apo-FPN dataset as templates. These particles were
605 extracted in a 360-pixel box, and binned to 64 pixels. 2D classification was run in cryoSPARC
606 with default settings except: number of 2D classes 200, initial classification uncertainty factor 4,
607 number of online-EM iterations 40, batch size per class 300. Objectively “good” (showing clear
608 Fab and receptor density) class averages were selected for 3D classification. Class averages

609 that were not classified as “good”, but were clearly not ice contamination, were run through a
610 second round of 2D classification with default settings except: number of 2D classes 200,
611 number of online-EM iterations 40, batch size per class 300. All “saved” class averages from the
612 second rounds of 2D classification in cryoSPARC were sent to 3D classification. These particles
613 were subjected to two rounds of heterogeneous refinement in cryoSPARC that serves as a
614 “trash collector”. Four initial models were used, three generated from an early round of ab initio
615 model generation and our final apo-FPN-Fab45D8 structure. All initial models were filtered to
616 30Å before refinement. Particles were unbinned and a final heterogeneous refinement was
617 performed with three good initial models of apo-FPN-Fab45D8. Non-uniform refinement was run
618 with default settings and no resolution limit, on the most populated class resulting in angle and
619 shift assignments for 310,647 particles. The particles were exported into RELION format using
620 `csparc.py`, converted into an image stack, and imported into `cisTEM`⁶⁵ as a refinement package.
621 The particles were reconstructed and half-maps were generated using the “generate 3D”
622 command. These half-maps, as well as the half-maps from cryoSPARC were run through our
623 lab’s directional Fourier shell correlation (dFSC) program⁶⁶ clearly showing a more distributed
624 range of views in the map generated by `cisTEM`. Maps were sharpened in `cisTEM`. Resolutions
625 are reported using the FSC = 0.143 cut-off⁶⁷ and were estimated in cryoSPARC and `cisTEM`.
626

627 **Model building and refinement**

628 For apo-FPN, a homology model of human FPN in the outward open state was built using
629 `Modeller`⁶⁸, with a previously determined X-ray crystal structure of outward open bbFPN (PDB
630 ID: 5AYN)²³ as a template. After truncating putatively flexible regions (N-terminus, ICL3, ECL5,
631 and C-terminus), the resulting model was fit into the 3.2 Å cryo-EM map of FPN:Fab45D8 using
632 `Chimera`⁶⁹. The initial template was manually rebuilt in `Coot`⁵² and iteratively refined with real
633 space refinement implemented in `Phenix`⁵³. Model geometry was assessed using `MolProbity`⁵⁴.
634 Further validation was performed with `EMRinger`⁷⁰ to compare the map and final model.
635 Map-to-model FSCs were calculated within `Phenix`. Figures were prepared in `Chimera`⁶⁹ and
636 `PyMol`.

637

638 To dock hepcidin into the Co²⁺/hepcidin-bound and apo-FPN difference density, we used a
639 previously determined X-ray crystal structure of hepcidin bound to a neutralizing Fab as a
640 starting model²¹. Hepcidin, without the first two residues, was manually placed within the
641 difference density in `Coot`, then real-space refined to conform to the difference density

642 with maintaining the disulfide connectivity and secondary structure observed in the
643 starting model. The resulting model docked to FPN has an overall RMSD of 1.2 Å
644 compared to the starting model for regions with defined secondary structure.

645

646 **Molecular dynamics simulations**

647 The structure of the outward-open apo conformation of human FPN was used as the starting
648 coordinates for all simulations. Three different conditions were simulated (Supplementary Table
649 3): (1) the iron-absent condition, where no iron was added; (2) the iron-bound condition, where
650 an Fe²⁺ ion was placed in the proposed iron binding site 5.6 Å from D325 α-carbon, 6.9 Å from
651 D504 α-carbon, and 7.8 Å from H507 α-carbon; (3) the iron-in-bulk-solvent condition, where 15
652 Fe²⁺ ions were placed randomly in the water box outside the protein using Dabble⁷¹.

653

654 Simulation coordinates were prepared by removing non-FPN molecules from the initial
655 structure. Prime (Schrödinger) was used to model missing side chains, and neutral acetyl and
656 methylamide groups were added to cap protein termini. The unresolved loops between
657 TM6-TM7 and TM9-TM10 (residues 239-290 and 394-451 respectively, inclusively) were not
658 modeled. The termini surrounding these loops were capped. PropKa was used to determine the
659 dominant protonation state of all titratable residues at pH 7^{72,73}. The structure was internally
660 hydrated using Dowser⁷⁴. Dabble was used to additionally fill the extracellular cavity⁷¹. The
661 structure was aligned using the Orientation of Proteins in Membranes (OPM) server⁷⁵.

662

663 Using Dabble, the protein was inserted into a pre-equilibrated
664 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) membrane bilayer. For all simulations except
665 condition three (iron in bulk solvent), sodium and chloride ions were added at 150 mM to
666 neutralize the system. For condition three, chloride ions were added to neutralize the system
667 resulting in a concentration of 108 mM. A periodic box was used with dimensions 90 x 90 Å in
668 the x-y plane and a water buffer of 10 Å above and below the protein to the periodic boundary.
669 We used the CHARMM36m parameters for lipids, proteins, sodium and chloride ions, and the
670 TIP3P model for waters⁷⁶⁻⁷⁸. The Fe²⁺ Lennard-Jones parameters were obtained from Li *et. al.*'s
671 compromise model⁷⁹.

672

673 All simulations were run on a single Graphical Processing Unit (GPU) using the Amber18
674 Compute Unified Device Architecture (CUDA) version of particle-mesh Ewald molecular

675 dynamics (PMEMD)^{80,81}. For each condition, 6 replicates were run. For each independent
676 replicate, the system was minimized with 500 steps of steepest descent followed by 500 steps
677 of conjugate gradient descent three times. 10 and 5 kcal mol⁻¹ · Å² harmonic restraints were used
678 on the protein, lipid, and Fe²⁺ ions for the first and second minimization, respectively. 1 kcal
679 mol⁻¹ · Å² harmonic restraints were used on the protein and Fe²⁺ ions for the final minimization.
680 The system was then heated from 0 K to 100 K over 12.5 ps in the NVP ensemble with a
681 Langevin thermostat and harmonically restraining the protein heavy atoms and Fe²⁺ ions with a
682 restraint of 10 kcal mol⁻¹ · Å². The system was further heated with the same restraints from 100 K
683 to 310 K in the NPT ensemble over 125 ps. The system was equilibrated with harmonic
684 restraints on protein heavy atoms and Fe²⁺ ions for 30 ns. The restraint strength started at 5 kcal
685 mol⁻¹ · Å² and was reduced by 1 kcal mol⁻¹ · Å² every 2 ns for the first 10 ns and then by 0.1 kcal
686 mol⁻¹ · Å² every 2 ns for the final 20 ns. Production simulations were performed at 310 K and 1
687 bar using the NPT ensemble, a Langevin thermostat and a Monte Carlo barostat. Every 200 ps
688 snapshots were saved. All simulations were run for at least 2.2 μs. These simulations used a
689 4-fs time step with hydrogen mass repartitioning⁸². Bond lengths to hydrogen atoms were
690 constrained using SHAKE^{82,83}. Non-bonded interactions were cut off at 9 Å.

691

692 **Simulation Analysis Methods**

693 MD snapshots were reimaged every 1 ns and centered using CPPTRAJ package in
694 AmberTools¹⁸⁸⁴. Simulations were visualized using Visual Molecular Dynamics and figures
695 prepared in PyMOL⁸⁵. Time traces from simulation were smoothed using a moving average with
696 a window size of 15 ns unless otherwise indicated and visualized with the PyPlot package from
697 Matplotlib. For all analysis in the manuscript that required structural alignment, we aligned to the
698 initial Ferroportin structure using the backbone atoms of residues 26-116, 127-228, 308-483,
699 and 492-543.

700

701 To investigate the localization of Fe²⁺ ions, the iron-in-bulk-solvent simulations (condition 3) were
702 analyzed. To visualize the density of Fe²⁺ ions, the position of Fe²⁺ ions was recorded every 10
703 ns for each of the 6 simulation replicates, each 2 μs in length. Each Fe²⁺ ion position was then
704 drawn as a point superimposed on the starting structure (Fig. 3d). To quantify the binding
705 events, the distance between iron and the closest side chain oxygen atom on D325 was
706 measured. This distance was graphed over 1.2 μs, including the equilibration time (Fig. 3d).

707

708 To investigate the dynamics and conformation of the TM7b region, the iron-absent (condition 1)
709 and iron-bound (condition 2) simulations were compared. The TM1-TM7b distance was
710 measured using distance between the C α of V51 and the C α of Y333. For each simulation, we
711 calculated the average of the distance over 2.2 μ s, excluding equilibration. The average over
712 the simulations for each condition was plotted with error bars representing the standard error of
713 the mean (s.e.m.) (Supplementary Fig. 7). The dynamics of D325 were also investigated. The
714 dynamics were visualized by overlaying representative frames showing the movement of D325,
715 the binding site, and TM7b. For iron-absent simulations, frames from a single replicate at 200,
716 350, 500, and 550 ns were overlaid (Supplementary Fig. 7). For iron-bound simulations, frames
717 from a single replicate at 200, 500, 725, 1000 ns were overlaid (Supplementary Fig. 7). The
718 conformational range of D325 was quantified by measuring the distance between C γ of D325
719 and C β of S47. This was visualized for one replicate for each condition over a time of 1 μ s
720 inclusive of equilibration (Supplementary Fig. 7). For each independent replicate, the mean of
721 the C γ D325 - C β S47 distance was calculated over 2.2 μ s. For each condition, the average
722 over the replicates was plotted with error bars representing the s.e.m. (Supplementary Fig. 7).
723 The flexibility of D325 was quantified by calculating the root-mean-square fluctuation (RMSF) of
724 the side-chain atoms of D325 using an in-house script (Supplementary Fig. 7). Statistical
725 significance was determined using the Mann-Whitney U test.

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727

728 **Data Availability**

729 All data generated or analyzed during this study are included in this published article and its
730 Supplementary Information. Crystallographic coordinates and structure factors for the Fab45D8
731 complex have been deposited in the Protein Data Bank under accession code 6W4V.
732 Coordinates for Fab45D8-FPN complex have been deposited in the Protein Data Bank under
733 accession code 6W4S and the maps have been deposited in the Electron Microscopy Data
734 Bank under accession code 21539. Coordinates for the FPN-Co²⁺-hepcidin-Fab45D8 complex
735 have been deposited in the Protein Data Bank under accession code 6WBV and the maps have
736 been deposited in the Electron Microscopy Data Bank under accession code 21599.

737

738 **Conflict of Interest**

739 Tara Arvedson is employed by Amgen and reports Amgen stock. None of the other authors
740 report conflicts of interest.

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