GLUT4 is the primary glucose transporter in adipose and skeletal muscle tissues. Its cellular trafficking is regulated by insulin signaling. Failed or reduced plasma membrane localization of GLUT4 is associated with diabetes. Here, we report the cryo-EM structures of human GLUT4 bound to a small molecule inhibitor cytochalasin B (CCB) at resolutions of 3.3 Å in both detergent micelles and lipid nanodiscs. CCB-bound GLUT4 exhibits an inward-open conformation. Despite the nearly identical conformation of the transmembrane domain to GLUT1, the cryo-EM structure reveals an extracellular glycosylation site and an intracellular helix that is invisible in the crystal structure of GLUT1. The structural study presented here lays the foundation for further mechanistic investigation of the modulation of GLUT4 trafficking. Our methods for cryo-EM analysis of GLUT4 will also facilitate structural determination of many other small size solute carriers.
Glucose, being the primary fuel, a versatile bio-precursor, and a signaling molecule, is tightly controlled for metabolic homeostasis via various mechanisms, such as hormonal regulation by insulin and glucagon. Insulin lowers blood sugar level by triggering cellular uptake of glucose. The major facilitator superfamily (MFS) glucose transporter GLUT4 mediates the rate-limiting glucose cellular uptake in adipocytes and muscle cells, and thus plays a vital role in insulin-responsive glucose metabolism.

At basal state, GLUT4 is primarily distributed on the membranes of the trans-Golgi network (TGN), endosomes and 50–70 nm tubulo-vesicular structures known as the GLUT4 storage vesicles (GSVs). Upon insulin stimulation, GLUT4 is quickly transported from these intracellular structures to the plasma membrane, resulting in rapid consumption of glucose from blood. Impairment of cellular glucose uptake due to compromised insulin availability or sensing underlies diabetes mellitus. Therefore, elucidating the structure and working mechanism of GLUT4 will both facilitate our understanding of the fundamental energy metabolism, and shed light on the development of potential intervention strategies for the deleterious disease.

GLUT4, encoded by SLC2A4, is one of the 14 members of the SLC2A family. Among the SLC2A members, GLUT4 is most closely related to GLUT1, with a sequence identity and similarity of 65% and 79%, respectively. We solved crystal structures of human GLUT1 and GLUT3 in the inward- and outward-facing conformations a few years ago. However, the unique sequences that mediate GLUT4’s membrane trafficking capability, including an FQFI motif on the amino (N) terminus and the LL and TELEY motifs on the carboxyl (C) terminus, are not conserved in the SLC2A family, entailing the need to solve the high-resolution structure of GLUT4.

Despite extensive efforts, we simply could not obtain diffracting crystals for GLUT4. Single particle cryo-electron microscopy (cryo-EM) is more powerful in grapping with low-yield samples with conformational heterogeneity. However, the size of GLUT4, with 509 residues in length, has represented a major technical obstacle for cryo-EM analysis.

In fact, small membrane proteins lacking relatively rigid bulky domains outside the membrane region represent one of the most challenging targets for cryo-EM, as the thick micelles or nanodiscs impede image processing and classification. They lack distinct features to facilitate the discrimination of extracellular/ luminal and intracellular sides. Proteins with pseudo-symmetries are even more challenging, as the repeats within the membrane cannot be distinguished at low resolution during data processing. Recently reported cryo-EM structures of small membrane proteins either have higher symmetry, or consist of a relatively rigid soluble domain. To overcome these technical barriers, binders like antibodies or nanobodies have to be selected to facilitate cryo-EM analysis.

One of our research interests has been to develop and optimize methods for solving high-resolution structures of small membrane proteins using advanced technology of cryo-EM. We started with proteins containing relatively flexible soluble domains, such as the monocarboxylate transporter MCT1 and the complex of Scap and Insig. Encouraged by these successful attempts, we took a direct assault on human GLUT4. Here, we report the near atomic resolution cryo-EM structures of GLUT4 bound to a small molecule inhibitor Cytochalasin B (CCB) in detergent micelles and in lipid nanodiscs.

**Results**

**Activity characterization and cryo-EM analysis of GLUT4.** The full-length wildtype human GLUT4 was fused with an N-terminal Flag tag and transiently expressed in HEK293F cells. After sequential purification through anti-FLAG affinity resin and size-exclusion chromatography (SEC) (Supplementary Fig. 2a), the transport activity of the peak fraction was examined in a proteoliposome-based counterflow assay. GLUT4 has a nominal $K_m$ of 5.4 mM and $V_{max}$ of 3.7 μmol/mg/min for D-glucose transport (Supplementary Fig. 2b). CCB inhibits the glucose transport activity of GLUT4 with an IC$_{50}$ of 3.7 μM (Supplementary Fig. 2c).

As analyzed above, GLUT4, which has a two-fold pseudo symmetry in the membrane and lacks a bulky soluble domain, presented many challenges for cryo-EM analysis. Our previous work with MCT1 and the Scap/Insig-2 complex suggests that the size of the micelles could have a direct impact on 2D and 3D classifications. We therefore set out to screen for detergents that gave rise to the best protein signal (Fig. 1).

We applied GLUT4 to a Superdex 200 column that was pre-equilibrated in the presence of the following detergents, 0.02% (w/v) glyco-diosgenin (GDN), 0.02% (w/v) n-dodecyl-β-D-maltoside (DDM) plus 0.002% (w/v) cholesteryl hemisuccinate tris salt (CHS), 0.01% (w/v) lauryl maltose neopentyl glycol (LMNG) plus 0.001% (w/v) CHS, and 0.1% (w/v) n-nonyl-β-D-glucopyranoside (β-NG). Indeed, different detergents gave rise to distinct elution peak volumes, earliest at 15 ml with GDN and latest at 15.7 ml with β-NG (Fig. 1c). We also successfully reconstituted the purified GLUT4 into nanodiscs of POPC plus cholesterol surrounded by the membrane scaffold protein MSP1D1. The elution volume of nanodisc-embedded GLUT4 is slightly earlier than β-NG (Fig. 1c).

Next, we examined the proteins purified in different conditions using cryo-EM. To stabilize the structure of GLUT4, 1 mM CCB was added to the purified proteins. Details for cryo-sample preparation and cryo-EM data acquisition are presented in detail in Methods. From the 2D class averages, it is immediately clear that GDN or DDM/CHS results in a weaker protein signal with relatively large and thick micelle. The other three conditions, β-NG, LMNG/CHS, and nanodisc, all present smaller size, and features characteristic of the transmembrane segments (TMs) are discernible (Fig. 1d). We, therefore, proceeded with these three conditions for 3D reconstruction.

For structural determination of small membrane proteins, a good reference map is critical for further classification and refinement. Using Ab-initio construction in cryoSPARC, we were able to obtain a decent initial model for the dataset of GLUT4 in β-NG (Supplementary Fig. 3), but not for that in LMNG/CHS or nanodisc. Details can be found in Methods. Following our previously developed “guided multi-reference 3D classification” and “seed”-facilitated 3D classification strategy, the resolutions of GLUT4 embedded in the LMNG/CHS micelles and nanodiscs both reached 3.3 Å after several rounds of 3D classification and refinement by cryoSPARC (Fig. 1e, Supplementary Figs. 4, 5 and Supplementary Table 2). The excellent EM map allowed for assignment of 464 side chains in the 12 TMs and the intracellular helical (ICH) domain (Fig. 1f, Supplementary Fig. 6, and Supplementary Table 2).

Inward-open structure of GLUT4 bound to CCB. In the structure of GLUT4, the amino terminal domain (NTD, containing TMs 1-6) and carboxyl terminal domain (CTD, containing TMs 7-12) enclose a large cavity that opens to the intracellular side, a state defined as inward-open (Fig. 2a, left). The inhibitor CCB is known to inhibit GLUTs with inward-open conformation, which is consistent with our present structure. CCB is accommodated in the big cleft between the NTD and CTD (Fig. 2a, right upper panel). CCB comprises three ring...
structures, a macrolide ring, a nine-membered bicyclic ring, and a phenyl ring, all of which are well-resolved in the cryo-EM map (Fig. 2a, right lower panel).

The macrolide ring of CCB is coordinated by Asn176 and Trp404 through polar interactions, and Phe38, Trp404, and Trp428 through hydrophobic interaction (Fig. 2b, c). The bicyclic ring is nestled in a hydrophobic cavity constituted by Ile42, Ile180, Ile184, Ile303, Phe307, and Phe395. The coordination is buttressed by hydrogen bonds with Gln298, Gln299, and Trp404. The phenyl ring interacts with Ile180, Gln177, and Pro401 via hydrophobic contacts (Fig. 2b–d).

Comparison of inward-open GLUT4 and GLUT1. We next compared GLUT4 to GLUT1, whose structures have also been captured in the inward-open state in the presence of CCB (PDB code: SEQJ) or β-NG (PDB code: 4PYP)12,31. The cryo-EM structure of GLUT4 can be superimposed to the crystal structures of GLUT1-CCB and GLUT1-NG with RMSD values of 1.13 Å and 1.09 Å over 439 and 435 aligned Cα atoms, respectively, with identical transmembrane domains (Fig. 3a). Among all the CCB-coordinating residues, two loci are different between the two proteins; Ile42 and Asn176 in GLUT4 are respectively substituted with Thr30 and His160 in GLUT1. The phenyl ring of CCB rotates by about 60 degrees in the two structures (Fig. 3b).

GLUT1-4 all contain a conserved N-linked glycosylation site on an extracellular helix designated TM1e, which is a bent extension of TM1. To facilitate crystallization, the glycosylation site in GLUT1, GLUT3, and GLUT5 was eliminated by single point mutation or deglycosylation treatment 12,13,31,32. In the cryo-EM map for GLUT4, a characteristic glycan density is observed contiguous with Asn57, exemplifying the unique

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**Fig. 1 Structural determination of human GLUT4.**

- **a** General strategies for structural resolution of solute carriers (SLCs) using single particle cryo-EM. While homo-oligomeric SLCs are convenient targets for cryo-EM analysis, relatively rigid soluble domains, either intrinsic to the protein, or binders like antibodies (Ab) or nanobodies (Nb), are usually required to increase the size of the protein for cryo-EM imaging. There has been no report on the cryo-EM structure of monomeric SLCs with transmembrane domain only.
- **b** The two-fold pseudo symmetry of monomeric major facilitator superfamily (MFS) transporters poses another tier of technical challenge for solving their cryo-EM structures. A canonical MFS fold contains 12 transmembrane helices (TMHs) organized to an amino terminal domain (NTD) and a C-terminal domain (CTD), which exhibit a two-fold pseudo symmetry around an axis that is perpendicular to the membrane plane. The lack of distinctive soluble domain and the pseudo symmetry imposes serious challenges for discriminating the orientations of the proteins embedded in the disc-like micelles or nanodiscs.
- **c** Detergent screening for optimal conditions for cryo-EM analysis of human GLUT4. To reduce the impact of the detergent micelles or nanodiscs, we screened for detergents that could yield smaller micelles. Shown here are overlaid SEC profiles of GLUT4 purified in the presence of 0.02% (w/v) GDN, 0.02% (w/v) DDM plus 0.002% (w/v) CHS, 0.01% (w/v) LMNG plus 0.001% (w/v) CHS, 0.1% (w/v) β-NG, and nanodiscs. Source data are provided.
- **d** Representative 2D class averages of GLUT4 in indicated detergents and nanodiscs. c Cryo-EM structural analysis of GLUT4. GLUT4 purified in β-NG gave rise to a decent initial reference and the 3D EM reconstruction was refined to 4.1 Å resolution. 3D EM reconstructions of GLUT4 in LMNG/CHS and nanodisc both reached higher resolutions to 3.3 Å.
- **e** Overall cryo-EM map of GLUT4 in a nanodisc. The density is domain colored, blue for the NTD, purple for the CTD, yellow for the intracellular helical (ICH) domain, and gray for the micelle. All density and structure figures were prepared in ChimeraX52.
power of cryo-EM in resolving posttranslational modifications (Fig. 3c).

Other than the observation of the extracellular glycosylation, ICH exhibits the most evident differences between GLUT4 and GLUT1. Our previous structural analysis of GLUT1 and GLUT3 defined five ICH helices, ICH1-ICH4 between the N and C domains and ICH5 after TM12.12,13. ICH3 and ICH4 display minor positional shifts between GLUT4 and GLUT1 (Fig. 3d). The major distinction occurs to ICH5, which is invisible in the structure of GLUT1, but clearly resolved in the EM map of GLUT4 (Fig. 3d and Supplementary Fig. 6). We examined the functional role of ICH5 based on the structural comparison of outward-facing GLUT3 and inward-open GLUT4.

ICH5 is important for the transport activity of GLUTs. The ICH domain undergoes major rearrangement between the outward-facing structure of GLUT3 and inward-open structure of GLUT4 (Fig. 4a). In the structure of GLUT3 (PDB code: 4ZW9), the ICH domain serves as a latch to secure the closure of the N and C domains on the intracellular side. Phe458 on ICH5 is surrounded by three Arg residues, Arg151 on TM5, Arg210 on ICH1, and Arg398 on TM11 (Fig. 4b, left). These residues are invariant between GLUT3 and GLUT4 (Supplementary Fig. 1). As ICH5 was invisible in previous structures, we did not get a chance to examine the rearrangement of this cation-π cluster. In the cryo-EM map for GLUT4, Phe476 (corresponding to Phe458 in GLUT3) is clearly resolved (Supplementary Fig. 6). The cation-π network is completely disassembled with ICH5 moving away (Fig. 4b, right).

To examine the functional relevance of this intracellular cluster, we substituted each of the Arg residues and Phe476 of GLUT4 with Ala and performed counterflow assay for these variants (Fig. 4c). GLUT4-F476A only retained half of the transport activity of WT, supporting an important role of this ICH5 residue in the alternating access transport process of GLUT4. Ala replacement of Arg169 (equivalent of GLUT3-Arg151), which also interacts with a Glu residue on TM10 and two backbone carbonyl oxygen groups on the loop between TM12 and ICH5, lost more than 70% of the activity. Mutation of Arg228 (equivalent of GLUT3-Arg210), which is mainly involved in bridging ICH1 and ICH3 other than binding to Phe476, reduced the activity by ~40%. Mutation of Arg416 has the least effect on the transport activity, with a reduction of approximately 20%. This result is consistent with the C domain-localization of Arg416, as there is barely any relative motion between the C domain and ICH5 during the alternating access cycle (Fig. 4a).

The assay results are consistent with the structural indication that ICH5 is important for the transport activity of GLUTs.

Discussion

GLUT4 plays a key role in reducing the glucose level in blood and insulin resistance. Insulin stimulates glucose uptake by acutely upregulating GLUT4 surface levels mainly through increased exocytosis of GSVs and decreased endocytosis. Single GLUT4 molecule during its lifetime goes through multiple cycles of exocytosis and endocytosis. Structural resolution of the WT full-length human GLUT4 lays an important framework for further investigation of GLUT4 regulation by insulin signaling. The cellular trafficking of GLUT4 has been shown to be regulated by posttranslational modifications. Cys223, which is at the end of the intracellular end of TM6, can be palmitoylated by DHHC7, an event that controls insulin-dependent translocation.
of GLUT4 to the plasma membrane (Fig. 5a). Future structural determination of palmitoylated GLUT4 may reveal the molecular basis for this regulation.

Four Ser residues, Ser10, Ser274, Thr486, and Ser488, are subject to phosphorylation. Among these, only Ser274 is resolved to be positioned immediately preceding ICH4, while the other three are on the invisible N or C terminal loops (Fig. 5a and Supplementary Fig. 1). Note that Ser488 is right before the LL motif that mediates interaction with adaptor proteins. Such arrangement provides a clue to the regulation of GLUT4 by Ser488 phosphorylation.

None of the GLUT4-unique sequences, 5FQQI8, 489LL490, and 498TELEY502, is visible, likely owing to the intrinsic flexibility of these segments (Fig. 5b). These motifs have been reported to interact with proteins like Golgi-localized γ-ear-containing ARF-binding protein (GGA), retromer, and AP1 adaptor complexes. Positioning of these motifs on the flexible termini ensures that their recognition by the adaptor proteins is independent of the transporter conformation.

In sum, our structural determination of intact human GLUT4 once again exemplifies the power of cryo-EM. It suggests that many other small size SLCs that have pseudo or no symmetry, can be directly analyzed by cryo-EM without the need for screening binders. Our study also marks an important step forward towards the mechanistic understanding of GLUT4 regulation by insulin signaling.

**Methods**

**Protein expression and purification.** Human GLUT4 (UniProt ID: P14762) cDNA or mutations were cloned into a pCAG vector with an N-terminal FLAG tag. 2 mg plasmid and 4 mg polyethylenimine (Polysciences) were preincubated in 50 ml fresh SMM 293-TII medium (Sino Biological) for 30 min before adding into one litre HEK293F cells at density of 2.0 × 10^6 cells per ml. After 48 h incubation at 37 °C under 5% CO2, cells were harvested and resuspended in the buffer containing 25 mM HEPES pH 7.4, 150 mM NaCl, 5 μg/ml aprotinin, 1 μg/ml pepstatin, and 5 μg/ml leupeptin. Cell membrane was solubilized with 2% (w/v) n-dodecyl-β-D-maltoside (DDM, Anatrace) at 4 °C for 2 h. After high-speed centrifugation at 20,000 g for 30 min, the supernatant was loaded onto anti-FLAG M2 resin (Sigma). Then the resin was rinsed with wash buffer containing 25 mM HEPES pH 7.4, 150 mM NaCl, and 0.02% (w/v) DDM. Protein was eluted with wash buffer plus 0.4 mg/ml FLAG peptide. Elution was subjected to Superdex 200 Increase 10/300 GL column (GE Healthcare) in buffer containing 25 mM HEPES pH 7.4, 150 mM NaCl, and 0.1% (w/v) n-nonyl-β-D-glucopyranoside (β-NG, Anatrace). Peak fractions were pooled for further experiments. For purification in other detergents, the detergent was changed from wash buffer to 0.02% (w/v) glyco-diosgenin (GDN, Anatrace), 0.01% (w/v) lauryl maltose neopentyl glycol (LMNG, Anatrace) plus 0.01% (w/v) cholesterol, 0.01% (w/v) glyximo-diosgenin (CDN, Anatrace), 0.01% (w/v) lauryl maltose neopentyl glycol (LMNG, Anatrace) plus 0.1% (w/v) sodium n-octyl-β-D-glucopyranoside (β-NG, Anatrace).

The membrane scaffold protein MSP1D1 with N-terminal 6 × His tag was expressed in E. coli BL21 (DE3) cells. Collected cells were lysed by sonication in lysis buffer (25 mM Tris-HCl pH 7.4, 130 mM NaCl, and 0.02% (w/v) DDM. Protein was eluted with wash buffer plus 0.4 mg/ml FLAG peptide. Elution was subjected to Superdex 200 Increase 10/300 GL column (GE Healthcare) in buffer containing 25 mM HEPES pH 7.4, 150 mM NaCl, and 0.1% (w/v) n-nonyl-β-D-glucopyranoside (β-NG, Anatrace). Peak fractions were pooled for further experiments. For purification in other detergents, the detergent was changed from wash buffer to 0.02% (w/v) glyco-diosgenin (GDN, Anatrace), 0.01% (w/v) lauryl maltose neopentyl glycol (LMNG, Anatrace) plus 0.01% (w/v) cholesterol, 0.01% (w/v) glyximo-diosgenin (CDN, Anatrace), 0.01% (w/v) lauryl maltose neopentyl glycol (LMNG, Anatrace) plus 0.1% (w/v) sodium n-octyl-β-D-glucopyranoside (β-NG, Anatrace).

The membrane scaffold protein MSP1D1 with N-terminal 6 × His tag was expressed in E. coli BL21 (DE3) cells. Collected cells were lysed by sonication in lysis buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl) supplemented with 5 μg/ml aprotinin, 1 μg/ml pepstatin, and 5 μg/ml leupeptin. After centrifugation at 20,000 g...

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**Fig. 3 Structural comparison of inward-open GLUT4 and GLUT1.** a Structural comparison of GLUT4 and GLUT1. Shown here are superimposed structures of GLUT4 (domain colored) with GLUT1 bound to CCB (silver, PDB code: 5EQI) or in the presence of β-NG (pink, PDB code: 4PYP). The TM region remains nearly identical. b Similar coordination of CCB by GLUT4 and GLUT1. Among all the CCB-coordinating residues, there are only two varied residues, Ile42 and Asn176 in GLUT4 are respectively substituted with Thr30 and His160 in GLUT1. c Cryo-EM analysis reveals the glycosylation site on GLUT4. The density for two sugar moieties (green) attach to Asn57 on the extracellular helix TM1e of GLUT4 is contoured at 4 σ. d Deviations of the ICH domain between GLUT4 and GLUT1. In contrast to the nearly identical structures of the TM region, there are deviations of ICH3 and ICH4 between GLUT4 and GLUT1. More importantly, the C-terminal ICH5 is resolved in GLUT4 only.
**Fig. 4 The ICH domain contributes to the intracellular gating during alternating access cycle of GLUTs.**

- **a** Conformational changes of the ICH domain between the outward-occluded GLUT3 and inward-open GLUT4 structures. The cryo-EM structure of GLUT4 is superimposed to the crystal structure of GLUT3 (PDB code: 4ZW9) relative to the N (left) and C (right) domains, respectively.
- **b** A conserved Phe residue on ICH5 serves as a local organizing center in the outward-facing state. Left, Phe458 on helix ICH5 forms cation-π interactions with multiple Arg residues in the N and C domains of outward-facing GLUT3. Right, rearrangement of the ICH domain in inward-open GLUT4. Phe476 in GLUT4 (equivalent of Phe458 in GLUT3) no longer interacts with any of the Arg residues in the inward-open state.
- **c** The interaction between Phe458 and N-domain Arg residues contributes to the transport activity of GLUTs. Shown here are normalized transport activities of GLUT4 variants in the liposome-based counterflow assay. Data are presented as mean with standard deviation, in three independent experiments. Source data are provided as a Source Data file.

**Fig. 5 The intracellular functional motifs on GLUT4.**

- **a** Posttranslational modification site on the intracellular segments of GLUT4. Residues that may be subject to phosphorylation and palmitoylation are shown as green and red spheres. Three potential phosphorylation sites, Ser10, Thr486, and Ser488, are invisible.
- **b** The intracellular motifs that are involved in the cellular trafficking of GLUT4 are localized on the flexible terminal loops that are not resolved in the cryo-EM structure. This arrangement may ensure cargo recognition independent of the functional states of GLUT4.
for 30 min at 4 °C, the supernatant was loaded onto the nickel-affinity resin. The resin was washed with lysis buffer plus 20 mM imidazole. Protein was eluted with lysis buffer and incubated with 0.5 mL Optiphase HISAFE 3 (PerkinElmer) overnight. Grids coated with a thin layer of homemade graphite were glow-discharged for 12 s using the low power setting.

The data that support this study are available from the corresponding authors upon reasonable request. The cryo-EM maps have been deposited in the Electron Microscopy Data Bank (EMDB) under accession number EMDB-32760 (GLUT4 bound to CCB in nanodiscs), EMDB-32761 (GLUT4 bound to CCB in LMNG/CHS) and the associated models have been deposited in the RCSB Protein Data Bank (PDB) under accession number 7WSN (GLUT4 bound to CCB in LMNG/CHS). Previously solved structures have been deposited in PDB under the accession code 4PPY (GLUT1 structure in the presence of β-NG), 5EQI (GLUT1 structure in the presence of CCB), and 4ZWW (GLUT3 structure in the presence of glucose). Source data are provided with this paper.

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Author contributions
N.Y. and C.Y. conceived the project. Y.Y., C.Y., and N.Y. designed all experiments. Y.Y., H.X., and A.Z. performed the experiments. F.K. and C.Y. contributed to the structure determination. All authors analyzed the data and contributed to manuscript preparation. N.Y., C.Y., and Y.Y. wrote the manuscript.

Competing interests
The authors declare no competing interests.

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