Structural basis for assembly and disassembly of the IGF/IGFBP/ALS ternary complex

Hyojin Kim1,3, Yaoyao Fu2,3, Ho Jeong Hong2, Seong-Gyu Lee2, Dong Sun Lee2 & Ho Min Kim1,2

Insulin-like growth factors (IGFs) have pleiotropic roles in embryonic and postnatal growth and differentiation. Most serum IGFs are bound in a ternary complex with IGF-binding protein 3 (IGFBP3) and acid-labile subunit (ALS), extending the serum half-life of IGFs and regulating their availability. Here, we report cryo-EM structure of the human IGF1/IGFBP3/ALS ternary complex, revealing the detailed architecture of a parachute-like ternary complex and crucial determinants for their sequential and specific assembly. In vitro biochemical studies show that proteolysis at the central linker domain of IGFBP3 induces release of its C-terminal domain rather than IGF1 release from the ternary complex, yielding an intermediate complex that enhances IGF1 bioavailability. Our results provide mechanistic insight into IGF/IGFBP3/ALS ternary complex assembly and its disassembly upon proteolysis for IGF bioavailability, suggesting a structural basis for human diseases associated with IGF1 and IGFLS gene mutations such as complete ALS deficiency (ACLSD) and IGF1 deficiency.
into the cleft formed by the palm/thumb-like NBP and the flat-shaped CBP. The structure of the IGF1/IGFIR complex explains how only one IGF1 molecule binds the Γ-shaped asymmetric IGF1R dimer for IGF1R activation and why IGF1 with longer C-domain loops than IGF2 exhibits stronger binding to IGF1R. Moreover, these structures also suggest that the IGF1 binding pocket in IGF1R is too small to accommodate the binary complex and that the IGF residues involved in high-affinity binding to the IGF1R primary site are engaged in interaction with IGFBP, thereby preventing binding of the IGF/IGFBP binary complex to the receptor. However, these structures provide limited information about the flexible regions of the binary complex, including the N-terminal thumb loop, CLD, and the C-terminal end of IGFBPs, or about the C-domain loop of IGF1 that may be involved in ternary complex formation or IGF release from the ternary complex.

ALS is produced almost exclusively by the liver and secreted into the circulation. It belongs to the leucine-rich repeat (LRR) superfamily consisting of 19 LRR motifs flanked by LRR N- and C-terminal modules (LRRNT and LRRCT, respectively) (Fig. 1a). Individuals with complete

---

**Fig. 1 | Overall structure of human IGF1/IGFBP3/ALS ternary complex.**

a Schematic diagram of human ALS, IGFBP3, and IGF1. Each domain is labeled above the diagram, and the amino acids are numbered under the diagram. In IGFBP3, the central linker domain, which was not resolved in the cryo-EM map, is shown as a dashed line. The boundary of the central linker domain is labeled with a red C and N, indicating the C-terminus of NBP3 and N-terminus of CBP3, respectively. b Size-exclusion chromatography (SEC) profile of human IGF1/IGFBP3/ALS ternary complex (left) and SDS-PAGE analysis of the SEC peak fractions stained with Coomassie blue (right). Pooled fractions for cryo-EM analysis are marked with the bar (violet). Similar results were observed in three independent experiments. c Overall structure of the human IGF1/IGFBP3/ALS ternary complex. Cryo-EM maps (top) and the corresponding cartoon models (bottom) of the ternary complex are shown. ALS, N-terminal domain of IGFBP3 (NBP3), C-terminal domain of IGFBP3 (CBP3), and IGF1 are in violet, blue, cyan, and yellow, respectively. The C-terminal end of NBP3 and N-terminal end of CBP3 are marked with red dots. Disulfide bridges (orange) and N-linked glycans (green) are shown as sticks.
ALS deficiency (ACLSD, OMIM #615961) have mutations in the IGFALS gene and exhibit severely reduced serum IGF1 and IGFBPs (particularly IGFBP3), leading to low birth weight and length, reduced head circumference and height, puberal delay, and insulin resistance\(^{12,17}\). These outcomes indicate that ALS is crucial for maintaining the integrity of the circulating IGF/IGFBP system and that the IGF/IGFBP/ALS ternary complex serves as an IGF reservoir facilitating the endocrine actions of IGFs. Of interest, ALS has no affinity for free IGFs or only a low affinity for apo IGFBP3 and IGFBP5\(^{18}\). The lack of a defined structure for IGF/IGFBP/ALS ternary complexes, however, has hindered understanding of the structural organization underlying a prolonged IGF half-life, as well as the distinct affinity of IGF/IGFBPs (IGFBP3 and IGFBP5) for ALS binding\(^{19,20}\).

Here, we determine the cryo-electron microscopy (cryo-EM) structure of the human IGF1/IGFBP3/ALS ternary complex at an overall resolution of 3.2 Å. Our structure reveals that the IGF1/IGFBP3 binary complex engages in long-range interactions with the entire concave surface of ALS. Also, interacting with the IGF1/IGFBP3 binary complex are the hook loop at LRRTC of ALS and the N-linked glycans attached to N368 of ALS. Our structural and biochemical analysis clearly shows that the N-terminal thumb of NB3 as well as the ε3 helix and C-terminal loop of CBP3 are key determinants for assembly of ALS with specific binary complexes (IGF/IGFBP3 or IGF/IGFBP5). Our results also indicate that the CLD of IGFBP3 sterically blocks ALS binding unless IGFBP3 binds to IGF1, and that proteolysis of IGFBP3 CLD in the ternary complex induces release of CBP3 rather than IGF1 from the ternary complex. Taken together, our findings provide mechanistic insight into assembly of the IGF/IGFBP3/ALS ternary complex and its disassembly for IGF bioavailability and allow us to interpret the structural effects of IGF1 and ALS mutations in ACLSD and IGF1 deficiency (OMIM #605747).

**Results**

**Overall structure of the human IGF1/IGFBP3/ALS ternary complex**

Using HEK293F cells, we co-expressed full-length human IGF1, IGFBP3, and ALS, and a stable ternary complex was co-purified by affinity chromatography and subsequent size-exclusion chromatography (SEC) (Fig. 1b). We determined the cryo-EM structure of the human IGF1/IGFBP3/ALS ternary complex at an overall resolution of 3.2 Å (Fig. 1c and Supplementary Figs. 1–2, and Supplementary Table 1). The atomic models of ALS were constructed de novo, and IGF1/IGFBP3 models were manually built after fitting the crystal structure of the IGF1/IGFBP3 complex (NP4 and CBP4 fragment without CLD; PDB: 2DSR)\(^{21}\) on the cryo-EM map. The glycan densities for five N-glycosylation sites of ALS (N64, N96, N368, N515, and N580) were clearly visible in the cryo-EM map (Supplementary Fig. 2c). The overall structure of the ternary complex had a “parachute” shape with 1:1 stoichiometry (IGF1:IGFBP3:ALS). The IGF1/IGFBP3 binary complex bound to almost the entire concave surface of the horseshoe-like ALS (Fig. 1c), and both IGF1 and IGFBP3 participated in the interaction with ALS. Consistent with the previously reported structure of IGF1 in complex with NP4 and CBP4 fragments\(^{22}\), IGF1 was clamped by the NP3 and CBP3 of full-length IGFBP3. Although the densities for IGFBP3 CLD (CLD3) were not well resolved in the cryo-EM map because of its structural flexibility, extra density around IGF1 under a lower threshold indicated that the CLD3 might act as a “mechanical flap” covering IGF1 not yet wrapped by NB3 and CBP3 (Supplementary Fig. 2e).

**Human ALS structure**

Human ALS, which belongs to the typical LRR family, consists of 19 LRR motifs with a consensus sequence (xLxxLxxNxLxxLxxxxFxx/Lx) flanked by LRRTN and LRRTC, and had a curved solenoid structure (Fig. 2a). A total of 21 parallel β-strands [LRRTN (βN), 19 LRRs (β1–19), and LRRTC (βC)] on the concave surface had uniform twist angles and radii throughout the entire curvature, giving ALS a flat horseshoe-like structure. The convex surface of the LRRT motifs was formed by well-organized loops except for LRRL8, which contained a 30 helix, and the unique disulfide bond (C273–C397) in the LRRT motif stabilized the convex portion of LRRL3 and LRRL4. The conserved phenylalanine-spiine and asparagine-ladder (except LRRL2, LRRL5, and LRRL7–19 for the phenylalanine-spiine, and LRRL2 and LRRL4 for the asparagine-ladder) were directed to the interior of the solenoid core (Fig. 2a). The LRRT (residues 28–74) and LRRTC (residues 530–605) were stabilized by disulfide bonds: C41–C47/C45–C60 and C566–C571/C540–C583/C542–C605, respectively. Five N-linked glycosylation sites of ALS were distributed on LRRLNT (N64), the LRR motif (N96 at the convex surface, N368 at the concave surface, and N515 at the ascending flank), and LRRTC (N580).

Of note, a long loop (residues 559–580) of LRRLCT and N-linked glycans at N368 extended from the concave surface, protruding into the central axis of the ALS curvature (Fig. 2a, b) (hereafter, we refer to this extended loop in LRRT as a “hook loop”). The tip and stem of the hook loop were stabilized by a disulfide bridge (C566–C571) and antiparallel β-sheet (residues 560–564 and 575–579), respectively. The bottom of the hook loop was anchored to the LRRT. In particular, RS60 at the beginning of the hook loop was tethered to DS93, RS95, and DS96 by forming an elaborate electrostatic network, and N579 at the end of the hook loop formed hydrogen bonds with RS95 and the main chain of LS31 on LRRT. In addition, the aromatic rings of F561, Y576, and Y578 and N-acetylglucosamine (NAG) attached to N580 were stacked with each other below the β-sheet of the hook loop (β CH1 and β CH2), and W532, P559, and V562 formed the hydrophobic networks above the β-sheet (Fig. 2b and Supplementary Figs. 3a and 4a). These extensive interactions conferred a stable and rigid structure on the hook loop, despite its 26 Å length. The significance of hook loop and glycan at N368 for interactions with the IGF1/IGFBP3 complex is described in a later section.

**Structure of full-length IGFBP3 in complex with IGF1**

The IGF1/full-length IGFBP3 complex within the ternary complex adopts similar global folds with the human IGF1/IGFBP4 complex (NP4 and CBP4 fragment without CLD; PDB ID: 2DSR), with a backbone root mean square deviation (RMSD) of 1.189 Å (Fig. 2c). Our cryo-EM structure clearly defined highly conserved disulfide bonds (3 in IGF1, 6 in NP3, and 3 in CBP3) crucial for rigidity of the core modules. The IGF1 B domain α helix (A8-V17) was wedged deep into the cleft formed by an edge of the NP3 palm and a flat surface of CBP3 (Fig. 2d, Supplementary Figs. 3 and 4b). The interactions between IGF1 and IGFBP3 were mainly mediated by hydrophobic contact. Residues L5, A13, F16, L54, and L57) on the IGF1 N-terminal loop (G1-L5) and one face of the IGF1 B domain α helix and IGF1 A domain α2 helix (L54-Y60) constituted long-range hydrophobic networks with residues on NP3 (P38, C54, I56, C67, L77, L80, and L81) (Fig. 2d). V11 on the other face of IGF1 B domain α helix also engaged in hydrophobic interactions with V208, I210, and P226 of CBP3 (Fig. 2d). At the entrance of the cleft, other hydrophobic residues of IGF1 (V44 and F49 on the A domain α1 helix) were bound to the hydrophobic flat surface of CBP3 consisting of C136, M190, L194, I210, and C213 (Fig. 2e; Supplementary Figs. 3 and 4b). These hydrophobic interactions were further tightened by electrostatic interactions [N212 (CBP3)-E9 (IGF1); K215 (CBP3)-T4 (IGF1); S227 (CBP3)-Q15 (IGF1)]. It is worth noting that D12 on the IGF1 B domain α helix made direct contact with both NB3 Y57 and CBP3 S227. For NB3 and CBP3 interaction, a hairpin-like loop of CBP3 between C224 and C235 made direct contacts with NB3 (Fig. 2e; Supplementary Figs. 3 and 4c). Similar to IGFBP4, the side chain of R232 on CBP3 stacked with the aromatic ring of conserved Y57 (NB3).
K228 and R230 on the tip of the hairpin-like loop formed additional charge interactions with NBP3 [K228 (CBP3)-E14 (NBP3); R230 (CBP3)-E37/T45/E59 (NBP3)] to further stabilize the NBP3–CBP3 interaction.

It has been reported that the extended N-terminal thumb of NBP is critical not only for regulation of IGF1 binding to IGF1R but also for interactions between NBP and CBP12. The thumb region consists of a short stretch of the very first N-terminal residues of IGFBPs that precede the first N-terminal cysteine (amino acids 1–12 in IGFBP3)\(^2\). Although most residues on the surface of the IGFBP3 cleft for IGF1 interaction are predominantly conserved in IGFBPs, the thumb regions of the different NBPs are of different amino acid (aa) lengths (NBP1, 4 aa; NBP2, 6 aa; NBP3, 12 aa; NBP4, 5 aa; NBP5, 6 aa; NBP6, 1 aa) (Supplementary Fig. 3b). Substitution of the IGFBP4 thumb with a corresponding region from IGFBP3 slightly enhanced the binding affinity to IGF1\(^3\), suggesting that the sequence of the N-terminal thumb may confer unique properties on each IGFBP. Of note, our cryo-EM
structure identified a unique feature of the IGFI/IGFBP3 binary complex in which the NBP3 thumb is sandwiched between CBP3 β4 (N201-L203) and IGFI B domain β strand (G22-Y24), forming a well-organized short β-sheet (Fig. 2F, Supplementary Figs. 3 and 4b). In particular, the NBP3 thumb (V10-V11) formed parallel and antiparallel β-sheets with CBP3 (N201-L203) and IGFI (G22-Y24), respectively, whereas the NBP4 thumb formed a short parallel β-sheet only with CBP4. Moreover, this short β-sheet shielded the hydrophobic core consisting of NBP3 (P9 and V11), CBP3 (L197-L203, and V208), and IGFI (F23 and F25). Another hydrophobic network [L7 and V10 (NBP3); Y24 and P28 (IGFI)] above this β-sheet would likely contribute to relocation of the IGFI C-domain loop toward a more suitable position for interaction with ALS (Fig. 2D). In the following sections, we describe the detailed interactions between the IGFI C-domain loop and ALS are described. Given the conserved three hydrophobic residues at positions 2, 3, and 6 with respect to the first N-terminal Cys residue (L7, V10, and V11 for IGFBP3; L1, F4, and V5 for IGFBP5) (Supplementary Fig. 3b), we speculate that the NBP3 thumb would similarly be involved in the relocation of the IGFI C-domain loop toward a more suitable position for interaction with ALS.

The C-domain loop of IGFI and CBP of IGFBP3 interact with ALS

Both IGFI and IGFBP3 were bound to ALS with a buried surface area of 593 Å² and 1396 Å², respectively (Fig. 3a), and the IGFI C-domain loop, a critical segment for recognizing IGF receptor, was held by ALS in the ternary complex (Fig. 3d). The interface between ALS and the IGFI/IGFBP3 binary complex could be divided into four distinct regions: IGFI patch, CBP3 helix patch, CBP3 loop patch, and hook loop patch, all evolutionarily conserved (Fig. 3a,b and Supplementary Tables 2–4). Most of the interacting interfaces, except the CBP3 loop patch, were mediated by electrostatic interactions (Fig. 3c).

The first IGFI patch was mediated by elaborated electrostatic networks involving residues of the IGFI C domain (T29, R36, and R37) and ALS residues on descending flanks of LRR3–LRR6 (I26, S171, D174, and E198) (Fig. 3d and Supplementary Fig. 4d). W173 on ALS was wedged between R36 and R37 on IGFI, forming a hydrophobic interaction (Pi-Alkyl). These interactions allocated two arginines (R36 and R37) in opposite directions, enabling another electrostatic interaction (Pi-Alkyl). These interactions allocated two arginines (R36 and R37) in opposite directions, enabling another electrostatic interaction (Pi-Alkyl).

The second and third regions were formed by long-range interactions of CBP3 with the concave surface of ALS LRR5-LRRCT. In particular, the α3 helix of CBP3, forming the edge of the flat CBP3 segment, traversed from the ascending flank of LRR5 to the descending flank of LRR12 with a ~45-degree tilt toward the LRR β-strand. Extensive hydrogen bonds and ionic interactions of ALS LRR5–LRR12 with CBP3 α3 and its flanking loops were observed in the CBP3 helix patch (Fig. 3e and Supplementary Fig. 4e). The CBP3 loop patch was composed of contacts between ALS LRR13-LRRCT and the CBP3 α-helical loop following the β strand (P244–K264). The LRR3-LRR4 and LRR9-LRRCT of ALS flanked both sides of the CBP3 loop through electrostatic interactions, stabilizing the weak central hydrophobic network.

To investigate the significance of these interacting interfaces for ternary complex formation [ALS LRR3–6 motif/IGFI C-domain loop (IGFI patch), ALS LRR5–12 motif/CBP3 α3 helix (CBP3 helix patch), ALS LRR3-LRRCT and CBP3 C-terminal loop (CBP3 loop patch) and ALS hook loop/CBP3 loops/NBP3 α helix (hook loop patch)], we selected key interacting residues and introduced mutations (ALS W173A and IGFI R36E/R37E) for the IGFI patch; IGFBP3 R188E for the CBP3 helix patch, ALS Δ561–578 deletion for the hook loop patch) (Supplementary Fig. 6). Then, we co-expressed the different combinations of IGFI-Strep-tag, IGFBP3, and ALS-His as indicated in Supplementary Fig. 6b (table) using Exp293-F cells and monitored the components in the resulting complex by pull-down of IGFI with Strep-Tactin resin. Although all mutants were well expressed and the binary complex (IGF1 WT/IGFBP3 WT) was properly assembled, the amount of ALS bound to these binary complexes were significantly reduced (Supplementary Fig. 6b), implying that these residues in each interacting patch are indeed critical for ternary complex formation. Particularly, mutations in the CBP3 helix patch and CBP3 loop patch (IGFBP3 R188E for the CBP3 helix patch, and ALS R414A for the CBP3 loop patch; ALS Δ561–578 deletion for the hook loop patch) (Supplementary Fig. 6). Then, we co-expressed the different combinations of IGFI-Strep-tag, IGFBP3, and ALS-His as indicated in Supplementary Fig. 6b (table) using Exp293-F cells and monitored the components in the resulting complex by pull-down of IGFI with Strep-Tactin resin. Although all mutants were well expressed and the binary complex (IGF1 WT/IGFBP3 WT) was properly assembled, the amount of ALS bound to these binary complexes were significantly reduced (Supplementary Fig. 6b), implying that these residues in each interacting patch are indeed critical for ternary complex formation. Particularly, mutations in the CBP3 helix patch and CBP3 loop patch (IGFBP3 R188E for the CBP3 helix patch, and ALS R414A for the CBP3 loop patch) completely abolished ALS binding to the binary complex, indicating that interactions between CBP3 and the ALS concave surface are especially crucial for ternary complex formation and partially explaining why IGFI alone could not bind to ALS. Collectively, these results suggest that the IGFI/IGFBP3 binary complex mainly binds to the ALS concave surface, with
Fig. 3 | Detail of interactions between ALS and the IGF1/IGFBP3 binary complex. a The surface representation of ALS (violet) in two orthogonal views. ALS residues for IGF1 and CBP3 interaction are shown in yellow and cyan, respectively. b Sequence conservations were calculated by the Consurf server and are presented on the surfaces of ALS and the IGF1/IGFBP3 binary complex. The structures are shown as open-book views and surface representations, with color maps reflecting conservation (green, highly conserved; white, less conserved). The orientation of the surface view of ALS is identical to that in a (bottom). c Electrostatic potential of ALS and the IGF1/IGFBP3 binary complex, calculated according to the Poisson-Boltzmann equation in PyMOL. Blue and red represent positively and negatively charged residues, respectively. The orientation of the surface view of ALS and the IGF1/IGFBP3 binary complex are identical to that in b. 

a–c The IGF1, CBP3 helix, CBP3 loop, and hook loop patches are marked with circles colored yellow, red, cyan, and lime, respectively. d–g Close-up views of interactions between ALS and the IGF1/IGFBP3 binary complex. Interactions (d) between the ALS LRR3–6 motif and IGF1 C-domain loop (IGF1 patch), (e) between the concave surface of the ALS LRR5–12 motif and CBP3 α3 helix (CBP3 helix patch), (f) between the concave surface of the ALS LRR13-LRRCT and CBP3 C-terminal loop (CBP3 loop patch), and (g) interactions among the ALS hook loop, CBP3 loops, and NBP3 α1 helix (hook loop patch). Key residues involved in each interaction are displayed as sticks and labeled.
secretion (Fig. 4c), which are consistent with a previous analysis using a ternary complex mechanism to explain the misfolding and aberrant interactions with the IGF1/IGFBP3 binary complex. Our cryo-EM studies on the ALS structural model revealed that none of the residues are involved in direct interactions (C60S, C540R, P73L, and P586L), whereas the N84S, L127P, L134Q, L172F, L241P, L244F, N276S, and L409F residues are involved in direct interactions with the IGF1/IGFBP3 binary complex. Our cryo-EM structure clearly showed that these mutations can affect the structural integrity of ALS, possibly causing its misfolding and aberrant secretion (Fig. 4c), which are consistent with a previous analysis using the predicted ALS structural model. In particular, the mutation on Leu and Asn residues (N84S, L127P, L134Q, L172F, L241P, L244F, N276S, and L409F) as part of the LRR consensus sequence (xLxxLxNlxLxLxxxxFxx/Lx) are likely to introduce a main chain distortion or disrupt the LRR hydrophobic core, affecting the entire LRR architecture of the ALS protein. Similar to this effect, substitution of T145 and L213 residues to long charged Lys and bulky hydrophobic Phe, respectively, which point toward the hydrophobic core of the LRR solenoid interior, would introduce steric hindrance with surrounding residues and destroy the regular fold of the LRR module. The highly conserved C60 and C540 residues participate in disulfide bridge formation with C45 and C583, respectively, and the P73 and P586 residues contribute to maintaining structural rigidity. Therefore, mutations at these residues (C60S, C540R, P73L, and P586L) could destabilize LRRNT and LRRCT (Fig. 4c). Indeed, previous studies on the in vitro expression of ALS variants demonstrated that N276S, L409F, and C540R are not synthesized, whereas L213F is partially expressed but cannot be secreted. Although D440 and D448 of ALS are not essential to ALS/IGFBP3 interactions, D440N and possibly D488N could introduce a new N-glycosylation site that leads to impairment of secretion of ALS and its ability to form a ternary complex, as reported previously. The S490W mutation is predicted to cause loss of the hydrogen bond network with neighboring S466, R467, D488, H491, S512, and R514 on the surface of the LRR17–LRR19 ascending flank (Fig. 4c). Two in-frame mutants, S195_R197dup and L437_L439dup, insert three amino acids at LRR6 and LRR16, respectively. The resulting altered length of the LRR modules might impair alignment of the hydrophobic residues on the consensus motif, perturbing the solenoid-like structure of ALS. Moreover, S195_R197dup and L437_L439dup lie adjacent to the ternary complex interface, probably disrupting ternary complex formation.

IGF1 deficiency and several bio-inactive IGF1 mutations (R36Q, V44M, and Y60H) also have been identified in patients with growth failure and postnatal growth retardation, and the glycan attached to N368 of ALS.
microcephaly, and sensorineural deafness\textsuperscript{22,26}. Taken together, our findings provide a structural basis for ACLSD and the impaired growth seen with genetic defects in the human IGF1/IGFBP3/ALS ternary complex.

The CLD of IGFBP3 is crucial for sequential assembly of IGF1 and IGFBP3 with ALS

Although the high-resolution cryo-EM structure of IGF1/IGFBP3/ALS elucidated interactions among the components of the ternary complex, the structure itself is insufficient to explain the assembly process for the IGF1/IGFBP3/ALS ternary complex. To address this gap, we co-expressed the different combinations of IGF1, IGFBP3, and ALS in Expi 293-F cells and monitored the components in the resulting complex (Fig. 5a). As His-tag and Strep-tag are attached to the C-terminus of ALS and IGFBP3, respectively, we could pull down ALS or IGFBP3 as well as the complexes containing ALS or IGFBP3 using Ni-NTA or Strep-Tactin resin. When three proteins were co-expressed, IGF1, IGFBP3, and ALS bands were all observed in both His and Strep pull-down, indicating proper assembly of the ternary complex. As IGFBP3 engages in long-range interactions with the concave surface and hook loop of ALS in the ternary complex (Fig. 3), we speculated that IGFBP3 in the absence of IGF1 could bind to ALS. However, when ALS and IGFBP3 were co-expressed without IGF1, only ALS in His pull-down or IGFBP3 in Strep pull-down was detected, indicating that ALS and IGFBP3 cannot form a binary complex without IGF1. The results of co-expression of ALS and IGF1 also demonstrated that IGF1 alone cannot form a complex with ALS, consistent with previous reports\textsuperscript{11}. Moreover, we found that CLD3-CBP3 cannot bind to both ALS and IGF1 (Fig. 5b), suggesting that NBP3 is required for IGF1/IGFBP3 binary complex formation. Unfortunately, we could not test whether CBP3 alone is sufficient for ALS

Fig. 5 | Critical role of CLD3 for sequential assembly of IGF1/IGFBP3/ALS ternary complex. a, SDS-PAGE analysis of the co-expression test. ALS-His, IGFBP3-Strep, and IGF1 (IGF1 or IGF1-His) were co-expressed and pulled down with Ni-NTA or Strep-Tactin resin, as indicated. b, Co-expression of CLD3-CBP3 with ALS and/or IGFBP3 with ALS, CLD3-CBP3-Strep, and IGF1 (IGF1 or IGF1-His) were co-expressed and pulled down with Ni-NTA or Strep-Tactin resin, as indicated. c, Fluorescence-detection size-exclusion chromatography (FSEC) after in vitro reconstitution of IGF1, ALS-His, CLD3-CBP3-Strep, and IGF1 (IGF1 or IGF1-His) were co-expressed and pulled down with Ni-NTA or Strep-Tactin resin, as indicated. d, Fluorescence signal (GFP) fused to IGF1 was monitored to examine the complex formation. The elution profiles for standard IGF1-GFP results were observed in three independent experiments.
binding, because we were unable to express CBP3 probably due to the exposed hydrophobic residues on its flat surface.

To assess the quantitative efficiency of IGF complex assembly, we performed fluorescence-detection SEC (FSEC) after in vitro reconstitution with different combinations of recombinant proteins (ALS, IGFBP3, CLD-CBP3, IGFI-GFP, and IGFI-GFP/IGFBP3 binary complex) and monitored fluorescence signal from the green fluorescent protein (GFP) fused to IGFI. Similar to co-expression and pull-down analysis, we found that IGFI-GFP show no affinity for ALS and CLD-CBP3, and that assembly of the IGFI/IGFBP3 binary complex should precede formation of the IGFI/IGFBP3/ALS ternary complex (Fig. 5c).

The CLD sequences of six members of IGFBP are less conserved compared with their NBP s and CBPs (Supplementary Fig. 3b). Therefore, we next questioned whether CLD could influence the specific binding of the IGFI/IGFBP3 binary complex to ALS. To this end, we replaced the CLD of IGFBP3 with that of a different IGFBP and used co-expression and pull-down assays to examine whether these hybrid-BPs could form a ternary complex with IGFI and ALS. Similar to IGFBP3, all versions of the hybrid-BP could form the ternary complex with IGFI and ALS (Fig. 5d), indicating that the CLD does not confer specificity of each IGFBP for ternary complex formation with ALS. Surprisingly, we found that even in the absence of IGFI, ALS could bind to hybrid-BP1, -2, -4, and -6, but not to IGFBP5 for hybrid-BPs (Fig. 5e), suggesting that the CLDs of IGFBP3 and IGFBP5 sterically block the interface for ALS binding. Collectively, our results imply that IGFI binding to the CLD of CBP3 and CBP5 may induce conformational changes in CLD3, facilitating ALS binding to the pre-assembled IGFI/IGFBP3 binary complex for formation of the IGFI/IGFBP3/ALS ternary complex.

IGFBP3 CLD proteolysis by thrombin and ADAM12 induces IGFBP3 CBP release and produces the intermediate ternary complex

Proteolysis at the IGFBP CLD by proteases such as metalloproteinase, cathepsins, and serine proteases is crucial for activation of IGFR signaling in a target tissue or cell. To check whether IGFBP proteolysis causes IGFI release from the ternary complex, we incubated the purified IGFI-GFP/IGFBP3 binary complex or IGFI-GFP/IGFBP3/ALS ternary complex with thrombin, which cleaves R97-A98 and R206-G207 of IGFBP3, and used FSEC to monitor IGFI-GFP release from the complex. As expected, IGFI-GFP was released from the binary complex after thrombin digestion and eluted at the peak position, corresponding to free IGFI-GFP (Fig. 6a, middle). Strikingly, IGFI-GFP peak released from the ternary complex after thrombin digestion was barely detected (Fig. 6a, bottom and Supplementary Fig. 7a), in contrast to previous reports. Instead, the fluorescence peak was slightly shifted to the smaller complex (~140 kDa) (Fig. 6a), suggesting that IGFI is not released from the ternary complex after IGFBP3 proteolysis, but that some other components of the ternary complex might be released. To identify the released component, we performed immunoblot analysis on the SEC fraction of the IGFI/myc-IGFBP3-strep/ALS ternary complex after cleavage with thrombin, using antibodies against ALS and IGFI as well as anti-myc and anti-Strep antibody for NBP3 and CBP3 detection, respectively. Of interest, the CBP3 band was not detected after thrombin digestion and subsequent SEC, whereas ALS, IGFI, and cleaved NBP3 remained in the ternary complex (Fig. 6b). These results indicate that CBP3, not IGFI, is released from the ternary complex after IGFBP3 proteolysis. FSEC analysis with IGFI/IGFBP3-GFP/ALS demonstrated that the amount of released CBP3-GFP from the ternary complex upon thrombin proteolysis was increased in time-dependent manner (Fig. 6c).

It has been known that a disintegrin and metalloprotease 12 (ADAM12) and pregnancy-associated plasma protein A2 (PAPP-A2) in pregnancy serum are also responsible for proteolysis of IGFBP3 and IGFBP5, and mutations in PAPP-A2 lead to short stature because of low IGFI availability (OMIM# 619489). Therefore, we further analyzed IGFBP3 proteolysis by ADAM12 and PAPP-A2, and subsequent CBP3 release from the ternary complex. Similar to thrombin, ADAM12 efficiently degraded IGFBP3 in the ternary complex, which in turn induced the release of CBP3-GFP, but not IG-FGF (Supplementary Fig. 7b–d). However, compared with thrombin and ADAM12, the efficiency of IGFBP3 cleavage by PAPP-A2 was significantly reduced (Supplementary Fig. 7b), which are consistent with previous reports. Interestingly, even after PAPP-A2 treatment to the IGFI/IGFBP3/ALS ternary complex, neither CBP3-GFP nor IG-FGF were released (Supplementary Fig. 7c, d).

We then questioned whether the resulting intermediate complex (IGFI/NBP3/ALS) after proteolysis can activate IGFR signaling. To this end, we treated HEK293 cells with the SEC fraction containing this intermediate ternary complex upon proteolysis with thrombin, ADAM12, or PAPP-A2 and monitored phosphorylation of IGFR. Indeed, the original ternary complex itself did not induce IGFR phosphorylation, but the SEC fraction containing the intermediate ternary complex after proteolysis with thrombin and ADAM12 could activate IGFR signaling at a level similar to that with free IGFI (Fig. 6d and Supplementary Fig. 7e). Of note, the intermediate ternary complex after PAPP-A2 proteolysis, which retained CBP3, could activate IGFR signaling, but with less potency as compared with those of the intermediate ternary complex lacking CBP3 after thrombin and ADAM12 proteolysis (Fig. 6d and Supplementary Fig. 7e). Interestingly, the intermediate ternary complex lacking CBP3 after thrombin proteolysis could not directly bind to the purified IGFR ectodomain dimer (IGFR ectodomain-leucine zipper), whereas IGFI alone could do so (Supplementary Fig. 7f). Moreover, the presence of the IGFR ectodomain alone could not promote the quick dissociation of IGFI from this intermediate ternary complex (Supplementary Fig. 7g). These results suggest that after proteolysis of IGFBP3 CLD and CBP3 release, IGFI needs to be further dissociated from the destabilized IGFI/IGFBP3-NBP3/ALS complex for IGFR activation, which may be mediated by some other unknown factors at the extracellular matrix (ECM) or plasma membrane.

To elucidate the specific role of the IGFBP CLD on CBP release, we incubated ternary complexes containing hybrid-BPs, IGFI, and ALS with thrombin and treated HEK293 cells with them. Strikingly, although all CLDs in hybrid-BPs were properly cleaved by thrombin, only the ternary complex containing IGFBP3 or hybrid-BP5 strongly activate IGFR upon thrombin digestion (Fig. 6e, f and Supplementary Fig. 7g). Consistent with FSEC results of IGFI-GFP/IGFBP3/ALS upon proteolysis, IGFI-GFP is not released from the IGFGF/GFP/hybrid-BPs/ALS ternary complex after CLD proteolysis with thrombin, ADAM12 and PAPP-A2 (Fig. 6g and Supplementary Fig. 7h–j). More importantly, the amount of released CBP3-GFP from the ternary complex containing hybrid-BPs after thrombin digestion was highly correlated with their ability for IGFR phosphorylation (hybrid-BP5 > hybrid-BP2 and 4 > hybrid-BP1 and -6) (Fig. 6h), suggesting that the release of CBP3 from the ternary complex after CLD proteolysis destabilizes the intermediate complex and further enhances IGFI bioavailability. Taken together, our findings indicate that the CLDs of IGFBP3 and IGFBP5 play a crucial role not only in sequential assembly of the ternary complex, but also in disassembly of CBP3 after CLD proteolysis for IGF bioavailability.

Discussion

In this study, we determined the cryo-EM structure of the human IGFI/IGFBP3/ALS ternary complex. We show that the IGFI/IGFBP3 binary complex, in which IGFI is clamped by the NBP and CBP of IGFBP3, binds to the concave surface of the horseshoe-like ALS, forming a parachute-like ternary complex. We also identified the important structural features of ALS such as the long hook loop on LRR1CT and the glycans attached to N368, which have not been assessed by previous in silico structural model. Moreover, our in vitro biochemical
**Fig. 6 | Release of CBP3 from the ternary complex after CLD3 proteolysis.**

**a** Fluorescence-detection size-exclusion chromatography (FSEC) of binary or ternary complexes after 10 h proteolysis by thrombin. The elution profile for standard IGF1-GFP (yellow), IGF1-GFP-strep/IGFBP3 binary complex (blue), and IGF1-GFP/IGFBP3-GFP/ALS-His ternary complex (red) are indicated as a control (top). No treatment for negative control (Ctrl). Data from three independent experiments (n = 3) were analyzed and relative pIGF1R/IGF1R (Fold to Ctrl) were expressed as mean ± SD (ns, not significant vs. ctrl; "P < 0.0001 vs. ctrl; **"P < 0.001 vs. ctrl; ***P < 0.01 vs. ctrl; ****P < 0.002 vs. ctrl). P-values by one-way ANOVA test followed by Sidak's multiple comparisons test.

**b** Immunoblot analysis of IGF1/myc-IGFBP3-strep/ALS ternary complex after 10 h proteolysis by thrombin and subsequent SEC. Anti-myc and anti-Strep antibody were used to detect NBP3 and CBP3, respectively. No thrombin treatment served as negative control (None). Data from three independent experiments (n = 5) were analyzed and relative pIGF1R/IGF1R (Fold to Ctrl) were expressed as mean ± SD (ns, not significant vs. ctrl vs. ADAM12 + thrombin for each group). P-values by one-way ANOVA test followed by Sidak's multiple comparisons test.

**c** SDS-PAGE analysis of the IGF1/hybrid-BPs/ALS ternary complex after thrombin digestion. F Quantification of immunoblot analysis for IGF1R phosphorylation with intermediated ternary complex containing hybrid-BPs. IGF1-His (5 nM, positive control), IGF1-His/IGFBP3/ALS ternary complexes (5 nM), or IGF1-His/hybrid-BPs/ALS ternary complexes (5 nM) with and without thrombin digestion were treated on HEK293A cells. No treatment for negative control (Ctrl). Data from five independent experiments (n = 5) were analyzed and relative pIGF1R/IGF1R (Fold to Ctrl) were expressed as mean ± SD (ns, not significant; "P < 0.0001 vs. IGF1-His or thrombin 10 hr). P-values by one-way ANOVA test followed by Sidak's multiple comparisons test.

**d** Fluorescence Intensity (mV)

**e** SDS-PAGE analysis of the IGF1/hybrid-BPs/ALS ternary complex after thrombin digestion.

**f** Fluorescence Intensity (mV)

**g** Fluorescence Intensity (mV)

**h** Fluorescence Intensity (mV)
experiments with hybrid-BPs showed that CLD in IGFBP3 is crucial for sequential assembly of the ternary complex and CBP3 release from the complex after proteolysis for IGFR activation.

The IGFI/IGFBP binary complex, which adopts similar global folds with the IGFI/IGFBP4 (NB4/CBP4) complex, binds to the concave surface of ALS through the IGFI C-domain loop and the IGFBP α3 helix and C-terminal loop. A 3D structural comparison using the Dali server indicated that the overall structure of ALS is similar to that of other LRR superfamily proteins such as drosophila Toll (PDB ID: 4LXR; Z-score: 29.3; RMSD: 4.9), LINGO1 (PDB ID: 4OQT; Z-score: 28.2; RMSD: 4.9), and LRIM1 (PDB ID: 3JOA; Z-score: 24.0; RMSD: 3.5), but ALS structure is unique in that it has a long hook loop protruding into the central axis of the LRR curvature. Although this hook loop does not directly interact with IGFI, it further stabilizes the interaction between ALS and the binary complex by intercalating between NBP3 and CBP3. Our ternary complex structure clearly shows that the hydrophobic part of the IGFI B domain as well as Y31, R36, and R37 of the IGFI C domain, which are crucial for IGFR binding, are buried, indicating that the ternary complex sterically protects IGFI from IGFR binding. The residues on IGFBP3 for IGFBP3 interactions are highly conserved in IG2, suggesting that the IGFI/IGFBP3 binary complex would adopt a conformation comparable to that of IGFI/IGFBP3. However, the C domain of IGFI is shorter than that of IGFBP3, and only two of four residues needed for interaction between ALS and IGFI are conserved in IG2, explaining its weaker affinity compared with IGFI for ALS/IGFBP3.

We also provide a structural basis for the role of N-linked glycan attached to ALS in ternary complex formation. Our structural analysis of the human IGFI/IGFBP3/ALS ternary complex identified five N-linked glycosylation sites on ALS (N64, N96, N368, N515, and N580) among seven potential candidates. Janosi et al. have shown that individual mutations of these N-linked glycosylation sites do not have a major effect on ALS binding to the IGFI/IGFBP3 complex, while enzymatic removal of these glycans abolishes complex formation. These findings indicate that a number of N-linked glycans of ALS rather than any single specific carbohydrate chain are responsible for its ability to form a ternary complex. Indeed, N-acetylgalcosamine (NAG) attached to ALS N550 contributes to stabilizing the ALS hook loop, allowing it to interact with IGFBP3. Moreover, although only one mannose and two NAGs attached to ALS N365 are visible in cryo-EM density, further extended glycans from the terminal mannose, such as galactose and sialic acid, are likely to interact with positively charged residues of IGFBP3, such as H196 and R206, supporting previous findings that removal of sialic acids from ALS decreases its affinity for complex formation by 50–80%. On the other hand, we could not observe N-linked glycans on IGFBP3 because three glycosylation sites (N89, N109, and N172) are non-essential to ALS or IGFBP binding, but they may modulate other biological activities of IGFBP3 such as ECM binding.

Among six members of the IGFBP family, only IGFBP3 and IGFBP5 can form a ternary complex with IGF and ALS. Our cryo-EM structure of the human IGFI/IGFBP3/ALS ternary complex provides a clear understanding of what would confer this unique ability on IGFBP3 and IGFBP5. First, the hydrophobic long N-terminal thumb in IGFBP3 (also in IGFBP5) provides local steric constraints on IGFI C-domain loop, thus facilitating relocation and anchoring of the IGFI C-domain toward ALS in a suitable configuration (Figs. 2f and 7a). As the C-domain loops of IGFI are partially visible in previous apo IGFI structures due to its high flexibility, we were unable to compare entire C-domain loops in apo-IGFI with those in the ternary complex. However, a structural comparison of the IGFI C-domain loop in the ternary complex with that of the IGFI/NBP4 complex (PDB ID: 1WQO) indicates that the protruding C domain of IGFI in IGFI/NBP4 complex is likely to trigger a steric clash, disrupting potential interactions with ALS, whereas the IGFI C-domain loops tightly contacts ALS in the IGFI/IGFBP3/ALS ternary complex (Fig. 7a). Second, the C-terminal loop of CBP3 following the β7 strand (P244-K264), which interacts with the ALS concave surface, is structurally flexible and extended (Figs. 3f and 7b).

In contrast, the corresponding regions in other CBP/IGFI complexes are rigid secondary structures (310 turn in CBP4 (PDB ID: 2DSR) and β-strand in CBP1 (PDB ID: 3O97), taking on a more compact conformation and hindering close contacts with the ALS concave surface (Fig. 7b and Supplementary Fig. 5). Previous domain swapping experiments with IGFBP2 or IGFBP6, which cannot form ternary complexes, have demonstrated that the CBP of IGFBP3 and IGFBP5 are crucial for ALS binding. Indeed, the apo-structures of CBP2 and CBP6, particularly the structure of their C-terminal loop region, are quite different from that of CBP3 (Ca RMSD CBP3 vs. CBP2: 3.11Å; CBP3 vs. CBP6: -3.789Å). Lastly, the key residues of IGFBP3 constituting CBP3 helix patch interactions, such as R188, E191, and K198, are highly conserved in IGFBP5 but not in other IGFBPs. These unique features of IGFBP3 and IGFBP5 enable their specific binding to ALS and IGF, thus forming the ternary complex.

Previous studies showed that isolated NBP and CBP of IGFBPs have significantly lower affinities for IGFs than do intact IGFBPs, suggesting that the flexible CLD of IGFBPs plays a role in formation of the IGF/IGFBP binary complex, perhaps by controlling inter-domain movement for their cooperative binding to IGF. As all cases of hybrid-BP, in which CLDs of IGFBP3 are replaced by those of other IGFBPs, can form the ternary complex with IGFI and ALS, a less conserved CLD could not be the reason for preferential binding of ALS only to IGFBP3 and IGFBP5. Instead, the current study implies a unique role for the IGFBP3 CLD in sequential assembly of the ternary complex. Our co-expression and pull-down analysis results demonstrate that ALS has a low affinity for uncomplexed IGFBP3 and hybrid-BPs, but a high affinity for uncomplexed hybrid-BPs (hybrid-BP1, 2, 4, and 6). This finding suggests that the CLDs of IGFBP3 and IGFBP5 sterically block the ALS-binding interface and that binary complex assembly may induce conformational changes in CLD3, thus exposing the CBP surface for ALS binding to form the ternary complex (Fig. 7c, top). These steps illustrate why the assembly of the binary complex (IGFI/IGFBP3 and IGFI/IGFBP5) should precede the formation of the ternary complex (IGFI/IGFBP3/ALS and IGFI/IGFBP5/ALS). However, we do not exclude the possibility that the unique features such as amino acid composition and length of the IGFBP3 CLD and/or the conformational changes in the CBP3 loop patch of IGFBP3 upon IGFI binding are also involved in sequential assembly of the ternary complex.

It has long been known that IGFBP proteolysis of the central linker domain leads to increased concentrations of bioavailable IGF and subsequent activation of the IGFR. Surprisingly, we found the intermediate ternary complex after proteolysis by thrombin and ADAM12, in which IGFI is retained, but IGFBP3 CBP3 is released, can stimulate IGFR phosphorylation. These intermediate ternary complexes can be explained by the previous results showing that CBPs have lower affinities than NBPs for IGFI. Moreover, with FSEC and IGFR activation analysis using hybrid-BPs, we showed that CLD3 and CLD5 have unique properties for CBP dissociation from the ternary complex. Because of the different exclusive digestion sites, proteolysis with PAPP-A2 produced different fragments of IGFBP3 and the intermediate complex containing CBP3. Moreover, the IGFR activation potency of the PAPP-A2-treated ternary complex was less than those treated with thrombin and ADAM12, probably due to the limited proteolytic activity of PAPP-A2 against IGFBP3. However, our results suggest that proteolysis of the IGFBP3 CLD is required for IGFR activation and that after the IGFBP3 CBP falls apart from the ternary complex by proteolysis (by thrombin or ADAM12), the remaining IGFI/IGFBP3-NBP/ALS complex will become relatively unstable, thereby further enhancing IGFI bioavailability. We also postulate that the potential conformational changes of IGFBP3 CLD after proteolysis by thrombin
ADAM12 can cause subsequent dissociation of CBP from the ternary complex (Fig. 7c, bottom). It is still unclear how the CLD3 of uncomplexed IGFBP3 sterically blocks the ALS-binding interface and how the cleaved CLD3 (or CLD5) triggers dissociation of CBP3 (or CBP5) from the ternary complex. Further experiments, such as high-speed atomic force microscopy analysis and time-resolved cryo-EM of the intermediate ternary complex, are needed to ascertain the precise dynamics of IGFBP CLD in the binary and ternary complexes.

In conclusion, our comprehensive investigation of IGF1/IGFBP3/ALS ternary complexes provides insight into their sequential and specific assembly as well as CLD proteolysis-mediated intermediate ternary complex formation for IGF1R activation. Given the crucial role of the IGF axis in growth and proliferation and the genetic defects in IGF and ALS associated with various human diseases, our study also can pave the way for identifying eventual therapeutic targets of IGF axis dysfunction.

**Methods**

**Cell lines and cell culture**

HEK293A cell (#R70507, Thermo Fisher Scientific) were maintained in Dulbecco’s Modified Eagle Medium (DMEM; #LM 001-05, Welgene) supplemented with 10% fetal bovine serum (FBS; #12484020, Thermo Fisher Scientific) and 1% Antibiotic-Antimycotic (#15240062, Thermo Fisher Scientific) and cultured at 37 °C in a humidified 5% CO2 incubator. Expi293-F cells (A14527, Thermo Fisher Scientific) were maintained in Expi293 Expression Medium (A1435102, Thermo Fisher Scientific) and cultured in a humidified shaking incubator at 37 °C and 8% CO2.

**Constructs for recombinant protein expression and mutagenesis**

Human IGF1 (residues M1-A118) followed by a stop codon, thrombin cleavage sequence, and Protein A-tag or Twin-Strep-tag were cloned
into the HindIII and XhoI sites of a pcDNA3.1 vector (#V79020, Invitrogen). For the GFP-fused IGF1 construct, human IGF1 followed by a GGGS linker, EGFP (M1-239K), and Twin-Strep-tag was cloned into the HindIII and XhoI sites of the pcDNA3.1 vector. Human IGFBP3 (residues G28-K291) followed by a stop codon or Twin-Strip-tag, and human ALS (residues A28-C605) followed by a stop codon. The His-tag were cloned into the BamH I and NotI sites of the pcDNA 3.1 vector containing the signal sequence for protein secretion. To generate recombinant CLD3-CBP3, the CLD of IGFBP3 (residues SI18-E209), CBP of IGFBP3 (residues 210Y-291K), and Twin-Strep-tag were cloned into the BamH I and NotI sites of the pcDNA 3.1 vector containing the signal sequence for protein secretion and Twin-Strip-tag. To generate recombinant hybrid-BPs, we performed overlap PCR using PCR fragments of the NBP and CBP of IGFBP3 and CLD of each human IGF1 [IGFBP1 (residues S108–W172), IGFBP2 (residues R135–A223), IGFBP4 (residues A104–P170), IGFBP5 (residues K104–E188), or IGFPB6 (residues R108–E159)] and subsequently cloned them into the BamHI and NotI sites of the pcDNA 3.1 vector containing the Twin-Strip-tag. Constructs and primer information are listed in Supplementary Tables 5 and 6. Mutants of IGF1, IGFBP3, and ALS at interacting interface were constructed from IGF1-Strep, IGFBP3, and ALS-His, respectively, using QuickChange PCR methods.

Expression and purification of the IGF1/IGFBP3/ALS ternary complex for cryo-EM

A total of 1 μg of plasmid DNA (IGF1-proteinA: IGFBP3: ALS ratio of 1:2:4) was transfected into 2.5 × 10^6 Exp293-F cells (#A14527, Thermo Fisher Scientific) using Mesifectamine (#A14524, Thermo Fisher Scientific), and cells were cultured in Exp293 expression medium (#A14351, Thermo Fisher Scientific) at 37 °C and 8% CO2 with shaking (orbital shaker, 120 rpm) for 4 days. After centrifugation to remove the cells, the culture supernatant was loaded onto IGG Sepharose 6 Fast Flow (#17-0969-C1, Thermo). After washing with 10 column volumes of wash buffer (20 mM Tris-HCl pH 8.0, 200 mM NaCl), the Protein A–fused ternary complex (IGF1-proteinA/IGFBP3/ALS-bound resin was incubated with thrombin (1% [v/v] in wash buffer) at room temperature for 2 h to remove the C-terminal Protein A tag. The eluted IGF1/IGFBP3/ALS complex was concentrated to 0.8 mg/ml using an Amicon Ultra centrifugal filter (#UFC8030, Millipore) and further purified by SEC using a Superdex 200 Increase 10/300 GL column (20 mM Tris-HCl pH 8.0, 200 mM NaCl). The peak fractions were concentrated to 0.6 mg/ml using an Amicon Ultra centrifugal filter for cryo-EM analyses.

Cryo-EM sample preparation and data collection

Quantifoil RL2/1.3 300 mesh gold holey carbon grids (Cat.# Q3100AR.1, Electron Microscopy Sciences) were glow-discharged with a PELCO easiGlow Glow Discharge Cleaning system (Ted Pella) for 90 s at 15 mA, and then 2.5 μl of the purified IGF1/IGFBP3/ALS complex was applied to the grid in 100% humidity at 4 °C. After 2 s of blotting, the grid was plunged into liquid ethane using a Vitrobot MkIV (Thermo Fisher Scientific) for cryo-EM. Micrographs were acquired from a Titan Krios G4 TEM operated at 300 keV with a K3 direct electron detector (Gatan) at the IFB Institute for Basic Science (IBS), using a lit width of 20 eV on a GIF-Digitizer (Gatan) at the Institute of High Energy Physics, Chinese Academy of Sciences, Beijing. Images were recorded using a K3 Direct electron detector at 30 mrad acceptance angle, 240,000 total dose. Detailed image acquisition parameters are summarized in Supplementary Table 1.

Image processing, model building, and refinement

The detailed image processing workflow and statistics are summarized in Supplementary Fig. 1b and Supplementary Table 1. Raw movies were motion-corrected using MotionCor228, and the CTF parameters were estimated by CTFIND429. All other image processing was performed using cryoSPARC v.325. Initial, a total of 1,030,000 particles were picked from a blob picker of cryoSPARC from a few micrographs. 2D class averages representing projections in different orientations selected from the initial 2D classification were used as templates for automatic particle picking from the full datasets. The resulting 16,780,000 extracted particles were binned two times, and subsequent 2D classification, Ab initio, and Heterogeneous refinement for clean-up were performed in cryoSPARC. A total of 638,058 particles from the 3D classes showing good secondary structural features were selected and re-extracted into the original pixel size (300 × 300 pixel boxes). Non-uniform refinement was performed on the resulting volume using per-particle defocus and per-group CTF optimizations applied283,284. The final refinement yielded a map at an overall 3.2 Å resolution (with mask). The mask-corrected Fourier shell correlation (FSC) curves were calculated in cryoSPARC, and reported resolutions were based on the gold-standard Fourier shell correlation (FSC) = 0.143 criterion28. Local resolutions of density maps were estimated by BLoC. Model building for IGF1 and IGF1 was initiated by docking the crystal structure of IGF1/IGFBP4 complex (PDB 2DSR [https://www.wwpdb.org/pdb/2DSR]) into the cryo-EM map using the Phenix package (v1.19.2)27. The Calpha chain and side chains of ALS were manually built in the cryo-EM density map using Coot (v0.8.9.2)28. The model was manually adjusted in Coot and refined against the map by using the real space refinement in the Phenix package. Model building of ambiguously resolved parts was aided by a protein model generated from AlphaFold225,26, and a post-processed map generated from DeepEMHancer26. The refinement statistics from Phenix validation are summarized in Supplementary Table 1. All figures for cryo-EM maps and structural models were generated using UCSF Chimera (v1.14), ChimeraX (v1.3), and PyMOL (v2.3.1)28–30.

Co-expression and pull-down assays

A total of 1 μg of plasmid DNA (ALS-His:IGFBP3-Strep:IGF1 = 1:1:2. ALS-His:IGFBP3-Strep = 1:1, ALS-His:IGFBP3-Strep = 1:1, ALS-His:CLD3-CBP3-Strep = 1:1, CLD3-CBP3-Strep:IGF1-His = 1:1, ALS-His:CLD3-CBP3-Strep:IGF1-His = 1:1, ALS-His:CLD3-CBP3-Strep:IGF1-His = 1:1, or ALS-His:CLD3-CBP3-Strep:IGF1-His = 1:1, 2:1) were transfected into 2.5 × 10^6 Exp293-F cells using Mesifectamine and cells were cultured in Exp293 expression medium at 37 °C and 8% CO2 with shaking (120 rpm) for 4 days. Similarly, a total of 1 μg of plasmid DNA ([ALS-His (mutant or WT);IGFBP3 (mutant or WT);IGF1-strep (mutant or WT) = 1:2:2] were transfected for complex formation analysis of mutants. Cells then were pelleted by centrifugation and the supernatants loaded onto Ni-NiTA Agarose (#30210, Qiagen). The protein-bound resins were washed with 10 column volumes of wash buffer (20 mM Tris-HCl pH 8.0, 200 mM NaCl, 20 mM imidazole), and proteins were eluted with elution buffer (20 mM Tris-HCl pH 8.0, 200 mM NaCl, 300 mM imidazole). For Strep-Tactin purification, 10 μl buffer W (#2-1003-100, ika) and BioLock (#2-0205-050, ika) were added to supernatants and incubated for 20 min with agitation at 4 °C to prevent unwanted biotinylated proteins from binding to Strep-Tactin. After centrifugation (3000 x g, 20 min), the supernatants were loaded to Strep-Tactin Sepharose (#2-1201-01S, ika) and washed with 10 column volumes of wash buffer (20 mM Tris-HCl pH 8.0, 200 mM NaCl). Then, proteins were eluted with elution buffer (20 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM desthiobiotin).

In vitro reconstitution of the binary or ternary complex and FSEC after proteolysis

To test the complex formation, the purified IGF1-GFP-Strep/IGFBP3 or IGF1-GFP-Strep alone were incubated with purified ALS (molar ratio of

https://doi.org/10.1038/s41467-022-32214-2
For 16 h at 4 °C with shaking. Also, the purified IGFBP3-Strep or CLD3-CBP3-Strep were incubated with purified IGF1-GFP-IGFBP3 (molar ratio of 1:1) for 16 h at 4 °C with shaking. Then, the mixtures were subjected to SEC using a Superdex 200 increase 10/300 GL column (#GE28-9909-44, Cytiva) equilibrated with 20 mM Tris pH 8.0, 200 mM NaCl. The flow path from the SEC column was directly connected to a Fluorescence Detector (FP-4025, JASCO) to detect the GFP fluorescence signal (485 nm/507 nm). To trace IGF1-GFP after proteolysis, the purified IGF1-GFP/IGFBP3 binary complex (Fig. 6a), IGF1-GFP/IGFBP3/ALS ternary complex (Fig. 6a and Supplementary Fig. 7a, c), or IGF1-GFP/hybrid-BPs/ALS ternary complexes (Fig. 6g and Supplementary Fig. 7i) were incubated with bovine thrombin (#91-035-020, BioPharm, 0.2 μg of thrombin for 1 μg of IGF1/IGFBP3-ALF) or human PAPP-A2 (#1668-ZN-020, R&D Systems, 0.2 μg of PAPP-A2 for 1 μg of IGF1/IGFBP3/ALS or IGF1-GFP/hybrid-BPs/ALS ternary complexes in 20 mM Tris pH 8.0, 200 mM NaCl for 16 h at 37 °C with shaking (Fig. 6d and Supplementary Fig. 7j) was incubated with bovine thrombin (#91-035-020, BioPharm, 0.2 μg of thrombin for 1 μg of IGF1/IGFBP3-ALF in 20 mM Tris pH 8.0, 200 mM NaCl for 10 h at 37 °C with shaking), human ADAM12 (#4416-AD-020, R&D Systems, 0.2 μg of ADAM12 for 1 μg of IGF1/IGFBP3-ALF in 20 mM Tris pH 8.0, 200 mM NaCl for 16 h at 37 °C with shaking) or human PAPP-A2 (#1668-ZN-020, R&D Systems, 0.2 μg of PAPP-A2 for 1 μg of IGF1/IGFBP3-ALF in 20 mM Tris pH 8.0, 200 mM NaCl for 16 h at 37 °C with shaking), followed by FSEC. To trace CBP3-GFP after proteolysis, the purified IGF1-GFP/CBP3-Strep ternary complex, IGF1-GFP/IGFBP3 binary complex (Fig. 6a), IGF1-GFP/IGFBP3/ALS or IGF1-GFP/hybrid-BPs/ALS ternary complexes (Fig. 6g and Supplementary Fig. 7i) were incubated with bovine thrombin (#91-035-020, BioPharm, 0.2 μg of thrombin for 1 μg of IGF1/IGFBP3-ALF in 20 mM Tris pH 8.0, 200 mM NaCl for 16 h at 37 °C with shaking) or human PAPP-A2 (#1668-ZN-020, R&D Systems, 0.2 μg of PAPP-A2 for 1 μg of IGF1/IGFBP3-ALF in 20 mM Tris pH 8.0, 200 mM NaCl for 16 h at 37 °C with shaking) (Fig. 6d, e, f and Supplementary Fig. 7b, e, g, h). Thereafter, cells were washed twice with PBS, and lysed in 100 μl of lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, protease inhibitor, phosphatase inhibitor). After incubation at 4 °C for 60 min, cell lysates were cleared by centrifugation at 15,000 × g for 20 min, after which the protein concentration in the supernatant was quantified by the Bradford assay. Then, the cell lysates were treated with SDS sample buffer and subjected to SDS-PAGE. Proteins were transferred to a PVDF membrane (#88518, Thermo Fisher Scientific). IGFR phosphorylation was assessed by first blocking the blot by incubation with 5% (w/v) skim milk in TBS-T at RT for 1 h, and then incubating it with an anti-phospho-IGFR primary antibody (Y1135/1136) (1:1000 dilution, #3024, Cell Signaling) followed by incubation with HRP-conjugated goat anti-rabbit IgG antibody (1:5000 dilution, #62-6520, Thermo Fisher Scientific). Immunoreactive phospho-IGFR was visualized using ECL-WestLumiOne (#2332632, ATTO). After staining, the membrane was incubated in stripping buffer (Thermo Fisher Scientific) to block any non-specific binding. The membrane was re-probed with a rabbit anti-IGF1 receptor antibody (1:1000 dilution, #3027, Cell Signaling), followed by incubation with HRP-conjugated goat anti-rabbit IgG antibody (1:5000 dilution, #31460, Thermo Fisher Scientific) to determine the amount of total IGFR. Another blocked membrane was incubated with an anti-beta-actin (1:1000 dilution, #sc-4778, Santa Cruz) followed by incubation with HRP-conjugated goat anti-mouse IgG antibody (1:5000 dilution, #62-6520, Thermo Fisher Scientific). Immunoreactive IGFR and beta-actin were visualized using ECL-WestLumiOne (#2332632, ATTO).

PULL-DOWN AND IMMUNOBLOTTING FOR ANALYSIS OF INTERMEDIATE TERNARY COMPLEX WHICH BINDS TO IGFR

After incubation of the purified IGFR ectodomain-leucine zipper-strep (IGRIR-zip) with IGF1, and intermediate ternary complex (IGF1/IGFBP3/ALS after thrombin proteolysis, 0.2 μg of thrombin for 1 μg of IGF1-GFP/IGFBP3/ALS in 20 mM Tris pH 8.0, 200 mM NaCl for 10 h at 37 °C with shaking), the mixture was loaded onto Strep-Tactin Sepharose (#2-1201-025, Iba) and washed with 10 column volumes of wash buffer (20 mM Tris-HCl pH 8.0, 80 mM NaCl). Strep-Tactin resin and flow through sample were treated with sodium dodecyl sulfate (SDS) sample buffer and subjected to denaturing polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (#88518, Thermo Fisher Scientific), which in turn was blocked by incubation with 5% (w/v) skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) at room temperature (RT) for 1 h, and then incubated with primary antibodies [His-tag (1:1000 dilution, #25864s, Cell Signaling); Strep-tag II (1:1000 dilution, #ab76949, Abcam); IgG (1:1000 dilution, #25864s, Cell Signaling); CBP3 (1:1000 dilution, #sc-374365, Santa Cruz); ALS (1:1000 dilution, #sc-377131, Santa Cruz); IGF1 (1:1000 dilution, #MAI-088, Thermo Fisher)] at 4 °C for 12 h. After being washed with TBS-T buffer three times, the membrane was incubated at RT for 30 min with horseradish peroxidase (HRP)-conjugated anti-mouse antibodies (#62-6520, Thermo Fisher Scientific) for anti-IGFBP3 (sc-374365), anti-ALS, and anti-IGF1 primary antibodies, or HRP-conjugated anti-rabbit antibodies (#31460, Thermo Fisher Scientific) for anti-Myc-tag, anti-Strep-tag II, and anti-IGFBP3 (#25864s) primary antibodies.
Quantification and statistical analysis
Data in figures and tables are reported as mean ± standard deviation (SD) with the number of biological and technical replicates indicated in the figure and table legends, where “n” represents the number of biological replicates. Immunoblotting was assessed using ImageJ and GraphPad Prism v9.0.

 Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

 Data availability
The atomic coordinates for the IGF1/IGFBP3/ALS ternary complex have been deposited to the RCSB Protein Data Bank under the accession number 7WBO. The cryo-EM map of the ternary complex has been deposited on Microscopy Data Bank under the accession number EMD-32735. The source data for all figures and supplementary figures are available as Source Data file. Reporting Summary for this article is provided as Supplementary Information file. All the other data and materials used for the analysis are available from the corresponding author upon reasonable request. Source data are provided with this paper.

 References
1. LeRoith, D. & Yakar, S. Mechanisms of disease: metabolic effects of growth hormone and insulin-like growth factor 1. Nat. Clin. Pr. Endocrinol. Metab. 3, 302–310 (2007).
2. Baxter, R. C. Circulating binding proteins for the insulin-like growth factors. Trends Endocrinol. Metab. 4, 91–96 (1993).
3. Twigg, S. M. & Baxter, R. C. Insulin-like growth factor (IGF)-binding protein 5 forms an alternative ternary complex with IGFs and the acid-labile subunit. J. Biol. Chem. 273, 6074–6079 (1998).
4. Rajaram, S., Baylink, D. J. & Mohan, S. Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. Endocr. Rev. 18, 801–831 (1997).
5. Guler, H. P., Zapf, J., Schmid, C. & Froesch, E. R. Insulin-like growth factors I and II in healthy man. Estimations of half-lives and production rates. Acta Endocrinol. (Copenh) 121, 753–758 (1989).
6. Baxter, R. C. IGF binding proteins in cancer: mechanistic and clinical insights. Nat. Rev. Cancer 14, 329–341 (2014).
7. Berryman, D. E., Glad, C. A., List, E. O. & Johansson, G. The GH/IGF-1 axis in obesity: pathophysiology and therapeutic considerations. Nat. Rev. Endocrinol. 9, 346–356 (2013).
8. Brooks, A. J. & Waters, M. J. The growth hormone receptor: mechanism of activation and clinical implications. Nat. Rev. Endocrinol. 6, 515–525 (2010).
9. Vajdos, F. & et al. Crystal structure of human insulin-like growth factor-I: detergent binding inhibits binding protein interactions. Biochemistry 40, 11022–11029 (2001).
10. Bunn, R. C. & Fowlkes, J. L. Insulin-like growth factor binding protein proteolysis. Trends Endocrinol. Metab. 14, 176–181 (2003).
11. Zeslawski, W. et al. The interaction of insulin-like growth factor-I with the N-terminal domain of IGFBP-5. EMBO J. 20, 3638–3644 (2001).
12. Sitar, T., Popowicz, G. M., Siwanowicz, I., Huber, R. & Holak, T. A. Structural basis for the inhibition of insulin-like growth factors by insulin-like growth factor-binding proteins. Proc. Natl Acad. Sci. USA 103, 13028–13033 (2006).
13. Li, J., Choi, E., Yu, H. & Bai, X. C. Structural basis of the activation of type 1 insulin-like growth factor receptor. Nat. Commun. 10, 4567 (2019).
14. Boisclair, Y. R., Rhoads, R. P., Ueki, I., Wang, J. & Ooi, G. T. The acid-labile subunit (ALS) of the 150 kDa IGF-binding protein complex: an important but forgotten component of the circulating IGF system. J. Endocrinol. 170, 63–70 (2001).
15. Domene, H. M., Hwa, V., Jasper, H. G. & Rosenfeld, R. G. Acid-labile subunit (ALS) deficiency. Best. Pr. Res. Clin. Endocrinol. Metab. 25, 101–113 (2011).
16. Domene, S. & Domene, H. M. The role of acid-labile subunit (ALS) in the modulation of GH-IGF-I action. Mol. Cell Endocrinol. 518, 111006 (2020).
17. Domene, H. M. et al. Deficiency of the circulating insulin-like growth factor system associated with inactivation of the acid-labile subunit gene. N. Engl. J. Med. 350, 570–577 (2004).
18. Baxter, R. C., Martin, J. L. & Beniach, V. A. High molecular weight insulin-like growth factor binding protein complex. Purification and properties of the acid-labile subunit from human serum. J. Biol. Chem. 264, 11843–11848 (1989).
19. Janosi, J. B., Firth, S. M., Bond, J. J., Baxter, R. C. & Delhanty, P. J. N-Linked glycosylation and sialylation of the acid-labile subunit. Role in complex formation with insulin-like growth factor (IGF)-binding protein-3 and the IGFs. J. Biol. Chem. 274, 5292–5298 (1999).
20. David, A., Kelley, L. A. & Sternberg, M. J. A new structural model of the acid-labile subunit: pathogenetic mechanisms of short stature-causing mutations. J. Mol. Endocrinol. 49, 213–220 (2012).
21. Siwanowicz, I. et al. Structural basis for the regulation of insulin-like growth factors by IGF binding proteins. Structure 13, 155–167 (2005).
22. Bayne, M. L., Applebaum, J., Cicchetti, G. G., Miller, R. E. & Cascieri, M. A. The roles of tyrosines 24, 31, and 60 in the high affinity binding of insulin-like growth factor-I to the type 1 insulin-like growth factor receptor. J. Biol. Chem. 265, 15648–15652 (1990).
23. Zhang, W., Gustafson, T. A., Rutter, W. J. & Johnson, J. D. Positively charged side chains in the insulin-like growth factor-1 C- and D-regions determine receptor binding specificity. J. Biol. Chem. 269, 10609–10613 (1994).
24. Martucci, L. C. et al. Assessment of pathogenicity of natural IGFLAS gene variants by in silico bioinformatics tools and in vitro functional studies. Mol. Cell Endocrinol. 429, 19–28 (2016).
25. Firth, S. M., Yan, X. & Baxter, R. C. D440N mutation in the acid-labile subunit of insulin-like growth factor complex exerts secretion and complex formation. Mol. Endocrinol. 25, 307–314 (2011).
26. Savage, M. O., Hwa, V., David, A., Rosenfeld, R. G. & Metherell, L. A. Genetic defects in the growth hormone-IGF-I axis causing growth hormone insensitivity and impaired linear growth. Front. Endocrinol. (Lausanne) 2, 95 (2011).
27. Shaheen, R. et al. Genomic analysis of primordial dwarfish reveals novel disease genes. Genome Res. 24, 291–299 (2014).
28. Keselman, A. C. et al. A homozygous mutation in the highly conserved Tyr60 of the mature IGF1 peptide broadens the spectrum of IGF1 deficiency. Eur. J. Endocrinol. 181, K43–K53 (2019).
29. Wajlenkamp, M. J. et al. Homozygous and heterozygous expression of a novel insulin-like growth factor-I mutation. J. Clin. Endocrinol. Metab. 90, 2855–2864 (2005).
30. Netchine, I. et al. Partial primary deficiency of insulin-like growth factor (IGF)-I activity associated with IGF1 mutation demonstrates its critical role in growth and brain development. J. Clin. Endocrinol. Metab. 94, 3913–3921 (2009).
31. Booth, B. A., Boes, M. & Bar, R. S. IGFBP-3 proteolysis by plasmin, thrombin, serum: heparin binding, IGF binding, and structure of fragments. Am. J. Physiol. 271, E465–E470 (1996).

32. Firth, S. M. & Baxter, R. C. Cellular actions of the insulin-like growth factor binding proteins. Endocrinol. Rev. 23, 824–854 (2002).

33. Lee, C. Y. & Rechler, M. M. Proteolysis of insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3) in 150-kilodalton IGFBP complexes by a cation-dependent protease activity in adult rat serum promotes the release of bound IGF-I. Endocrinology 137, 2051–2058 (1996).

34. Gaidamauskas, E. et al. IGF dependent modulation of IGF binding protein (IGFBP) proteolysis by pregnancy-associated plasma protein-A (PAPP-A); multiple PAPP-A-IGFBP interaction sites. Biochim. Biophys. Acta 1830, 2701–2709 (2013).

35. Shi, Z., Xu, W., Loechel, F., Wewer, U. M. & Murphy, L. J. ADAM12, a disintegrin metallopeptase, interacts with insulin-like growth factor-binding protein-3. J. Biol. Chem. 275, 18574–18580 (2000).

36. Dauber, A. et al. Mutations in pregnancy-associated plasma protein A2 cause short stature due to low IGF-I availability. EMBO Mol. Med. 8, 363–374 (2016).

37. Overgaard, M. T. et al. Pregnancy-associated plasma protein-A2 (PAPP-A2), a novel insulin-like growth factor-binding protein-5 proteasome. J. Biol. Chem. 276, 21849–21853 (2001).

38. Xu, Y. et al. How IGF-II binds to the human type 1 insulin-like growth factor receptor. Structure 20, 786–798 (2020).786.

39. Holm, L. & Laakso, L. M. Dal server update. Nucleic Acids Res. 44, W351–W355 (2016).

40. Denley, A., Cosgrove, L. J., Booker, G. W., Wallace, J. C. & Forbes, B. E. Molecular interactions of the IGF system. Cytokine Growth Factor Rev. 16, 421–439 (2005).

41. Holman, S. R. & Baxter, R. C. Insulin-like growth factor binding protein-3: factors affecting binary and ternary complex formation. Growth Regul. 6, 42–47 (1996).

42. Firth, S. M. & Baxter, R. C. Characterisation of recombinant glycosylation variants of insulin-like growth factor binding protein-3. J. Endocrinol. 160, 379–387 (1999).

43. Brzozowski, A. M. et al. Structural origins of the functional divergence of human insulin-like growth factor-I and insulin. Biochemistry 41, 9389–9397 (2002).

44. Hashimoto, R. et al. Binding sites and binding properties of binary and ternary complexes of insulin-like growth factor-II (IGF-II), IGF-binding protein-3, and acid-labile subunit. J. Biol. Chem. 272, 27936–27942 (1997).

45. Twigg, S. M., Kiefer, M. C., Zapf, J. & Baxter, R. C. Insulin-like growth factor-binding protein 5 complexes with the acid-labile subunit. Role of the carboxy-terminal domain. J. Biol. Chem. 273, 28791–28798 (1998).

46. Kuang, Z. et al. Cooperativity of the N- and C-terminal domains of insulin-like growth factor (IGF) binding protein 2 in IGF binding. Biochemistry 46, 13720–13732 (2007).

47. Payet, L. D., Wang, X. H., Baxter, R. C. & Firth, S. M. Amino- and carboxy-terminal fragments of insulin-like growth factor (IGF) binding protein-3 cooperate to bind IGFs with high affinity and inhibit IGF receptor interactions. Endocrinology 144, 2797–2806 (2003).

48. Forbes, B. E., McCarthy, P. & Norton, R. S. Insulin-like growth factor binding proteins: a structural perspective. Front. Endocrinol. (Lausanne) 3, 38 (2012).

49. Sun, M. et al. Practical considerations for using K3 cameras in CDS mode for high-resolution and high-throughput single particle cryo-EM. J. Struct. Biol. 213, 107745 (2021).

50. Zheng, S. Q. et al. MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. Nat. Methods 14, 331–332 (2017).

51. Rohou, A. & Grigorieff, N. CTFFind4: Fast and accurate defocus estimation from electron micrographs. J. Struct. Biol. 192, 216–221 (2015).

52. Punjani, A., Rubinstein, J. L., Fleet, D. J. & Brubaker, M. A. cryoS-PARC: algorithms for rapid unsupervised cryo-EM structure determination. Nat. Methods 14, 290–296 (2017).

53. Punjani, A., Zhang, H. & Fleet, D. J. Non-uniform refinement: adaptive regularization improves single-particle cryo-EM reconstruction. Nat. Methods 17, 1214–1221 (2020).

54. Rubinstein, J. L. & Brubaker, M. A. Alignment of cryo-EM movies of individual particles by optimization of image translations. J. Struct. Biol. 192, 188–195 (2015).

55. Rosenthal, P. B. & Henderson, R. Optimal determination of particle orientation, absolute hand, and contrast loss in single-particle electron cryomicroscopy. J. Mol. Biol. 333, 721–745 (2003).

56. Kučukelbir, A., Sigworth, F. J. & Tagare, H. D. Quantifying the local resolution of cryo-EM density maps. Nat. Methods 11, 63–65 (2014).

57. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. Sect. D: Biol. Crystallogr. 66, 212–221 (2010).

58. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr. Sect. D: Biol. Crystallogr. 60, 2126–2132 (2004).

59. Jumper, J. et al. Highly accurate protein structure prediction with AlphaFold. Nature 596, 583–589 (2021).

60. Tynysuvunakool, K. et al. Highly accurate protein structure prediction for the human proteome. Nature 596, 590–596 (2021).

61. Sanchez-Garcia, R. et al. DeepEMhancer: a deep learning solution for cryo-EM volume post-processing. Commun. Biol. 4, 874 (2021).

62. Petersen, E. F. et al. UCSF ChimeraX—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612 (2004).

63. Petersen, E. F. et al. UCSF ChimeraX: Structure visualization for researchers, educators, and developers. Protein Sci. 30, 70–82 (2021).

64. Schrodinger, LLC. The PyMOL Molecular Graphics System, Version 1.8) (Schrodinger, LLC, 2015).

65. Ashkenazy, H. et al. ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules. Nucleic Acids Res. 44, W344–W350 (2016).

66. Williams, C. et al. An exon splice enhancer primes IGF2/IGF2R binding site structure and function evolution. Science 338, 1209–1213 (2012).

Acknowledgements
We are grateful to the staff of the Research Solution Center at IBS for help with cryo-EM data collection. Computational work for this research was performed on the data analysis hub, Olaf, in the IBS Research Solution Center. We also thank Global Science experimental Data hub Center (GSDC) at Korea Institute of Science and Technology Information (KISTI) for computing resources and technical support, and Dr. Jin Young Kang, Jinwoo Kim at KAIST, Department of chemistry for image processing counsel. This work was supported by grants from the Institute for Basic Science (IBS-R030-C1 to H.M.K.).

Author contributions
H.K., Y.F., and H.M.K. designed the experiments and analyzed the data; H.J.H. and D.S.L. purified proteins; H.K., Y.F., and S.-G.L. determined the cryo-EM structure; and H.K., Y.F., and H.M.K. wrote the manuscript.

Competing interests
The authors declare no competing interests.
Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-32214-2.

Correspondence and requests for materials should be addressed to Ho Min Kim.

Peer review information Nature Communications thanks Xiaochen Bai, Horacio Domené and Briony Forbes for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022