### <sup>1</sup>**Structural basis of the excitatory amino acid transporter 3 substrate recognition.**

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- <sup>9</sup>**Abstract:**
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11 Excitatory amino acid transporters (EAATs) reside on cell surfaces and uptake substrates,<br>12 including L-glutamate, L-aspartate, and D-aspartate, using ion gradients. Among five EAATs, 12 including L-glutamate, L-aspartate, and D-aspartate, using ion gradients. Among five EAATs,<br>13 EAAT3 is the only isoform that can efficiently transport L-cysteine, a substrate for glutathione 13 EAAT3 is the only isoform that can efficiently transport L-cysteine, a substrate for glutathione<br>14 synthesis. Recent work suggests that EAAT3 also transports the oncometabolite R-2-14 synthesis. Recent work suggests that EAAT3 also transports the oncometabolite R-2-<br>15 hydroxyglutarate (R-2HG). Here, we examined the structural basis of substrate promiscuity by 15 hydroxyglutarate (R-2HG). Here, we examined the structural basis of substrate promiscuity by<br>16 determining the cryo-EM structures of EAAT3 bound to different substrates. We found that L-16 determining the cryo-EM structures of EAAT3 bound to different substrates. We found that L-<br>17 cysteine binds to EAAT3 in thiolate form, and EAAT3 recognizes different substrates by fine-17 cysteine binds to EAAT3 in thiolate form, and EAAT3 recognizes different substrates by fine-18 tuning local conformations of the coordinating residues. However, using purified human EAAT3,<br>19 we could not observe R-2HG binding or transport. Imaging of EAAT3 bound to L-cysteine 19 we could not observe R-2HG binding or transport. Imaging of EAAT3 bound to L-cysteine<br>20 revealed several conformational states, including an outward-facing state with a semi-open gate 20 revealed several conformational states, including an outward-facing state with a semi-open gate<br>21 and a disrupted sodium-binding site. These structures illustrate that the full gate closure, coupled 21 and a disrupted sodium-binding site. These structures illustrate that the full gate closure, coupled<br>22 with the binding of the last sodium ion. occurs after substrate binding. Furthermore, we observed 22 with the binding of the last sodium ion, occurs after substrate binding. Furthermore, we observed<br>23 that different substrates affect how the transporter distributes between a fully outward-facing 23 that different substrates affect how the transporter distributes between a fully outward-facing<br>24 conformation and intermediate occluded states on a path to the inward-facing conformation. 24 conformation and intermediate occluded states on a path to the inward-facing conformation,<br>25 suggesting that translocation rates are substrate-dependent. 25 suggesting that translocation rates are substrate-dependent.<br>26

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# <sup>27</sup>**Introduction:**

29 EAATs belong to the Solute Carrier 1 (SLC1) family uptake substrates into cells against their<br>30 concentration gradients by symporting them with three sodium ions (Na<sup>+</sup>) and a proton (H<sup>+</sup>) and 30 concentration gradients by symporting them with three sodium ions  $(Na^+)$  and a proton  $(H^+)$  and 31 counter-transporting a potassium ion  $(K^+)^{1-3}$ . There are 5 EAAT subtypes in humans, sharing

similar molecular mechanisms but expressed in different tissues and cell types<sup>4</sup>. EAAT1 and<br>33. EAAT2 are the principal glial glutamate transporters, with EAAT2 responsible for the uptake of EAAT2 are the principal glial glutamate transporters, with EAAT2 responsible for the uptake of<br>34 up to 80-90% of the neurotransmitter into astrocytes following rounds of synaptic transmission<sup>5</sup>. up to 80-90% of the neurotransmitter into astrocytes following rounds of synaptic transmission<sup>5</sup>.<br>35. EAAT4 and EAAT5 are expressed in Purkinje cells of the cerebellum and retina; they display EAAT4 and EAAT5 are expressed in Purkinje cells of the cerebellum and retina; they display<br>36 lower glutamate transport but higher chloride conductance ability<sup>6,7</sup>. By contrast, EAAT3 is lower glutamate transport but higher chloride conductance ability<sup>6,7</sup>. By contrast, EAAT3 is<br>37. expressed in neurons throughout the brain and peripheral tissues, such as epithelial cells of the 37 expressed in neurons throughout the brain and peripheral tissues, such as epithelial cells of the<br>38 intestine and kidney and endothelial cells of capillaries<sup>8</sup>. All EAATs can uptake L-Glu, L-Asp, intestine and kidney and endothelial cells of capillaries<sup>8</sup>. All EAATs can uptake L-Glu, L-Asp, 39 and D-Asp. L-Glu is the brain's most abundant free amino acid; it mediates transmission at most<br>40 fast excitatory synapses and is a metabolic hub linking energy metabolism and amino acid 40 fast excitatory synapses and is a metabolic hub linking energy metabolism and amino acid<br>41 biosynthesis in neurons<sup>9</sup>. Under normal conditions, most L-Glu is sequestered inside brain cells, biosynthesis in neurons<sup>9</sup>. Under normal conditions, most L-Glu is sequestered inside brain cells,<br>42 and its excess in the extracellular space can lead to excitotoxicity. L-Asp also fits the criteria of 42 and its excess in the extracellular space can lead to excitotoxicity. L-Asp also fits the criteria of an excitatory neurotransmitter because it excites the NMDA subtype of ionotropic glutamate 43 an excitatory neurotransmitter because it excites the NMDA subtype of ionotropic glutamate<br>44 receptors<sup>10</sup>, but its role in neurotransmission has been questioned<sup>11</sup>. D-Asp, found in the brain receptors<sup>10</sup>, but its role in neurotransmission has been questioned<sup>11</sup>. D-Asp, found in the brain<br>45 and neuroendocrine tissues, shows neuromodulatory activity and may also be a 45 and neuroendocrine tissues, shows neuromodulatory activity and may also be a<br>46 neurotransmitter<sup>12,13</sup>. It is present in high concentrations in the mammalian brain during neurotransmitter<sup>12,13</sup>. It is present in high concentrations in the mammalian brain during development but drops sharply postnatally. 47 development but drops sharply postnatally.<br>48

EAAT3 is the only EAAT subtype able to transport L-Cys efficiently<sup>14,15</sup>. Neutral SLC1 amino<br>50. acid transporters (Alanine, Serine, Cysteine Transporters, or ASCTs) can also transport L-50 acid transporters (Alanine, Serine, Cysteine Transporters, or ASCTs) can also transport L-<br>51 Cys<sup>16,17</sup>, while system xc- transporter from the SLC7 family exchanges oxidized L-cystine for  $Cys<sup>16,17</sup>$ , while system xc- transporter from the SLC7 family exchanges oxidized L-cystine for glutamate<sup>18</sup>. These transporters are enriched in astrocytes<sup>19-21</sup>, whereas EAAT3 mediates about 52 glutamate<sup>18</sup>. These transporters are enriched in astrocytes<sup>19-21</sup>, whereas EAAT3 mediates about<br>53 90% of L-Cvs uptake into neurons<sup>22,23</sup>. In so doing, EAAT3 protects them from oxidative stress 90% of L-Cys uptake into neurons<sup>22,23</sup>. In so doing, EAAT3 protects them from oxidative stress<br>54. because L-Cys is a rate-limiting precursor for antioxidant glutathione (GSH) synthesis. Cysteine 54 because L-Cys is a rate-limiting precursor for antioxidant glutathione (GSH) synthesis. Cysteine<br>55 is also a substrate for producing the gaseous signaling molecule hydrogen sulfide (H<sub>2</sub>S), a is also a substrate for producing the gaseous signaling molecule hydrogen sulfide  $(H_2S)$ , a 56 substrate for the post-translational persulfidation of cysteine residues. This evolutionarily<br>57 conserved modification protects proteins from oxidative stress and can extend the organism's 57 conserved modification protects proteins from oxidative stress and can extend the organism's<br>58 life<sup>24,25</sup>. EAAT3 deficiency may contribute to a plethora of neurologic pathologies, including 158. life<sup>24,25</sup>. EAAT3 deficiency may contribute to a plethora of neurologic pathologies, including ischemic stroke, epilepsy, Parkinson's, Huntington's, and Alzheimer's diseases<sup>26</sup>. Indeed, ischemic stroke, epilepsy, Parkinson's, Huntington's, and Alzheimer's diseases<sup>26</sup>. Indeed, decreased levels of GSH, present in 2-3 mM concentration in the healthy brain, are an early 60 decreased levels of GSH, present in 2-3 mM concentration in the healthy brain, are an early<br>61 biomarker of brain aging and Parkinson's disease<sup>27</sup>. Furthermore, inhibition of EAAT3 by biomarker of brain aging and Parkinson's disease<sup>27</sup>. Furthermore, inhibition of EAAT3 by 62 morphine decreases the cell methylation potential and DNA methylation, leading to epigenetic changes implicated in morphine addiction<sup>28</sup>. 63 changes implicated in morphine addiction<sup>28</sup>.<br>64 EAAT3-mediated L-Glu and L-Asp upta

64 EAAT3-mediated L-Glu and L-Asp uptake outside the central nervous system promotes<br>65 metabolic activity, and the amino acids serve as nucleotide precursors<sup>29</sup>. EAAT3 is also required metabolic activity, and the amino acids serve as nucleotide precursors<sup>29</sup>. EAAT3 is also required<br>66 for rapid metabolic reprogramming in activated B cells<sup>30</sup> and cancer cells<sup>31</sup>. Recently, EAAT3 for rapid metabolic reprogramming in activated B cells<sup>30</sup> and cancer cells<sup>31</sup>. Recently, EAAT3<br>67 has been identified as the "oncometabolite" R-2-hydroxyglutarate (R-2HG) transporter<sup>32</sup>. Tumor has been identified as the "oncometabolite" R-2-hydroxyglutarate (R-2HG) transporter<sup>32</sup>. Tumor cells produce and secrete R-2HG, which acts as a signaling molecule on the surrounding cells, cells produce and secrete R-2HG, which acts as a signaling molecule on the surrounding cells, modulating the tumor microenvironment<sup>33</sup> and might enter endothelial cells via EAAT3,<br>70 stimulating angiogenesis. 70 stimulating angiogenesis.<br>71

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72 EAAT3 is a homotrimer, with each protomer comprised of the central trimeric scaffold and<br>73 peripheral transport domains. During uptake, the transport domain undergoes  $\sim$ 15 Å 73 peripheral transport domains. During uptake, the transport domain undergoes ~15 Å<br>74 transmembrane movement combined with a rotation alternating between the outward- and 74 transmembrane movement combined with a rotation alternating between the outward- and<br>75 inward-facing states (OFS and IFS): the scaffold domain remains mostly immobile<sup>34,35</sup>. All inward-facing states (OFS and IFS); the scaffold domain remains mostly immobile<sup>34,35</sup>. All<br>76 SLC1 family proteins<sup>36-45</sup> and its archaeal homologues<sup>46-52</sup> share this elevator mechanism. A SLC1 family proteins<sup>36-45</sup> and its archaeal homologues<sup>46-52</sup> share this elevator mechanism. A<br>77 substrate molecule, three Na<sup>+</sup> ions, and a proton bind to the transport domain in the OFS and substrate molecule, three Na<sup>+</sup> ions, and a proton bind to the transport domain in the OFS and<br>78 dissociate in the IFS; a K<sup>+</sup> ion binds instead to the IFS and dissociates from the OFS to complete dissociate in the IFS; a  $K^+$  ion binds instead to the IFS and dissociates from the OFS to complete<br>79 the cycle. The first cryo-EM study on the glycosylation mutant of human EAAT3, hEAAT3g, 79 the cycle. The first cryo-EM study on the glycosylation mutant of human EAAT3, hEAAT3g,<br>80 revealed that the transporter preferentially resided in the IFS in the presence of saturating  $Na<sup>+</sup>$ revealed that the transporter preferentially resided in the IFS in the presence of saturating  $Na<sup>+</sup>$  concentrations<sup>35</sup>. L-Asp showed a very low affinity for the IFS and a greater affinity for the OFS; concentrations<sup>35</sup>. L-Asp showed a very low affinity for the IFS and a greater affinity for the OFS;<br>82. therefore, we observed growing populations of L-Asp-bound OFS in increasing L-Asp 82 therefore, we observed growing populations of L-Asp-bound OFS in increasing L-Asp<br>83 concentrations. In contrast, IFS remained substrate-free. To increase the population of the OFS 83 concentrations. In contrast, IFS remained substrate-free. To increase the population of the OFS<br>84 and observe a lower affinity L-Glu binding, we developed a crosslinking protocol constraining a 84 and observe a lower affinity L-Glu binding, we developed a crosslinking protocol constraining a<br>85 double cysteine K269C/W441C mutant of EAAT3g in the OFS (hEAAT3-X). The crosslinked double cysteine K269C/W441C mutant of EAAT3g in the OFS (hEAAT3-X). The crosslinked 86 protein showed a mixture of the OFS and an atypical intermediate outward-facing state (iOFS\*),<br>87 in which the transport domain moves closer to IFS. The intermediate state exhibited a higher 87 in which the transport domain moves closer to IFS. The intermediate state exhibited a higher<br>88 substrate affinity, with L-Glu favoring iOFS\* over OFS<sup>34</sup>. substrate affinity, with L-Glu favoring  $i$ OFS<sup>\*</sup> over OFS<sup>34</sup>.

89<br>90 90 Here, we used hEAAT3-X to examine the structural basis of how EAAT3 recognizes diverse<br>91 substrates. We combined these studies with ligand-mediated thermal stabilization experiments on 91 substrates. We combined these studies with ligand-mediated thermal stabilization experiments on<br>92 bEAAT3g to probe substrate binding in solution and solid-supported membrane (SSM) hEAAT3g to probe substrate binding in solution and solid-supported membrane (SSM)

93 electrophysiology to test substrate transport. The substrates showed thermal stabilization of the transporters in the order L-Asp > D-Asp > L-Glu > L-Cys, which likely reflects how tightly they 94 transporters in the order L-Asp > D-Asp > L-Glu >L-Cys, which likely reflects how tightly they<br>95 bind. Notably, L-Cys showed thermal stabilization only at elevated pH, suggesting it binds in the 95 bind. Notably, L-Cys showed thermal stabilization only at elevated pH, suggesting it binds in the<br>96 thiolate form. We observed no hEAAT3 stabilization by R-2HG. SSM electrophysiology showed 96 thiolate form. We observed no hEAAT3 stabilization by R-2HG. SSM electrophysiology showed<br>97 transport currents for Asp. Glu. and L-Cvs. while R-2HG produced no currents. CrvoEM 97 transport currents for Asp, Glu, and L-Cys, while R-2HG produced no currents. CryoEM<br>98 imaging of hEAAT3-X in the presence of L-Asp and D-Asp showed transporters predominantly 98 imaging of hEAAT3-X in the presence of L-Asp and D-Asp showed transporters predominantly<br>99 in iOFS<sup>\*</sup> and bound to the amino acids. In contrast, hEAAT3-X, in the presence of R-2HG, in iOFS<sup>\*</sup> and bound to the amino acids. In contrast, hEAAT3-X, in the presence of R-2HG,<br>100 pictured the transporter in OFS with an empty and open substrate-binding site, consistent with 100 pictured the transporter in OFS with an empty and open substrate-binding site, consistent with<br>101 the biophysical results suggesting that R-2HG is not a transported substrate. Imaging hEAAT3-X 101 the biophysical results suggesting that R-2HG is not a transported substrate. Imaging hEAAT3-X<br>102 in the presence of L-Cys revealed an ensemble of OFS, iOFS<sup>\*</sup>, and a slightly shifted iOFS. The in the presence of L-Cys revealed an ensemble of OFS, iOFS<sup>\*</sup>, and a slightly shifted iOFS. The<br>103, iOFS and iOFS<sup>\*</sup> featured the full complement of bound L-Cys and symported ions. In contrast, iOFS and iOFS<sup>\*</sup> featured the full complement of bound L-Cys and symported ions. In contrast,<br>104 OFS, while bound to L-Cys and two Na<sup>+</sup> ions (at Na1 and Na3 sites), featured a semi-open OFS, while bound to L-Cys and two Na<sup>+</sup> ions (at Na1 and Na3 sites), featured a semi-open<br>105 extracellular gate (helical hairpin 2, HP2) and a disrupted Na2 site. Our work provides the 105 extracellular gate (helical hairpin 2, HP2) and a disrupted Na2 site. Our work provides the<br>106 structural basis of promiscuous substrate recognition by EAAT3 and suggests that the substrate 106 structural basis of promiscuous substrate recognition by EAAT3 and suggests that the substrate<br>107 binding occurs before the last  $Na<sup>+</sup>$  bounding at the Na2 site and the coupled gate closure. 107 binding occurs before the last  $Na<sup>+</sup>$  bounding at the Na2 site and the coupled gate closure.<br>108

# <sup>109</sup>**Results:**

## **110 Purified hEAAT3g binds and transports diverse substrates.**<br>**111** To compare the binding of different substrates to hEAAT3,

111 To compare the binding of different substrates to hEAAT3, we purified the transporter and<br>112 measured its temperature-induced denaturation in the absence and presence of substrates (Fig. 112 measured its temperature-induced denaturation in the absence and presence of substrates (**Fig.** 113 **1a-c**). hEAAT3g in 200 mM NaCl at pH 7.4 denatured at  $69.2 \pm 0.2$  °C. Additions of 10 mM L-113 **1a-c**). hEAAT3g in 200 mM NaCl at pH 7.4 denatured at  $69.2 \pm 0.2$  °C. Additions of 10 mM L-<br>114 Asp, D-Asp, and L-Glu increased the denaturation temperature by  $3.8 \pm 0.1$ ,  $2.4 \pm 0.2$ , and 114 Asp, D-Asp, and L-Glu increased the denaturation temperature by  $3.8\pm0.1$ ,  $2.4\pm0.2$ , and 115  $1.0\pm0.1$  °C, respectively. In contrast, 10 mM L-Cys, 10 mM D-Glu, or R-2HG did not 1.0 $\pm$ 0.1 °C, respectively. In contrast, 10 mM L-Cys, 10 mM D-Glu, or R-2HG did not significantly stabilize the transporter, suggesting that they bind weaker or not at all (Fig. 1b, c). 116 significantly stabilize the transporter, suggesting that they bind weaker or not at all (**Fig. 1b, c**).<br>117 To test L-Cys and R-2HG further, we increased their concentrations to 100 mM at pH 7.4 and 117 To test L-Cys and R-2HG further, we increased their concentrations to 100 mM at pH 7.4 and<br>118 8.8 for L-Cys. We observed no significant stabilization by either substrate at pH 7.4. However, at 118 8.8 for L-Cys. We observed no significant stabilization by either substrate at pH 7.4. However, at pH 8.8. L-Cys stabilized the transporter by  $4.2 \pm 0.6^{\circ}$ C (Fig. 1c). These data suggest that L-Cys 119 pH 8.8, L-Cys stabilized the transporter by  $4.2 \pm 0.6^{\circ}$ C (**Fig. 1c**). These data suggest that L-Cys<br>120 binds to the transporter as thiolate. Surprised by the apparent lack of R-2HG binding, we tested 120 binds to the transporter as thiolate. Surprised by the apparent lack of R-2HG binding, we tested<br>121 whether hEAAT3g reconstituted into liposomes transported R-2HG in solid-supported 121 whether hEAAT3g reconstituted into liposomes transported R-2HG in solid-supported<br>122 membrane electrophysiology (SSME). R-2HG carries one less positive charge than L-Glu and D-122 membrane electrophysiology (SSME). R-2HG carries one less positive charge than L-Glu and D-<br>123 Glu, but its transport should result in a net uptake of one positive charge and be electrogenic. <sup>123</sup>Glu, but its transport should result in a net uptake of one positive charge and be electrogenic.

- 124 Nevertheless, we observed no capacitance peaks upon perfusion of R-2HG. In contrast, perfusion of L- and D-Asp, L-Glu, and L-Cys over the same SSM chip produced robust peaks, and
- 125 of L- and D-Asp, L-Glu, and L-Cys over the same SSM chip produced robust peaks, and perfusion of D-Glu produced a small but reproducible capacitance current (**Fig. 1d**).
- perfusion of D-Glu produced a small but reproducible capacitance current (**Fig. 1d**).



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130 <sup>131</sup>**Figure 1. Ligand-dependent thermal stability and transport activity of hEAAT3g.** (**a**), 132 Chemical structures of EAAT3 amino acid substrates and R-2HG. (**b**), Representative melting<br>133 curves of hEAAT3g in 200 mM NaCl (dotted line) and the presence of amino acids, as indicated 133 curves of hEAAT3g in 200 mM NaCl (dotted line) and the presence of amino acids, as indicated next to the graph. Shown are the first derivatives of the fluorescence emission intensity ratio at 134 next to the graph. Shown are the first derivatives of the fluorescence emission intensity ratio at  $350$  and  $330$  nm  $(A_{350}/A_{330})$ , with peaks corresponding to the inflections of the sigmoidal melting 135 350 and 330 nm (A<sub>350</sub>/A<sub>330</sub>), with peaks corresponding to the inflections of the sigmoidal melting<br>136 curves and termed melting temperatures (Tm) (c). Tm increases ( $\Delta T$ m) in the presence of 136 curves and termed melting temperatures (Tm) (**c**), Tm increases (ΔTm) in the presence of potential substrates compared to NaCl alone. The results for two independent protein 137 potential substrates compared to NaCl alone. The results for two independent protein<br>138 preparations (except for D-Glu, which was prepared once), each with multiple technical repeats, 138 preparations (except for D-Glu, which was prepared once), each with multiple technical repeats, are shown; the error bars are the standard deviations. (d), Examples of SSME-measured transient 139 are shown; the error bars are the standard deviations. (**d**), Examples of SSME-measured transient<br>140 currents when immobilized hEAAT3g proteoliposomes were perfused with 3 mM of potential 140 currents when immobilized hEAAT3g proteoliposomes were perfused with 3 mM of potential<br>141 substrates. All experiments were performed using two independent protein purification and 141 substrates. All experiments were performed using two independent protein purification and<br>142 reconstitutions, and at least three sensors were used to measure each reconstitution. The color 142 reconstitutions, and at least three sensors were used to measure each reconstitution. The color scheme is the same in (b-d): L-Asp, blue; D-Asp, red; L-Glu, green; D-Glu, cyan; L-Cys, brown; 143 scheme is the same in (**b-d**): L-Asp, blue; D-Asp, red; L-Glu, green; D-Glu, cyan; L-Cys, brown; R-2HG, purple. R-2HG, purple. 145

146 **Structures of hEAAT3-X bound to substrates.**<br>147 To examine substrate binding structurally, we in 147 To examine substrate binding structurally, we introduced K269C/W441C into Cysmini EAAT3<br>148 as previously described<sup>34,53,54</sup>. Hg<sup>2+</sup>-mediated cross-linking traps the transporter in iOFS\*, iOFS, as previously described<sup>34,53,54</sup>. Hg<sup>2+</sup>-mediated cross-linking traps the transporter in iOFS\*, iOFS,<br>and OFS (hEAAT3-X), which show high-affinity L-Asp and L-Glu binding and are ideal for 149 and OFS (hEAAT3-X), which show high-affinity L-Asp and L-Glu binding and are ideal for<br>150 examining varying potential substrates. Following cross-linking, we purified hEAAT3-X by SEC 150 examining varying potential substrates. Following cross-linking, we purified hEAAT3-X by SEC<br>151 in 100 mM NMDG-Cl (Apo condition), split the eluted protein into two samples, and 151 in 100 mM NMDG-Cl (Apo condition), split the eluted protein into two samples, and<br>152 supplemented them with 200 mM NaCl and 10 mM L-Asp or R-2HG before freezing cryo-EM 152 supplemented them with 200 mM NaCl and 10 mM L-Asp or R-2HG before freezing cryo-EM<br>153 grids. Data processing on the L-Asp sample yielded a well-resolved map at 2.87 Å resolution. 153 grids. Data processing on the L-Asp sample yielded a well-resolved map at 2.87 Å resolution.<br>154 The map revealed iOFS\* conformation with a closed substrate gate (helical hairpin 2, HP2) and a 154 The map revealed iOFS\* conformation with a closed substrate gate (helical hairpin 2, HP2) and a<br>155 well-resolved density corresponding to the bound L-Asp (Fig. 2a, c, Supplementary Fig. 1, 155 well-resolved density corresponding to the bound L-Asp (**Fig. 2a, c, Supplementary Fig. 1, Supplementary Table 1**); we found no additional minor conformations in 3D classifications. In **Supplementary Table 1**); we found no additional minor conformations in 3D classifications. In contrast, the R-2HG dataset yielded a 3.07 Å resolution OFS map featuring a wide-open HP2 157 contrast, the R-2HG dataset yielded a 3.07 Å resolution OFS map featuring a wide-open HP2<br>158 gate, nearly identical to the OFS observed in  $Na<sup>+</sup>$  buffers without substrates (**Fig. 2b. d.**) gate, nearly identical to the OFS observed in  $Na^+$  buffers without substrates (**Fig. 2b, d, 359 Supplementary Fig. 2. Supplementary Table 1**). We found 8 % protomers in iOFS with no **Supplementary Fig. 2, Supplementary Table 1**). We found 8 % protomers in iOFS with no<br>160 density corresponding to R-2HG (**Supplementary Fig. 2c**); this conformation is nearly identical 160 density corresponding to R-2HG (**Supplementary Fig. 2c**); this conformation is nearly identical<br>161 to the minor state observed in Na<sup>+</sup> buffer without substrate<sup>34</sup>. 161 to the minor state observed in Na<sup>+</sup> buffer without substrate<sup>34</sup>.

163 Next, we prepared another batch of apo hEAAT3-X, which we supplemented with 200 mM NaCl<br>164 and 10 mM L-Cys or D-Asp. Because L-Cys can break  $Hg^{2+}$ -mediated cysteine crosslink, we and 10 mM L-Cys or D-Asp. Because L-Cys can break  $Hg^{2+}$ -mediated cysteine crosslink, we rapidly mixed ice-cold EAAT3-X with L-Cys and froze grids immediately, in less than 10 165 rapidly mixed ice-cold EAAT3-X with L-Cys and froze grids immediately, in less than 10<br>166 seconds. Processing of the D-Asp dataset produced a 2.73 Å resolution density map with 166 seconds. Processing of the D-Asp dataset produced a 2.73 Å resolution density map with<br>167 resolved scaffold and transport domains corresponding to iOFS\* (Fig. 2e, Supplementary Fig. 3, 167 resolved scaffold and transport domains corresponding to iOFS\* (**Fig. 2e, Supplementary Fig. 3, Supplementary Table. 1**), and 3D classification did not reveal the presence of any other states. **Supplementary Table. 1**), and 3D classification did not reveal the presence of any other states.<br>169 Interestingly, we previously found that for hEAAT3-X bound to L-Glu, about 14% of protomers 169 Interestingly, we previously found that for hEAAT3-X bound to L-Glu, about 14% of protomers<br>170 were in the OFS conformation, with the remainder in iOFS\*. In contrast, we found no OFS 170 were in the OFS conformation, with the remainder in iOFS\*. In contrast, we found no OFS<br>171 structural classes in the current L-Asp or D-Asp datasets. Thus, we hypothesize that ligands can 171 structural classes in the current L-Asp or D-Asp datasets. Thus, we hypothesize that ligands can<br>172 affect the transport domain distribution of the EAAT3-X. 172 affect the transport domain distribution of the EAAT3-X.<br>173





174<br>175 **Figure 2. The structures of hEAAT3-X with 10 mM substrates.** The overall structure of hEAAT3-X with 10 mM L-Asp (a), R-2HG (b), D-Asp (e), or L-Cys (f); The orange dashed 176 hEAAT3-X with 10 mM L-Asp (**a**), R-2HG (**b**), D-Asp (**e**), or L-Cys (**f**); The orange dashed ovals highlight the transport domain density of iOFS\*-D-Asp and iOFS\*-L-Cys. The scaffold 177 ovals highlight the transport domain density of iOFS\*-D-Asp and iOFS\*-L-Cys. The scaffold<br>178 domains are colored in wheat, the lipid densities are gray, and the transport domains are 178 domains are colored in wheat, the lipid densities are gray, and the transport domains are<br>179 multicolored with L-Asp, light blue; R-2HG, green; D-Asp, pink; and L-Cys, dark blue. (c, d), 179 multicolored with L-Asp, light blue; R-2HG, green; D-Asp, pink; and L-Cys, dark blue. (**c, d**), 180 The structures of iOFS\*-L-Asp (**c**) and OFS-R2HG (**d**) transport domains. Helical hairpin 1 180 The structures of iOFS<sup>\*</sup>-L-Asp (**c**) and OFS-R2HG (**d**) transport domains. Helical hairpin 1<br>181 (HP1) and HP2, which define the location of the substrate-binding site, are colored vellow-181 (HP1) and HP2, which define the location of the substrate-binding site, are colored yellow-<br>182 orange and red, respectively. HP2 of iOFS\*-L-Asp is closed, with the bound L-Asp colored in 182 orange and red, respectively. HP2 of iOFS\*-L-Asp is closed, with the bound L-Asp colored in teal (c); The HP2 of OFS-R2HG is wide open, and the ligand-binding cavity, emphasized by the 183 teal (c); The HP2 of OFS-R2HG is wide open, and the ligand-binding cavity, emphasized by the black dotted oval, is empty (d). The contour levels of the iOFS<sup>\*</sup>-L-Asp, OFS-R-2HG, iOFS<sup>\*</sup>-D-184 black dotted oval, is empty (**d**). The contour levels of the iOFS<sup>\*</sup>-L-Asp, OFS-R-2HG, iOFS<sup>\*</sup>-D-<br>185 Asp, and iOFS<sup>\*</sup>-L-Cys trimer maps are 0.614, 0.34, 0.614, and 0.62, respectively; the gray 185 Asp, and iOFS\*-L-Cys trimer maps are 0.614, 0.34, 0.614, and 0.62, respectively; the gray dashed lines represent an approximate position of the lipid bilayer. 186 dashed lines represent an approximate position of the lipid bilayer.<br>187

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### 188 189 **Conformational ensemble of L-Cys-bound hEAAT3-X.**<br>190 Processing the L-Cys dataset yielded a density map at

190 Processing the L-Cys dataset yielded a density map at 2.36 Å resolution with applied C3<br>191 symmetry. The map showed a well-resolved scaffold domain density but a blurred transport 191 symmetry. The map showed a well-resolved scaffold domain density but a blurred transport<br>192 domain density (Fig. 2f. Supplementary Fig. 4). Because we observed no such blurring in the 192 domain density (**Fig. 2f, Supplementary Fig. 4**). Because we observed no such blurring in the<br>193 D-Asp dataset, which was prepared simultaneously, we reasoned that it was not due to damaged 193 D-Asp dataset, which was prepared simultaneously, we reasoned that it was not due to damaged<br>194 protein and might reflect protein dynamics. To uncover the complete conformational ensemble 194 protein and might reflect protein dynamics. To uncover the complete conformational ensemble<br>195 of L-Cys-bound EAAT3-X, we performed symmetry expansion and optimized the parameters of 195 of L-Cys-bound EAAT3-X, we performed symmetry expansion and optimized the parameters of the local 3D classification in Relion<sup>55</sup>. When the class number,  $K$ , and the regularization the local 3D classification in Relion<sup>55</sup>. When the class number,  $K$ , and the regularization parameter,  $T$ , were set to 20 and 40, we identified 4 distinct structural classes. Further local 197 parameter, *T*, were set to 20 and 40, we identified 4 distinct structural classes. Further local<br>198 refinement produced EM maps corresponding to OFS, iOFS, iOFS<sup>\*</sup>, and IFS with resolutions of 198 refinement produced EM maps corresponding to OFS, iOFS, iOFS\*, and IFS with resolutions of<br>199 2.58, 2.99, 2.60, and 2.94 Å. (Fig. 3, Supplementary Figs. 5, 6, Supplementary Table 1). The <sup>199</sup>2.58, 2.99, 2.60, and 2.94 Å. (**Fig. 3, Supplementary Figs. 5, 6, Supplementary Table 1**). The IFS presence indicates that the  $Hg^{2+}$  crosslink is disrupted in a fraction of EAAT3-X molecules<br>201 during grid preparation. Aided by the substantial number of expanded particles (3.3 million), the 201 during grid preparation. Aided by the substantial number of expanded particles (3.3 million), the<br>202 EM map of the lowly populated iOFS class, comprising 1.8% of particles, is well-resolved. We 202 EM map of the lowly populated iOFS class, comprising 1.8% of particles, is well-resolved. We<br>203 could not sort out iOFS with smaller K values, such as 5 and 10. 203 could not sort out iOFS with smaller *K* values, such as 5 and 10.<br>204

205 205 We observed strong non-protein density in the substrate-binding pocket of OFS, iOFS, and iOFS\* maps, which we modeled as L-Cys (**Fig. 3a-c**). In contrast, there was no ligand density in 206 iOFS<sup>\*</sup> maps, which we modeled as L-Cys (**Fig. 3a-c**). In contrast, there was no ligand density in<br>207 the IFS map (**Fig. 3d**). Furthermore, the HP2 gate in the IFS map is wide open, suggesting that it 207 the IFS map (**Fig. 3d**). Furthermore, the HP2 gate in the IFS map is wide open, suggesting that it<br>208 is bound to Na<sup>+</sup> ions only, consistent with the low substrate affinity of the IFS we previously 208 is bound to Na<sup>+</sup> ions only, consistent with the low substrate affinity of the IFS we previously<br>209 reported for hEAAT3g<sup>35</sup>. The overall structure of L-Cys-bound iOFS\* (iOFS\*-L-Cys) is reported for  $hEAAT3g^{35}$ . The overall structure of L-Cys-bound iOFS\* (iOFS\*-L-Cys) is<br>210 remarkably similar to iOFS\*-L-Glu; the RMSD calculated by the whole structure alignment is 210 remarkably similar to iOFS\*-L-Glu; the RMSD calculated by the whole structure alignment is<br>211 0.628 Å. The superposition of iOFS-L-Cys and iOFS\*-L-Cys aligned on the scaffold domain 211 0.628 Å. The superposition of iOFS-L-Cys and iOFS\*-L-Cys aligned on the scaffold domain<br>212 shows that the iOFS-L-Cys transport domain is positioned more outward than in iOFS\*. It 212 shows that the iOFS-L-Cys transport domain is positioned more outward than in iOFS\*. It<br>213 corresponds more closely to the iOFS observed in potassium-bound EAAT3-X, iOFS- $K^{+34}$ corresponds more closely to the iOFS observed in potassium-bound EAAT3-X, iOFS- $K^{+34}$ <br>214 (**Supplementary Fig. 7**). <sup>214</sup>(**Supplementary Fig. 7**).



216<br>217 **Figure 3. Conformational ensemble of hEAAT3-X with 10 mM L-Cys. (a), The overall structure of OFS-L-Cys. The scaffold domain is colored in gray and shown as a cartoon; the** 218 structure of OFS-L-Cys. The scaffold domain is colored in gray and shown as a cartoon; the transport domain is colored in light gray, with the HP1 and HP2 colored in yellow-orange and 219 transport domain is colored in light gray, with the HP1 and HP2 colored in yellow-orange and red, respectively; the density of L-Cys is colored in teal. The transport domains of iOFS-L-Cys 220 red, respectively; the density of L-Cys is colored in teal. The transport domains of iOFS-L-Cys (c), and IFS-Na<sup>+</sup> (d) are colored as in (a). For clarity, their scaffold domains, (**b**), iOFS\*-L-Cys (**c**), and IFS-Na<sup>+</sup> (**d**) are colored as in (**a**). For clarity, their scaffold domains, which were aligned to OFS-L-Cys, are not shown. The contour levels of these maps are 0.65, 222 which were aligned to OFS-L-Cys, are not shown. The contour levels of these maps are 0.65, 0.54, 0.61, and 0.43, respectively.

0.54, 0.61, and 0.43, respectively.

## 224 **Structural basis of ligands recognition by EAAT3.**<br>225 The iOFS<sup>\*</sup>-Cys structure shows that L-Cys is coording

225 The iOFS\*-Cys structure shows that L-Cys is coordinated identically to L-Glu. Its main chain<br>226 carboxylate interacts with the sidechain of N451 in TM8 and the main chain and sidechain 226 carboxylate interacts with the sidechain of N451 in TM8 and the main chain and sidechain<br>227 oxygens of S333 in HP1, and its amino group interacts with the sidechain of D444 in TM8. The 227 oxygens of S333 in HP1, and its amino group interacts with the sidechain of D444 in TM8. The<br>228 L-Cys sidechain sulfur atom is 2.9 Å away from the guanidinium group of R447 (**Fig. 4b, d**), 228 L-Cys sidechain sulfur atom is 2.9 Å away from the guanidinium group of R447 (**Fig. 4b, d**),<br>229 which typically coordinates the sidechain carboxylate of L-Glu, consistent with the bound L-Cys 229 which typically coordinates the sidechain carboxylate of L-Glu, consistent with the bound L-Cys<br>230 being in thiolate form. Further comparison between EAAT3-X bound to L- and D-Asp, L-Glu, 230 being in thiolate form. Further comparison between EAAT3-X bound to L- and D-Asp, L-Glu,<br>231 and L-Cys shows that the R447 sidechain moves slightly outward and assumes a different 231 and L-Cys shows that the R447 sidechain moves slightly outward and assumes a different<br>232 rotamer in the L-Glu- and L-Cys-bound structures compared with the L-Asp- and D-Asp-bound 232 rotamer in the L-Glu- and L-Cys-bound structures compared with the L-Asp- and D-Asp-bound<br>233 conformations (**Fig. 4**). The superposition of EAAT3-X substrate-binding pockets shows that L-233 conformations (**Fig. 4**). The superposition of EAAT3-X substrate-binding pockets shows that L-<br>234 Glu, L-Cys, and L-Asp bind to EAAT3 in similar poses with their amino groups pointing toward 234 Glu, L-Cys, and L-Asp bind to EAAT3 in similar poses with their amino groups pointing toward<br>235 HP2 and interacting with D444. In contrast, the D-Asp's amino group points toward TM8 while 235 HP2 and interacting with D444. In contrast, the D-Asp's amino group points toward TM8 while<br>236 still interacting with D444 (Fig. 4). The subtle binding pose difference between L- and D-Asp is 236 still interacting with D444 (**Fig. 4**). The subtle binding pose difference between L- and D-Asp is<br>237 consistent with the previous structural study on  $Gltr<sub>v</sub><sup>56</sup>$ . Thus, EAAT3 recognizes diverse consistent with the previous structural study on  $Glt_{Tk}^{56}$ . Thus, EAAT3 recognizes diverse 238 substrates by fine-turning sidechain conformations in the binding pocket and subtle changes in<br>239 the substrate poses. 239 the substrate poses.<br>240





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243 244 **Figure 4. The substrate-binding pocket of hEAAT3-X with different substrates.** Binding pockets with L-Asp (a), L-Glu (b, PDB: 8CTC), D-Asp (c), and L-Cys (d). The substrates and 245 pockets with L-Asp (**a**), L-Glu (**b**, PDB: 8CTC), D-Asp (**c**), and L-Cys (**d**). The substrates and interacting residues are shown as sticks. Dashed black lines show the interactions between the 246 interacting residues are shown as sticks. Dashed black lines show the interactions between the residues and the substrates. The transport domains are superposed on their cytoplasmic halves 247 residues and the substrates. The transport domains are superposed on their cytoplasmic halves<br>248 (residues 314-372 and 442-465). (residues 314-372 and 442-465).

## 250 **Partially open gate in the outward-facing L-Cys-bound state.**<br>251 HP2 gate occludes substrates in the binding site of EAATs befo

251 HP2 gate occludes substrates in the binding site of EAATs before their translocation across the membrane. The superposition of the transport domains (residues 80-120 and 280-470) of L-Cys-252 membrane. The superposition of the transport domains (residues 80-120 and 280-470) of L-Cys-<br>253 bound iOFS\* and iOFS with OFS produced RMSDs of 0.607 Å and 0.692 Å, suggesting that 253 bound iOFS\* and iOFS with OFS produced RMSDs of 0.607 Å and 0.692 Å, suggesting that overall transport domains are almost identical in the three states. We found well-defined density 254 overall transport domains are almost identical in the three states. We found well-defined density<br>255 at the three sodium sites in iOFS<sup>\*</sup>, and the surrounding residues feature appropriate geometry to 255 at the three sodium sites in iOFS\*, and the surrounding residues feature appropriate geometry to<br>256 coordinate Na<sup>+</sup> (**Supplementary Fig. 8a**). Thus, iOFS\*-L-Cys is in the fully-bound occluded coordinate Na<sup>+</sup> (**Supplementary Fig. 8a**). Thus, iOFS\*-L-Cys is in the fully-bound occluded<br>257 state with L-Cys, three Na<sup>+</sup> ions, and a closed HP2. iOFS shows nearly identical geometry of the state with L-Cys, three Na<sup>+</sup> ions, and a closed HP2. iOFS shows nearly identical geometry of the sodium-binding sites, an excess density corresponding to L-Cys, and a closed HP2, suggesting it 258 sodium-binding sites, an excess density corresponding to L-Cys, and a closed HP2, suggesting it<br>259 is also a fully-bound occluded state, even if the resolution is insufficient to visualize  $Na<sup>+</sup>$  ions is also a fully-bound occluded state, even if the resolution is insufficient to visualize  $Na<sup>+</sup>$  ions<br>260 unambiguously. By contrast, in OFS, we could find extra densities at the substrate-binding site, 260 unambiguously. By contrast, in OFS, we could find extra densities at the substrate-binding site,<br>261 the Na1 and Na3 sites, but not the Na2 site. The HP2 tip (i.e., the GVPN<sub>410-413</sub> loop between the 261 the Na1 and Na3 sites, but not the Na2 site. The HP2 tip (i.e., the GVPN<sub>410-413</sub> loop between the two helical arms of HP2) is positioned roughly in the middle between the wide-open OFS-Na<sup>+</sup> two helical arms of HP2) is positioned roughly in the middle between the wide-open OFS-Na<sup>+</sup><br>263 state and the fully-bound, closed iOFS\*-L-Cys state: it moves away from the substrate-binding 263 state and the fully-bound, closed iOFS\*-L-Cys state; it moves away from the substrate-binding<br>264 pocket by about 4.5 Å compared to the iOFS\* structure (**Fig. 5a**). Thus, the substrate-binding 264 pocket by about 4.5 Å compared to the iOFS<sup>\*</sup> structure (**Fig. 5a**). Thus, the substrate-binding<br>265 pocket is exposed to solvent (**Fig. 5b, c**), and we found two extra densities assigned to water 265 pocket is exposed to solvent (**Fig. 5b, c**), and we found two extra densities assigned to water<br>266 molecules in the pocket. While OFS-L-Cys lacks interactions between L-Cys and HP2, which 266 molecules in the pocket. While OFS-L-Cys lacks interactions between L-Cys and HP2, which<br>267 are present in iOFS\*-L-Cys (**Supplementary Fig. 8c, d**), the remainder of L-Cys coordination is 267 are present in iOFS<sup>\*</sup>-L-Cys (**Supplementary Fig. 8c, d**), the remainder of L-Cys coordination is<br>268 preserved (**Supplementary Fig. 8e, f**). In the iOFS<sup>\*</sup>-L-Cys structure, residues SASIGA<sub>403-408</sub> 268 preserved (**Supplementary Fig. 8e, f**). In the iOFS\*-L-Cys structure, residues SASIGA<sub>403-408</sub> form the last 2 helical turns of the HP2a arm, and the main chain oxygen atoms of S405, I406, 269 form the last 2 helical turns of the HP2a arm, and the main chain oxygen atoms of S405, I406, 270 and A408 coordinate the Na<sup>+</sup> at the Na2 site with the sulfur of M367 and main chain oxygen of 270 and A408 coordinate the Na<sup>+</sup> at the Na2 site with the sulfur of M367 and main chain oxygen of 271 T364 in TM7a (**Fig. 5d, e**). The side chain of the conserved S405 residue points toward TM7a, 271 T364 in TM7a (**Fig. 5d, e**). The sidechain of the conserved S405 residue points toward TM7a,<br>272 forming a water-mediated hydrogen bond and stabilizing the closed HP2 configuration. In 272 forming a water-mediated hydrogen bond and stabilizing the closed HP2 configuration. In contrast, the SASIGA<sub>403-408</sub> region is unwound in the OFS-L-Cys structure; the S405 side chain 273 contrast, the SASIGA<sub>403-408</sub> region is unwound in the OFS-L-Cys structure; the S405 side chain<br>274 weakly interacts with the L-Cys thiolate group (**Supplementary Fig. 8c, d**). The geometry of the 274 weakly interacts with the L-Cys thiolate group (**Supplementary Fig. 8c, d**). The geometry of the<br>275 Na2 site is disrupted with distances to the main chain oxygen atoms of S405, I406, and A408 275 Na2 site is disrupted with distances to the main chain oxygen atoms of S405, I406, and A408<br>276 being 1.3, 3.8, and 5.5 Å, respectively (Fig. 5f. supplementary Fig. 8a. b). These features 276 being 1.3, 3.8, and 5.5 Å, respectively (**Fig. 5f, supplementary Fig. 8a, b**). These features suggest the OFS-L-Cys structure captures an intermediate before the last sodium binds at the 277 suggest the OFS-L-Cys structure captures an intermediate before the last sodium binds at the Na2 site and the HP2 gate closes (Supplementary Movie 1). Na2 site and the HP2 gate closes (**Supplementary Movie 1**).





282<br>283 **Figure 5. The partially open HP2 gate in OFS-L-Cys.** (a), The tip of HP2 of OFS-L-Cys<br>284 (pastel blue) is in between the wide-open HP2 observed in Na<sup>+</sup>-only bound OFS (white, PDB: (pastel blue) is in between the wide-open HP2 observed in  $Na^+$ -only bound OFS (white, PDB: 285  $\,$  8CV2) and the fully closed HP2 in iOFS\*-L-Cys (slate blue). The distances between  $\alpha$  carbons 285 8CV2) and the fully closed HP2 in iOFS\*-L-Cys (slate blue). The distances between  $\alpha$  carbons 286 of P412 in the HP2 tip of the three states are shown as dashed red lines. The transport domains 286 of P412 in the HP2 tip of the three states are shown as dashed red lines. The transport domains are superposed as in Figure 4. Only L-Cys in iOFS\* is shown as sticks for clarity. (b, c), The 287 are superposed as in Figure 4. Only L-Cys in iOFS<sup>\*</sup> is shown as sticks for clarity. (**b, c**), The surface representation of iOFS<sup>\*</sup>-L-Cys (**b**) and OFS-L-Cys (**c**) binding sites. HP2 (red) occludes 288 surface representation of iOFS<sup>\*</sup>-L-Cys (**b**) and OFS-L-Cys (**c**) binding sites. HP2 (red) occludes the pocket in iOFS<sup>\*</sup> (**b**) but allows solvent access in OFS (**c**). (**d**), The Na2 site in the three states 289 the pocket in iOFS\* (**b**) but allows solvent access in OFS (**c**). (**d**), The Na2 site in the three states with protein structures colored as in (**a**). The red box shows the part of the structure enlarged in **e** 290 with protein structures colored as in (**a**). The red box shows the part of the structure enlarged in **e** 291 and **f**. (**e**, **f**), The formed Na2 site with the bound Na<sup>+</sup> ion in iOFS<sup>\*</sup>-L-Cys (**e**), and the distorted and **f**. (**e, f**), The formed Na2 site with the bound Na<sup>+</sup> ion in iOFS\*-L-Cys (**e**), and the distorted Na2 site in OFS-L-Cys (**f**). The dashed black lines represent the interactions between residues 292 Na2 site in OFS-L-Cys (**f**). The dashed black lines represent the interactions between residues and the ion (**e**) or the distance between the main chain oxygens of 1406 and A408 and the site of 293 and the ion (**e**) or the distance between the main chain oxygens of I406 and A408 and the site of Na2 binding, shown as a transparent purple sphere (**f**). Na2 binding, shown as a transparent purple sphere (**f**).

### <sup>296</sup>**Discussion**

297<br>298 298 EAAT3, an electrogenic acidic amino acid and cysteine transporter, orchestrates amino acid<br>299 metabolism and protects cells from oxidative stress. Our structures visualize hEAAT3 299 metabolism and protects cells from oxidative stress. Our structures visualize hEAAT3<br>200 recognizing four substrates: L-Asp, D-Asp, L-Glu, and L-Cys. Supported by the binding assays. 300 recognizing four substrates: L-Asp, D-Asp, L-Glu, and L-Cys. Supported by the binding assays,<br>301 they suggest that EAAT3 transports L-Cys in thiolate form, consistent with previous studies<sup>14</sup>. they suggest that EAAT3 transports L-Cys in thiolate form, consistent with previous studies<sup>14</sup>.<br>302. The transporter coordinates acidic amino acids and L-Cys thiolate by fine-tuning the position of 302 The transporter coordinates acidic amino acids and L-Cys thiolate by fine-tuning the position of<br>303 the same residues, especially the pivotal R447, which coordinates the substrate side-chain acidic 303 the same residues, especially the pivotal R447, which coordinates the substrate side-chain acidic<br>304 moiety. R447 is replaced with threonine and cysteine in the neutral amino acid transporters 304 moiety. R447 is replaced with threonine and cysteine in the neutral amino acid transporters<br>305 ASCT1 and 2, respectively, and a recently reported structure of ASCT2 with L-alanine<sup>45</sup> ASCT1 and 2, respectively, and a recently reported structure of ASCT2 with L-alanine<sup>45</sup> suggests that ASCT2 transports L-Cys in the thiol form. (**Supplementary Fig. 9a, b**). The 306 suggests that ASCT2 transports L-Cys in the thiol form. (**Supplementary Fig. 9a, b**). The EAAT3 R447C mutant does not bind or transport acidic amino acids while it still transports L-307 EAAT3 R447C mutant does not bind or transport acidic amino acids while it still transports L-<br>308 Cys and neutral amino acids via the electroneutral exchange mechanism, similar to ASCT2<sup>57</sup>. Cys and neutral amino acids via the electroneutral exchange mechanism, similar to  $\text{ASCT2}^{57}$ .<br>309. The main chain amino and carboxyl groups or the substrate are coordinated by the highly 309 The main chain amino and carboxyl groups or the substrate are coordinated by the highly<br>310 conserved D444 and N451, respectively. Together, D444, R447, and N451 are the critical 310 conserved D444 and N451, respectively. Together, D444, R447, and N451 are the critical<br>311 determinants of substrate specificity. 311 determinants of substrate specificity.<br>312

312 313 R-2HG is an oncometabolite that rewires the metabolism of cancer cells by inhibiting α-KG-<br>314 dependent dioxygenases and changing epigenetic modification patterns<sup>58</sup>. R-2HG might also dependent dioxygenases and changing epigenetic modification patterns<sup>58</sup>. R-2HG might also<br>315. promote tumor growth through other mechanisms<sup>59,60</sup>. Recently, it was proposed that R-2HG promote tumor growth through other mechanisms<sup>59,60</sup>. Recently, it was proposed that R-2HG<br>316. enters cells and their mitochondria through EAAT3 localized to the plasma and mitochondrial 316 enters cells and their mitochondria through EAAT3 localized to the plasma and mitochondrial<br>317 membranes, respectively<sup>32</sup>. This proposal prompted us to examine R-2HG binding and transport membranes, respectively<sup>32</sup>. This proposal prompted us to examine R-2HG binding and transport<br>318. using purified protein. We found that up to 100 mM R-2HG did not significantly thermally 318 using purified protein. We found that up to 100 mM R-2HG did not significantly thermally<br>319 stabilize hEAAT3g in differential scanning fluorimetry experiments, suggesting that it binds 319 stabilize hEAAT3g in differential scanning fluorimetry experiments, suggesting that it binds<br>320 weakly or does not bind. The SSME assays performed with 3 mM substrates, a saturating 320 weakly or does not bind. The SSME assays performed with 3 mM substrates, a saturating<br>321 concentration for L-Asp, showed similar transport currents for L-Asp, D-Asp, and L-Glu and a 321 concentration for L-Asp, showed similar transport currents for L-Asp, D-Asp, and L-Glu and a<br>322 smaller current for L-Cys (Fig. 1c). The D-Glu transport current was shallow, persisting much 322 smaller current for L-Cys (**Fig. 1c**). The D-Glu transport current was shallow, persisting much<br>323 longer ligand perfusion time, suggesting that D-Glu transport is very slow. Indeed, D-Glu is a 323 longer ligand perfusion time, suggesting that D-Glu transport is very slow. Indeed, D-Glu is a<br>324 low-affinity EAAT3 substrate with Km of ~1.8 mM, approximately 60-fold higher than L-Glu<sup>61</sup>. 124 Iow-affinity EAAT3 substrate with Km of ~1.8 mM, approximately 60-fold higher than L-Glu<sup>61</sup>.<br>125. In contrast, R-2HG produced no current (Fig. 1c). Finally, R-2HG added at 10 mM did not bind 325 In contrast, R-2HG produced no current (**Fig. 1c**). Finally, R-2HG added at 10 mM did not bind<br>326 to EAAT3-X in cryo-EM imaging experiments. R-2HG is an analog of D-Glu, in which an to EAAT3-X in cryo-EM imaging experiments. R-2HG is an analog of D-Glu, in which an 327 alcohol moiety replaces the amino group. Compared to D-Glu, R-2HG loses a critical salt bridge<br>328 between the amino group and D444. Mutations of D444 in EAAT3 cause a dramatic reduction of 328 between the amino group and D444. Mutations of D444 in EAAT3 cause a dramatic reduction of affinity for amino acids<sup>62,63</sup>, suggesting that EAAT3 would bind R-2HG even weaker than D-Glu. affinity for amino acids<sup>62,63</sup>, suggesting that EAAT3 would bind R-2HG even weaker than D-Glu.<br>330 Thus, our results and structural considerations do not support the hypothesis that EAAT3 is the 330 Thus, our results and structural considerations do not support the hypothesis that EAAT3 is the<br>331 R-2HG transporter in cancer cells. However, it should be noted that R-2HG concentrations in  $R-2HG$  transporter in cancer cells. However, it should be noted that R-2HG concentrations in<br>332 tumors can reach 30 mM<sup>60</sup>, and it is, in principle, possible that EAAT3 transports R-2HG with tumors can reach 30 mM<sup>60</sup>, and it is, in principle, possible that EAAT3 transports R-2HG with<br>333 very low affinity. very low affinity.

334<br>335 335 L-Cys is a rate-limiting substrate of GSH biosynthesis and, therefore, is an important metabolite<br>336 in maintaining the cell redox status, methylation potential, and protection against oxidative stress 336 in maintaining the cell redox status, methylation potential, and protection against oxidative stress<br>337 in all cell types. In the bloodstream, ~95 % of L-Cys is oxidized to cystine, which can be taken 337 in all cell types. In the bloodstream, ~95 % of L-Cys is oxidized to cystine, which can be taken<br>338 up by SLC7A11 transporter system xc- into glial cells and reduced to L-Cys. Interestingly, up by SLC7A11 transporter system xc- into glial cells and reduced to L-Cys. Interestingly,  $339$  ASCT2, which could also contribute to L-Cys uptake into glia, has a similar Km of ~20  $\mu$ M for <br>340 L-Cys and other neutral amino acids but a nearly 10-fold lower Vmax, suggesting L-Cys is not 340 L-Cys and other neutral amino acids but a nearly 10-fold lower Vmax, suggesting L-Cys is not<br>341 an efficient substrate<sup>17</sup>. EAAT2, highly expressed in glial cells, does not uptake L-Cys well an efficient substrate<sup>17</sup>. EAAT2, highly expressed in glial cells, does not uptake L-Cys well<br>342. because of its low affinity for the amino acid with Km of ~1-2 mM, much higher than ~250 µM 342 because of its low affinity for the amino acid with Km of ~1-2 mM, much higher than ~250  $\mu$ M<br>343 concentration of L-Cys and its derivatives in the plasma<sup>64</sup>. EAAT3 is the main L-Cys transporter concentration of L-Cys and its derivatives in the plasma<sup>64</sup>. EAAT3 is the main L-Cys transporter<br>344 in the neurons with a Km of ~100-200  $\mu$ M<sup>23</sup>, about 10-fold above L-Glu, and a similar Vmax. 344 in the neurons with a Km of ~100-200  $\mu$ M<sup>23</sup>, about 10-fold above L-Glu, and a similar Vmax.<br>345 Interestingly, the comparison between L-Glu-bound EAAT3 and EAAT2 and L-Cys-bound 345 Interestingly, the comparison between L-Glu-bound EAAT3 and EAAT2 and L-Cys-bound<br>346 EAAT3 does not reveal significant structural differences between EAAT2 and EAAT3 that 346 EAAT3 does not reveal significant structural differences between EAAT2 and EAAT3 that<br>347 would explain similar affinity for L-Glu and drastically different affinities for L-Cys. Thus, 347 would explain similar affinity for L-Glu and drastically different affinities for L-Cys. Thus,<br>348 allosteric effects outside of the binding site might contribute to different substrate specificities. 348 allosteric effects outside of the binding site might contribute to different substrate specificities.<br>349 Indeed, previous studies in an archaeal homolog Glt<sub>Ph</sub> suggested that differences in protein 349 Indeed, previous studies in an archaeal homolog Glt<sub>Ph</sub> suggested that differences in protein<br>350 packing and dynamics might contribute to substrate affinity and selectivity <sup>65,66</sup>. packing and dynamics might contribute to substrate affinity and selectivity  $65,66$ .

351

352 Kinetic studies on EAATs and their homologs suggest that substrate and ion binding proceeds<br>353 via partially bound intermediates, such as the transporter bound to the substrate and one or two 353 via partially bound intermediates, such as the transporter bound to the substrate and one or two<br>354 sodium ions, before forming the transport-component complex of the substrate and three sodium 354 sodium ions, before forming the transport-component complex of the substrate and three sodium<br>355 ions. EAATs bind substrates rapidly on the sub-millisecond time scale but transport them slower, 355 ions. EAATs bind substrates rapidly on the sub-millisecond time scale but transport them slower,<br>356 with turnover times estimates in milliseconds to tens of milliseconds, resulting in biphasic with turnover times estimates in milliseconds to tens of milliseconds, resulting in biphasic 357 electrical currents comprised of the binding peak currents and the lower steady-state currents<sup>67,68</sup>.

358 The initial binding is weak, with Kd of ~140  $\mu$ M for EAAT2 significantly higher than the<br>359 transporter Km of 10-20  $\mu$ M<sup>69</sup>. Our structure of EAAT3 in OFS with bound L-Cys and partially<br>360 open HP2 gate with cle transporter Km of 10-20  $\mu$ M<sup>69</sup> 359 transporter Km of 10-20  $\mu$ M<sup>o9</sup>. Our structure of EAAT3 in OFS with bound L-Cys and partially<br>360 open HP2 gate with clear densities at the Na1 and Na3 sites but a distorted empty Na2 site might<br>361 directly visuali

360 open HP2 gate with clear densities at the Na1 and Na3 sites but a distorted empty Na2 site might<br>361 directly visualize the proposed low-affinity binding intermediate.<br>362 Interestingly, the transporter has demonstrate 361 directly visualize the proposed low-affinity binding intermediate.<br>362 Interestingly, the transporter has demonstrated different conform<br>364 on the substrate. Thus, in L- and D-Asp, we only observed the contrast, the t 362<br>363<br>364<br>365<br>366<br>367 363 Interestingly, the transporter has demonstrated different conformational preferences depending<br>364 on the substrate. Thus, in L- and D-Asp, we only observed the EAAT3-X in the iOFS\*. In<br>365 contrast, the transporter bo 364 on the substrate. Thus, in L- and D-Asp, we only observed the EAAT3-X in the iOFS\*. In contrast, the transporter bound to L-Glu populated iOFS\* and OFS with closed HP2, while the transporter bound to L-Cys populated iO 365 contrast, the transporter bound to L-Glu populated iOFS\* and OFS with closed HP2, while the<br>366 transporter bound to L-Cys populated iOFS\*, iOFS, and OFS with the partially open HP2 gate.<br>367 These observations should transporter bound to L-Cys populated iOFS\*, iOFS, and OFS with the partially open HP2 gate.<br>367 These observations should be taken cautiously because the grids were not prepared identically in<br>368 all cases: the L-Cys grid 367 These observations should be taken cautiously because the grids were not prepared identically in<br>368 all cases: the L-Cys grids were prepared by rapidly freezing the protein seconds after adding the<br>369 substrate, whil 368 all cases: the L-Cys grids were prepared by rapidly freezing the protein seconds after adding the<br>369 substrate, while others were prepared using protein equilibrated with substrates. Nevertheless,<br>370 the observed di 369 substrate, while others were prepared using protein equilibrated with substrates. Nevertheless,<br>370 the observed differences suggest that to the relative energies of transporter states along the<br>371 transport cycle de 370 the observed differences suggest that to the relative energies of transporter states along the<br>371 transport cycle depend on the substrates. If so, we would speculate that the transporters might<br>372 show substrate-dep 371 transport cycle depend on the substrates. If so, we would speculate that the transporters might<br>372 show substrate-dependent transport rates, as was shown for  $EmrE^{70}$ .<br>373 **Methods** show substrate-dependent transport rates, as was shown for  $EmrE^{70}$ . show substrate-dependent transport rates, as was shown for EmrE<sup>70</sup>.<br>373<br>374 **Methods**<br>375 **Protein expression and purification.** 

373<br>374<br>375<br>376<br>377<br>378 374 **Methods**<br>375 **Protein e**<br>377 The hEA<br>378 described. 376<br>377<br>378<br>379<br>380 **Protein expression and purification.**<br>
377 The hEAAT3g and Cysmini K269C.<br>
378 described. In brief, hEAAT3 construc<br>
379 Isolated membrane pellets were solub<br>
380 10mM L-Asp, 10mM EDTA, 10mM 377 The hEAAT3g and Cysmini K269C/W441C EAAT3g proteins were purified as previously<br>378 described. In brief, hEAAT3 constructs were expressed in suspension FreeStyle<sup>TM</sup> 293-F cells.<br>379 Isolated membrane pellets were sol described. In brief, hEAAT3 constructs were expressed in suspension FreeStyle<sup>TM</sup> 378 described. In brief, hEAAT3 constructs were expressed in suspension FreeStyle<sup>1M</sup> 293-F cells.<br>379 Isolated membrane pellets were solubilized in a buffer containing 50 $\Box$ mM Tris-Cl at pH $\Box$ 8.0,<br>380 1 $\Box$ mM L-Asp, 1379 Isolated membrane pellets were solubilized in a buffer containing 50 $\Box$ mM Tris-Cl at pH $\Box$ 8.0,<br>380 1 $\Box$ mM L-Asp, 1 $\Box$ mM EDTA, 1 $\Box$ mM tris(2-carboxyethyl) phosphine (TCEP), 10% glycerol,<br>381 1:200 dilution of pro 380 1 $\Box$ mM L-Asp, 1 $\Box$ mM EDTA, 1 $\Box$ mM tris(2-carboxyethyl) phosphine (TCEP), 10% glycerol, 1:200 dilution of protease inhibitor cocktail (catalog no. P8340, Sigma-Aldrich), 1 $\Box$ mM phenylmethylsulfonyl fluoride (PMSF), 381 1:200 dilution of protease inhibitor cocktail (catalog no. P8340, Sigma-Aldrich),  $1 \square mM$ <br>382 phenylmethylsulfonyl fluoride (PMSF), 1% dodecyl-β-D-maltopyranoside (DDM, Anatrace) and<br>383 0.2% cholesteryl hemisuccinate 382 phenylmethylsulfonyl fluoride (PMSF), 1% dodecyl-β-D-maltopyranoside (DDM, Anatrace) and<br>383 0.2% cholesteryl hemisuccinate (CHS; Sigma-Aldrich) at 4<sup>0</sup>°C, overnight. The insoluble<br>384 material was removed by centrif 383 0.2% cholesteryl hemisuccinate (CHS; Sigma-Aldrich) at  $4\degree$ C, overnight. The insoluble material was removed by centrifugation, and the supernatant was incubated with Strep-Tactin Sepharose resin (GE Healthcare) for 384 material was removed by centrifugation, and the supernatant was incubated with Strep-Tactin<br>385 Sepharose resin (GE Healthcare) for  $1 \Box h$  at  $4 \Box^{\circ}C$ . The resin was washed with a buffer<br>386 containing 50 $\Box$ mM Tris 385 Sepharose resin (GE Healthcare) for  $1 \Box h$  at  $4 \Box^{\circ}C$ . The resin was washed with a buffer containing 50 $\Box$ mM Tris-HCl at pH $\Box$ 8.0, 200 $\Box$ mM NaCl, 0.06% glyco-diosgenin (GDN, Anatrace),  $1 \Box$ mM TCEP, 5% glycerol 386 containing 50 $\Box$ mM Tris-HCl at pH $\Box$ 8.0, 200 $\Box$ mM NaCl, 0.06% glyco-diosgenin (GDN, Anatrace), 1 $\Box$ mM TCEP, 5% glycerol and 1 $\Box$ mM L-Asp (wash buffer). The protein was eluted with a wash buffer supplemented with 387 Anatrace),  $1 \Box$ mM TCEP, 5% glycerol and  $1 \Box$ mM L-Asp (wash buffer). The protein was eluted<br>388 with a wash buffer supplemented with  $2.5 \Box$ mM D-desthiobiotin (elution buffer). The N-terminal 388 with a wash buffer supplemented with  $2.5 \square$  mM D-desthiobiotin (elution buffer). The N-terminal variable supplemented with  $2.5 \square$  mM D-desthiobiotin (elution buffer). The N-terminal variable supplemented with  $2.5 \square$ 

389 Strep II and GFP tag was cleaved by overnight PreScission protease digestion at  $4\degree$ C.<br>390 hEAAT3g and Cysmini K269C/W441C EAAT3g were purified by size-exclusion<br>391 chromatography (SEC) in a buffer containing 20 mM 390 hEAAT3g and Cysmini K269C/W441C EAAT3g were purified by size-exclusion<br>391 chromatography (SEC) in a buffer containing 20 mM HEPES-Tris at pH $\Box$ 7.4, 1 mM L-Asp,<br>392 and 0.01% GDN with/without 1mM TCEP. The Cysmini K2 391 chromatography (SEC) in a buffer containing  $20 \Box \text{m}$ M HEPES-Tris at pH $\Box$ 7.4,  $1 \Box \text{m}$ M L-Asp,<br>392 and 0.01% GDN with/without 1mM TCEP. The Cysmini K269C/W441C EAAT3g protein was<br>393 concentrated to ~0.5 mg/ml a 392 and 0.01% GDN with/without 1mM TCEP. The Cysmini K269C/W441C EAAT3g protein was<br>393 concentrated to ~0.5 mg/ml and incubated with a 20-fold molar excess of HgCl<sub>2</sub> for 15 min at<br>394 room temperature. Then, crosslinked 393 concentrated to ~0.5 mg/ml and incubated with a 20-fold molar excess of HgCl<sub>2</sub> for 15 min at<br>394 room temperature. Then, crosslinked hEAAT3-X was purified by SEC in a buffer containing 20<br>395 mM Hepes-Tris pH 7.4, 10 394 room temperature. Then, crosslinked hEAAT3-X was purified by SEC in a buffer containing 20 mM Hepes-Tris pH 7.4, 100 mM N-methyl-D-glucamine (NMDG) chloride, and 0.01% GDN to remove sodium and L-Asp. The eluted protei 395 mM Hepes-Tris pH 7.4, 100 mM N-methyl-D-glucamine (NMDG) chloride, and 0.01% GDN to<br>396 remove sodium and L-Asp. The eluted protein was diluted ~1,000-fold into a buffer containing<br>397 20 mM Hepes-Tris pH 7.4, 200 mM 396 remove sodium and L-Asp. The eluted protein was diluted ~1,000-fold into a buffer containing<br>397 20 mM Hepes-Tris pH 7.4, 200 mM NaCl, and 0.01% GDN and concentrated to ~5 mg/ml using<br>398 100 kD MWCO concentrators (Am 397 20 mM Hepes-Tris pH 7.4, 200 mM NaCl, and 0.01% GDN and concentrated to ~5 mg/ml using<br>398 100 kD MWCO concentrators (Amicon). EAAT3-X in 200 mM NaCl was incubated with the<br>599 final concentration of 10 mM L-Lap, D-As 398 100 kD MWCO concentrators (Amicon). EAAT3-X in 200 mM NaCl was incubated with the<br>599 final concentration of 10 mM L-Lap, D-Asp, or R2-HG for about 1 hour on ice before making<br>590 grids. EAAT3-X in 200 mM NaCl was mixe 399 final concentration of 10 mM L-Lap, D-Asp, or R2-HG for about 1 hour on ice before making<br>300 grids. EAAT3-X in 200 mM NaCl was mixed with L-Cys at a final concentration of 10 mM and<br>301 put on grids immediately.<br>302 T

quared action and the U-Cys at a final concentration of 10 mM and<br>
401 put on grids immediately.<br>
402 **Thermostability assays.**<br>
403 Purified hEAAT3g was diluted ~4000-fold in a buffer containing 50 mM Hepes-Tris pH 7.4, 401 put on grids immediately.<br>402 **Thermostability assays.**<br>403 Purified hEAAT3g was di<br>404 mM NMDG, and 0.01%<br>405 concentrator. The concent 402 **Thermostability assays.**<br>403 Purified hEAAT3g was d:<br>404 mM NMDG, and 0.019<br>405 concentrator. The concent<br>406 Tris pH 7.4, 200 mM NaC 403 Purified hEAAT3g was diluted ~4000-fold in a buffer containing 50 mM Hepes-Tris pH 7.4, 100 mM NMDG, and 0.01% GDN and concentrated to ~100  $\mu$ M using a 100 kD MWCO concentrator. The concentrated protein was diluted 404 mM NMDG, and 0.01% GDN and concentrated to ~100  $\mu$ M using a 100 kD MWCO<br>405 concentrator. The concentrated protein was diluted 20-fold in a buffer containing 50 mM Hepes-<br>406 Tris pH 7.4, 200 mM NaCl, and 0.01% GDN, 405 concentrator. The concentrated protein was diluted 20-fold in a buffer containing 50 mM Hepes-<br>406 Tris pH 7.4, 200 mM NaCl, and 0.01% GDN, supplemented with 10 mM or 100 mM ligands. To<br>407 promote L-Cys binding, the 406 Tris pH 7.4, 200 mM NaCl, and 0.01% GDN, supplemented with 10 mM or 100 mM ligands. To<br>407 promote L-Cys binding, the concentrated protein was diluted 20-fold in a buffer containing 50<br>408 mM Tris-Cl, pH 8.8, 200 mM N 407 promote L-Cys binding, the concentrated protein was diluted 20-fold in a buffer containing 50 mM Tris-Cl, pH 8.8, 200 mM NaCl, and 0.01% GDN, supplemented with 100 mM L-Cys. The thermostability assay was performed usi 408 mM Tris-Cl, pH 8.8, 200 mM NaCl, and 0.01% GDN, supplemented with 100 mM L-Cys. The<br>409 thermostability assay was performed using Tycho NT.6 (NanoTemper Technologies). Protein<br>410 samples were heated from 35 °C to 95 409 thermostability assay was performed using Tycho NT.6 (NanoTemper Technologies). Protein<br>410 samples were heated from 35 °C to 95 °C at 30 °C per minute; the intrinsic protein fluorescence<br>411 was recorded at 330 nm an 410 samples were heated from 35 °C to 95 °C at 30 °C per minute; the intrinsic protein fluorescence<br>411 was recorded at 330 nm and 350 nm. The amplitude ratio, A350/A330 as a function of<br>412 temperature, and its first der 411 was recorded at 330 nm and 350 nm. The amplitude ratio, A350/A330 as a function of<br>412 temperature, and its first derivative were calculated by the Tycho NT.6 software. The inflection<br>413 temperature (Ti) corresponds t temperature, and its first derivative were calculated by the Tycho NT.6 software. The inflection<br>
413 temperature (Ti) corresponds to the peak of the derivative. All measurements were repeated at<br>
414 least thrice on indep temperature (Ti) corresponds to the peak of the derivative. All measurements were repeated at<br>
414 least thrice on independently prepared protein samples except the D-Glu sample.<br> **415** Proteoliposome reconstitution and s 114 least thrice on independently prepared protein samples except the D-Glu sample.<br>
115 **Proteoliposome reconstitution and solid-supported membrane electrophysiol**<br>
116 The proteoliposome reconstitution and SSME were perf

**Proteoliposome reconstitution and solid-supported membrane electrophysiology (SSME).**<br>416 The proteoliposome reconstitution and SSME were performed as previously described<sup>34</sup>. In brie<br>417 4 mg/ml liposomes comprising 5:5

The proteoliposome reconstitution and SSME were performed as previously described<sup>34</sup>. In brief, 4 mg/ml liposomes comprising 5:5:2 (w:w) 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, Avanti Polar Lipids), 1-pal

417 4 mg/ml liposomes comprising 5:5:2 (w:w) 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine<br>418 (POPC, Avanti Polar Lipids), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE, 418 (POPC, Avanti Polar Lipids), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE,

419 Avanti Polar Lipids) and CHS were extruded 11 times through 400 nm polycarbonate<br>420 membranes (Avanti Polar Lipids) in a buffer containing 50 mM Hepes-Tris, pH 7.4, 200 mM<br>421 NaCl, 1mM TCEP, 1 mM L-Asp. The resultin 420 membranes (Avanti Polar Lipids) in a buffer containing 50 mM Hepes-Tris, pH 7.4, 200 mM<br>421 NaCl, 1mM TCEP, 1 mM L-Asp. The resulting unilamellar liposomes were destabilized by<br>422 incubating with 5:1 (w:w) DDM-CHS at 421 NaCl, 1mM TCEP, 1 mM L-Asp. The resulting unilamellar liposomes were destabilized by<br>422 incubating with 5:1 (w:w) DDM-CHS at a 1:0.75 lipid-detergent ratio for 30 min at 23 °C. 0.4<br>423 mg purified hEAAT3g was incubat 422 incubating with 5:1 (w:w) DDM-CHS at a 1:0.75 lipid-detergent ratio for 30 min at 23 °C. 0.4<br>423 mg purified hEAAT3g was incubated with liposomes at a lipid-protein ratio (LPR) of 10 for 30<br>424 min at 23 °C. The deter 423 mg purified hEAAT3g was incubated with liposomes at a lipid-protein ratio (LPR) of 10 for 30 min at 23 °C. The detergent was removed by incubating with 100 mg fresh Bio-Beads SM-2 (Bio-Rad) for 1h at 23 °C, 1 h at 4 ° 424 min at 23 °C. The detergent was removed by incubating with 100 mg fresh Bio-Beads SM-2<br>425 (Bio-Rad) for 1h at 23 °C, 1 h at 4 °C (three times), overnight at 4 °C, and finally 2 h at 4 °C.<br>426 The proteoliposomes were 425 (Bio-Rad) for 1h at 23 °C, 1 h at 4 °C (three times), overnight at 4 °C, and finally 2 h at 4 °C.<br>426 The proteoliposomes were collected by centrifugation at 86,600 g for 45 min at 4 $\Box$ °C and were<br>427 resuspended in 426 The proteoliposomes were collected by centrifugation at 86,600 g for 45 min at  $4\square$  °C and were<br>427 resuspended in the SSME resting buffer containing 100 mM potassium phosphate, pH 7.4, 2 mM<br>428 MgSO<sub>4</sub>. The proteoli 427 resuspended in the SSME resting buffer containing 100 mM potassium phosphate, pH 7.4, 2 mM<br>428 MgSO<sub>4</sub>. The proteoliposomes were frozen in liquid nitrogen and thawed at room temperature.<br>429 The centrifugation and free MgSO<sub>4</sub>. The proteoliposomes were frozen in liquid nitrogen and thawed at room temperature.<br>
429 The centrifugation and freeze-thaw steps were repeated three times for buffer exchange. Then,<br>
430 the proteoliposomes were e The centrifugation and freeze-thaw steps were repeated three times for buffer exchange. Then,<br>
430 the proteoliposomes were extruded 11 times through a 400 nm polycarbonate membrane and<br>
431 immediately deposited onto the 430 the proteoliposomes were extruded 11 times through a 400 nm polycarbonate membrane and<br>431 immediately deposited onto the SF-N1 sensor 3mm (Nanion Technologies). The transport-<br>432 coupled currents were recorded on a S 431 immediately deposited onto the SF-N1 sensor 3mm (Nanion Technologies). The transport-<br>432 coupled currents were recorded on a SURFE2R N1 instrument (Nanion Technologies). The non-<br>433 activating buffer containing 100 m coupled currents were recorded on a SURFE2R N1 instrument (Nanion Technologies). The non-<br>activating buffer containing 100 mM sodium phosphate, pH 7.4, and 2 mM MgSO<sub>4</sub> flowed<br>through the sensor to build ion gradients acro 433 activating buffer containing 100 mM sodium phosphate, pH 7.4, and 2 mM MgSO<sub>4</sub> flowed<br>434 through the sensor to build ion gradients across the proteoliposomes. The transport-coupled<br>435 current was activated by flowin 434 through the sensor to build ion gradients across the proteoliposomes. The transport-coupled<br>435 current was activated by flowing the activation buffer containing 100 mM sodium phosphate, pH<br>436 7.4, 2 mM MgSO<sub>4</sub>, and 3 435 current was activated by flowing the activation buffer containing 100 mM sodium phosphate, pH<br>436 7.4, 2 mM MgSO<sub>4</sub>, and 3 mM ligands. At least three sensors were recorded for each independent<br>437 proteoliposome prepa 2436 7.4, 2 mM MgSO<sub>4</sub>, and 3 mM ligands. At least three sensors were recorded for each independent<br>
437 proteoliposome preparation.<br>
438 Cryo-EM sample preparation and data acquisition.<br>
439 3.5 µl of protein samples at

437 proteoliposome preparation.<br>438 Cryo-EM sample preparation<br>439 3.5 µl of protein samples at ~<br>440 carbon-coated 300 mesh gole<br>441 ethane using FEI Mark IV V **438 Cryo-EM sample preparation and data acquisition.**<br>
439 3.5 µl of protein samples at ~5 mg/ml were applied to g<br>
440 carbon-coated 300 mesh gold grids. The grids were blow<br>
441 ethane using FEI Mark IV Vitrobot at  $4^$ 439 3.5 μl of protein samples at ~5 mg/ml were applied to glow-discharged Quantifoil R1.2/1.3 holey<br>
440 carbon-coated 300 mesh gold grids. The grids were blotted for 3 s and plunge-frozen into liquid<br>
441 ethane using F 440 carbon-coated 300 mesh gold grids. The grids were blotted for 3 s and plunge-frozen into liquid<br>441 ethane using FEI Mark IV Vitrobot at 4°C and 100% humidity. For the hEAAT3-X with 10 mM<br>442 L-Asp sample, 13,349 movi 441 ethane using FEI Mark IV Vitrobot at 4°C and 100% humidity. For the hEAAT3-X with 10 mM<br>442 L-Asp sample, 13,349 movies were collected at a nominal magnification of 100,000-fold with a<br>443 calibrated pixel size of 1.1 442 L-Asp sample, 13,349 movies were collected at a nominal magnification of 100,000-fold with a<br>
443 calibrated pixel size of 1.16 Å. The nominal defocus value -1.0 ~ -2.5 µm and total dose 40 e<sup>-</sup>/Å<sup>2</sup><br>
444 (dose rate 7 calibrated pixel size of 1.16 Å. The nominal defocus value -1.0 ~ -2.5  $\mu$ m and total dose 40 e<sup>-</sup>/Å<sup>2</sup> 443<br>444<br>445<br>446<br>447<br>448 (dose rate 7.98 e $\angle$ Å<sup>2</sup> 444 (dose rate 7.98 e<sup>-</sup>/Å<sup>2</sup>/s) were applied to the data collection. For the hEAAT3-X with 10 mM R-<br>445 2HG dataset, 11,952 movies were collected at a nominal magnification of 105,000-fold with a<br>446 calibrated pixel siz 2HG dataset, 11,952 movies were collected at a nominal magnification of 105,000-fold with a calibrated pixel size of 0.8443 Å using the counting model. The nominal defocus value of ~0.8-<br>2.2 µm and the total dose of 50.54 446 calibrated pixel size of 0.8443 Å using the counting model. The nominal defocus value of ~0.8-<br>447 2.2  $\mu$ m and the total dose of 50.54  $e^t/\text{\AA}^2$  (dose rate 33.69  $e^t/\text{\AA}^2$ /s) were applied to data collection.<br>4 2.2 µm and the total dose of 50.54 e<sup>-</sup>/ $\AA$ <sup>2</sup> (dose rate 33.69 e<sup>-</sup>/ $\AA$ <sup>2</sup> 2.2  $\mu$ m and the total dose of 50.54  $e^{\frac{1}{A^2}}$  (dose rate 33.69  $e^{\frac{1}{A^2}}$ ) were applied to data collection.<br>448 For the hEAAT3-X with 10 mM D-Asp sample, 4,190 movies were collected at a nominal<br>449 magnification 448 For the hEAAT3-X with 10 mM D-Asp sample, 4,190 movies were collected at a nominal magnification of 64,000-fold with a calibrated pixel size of 1.076 Å using the counting model. A magnification of 64,000-fold with a c magnification of 64,000-fold with a calibrated pixel size of 1.076  $\AA$  using the counting model. A

nominal defocus value  $-0.5 \sim -2.0$  µm was applied to data collection, with the total dose 52.19 e<sup>-</sup> 450<br>451<br>452<br>453<br>454<br>455  $/\text{\AA}^2$  (dose rate 26.09e<sup>-</sup>/ $\text{\AA}^2$ 451 /Å<sup>2</sup> (dose rate 26.09e/Å<sup>2</sup>/s) distributed over 40 frames in each movie. For data collection on<br>452 hEAAT3-X with 10 mM L-Cys sample, subset A (5,765 movies) and subset B (3,757 movies)<br>453 were collected at a nomina 452 hEAAT3-X with 10 mM L-Cys sample, subset A (5,765 movies) and subset B (3,757 movies)<br>453 were collected at a nominal magnification of 105,000-fold with a calibrated pixel size of 0.4125<br>454 Å using the super-resoluti 453 were collected at a nominal magnification of 105,000-fold with a calibrated pixel size of 0.4125<br>454 Å using the super-resolution model. A nominal defocus value -0.8~-2.4 µm was applied to data<br>455 collection, with th 454 Å using the super-resolution model. A nominal defocus value -0.8~-2.4  $\mu$ m was applied to data<br>455 collection, with the total dose 58.25 e<sup>-</sup>/Å<sup>2</sup> (dose rate 29.12 e<sup>-</sup>/Å<sup>2</sup>/s, subset A) and 58.01 (dose rate<br>456 29.0 collection, with the total dose 58.25 e<sup>-</sup>/ $\AA$ <sup>2</sup> (dose rate 29.12 e<sup>-</sup>/ $\AA$ <sup>2</sup> 455 collection, with the total dose 58.25 e/ $\overrightarrow{A}^2$  (dose rate 29.12 e/ $\overrightarrow{A}^2$ /s, subset A) and 58.01 (dose rate<br>456 29.00 e/ $\overrightarrow{A}^2$ /s, subset B) e/ $\overrightarrow{A}^2$  distributed over 50 frames in each movie. The hEAAT3-29.00 e<sup>-</sup>/ $\AA^2$ /s, subset B) e<sup>-</sup>/ $\AA^2$ 29.00 e<sup>7</sup>/Å<sup>2</sup>/s, subset B) e<sup>7</sup>/Å<sup>2</sup> distributed over 50 frames in each movie. The hEAAT3-X with 10 mM L-Asp data was auto-collected using EPU on Glacios with Falcon4i camera at Weill Cornell Cryo-EM facility; other dat mM L-Asp data was auto-collected using EPU on Glacios with Falcon4i camera at Weill Cornell<br>458 Cryo-EM facility; other datasets were auto-collected using Leginon<sup>71</sup> on Titan Krios with Gantan<br>459 K3 camera at the Simons Cryo-EM facility; other datasets were auto-collected using Leginon<sup>71</sup> 458 Cryo-EM facility; other datasets were auto-collected using Leginon<sup>71</sup> on Titan Krios with Gantan<br>459 K3 camera at the Simons Electron Microscopy Center (SEMC) at New York Structural Biology<br>460 Center (SEMC-NYSBC, R-2 459 K3 camera at the Simons Electron Microscopy Center (SEMC) at New York Structural Biology<br>
460 Center (SEMC-NYSBC, R-2HG, and D-Asp datasets), and at New York University Langone's<br>
461 Cryo-EM laboratory (L-Cys dataset) 460 Center (SEMC-NYSBC, R-2HG, and D-Asp datasets), and at New York University Langone's<br>
461 Cryo-EM laboratory (L-Cys dataset) and. All microscopes were equipped with a 20 -eV energy<br>
462 filter.<br>
463 Cryo-EM image proc 461 Cryo-EM laboratory (L-Cys dataset) and. All microscopes were equipped with a 20 -eV energy<br>462 filter.<br>463 Cryo-EM image processing.<br>465 For the hEAAT3-X with 10 mM L-Asp dataset, the movies were aligned using MotionC

462 filter.<br>
463<br>
464 **Cryo-**<br>
465 For th<br>
466 impler 464<br>465<br>466<br>467<br>468 464 **Cryo-EM image processing.**<br>465 For the hEAAT3-X with 10 r<br>466 implemented in Relion 4, and<br>467 4.1<sup>73</sup>. Over 12 million particl<br>468 with a box size of 120 pixel For the hEAAT3-X with 10 mM L-Asp dataset, the movies were aligned using MotionCorr2<sup>72</sup> 466<br>467<br>468<br>469<br>470 466 implemented in Relion 4, and the micrograph CTF parameters were estimated using CtfFfind-<br>467 4.1<sup>73</sup>. Over 12 million particles were selected by Laplacia-of-Gaussian (LoG)<sup>74</sup> and extracted<br>468 with a box size of 120 4.1<sup>73</sup>. Over 12 million particles were selected by Laplacia-of-Gaussian  $(LoG)^{74}$  and extracted 467 4.1<sup>75</sup>. Over 12 million particles were selected by Laplacia-of-Gaussian (LoG)<sup>74</sup> and extracted<br>468 with a box size of 120 pixels (2-fold binning) from 12,021 micrographs. The particles were<br>469 divided into four par 468 with a box size of 120 pixels (2-fold binning) from 12,021 micrographs. The particles were<br>469 divided into four parts and imported into CryoSPARC v4<sup>75</sup> for 2D classification. 378,103<br>470 particles showing clear seco divided into four parts and imported into CryoSPARC v4<sup>75</sup> 469 divided into four parts and imported into CryoSPARC v4<sup>75</sup> for 2D classification. 378,103 particles showing clear secondary features were selected and used for 1 round of *ab initio* reconstruction; the resulting 211, particles showing clear secondary features were selected and used for 1 round of *ab initio*<br>reconstruction; the resulting 211,611 particles were subjected to nonuniform refinement<sup>76</sup><br>(hereafter NUR) with C1 symmetry to g reconstruction; the resulting 211,611 particles were subjected to nonuniform refinement<sup>76</sup> 471<br>472<br>473<br>474<br>475<br>476 472 (hereafter NUR) with C1 symmetry to generate a good template, while for generating 5 decoy<br>473 templates, 448,378 junk particles were selected and subjected to *ab initio* reconstruction for less<br>474 than 10 iteration templates, 448,378 junk particles were selected and subjected to *ab initio* reconstruction for less<br>than 10 iterations. More than 10 million particles after 2D selection that removed obvious non-<br>protein junks (2D cleanin 474 than 10 iterations. More than 10 million particles after 2D selection that removed obvious non-<br>
475 protein junks (2D cleaning) were further cleaned by heterogeneous refinement with 1 good<br>
476 template and 5 decoy n 475 protein junks (2D cleaning) were further cleaned by heterogeneous refinement with 1 good<br>476 template and 5 decoy noise volumes (heterogeneous refinement cleaning, HRC). 1,240,537<br>477 particles were refined to 4.84 Å 476 template and 5 decoy noise volumes (heterogeneous refinement cleaning, HRC). 1,240,537 particles were refined to 4.84 Å by NUR with C1 symmetry. Then, the particles were re-<br>478 imported into Relion through PyEM<sup>77</sup> a 477 particles were refined to 4.84 Å by NUR with C1 symmetry. Then, the particles were re-<br>478 imported into Relion through PyEM<sup>77</sup> and extracted with a box size of 240 pixels without<br>479 binning. These particles were im imported into Relion through PyEM<sup>77</sup> 478 imported into Relion through PyEM<sup> $\prime\prime$ </sup> and extracted with a box size of 240 pixels without binning. These particles were imported into CryoSPARC and subjected to HRC and NUR, generating a 3.30 Å map. The resulting 479 binning. These particles were imported into CryoSPARC and subjected to HRC and NUR, generating a 3.30 Å map. The resulting 1,217,462 particles were subjected to two rounds of 480 generating a 3.30 Å map. The resulting 1,217,462 particles were subjected to two rounds of  $\frac{1}{2}$  polishing in Relion, HRC, and NUR. The final 908,281 particles were refined to 2.87 Å. Then,<br>
482 the particles were expanded using C3 symmetry and applied to local 3D classification with a<br>
483 mask covering the protomer 482 the particles were expanded using C3 symmetry and applied to local 3D classification with a<br>483 mask covering the protomer in Relion. No other conformations were found following symmetry<br>484 expansion and local 3D clas mask covering the protomer in Relion. No other conformations were found following symmetry<br>
484 expansion and local 3D classification. For the hEAAT3-X with 10 mM R2-HG dataset, the<br>
485 movie alignments, and micrograph CT 484 expansion and local 3D classification. For the hEAAT3-X with 10 mM R2-HG dataset, the<br>485 movie alignments, and micrograph CTF estimation were performed in Relion 4. 3,622,598<br>486 particles were auto-picked using templ movie alignments, and micrograph CTF estimation were performed in Relion 4. 3,622,598<br>
particles were auto-picked using template picking and extracted with a box size of 160 pixels (2-<br>
fold binning). The particles were im particles were auto-picked using template picking and extracted with a box size of 160 pixels (2-<br>
487 fold binning). The particles were imported into CryoSPARC v4 for 2D classification, 2D<br>
488 cleaning, and HRC as the L-487 fold binning). The particles were imported into CryoSPARC v4 for 2D classification, 2D cleaning, and HRC as the L-Asp dataset. 1,233,807 particles, refined to 3.81 Å, were re-imported into Relion 4 and extracted with 488 cleaning, and HRC as the L-Asp dataset. 1,233,807 particles, refined to 3.81 Å, were re-imported<br>489 into Relion 4 and extracted with a box size of 320 pixels without binning. These particles were<br>490 further processe 489 into Relion 4 and extracted with a box size of 320 pixels without binning. These particles were<br>490 further processed as the L-Asp dataset; the final 3.07 Å map was reconstituted using 773,970<br>491 particles. Symmetry e 490 further processed as the L-Asp dataset; the final 3.07 Å map was reconstituted using 773,970 particles. Symmetry expansion and local 3D classification performed in CryoSPARC sorted out about 8% of monomers in a minor c 491 particles. Symmetry expansion and local 3D classification performed in CryoSPARC sorted out<br>492 about 8% of monomers in a minor conformation. For the hEAAT3-X with 10 mM D-Asp dataset,<br>493 the movies were aligned by Mo 492 about 8% of monomers in a minor conformation. For the hEAAT3-X with 10 mM D-Asp dataset,<br>493 the movies were aligned by MotionCorr2 implemented in Relion 3, and the micrograph CTF<br>494 parameters were estimated using Ct 493 the movies were aligned by MotionCorr2 implemented in Relion 3, and the micrograph CTF<br>494 parameters were estimated using CtfFfind-4.1. 3,346,010 particles were selected by LoG,<br>495 extracted with a box size of 256 pi parameters were estimated using CtfFfind-4.1. 3,346,010 particles were selected by LoG,<br>extracted with a box size of 256 pixels, and imported into CryoSPARC v3 for 2D classification.<br>496 719,954 particles showing secondary 495 extracted with a box size of 256 pixels, and imported into CryoSPARC v3 for 2D classification.<br>496 719,954 particles showing secondary features were selected and subjected to *ab initio*<br>497 reconstruction followed by 496 719,954 particles showing secondary features were selected and subjected to *ab initio*<br>497 reconstruction followed by NUR with C3 symmetry to generate a good template. 3,075,243<br>498 particles after 2D cleaning were su 497 reconstruction followed by NUR with C3 symmetry to generate a good template. 3,075,243 particles after 2D cleaning were subjected to two rounds of HRC using one good model and seven decoy volumes. 444,289 particles wer 498 particles after 2D cleaning were subjected to two rounds of HRC using one good model and<br>499 seven decoy volumes. 444,289 particles were selected and refined to 3.29 Å by NUR. After two<br>500 rounds of polishing in Reli 499 seven decoy volumes. 444,289 particles were selected and refined to 3.29 Å by NUR. After two<br>500 rounds of polishing in Relion, HRC, and NUR, 391,308 particles were refined to 2.73 Å by NUR<br>501 with C3 symmetry. Symme 500 rounds of polishing in Relion, HRC, and NUR, 391,308 particles were refined to 2.73 Å by NUR with C3 symmetry. Symmetry expansion and local 3D classification did not identify multiple conformations in this dataset. For 501 with C3 symmetry. Symmetry expansion and local 3D classification did not identify multiple<br>502 conformations in this dataset. For the hEAAT3-X with L-Cys subset A, 5,756 movies were<br>503 aligned using MotionCorr2 implem 502 conformations in this dataset. For the hEAAT3-X with L-Cys subset A, 5,756 movies were<br>503 aligned using MotionCorr2 implemented in Relion 3 with 2-fold binning. The micrograph CTF<br>504 parameters were estimated using C 503 aligned using MotionCorr2 implemented in Relion 3 with 2-fold binning. The micrograph CTF<br>504 parameters were estimated using CtfFfind-4.1. 2,538,702 particles were selected using LoG and<br>505 extracted with a box size 504 parameters were estimated using CtfFfind-4.1. 2,538,702 particles were selected using LoG and<br>505 extracted with a box size of 300 pixels. Particles were imported into CryoSPARC v3 for 2D<br>506 classification. The good t 505 extracted with a box size of 300 pixels. Particles were imported into CryoSPARC v3 for 2D classification. The good template was generated using particles showing 2D features as previously described. Separately, all th 506 classification. The good template was generated using particles showing 2D features as<br>507 previously described. Separately, all the particles after 2D classification were used in *ab initio*<br>508 reconstruction with le previously described. Separately, all the particles after 2D classification were used in *ab initio*<br>508 reconstruction with less than 10 iterations to generate 7 noise volumes. 2,268,928 particles after<br>509 2D cleaning we 508 reconstruction with less than 10 iterations to generate 7 noise volumes. 2,268,928 particles after 2D cleaning were further cleaned by HRC with one good template and 7 decoy noise volumes.<br>510 After that, 902,201 parti 509 2D cleaning were further cleaned by HRC with one good template and 7 decoy noise volumes.<br>510 After that, 902,201 particles were reconstituted to 2.8 Å with C3 symmetry by NUR. Then, the<br>511 particles were re-imported 510 After that, 902,201 particles were reconstituted to 2.8 Å with C3 symmetry by NUR. Then, the<br>511 particles were re-imported to Relion using PyEM and subjected to Bayesian polishing. The<br>511 particles were re-imported t 511 particles were re-imported to Relion using PyEM and subjected to Bayesian polishing. The

512 polished particles underwent one round of HRC and NUR to improve resolution. The second<br>513 round of polishing, HRC, and NUR procedures finally generated a 2.43 Å map with 653,778<br>514 particles. Subset B was processed 514 particles. Subset B was processed in parallel using a similar strategy. 1,641,561 particles were<br>515 extracted from 3,757 micrographs and imported into CryoSPARC for 2D classification. After 2D<br>516 cleaning, 1,474,916 514 particles. Subset B was processed in parallel using a similar strategy. 1,641,561 particles were<br>
515 extracted from 3,757 micrographs and imported into CryoSPARC for 2D classification. After 2D<br>
516 cleaning, 1,474,91 515 extracted from 3,757 micrographs and imported into CryoSPARC for 2D classification. After 2D cleaning, 1,474,916 particles underwent further cleaning through heterogeneous refinement. The resulting 614,371 particles w 516 cleaning, 1,474,916 particles underwent further cleaning through heterogeneous refinement. The<br>517 resulting 614,371 particles were refined to 2.92 Å by NUR with C3 symmetry. After two rounds<br>518 of polishing in Relio 517 resulting 614,371 particles were refined to 2.92 Å by NUR with C3 symmetry. After two rounds<br>518 of polishing in Relion, HRC, and NUR, 444,946 particles were refined to 2.54 Å. 1,112,764<br>519 particles from two subsets 519 particles from two subsets were combined and refined to 2.36 Å by NUR. These particles were<br>520 applied to symmetry expansion and local 3D classification. Individual classes of interest were<br>521 further subjected to lo 519 particles from two subsets were combined and refined to 2.36 Å by NUR. These particles were<br>520 applied to symmetry expansion and local 3D classification. Individual classes of interest were<br>521 further subjected to l

applied to symmetry expansion and local 3D classification. Individual classes of interest were<br>
521 further subjected to local refinement in CryoSPARC.<br>
522 **Model building and refinement.**<br>
523 hEAAT3-X structures with bo 521 further subjected to local refinement in CryoSPARC.<br>522 **Model building and refinement.**<br>523 hEAAT3-X structures with bound L-Glu in iOFS\*, 1<br>524 iOFS, and hEAAT3g with bound L-Asp (PDB acce<br>525 6X2Z respectively) were 522 **Model building and refinement.**<br>523 **hEAAT3-X** structures with bound<br>524 **iOFS**, and hEAAT3g with bound<br>525 6X2Z respectively) were fitted i<br>526 manually adjusted in COOT<sup>79</sup> ar hEAAT3-X structures with bound L-Glu in iOFS\*, hEAAT3-X bound to  $Na<sup>+</sup>$ 523 hEAAT3-X structures with bound L-Glu in iOFS\*, hEAAT3-X bound to Na<sup>+</sup> ions in OFS, and<br>524 iOFS, and hEAAT3g with bound L-Asp (PDB accession codes: 8CTC, 8CV2 and 8CV3, and<br>525 6X2Z respectively) were fitted into EM 524 iOFS, and hEAAT3g with bound L-Asp (PDB accession codes: 8CTC, 8CV2 and 8CV3, and 6X2Z respectively) were fitted into EM density maps using ChimeraX<sup>78</sup>. The models were manually adjusted in COOT<sup>79</sup> and subjected to 6X2Z respectively) were fitted into EM density maps using Chimera $X^{78}$ 525 6X2Z respectively) were fitted into EM density maps using ChimeraX<sup>78</sup>. The models were<br>526 manually adjusted in COOT<sup>79</sup> and subjected to real-space refinement in Phenix<sup>80</sup>. Structural<br>527 model validation was perfo manually adjusted in  $\text{COOT}^{79}$  and subjected to real-space refinement in Phenix<sup>80</sup> manually adjusted in COOT<sup>79</sup> and subjected to real-space refinement in Phenix<sup>80</sup>. Structural<br>527 model validation was performed in Phenix. All the structural figures were prepared using<br>528 ChimeraX.<br>**Acknowledgments:** W

model validation was performed in Phenix. All the structural figures were prepared using<br>528 ChimeraX.<br>529 Acknowledgments:<br>530 We thank Dr. Xiaoyu Wang, Dr. Qianyi Wu, Dr. Krishna Reddy, and Dr. Yun Huang for the<br>531 usef 528 ChimeraX.<br>529 Acknowled<br>530 We thank I<br>531 useful discu<br>532 NYU Lange 529 **Acknowledgments:**<br>530 We thank Dr. Xiaoy<br>531 useful discussions. V<br>532 NYU Langone's Cryc<br>533 for assistance with 530 We thank Dr. Xiaoyu Wang, Dr. Qianyi Wu, Dr. Krishna Reddy, and Dr. Yun Huang for the useful discussions. We thank Jing Wang at SEMC-NYSBC, Bing Wang and William Rice at NYU Langone's Cryo-EM laboratory, and Edwin Fluc 531 useful discussions. We thank Jing Wang at SEMC-NYSBC, Bing Wang and William Rice at NYU Langone's Cryo-EM laboratory, and Edwin Fluck at Weill Cornell Cryo-EM facility center for assistance with data collection. **Fundi** 532 NYU Langone's Cryo-EM laboratory, and Edwin Fluck at Weill Cornell Cryo-EM facility center<br>533 for assistance with data collection. **Funding**: This work was supported by HHMI and the<br>534 National Institute of Neurologi 533 for assistance with data collection. **Funding**: This work was supported by HHMI and the<br>534 National Institute of Neurological Disorders and Stroke R37NS085318 to Olga Boudker. Some<br>535 of this work was performed at th 534 National Institute of Neurological Disorders and Stroke R37NS085318 to Olga Boudker. Some<br>535 of this work was performed at the Simons Electron Microscopy Center at the New York<br>536 Structural Biology Center, with majo 535 of this work was performed at the Simons Electron Microscopy Center at the New York<br>536 Structural Biology Center, with major support from the Simons Foundation (SF349247). **Author**<br>537 **contribution and interest confl** 536 Structural Biology Center, with major support from the Simons Foundation (SF349247). **Author**<br>537 **contribution and interest conflict**: B.Q. performed the experiments; B.Q. and O.B. conceived<br>538 the projects, analyzed 537 **contribution and interest conflict**: B.Q. performed the experiments; B.Q. and O.B. conceived<br>538 the projects, analyzed data, and wrote the manuscript. Competing interests: The authors declare<br>539 no competing commerc the projects, analyzed data, and wrote the manuscript. Competing interests: The authors declare<br>539 no competing commercial interests. **Data availability**: The Cryo-EM maps and atomic<br>540 coordinates have been deposited in 539 no competing commercial interests. **Data availability**: The Cryo-EM maps and atomic<br>540 coordinates have been deposited in the Electron Microscopy Data Bank (EMDB) and Protein<br>541 Data Bank (PDB) under accession code: 540 coordinates have been deposited in the Electron Microscopy Data Bank (EMDB) and Protein<br>541 Data Bank (PDB) under accession code: EMD-46586, PDB-9D66 (hEAAT3-X with L-Asp<br>541 Data Bank (PDB) under accession code: EMD-4 541 Data Bank (PDB) under accession code: EMD-46586, PDB-9D66 (hEAAT3-X with L-Asp

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- 542 bound at iOFS\*); EMD-46587, (hEAAT3-X in sodium and R-2HG at OFS); EMD-46588, PDB-<br>9D67 (hEAAT3-X with D-Asp bound at iOFS\*); EMD-46589, PDB-9D68 (hEAAT3-X with L-<br>544 Cys bound at OFS, semi-open HP2); EMD-46590, PDB-9 543 9D67 (hEAAT3-X with D-Asp bound at iOFS\*); EMD-46589, PDB-9D68 (hEAAT3-X with L-<br>
544 Cys bound at OFS, semi-open HP2); EMD-46590, PDB-9D69 (hEAAT3-X with L-Cys bound at<br>
iOFS); EMD-46591, PDB-9D6A (hEAAT3-X with L-Cys
- 544 Cys bound at OFS, semi-open HP2); EMD-46590, PDB-9D69 (hEAAT3-X with L-Cys bound at iOFS); EMD-46591, PDB-9D6A (hEAAT3-X with L-Cys bound at iOFS\*); EMD-46592 (hEAAT3-X in sodium and L-Cys at IFS).<br>547 545 iOFS); EMD-46591, PDB-9D6A (hEAAT3-X with L-Cys bound at iOFS\*); EMD-46592<br>546 (hEAAT3-X in sodium and L-Cys at IFS).<br>547
- 546 (hEAAT3-X in sodium and L-Cys at IFS).<br>547
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