

1 **Fusion of DARPin to aldolase enables visualization of small**
2 **protein by cryoEM**

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4 Running title: Visualization of small protein by cryoEM

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Abstract

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In recent years, solving protein structures by single particle cryogenic electron microscopy (cryoEM) has become a crucial tool in structural biology. While exciting progress is being made towards the visualization of smaller and smaller macromolecules, the median protein size in both eukaryotes and bacteria is still beyond the reach of single particle cryoEM. To overcome this problem, we implemented a platform strategy in which a small protein target was rigidly attached to a large, symmetric base via a selectable adapter. Seven designs were tested. In the best construct, a designed ankyrin repeat protein (DARPin) was rigidly fused to tetrameric rabbit muscle aldolase through a helical linker. The DARPin retained its ability to bind its target, the 27 kDa green fluorescent protein (GFP). We solved the structure of this complex to 3.0 Å resolution overall, with 5 to 8 Å resolution in the GFP region. As flexibility in the DARPin limited the overall resolution of the target, we describe strategies to rigidify this element.

Author summary

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Single particle cryogenic electron microscopy (cryoEM) is a technique that uses images of purified proteins to determine their atomic structure. Unfortunately, the majority of proteins in the human and bacterial proteomes are too small to be analyzed by cryoEM. Over the years, several groups have suggested the use of a platform to increase the size of small protein targets. The platform is composed of a large protein base and a selectable adapter that binds the target protein. Here we report a platform based on tetrameric rabbit muscle aldolase that is fused to a Designed Ankyrin Repeat Protein (DARPin). Phage display libraries can be used to generate DARPins against target proteins. The residues mutated in a phage display library to generate a DARPin against a new target do not overlap with the DARPin-base fusion in the platform, thus changing the DARPin identity will not disrupt the platform design. The DARPin adapter used here is capable of binding Green Fluorescent Protein (GFP). We report the structure of GFP to 5 to 8 Å local resolution by single particle cryoEM. Our analysis demonstrates that flexibility in the DARPin-aldolase platform prevents us from achieving higher resolution in the GFP region. We suggest changes to the DARPin design to rigidify the DARPin-aldolase platform. This work expands on current platforms and paves a generally applicable way toward structure determination of small proteins by cryoEM.

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Introduction

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85 Single particle cryoEM can reveal the structures of large macromolecular complexes to near
86 atomic resolution. To solve a protein structure by single particle cryoEM, purified proteins are
87 rapidly frozen in a thin layer of vitreous ice. A transmission electron microscope is used to
88 collect projection images of the protein. Individual proteins are identified in the ice and their
89 orientations are computationally determined. The projection images are then combined to
90 calculate a 3D reconstruction of the protein.

91

92 A fundamental challenge in single particle cryoEM is that small proteins do not produce enough
93 contrast in noisy projection images to precisely determine their orientation. Richard Henderson
94 estimated that with ideal images, a 3 Å structure could be reconstructed for a 40 kDa protein (1).
95 Unfortunately, real electron micrographs are imperfect so this theoretical minimum of
96 macromolecular size has never been reached. The smallest protein to be solved to near atomic
97 resolution so far by cryoEM is hemoglobin (64 kDa) (2), but the median protein lengths in both
98 bacteria (27 kDa) and eukaryotes (36 kDa) are about two times smaller (3). Consequently, many
99 proteins in biology are beyond the reach of high-resolution structure determination by single
100 particle cryoEM.

101

102 Over the years, several strategies to overcome the size limit problem in single particle cryoEM
103 have been suggested. Two major themes have emerged to increase the target mass and improve
104 its orientation determination. First, the target can be decorated with antibody fragments (6) (7).
105 Second, the target can be rigidly attached to a large platform protein. The platform is typically
106 composed of a base protein and an adapter. The purpose of the base protein is to increase the
107 molecular weight, which facilitates accurate particle picking and precise particle orientation
108 determination. The adapter can be customized (a covalent fusion between the target and the base)
109 or general (a selectable adapter that facilitates non-covalent binding of the target to the platform
110 base). Covalent approaches have utilized direct fusions between the target protein and the base
111 either via a flexible linker adapter (4) or a helical junction adapter (5) (8). For the platform to be
112 successful, the adapter must be rigidly attached to the base. The flexible linker adapter was
113 therefore insufficient to determine the structure of the target (4), but the use of a helix-forming

114 peptide linker (8)(9) or direct concatenation of two helices (5)(10) has shown promise. Most
115 recently, Liu et al. demonstrated that a rigid, continuous α -helix could be formed by linking the
116 terminal α -helices of a designed ankyrin repeat protein (DARPin) and a nanocage subunit
117 through a helix-forming peptide linker (9) (8). Notably, Liu et al were able to show the structure
118 of the 17 kDa DARPin to 3.5 to 5 Å local resolution (8). Unfortunately, these strategies are
119 limited to target proteins with a terminal α -helix, and their implementation requires that the
120 length of the helical junction adapter must be customized for each new target. Utilizing a non-
121 covalent platform strategy with a selectable adapter (like an antibody or a DARPin) has the
122 potential to be generally applicable, regardless of the structure of the target, since the selectable
123 adapter could be raised against any target using phage display, while the invariant nature of the
124 adapter framework region would allow the one-time optimization of a rigid attachment point
125 between it and the base. Along these lines, Liu et al. suggested that their DARPin-nanocage
126 could display a small protein for structure determination by cryoEM (8), but so far no group has
127 demonstrated this.

128
129 Here we report the outcomes of a variety of new designs and report the structure of the first small
130 protein visualized through a base/selectable-adapter platform approach.

131

132 **Results**

133

134 *Platform strategy and design*

135 The goal of our study was to design a generally applicable platform to solve small protein
136 structures by single particle cryoEM. We explored several candidate base proteins and selectable
137 adapters (Fig S1). We favored bases that were easy to purify and that had already been solved to
138 high-resolution by single particle cryoEM. We reasoned that oligomeric and symmetric (as a
139 globular protein, or as a helical tube) bases would be best.

140

141 As selectable adapters, we first considered antibody fragments (Fabs and scFvs). Fabs have a
142 flexible elbow connecting two immunoglobulin regions, whereas scFvs are made up of one
143 immunoglobulin region. The Fab elbow could introduce flexibility, so we preferred the smaller,
144 more compact scFv. However, because the beta sandwich immunoglobulin fold of a scFv could

145 be difficult to rigidly fuse to the surface of a platform base, we identified Staphylococcus Protein
146 A (PrA) as a linker that could bind the invariant region of a scFv (11). As PrA is a three-helix
147 bundle, we reasoned that it could be rigidly attached to a base via a helical linker. Thus in one of
148 our designs, the C-terminal helix of PrA was fused to the N-terminal helix of the base protein.
149 Since PrA is capable of binding the invariant scFv framework, the base-PrA:scFv interfaces
150 would not need to be redesigned for each new target. Unfortunately, in our biochemical
151 experiments, we observed that the PrA:scFv interaction did not remain stable through a gel
152 filtration column, indicating that the binding affinity was not strong enough for our purposes.
153 Further mutagenesis to the PrA:scFv interface may strengthen the interaction. Regardless, a
154 fundamental concern with this design is that two non-covalent binding interactions are required
155 (PrA:scFv, and scFv:target), which could lead to occupancy issues. As a result, we moved to
156 DARPs as our selectable adapter (Fig 1B).

157

158 In our designs, the final alpha helix of the DARPin C-terminal cap (C-cap) was directly fused to
159 the first α -helix of the base (Fig 1C). All DARPin libraries use a C-cap to stabilize the protein, so
160 we expect it will be straightforward to swap in any DARPin built on the same framework (Fig
161 1B). In the base-DARPin platform design, only one non-covalent interaction is required
162 (between the DARPin and the target), which results in a more predictable and stable complex.
163 We chose a DARPin that formed a stable complex with GFP as a first test case (12) and screened
164 several base-DARPin candidates.

165

166 *Screening base candidates*

167 We performed expression trials for five of our base-DARPin candidates (Fig S1). These bases
168 included β -galactosidase (β -gal) (13), the vipA/vipB helical tube (14), the *E. coli* ribosome (15),
169 TibC (16), and aldolase (17).

170

171 Because β -gal tetramerization requires the N- and C-termini of each subunit (18), an internal
172 DARPin insertion was used, flanked by a helix-forming peptide (at the DARPin N-cap) and a
173 flexible linker (at the DARPin C-cap) (9). Biochemically the β -gal-DARPin platform formed a
174 stable complex with GFP, but no cryoEM density was observed for the DARPin or GFP in our 3
175 Å reconstruction. This means that the helical linker was flexible relative to the β -gal base.

176

177 We therefore focused on bases with a terminal α -helix that could be rigidly fused to the DARPin.
178 The vipA/vipB, ribosome L29, TibC, and aldolase proteins all had long terminal α -helices to
179 facilitate direct fusion. In our experiments, the helical tube vipA-DARPin/vipB platform
180 exhibited poor expression in *E. coli*, while the L29-PrA fusion did not integrate well into Δ L29
181 *E. coli* ribosomes (15) (Fig S1). The purified TibC-DARPin platform formed a stable complex
182 with GFP, but the complex demonstrated aggregation and preferred orientation on plunge frozen
183 grids. In contrast, the DARPin-aldolase platform was well-behaved.

184

185 In our DARPin-aldolase platform, the C-terminal α -helix of the DARPin was directly
186 concatenated to the N-terminal α -helix of aldolase (Fig 1C) (S2 Fig A). The D2 symmetry of the
187 DARPin-aldolase platform provided extensive space for the target and could potentially
188 accommodate a globular protein of up to 740 kDa without steric clash (Fig 1E, 1F) (S1 Movie).
189 The purified GFP:DARPin-aldolase complex was stable in a gel filtration column with an
190 apparent 1:1 stoichiometry of DARPin-aldolase to the target (GFP) (S2 Fig B and C).

191

192 *CryoEM analysis of the GFP:DARPin-aldolase complex*

193 To solve the structure of GFP bound to the DARPin-aldolase platform, we collected 1,681
194 micrographs on a Titan Krios (Fig S3). Because the thin ice forced a slight preferred orientation
195 issue, an additional 1,180 micrographs were collected at 26° tilt (see methods) (19). High quality
196 micrographs were selected after CTF correction (Fig S4) and particles were autopicked in Relion
197 (Fig S3). After 2D classification in cryoSPARC, classes with strong secondary structure were
198 selected for reconstruction. The GFP:DARPin-aldolase complex reconstruction yielded an
199 overall resolution of 3 Å with C1 symmetry (Fig S5B). Further classification suggested too much
200 conformational heterogeneity to apply D2 symmetry. The aldolase core and the helical linker
201 were resolved to near atomic resolution (Fig 2B, 2C, 2D). The DARPin and GFP exhibited a
202 local resolution of 4 to 8 Å, with discontinuous regions of higher resolution of 3.5 Å (Fig 2D)
203 (S2 Movie). Although the resolution in the GFP and DARPin portion was not sufficient to build
204 a model or assign sequence *de novo*, the static X-ray structures of GFP and the DARPin could be
205 reliably docked into the map (Fig 2A).

206

207 *DARPin framework caused conformational heterogeneity*

208 Because of the 5 to 8 Å local resolution range in the GFP portion of the map (Fig 2D), we
209 suspected that part of the GFP:DARPin-aldolase complex was flexible. To better understand the
210 conformational heterogeneity in the data, a mask was generated around a single DARPin/GFP
211 unit and Relion particle symmetry expansion was used to consider each subunit individually (Fig
212 3A, Fig S3) (21). The symmetry expanded particles were subjected to 3D classification without
213 alignment, a strategy in which the orientation parameters determined in the previous refinement
214 are used to classify the particles into subsets. For this focused classification, a spherical mask
215 that encompassed the aldolase surface was used to increase the signal. The resulting five classes
216 showed reasonable GFP:DARPin conformations (Fig 3B), but subsequent refinements were still
217 limited to 5 to 6 Å overall, which suggested that additional conformational heterogeneity
218 remained within the subsets. The majority of the particles (54%) were classified into class 2
219 (yellow), which appeared to lack a DARPin (Fig 3B). Class 2 was subjected to an additional
220 round of 3D classification where it revealed several reasonable but lower resolution
221 GFP:DARPin conformations (Fig S6). To investigate the heterogeneity in the focused classes,
222 we compared each class to class 4 (Fig 3C, 3D). In the different classes, the GFP:DARPin
223 density shows a clear rocking around the Y axis (Fig 3C) and around the Z axis (Fig 3D) relative
224 to the aldolase base. At this point, we wondered if any these displacements could be attributed to
225 the aldolase subunit. We performed a similar focused classification experiment with a mask
226 around the aldolase subunit and the helical linker, but no rotation or shift was observed in the
227 resulting subsets (data not shown). Thus, we concluded that the displacement likely arose in the
228 C-cap second helix that is fused into the helical linker, and other regions of the DARPin distal to
229 the linker.

230

231 **Discussion**

232

233 In this study, we designed and tested a variety of platforms capable of non-covalently binding a
234 small target protein via a selectable adapter for structure determination by single particle
235 cryoEM. In our best construct, we resolved our target protein (GFP) to 5 to 8 Å resolution.

236

237 Our DARPin-aldolase platform has several advantages over other strategies. It is simple to
238 express and purify. Aldolase has D2 symmetry and allows attachment of four targets without
239 steric clash. Aldolase can be reconstructed to 2.6 Å resolution with even a 200 keV microscope
240 (17). Because DARPins can be readily generated against a wide range of small protein targets,
241 the attachment of a DARPin to aldolase promises to be a generally applicable strategy. A recent
242 study of the insulin degrading enzyme (IDE) bound to Fabs was able to isolate several IDE
243 conformations using different Fabs (22). It stands to reason that different DARPins could also
244 stabilize different conformations of the target. Because switching DARPins in the platform
245 would be done by straight-forward DNA manipulations, our DARPin-aldolase platform has the
246 potential to resolve a series of conformations of the target protein.

247
248 Our biochemistry experiments suggested that the purified GFP:DARPin-aldolase complex was
249 very stable, and clear secondary structure was apparent in the 2D classes, yet heterogeneity
250 remained. Because the aldolase base and the helical linker region were resolved to near atomic
251 resolution (Fig 2B and 2C), the heterogeneity likely began in the DARPin C-cap. The DARPin
252 against GFP used here was from a first generation DARPin library. The C-cap of the first
253 generation DARPins was reported to be less stable than the other repeat modules (23). While the
254 crystal structure contained a well-resolved C-cap, the heterogeneity observed here suggests that
255 it is not yet sufficiently rigid to serve as an attachment point in a cryoEM platform (Fig 3).
256 Recent DARPin phage display libraries contain DARPins with reduced surface entropy and a
257 more stable C-cap sequence (23), however, and additional stabilizing surface interactions could
258 be introduced in future designs (28) (29), or even a second attachment point of the DARPin to
259 the base (at both N- and C-terminal caps of DARPin for instance). Together such improvements
260 could allow the DARPin-aldolase platform to reveal the structures of many small proteins to near
261 atomic resolution.

262

263 **Materials and Methods**

264

265 *Computational design*

266 Computational α -helix fusion was generated by manually docking the rabbit muscle aldolase
267 structure (PDB code: 5VY5) and GFP/DARPin complex (PDB code: 5MA6). In order to rigidly

268 join the aldolase and DARPin moiety together, we truncated the C-terminal flexible loop on
269 DARPin and N-terminal flexible loop on aldolase, respectively, exposing the two terminal α -
270 helices. The two terminal α -helices were manually concatenated and joined together to form an
271 ideal α -helix using building α -helix tool in UCSF Chimera (30). The model was inspected for the
272 orientation of DARPin relative to the aldolase, ensuring no steric clash and the providing enough
273 space for target protein attachment. All structural design figures were generated using
274 PyMOL1.8 (<https://pymol.org>).

275

276 *Cloning, protein expression, and purification of the recombinant DARPin-aldolase platform and* 277 *GFP*

278 The DARPin sequence was DARPin 3G86.32 (Fig S2A) (12). The cDNA expressing GFP and
279 our DARPin-aldolase fusion were synthesized at IDT DNA company. The cDNA of GFP and
280 DARPin-aldolase fusion were PCR-amplified and inserted into pACYCDuet and pET21b vector
281 for recombinant expression in *E. coli*, producing no-tag GFP protein and C-terminal His-tag of
282 DARPin-aldolase chimeric fusion. GFP and DARPin-aldolase were coexpressed in *E. coli*
283 BL21(DE3) (Lucigen) using autoinduction medium with trace elements (Formedium) at 30 °C
284 for overnight. Cells were harvested by centrifugation and the protein complex was then purified
285 with Ni-NTA affinity chromatography (Qiagen), and Superdex 200 chromatography (GE
286 healthcare). The purified GFP-DARPin-aldolase complex was concentrated to 2.5mg/ml in a
287 buffer containing 25 mM Tris-HCl pH 8.0 and 150 mM NaCl.

288

289 *CryoEM sample preparation and data collection*

290 Electron microscopy grids were prepared at Scripps Research Institute. Briefly, 3 μ L sample of
291 2.5 mg/ml GFP-DARPin-aldolase complex was applied to a plasma cleaned Au UltraFoil Grid
292 (300 Mesh, R2/2, Quantifoil) in a cold room (4°C, \geq 95% relative humidity). The grid was
293 manually blotted with a filter paper (Whatman No.1) for approximately 3 seconds before
294 plunging into liquid ethane using a manual plunger (17). The grids were screened in Talos
295 Arctica 200 kV with Falcon 3 (FEI) direct electron detector for ice thickness and sample
296 distribution. Micrographs of GFP-DARPin-aldolase complex were collected on Titan Krios
297 microscope (FEI) operating and 300 kV with energy filter (Gatan) and equipped with a K2
298 Summit direct electron detector (Gatan). For untilted data, Serial EM was used for automated

299 EM image acquisition (31). After calculating an efficiency score from early refinements using
300 cryoEF (19), additional data were collected at 26° using EPU software (FEI). A nominal
301 magnification of 165,000x was used for data collection, corresponding to a pixel size of 0.865 Å
302 at the specimen level, with the defocus ranging from -1.0 μm to -3.0 μm. Movies were recorded
303 in superresolution mode, with a total dose of ~40 e-/ Å², fractioned into 20 frames (0° tilt
304 images) or 40 frames (26° tilt images) under the does rate of 8.4 electron per pixel per second.

305

306 *Image processing and structure analysis*

307 Movies were decompressed and gain corrected with IMOD (32). Motion correction was
308 performed using program MotionCor2 (33), and exposure filtered in accordance with relevant
309 radiation damage curves (34). Micrographs with high CTF Figure of Merit scores and a
310 maximum resolution great than 3.6 Å were selected for further processing. Particles were
311 autopicked using 2D classes as references and extracted in RELION (35) and initial 2D
312 classification was performed in cryoSPARC (36). High quality 2D classes were selected for
313 further processing. The initial model was *de novo* generated and subsequent 3D refinement were
314 performed using cryoSPARC. The UCSF PyEM package (<https://github.com/asarnow/pyem>)
315 script was used to convert the cryoSPARC coordinates into Relion. Duplicate particles were
316 removed and particles were analyzed by 3D refinement, Bayesian Particle Polishing and CTF
317 Refinement in Relion. The data were binned to 1.5 Å/pixel, refined with D2 symmetry, and
318 symmetry expanded. Symmetry expanded particles were used in 3D classification without
319 alignment. All reconstructions were analyzed using USCF Chimera. The initial model was built
320 rigidly docking individual protein structures into the EM map using Chimera. The model was
321 then fit and adjusted manually in USCF Chimera and Coot (37). The figures were generated
322 using UCSF Chimera, and local resolution and final Fourier shell correlation were calculated
323 using ResMap (38) and cryoSPARC.

324

325 *Data deposition*

326 Density map of GFP:DARPin-aldolase complex has been deposited in the Electron Microscopy
327 Data Bank (EMDB) with access code: EMD-9277 and PDB 6MWQ.

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329

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331

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337 Beckman Institute Resource Center for Transmission Electron Microscopy at Caltech.

338

339

Figure captions

340

341 **Fig 1. The Design of the platform.**

342 (A) The platform base was homotetrameric rabbit muscle aldolase (PDB ID code 5VY5). One
343 subunit was depicted with rainbow coloring and N and C labels to indicate the orientation of the
344 monomer chain. The other three identical subunits are shown in yellow. Aldolase has D2
345 symmetry. (B) The selectable adapter was a Designed Ankyrin Repeat Protein (DARPin) (PDB
346 ID Code 5MA6). A DARPin is made up of a series of ankyrin repeat motifs (a beta turn followed
347 by two antiparallel alpha helices). Here a DARPin against GFP was used. The general structure
348 of the library this DARPin came from is an N-terminal cap (red) followed by a series of internal
349 binding modules (here module 1 (orange), module 2 (yellow) and module 3 (green)) that are
350 stabilized by a C-terminal cap (blue) (12). Shown below is a close-up view of the repetitive motif
351 of DARPin with its amino acid sequence (orange). Using a phage display library, DARPins can
352 be generated against a protein target. The selectable residues are depicted in black as X (any
353 amino acid except cysteine or proline) or Z (amino acids asparagine, histidine or tyrosine) (12).
354 (C) The final helix of the C-terminal cap of the DARPin (orange) was directly fused to the first
355 alpha helix of aldolase (yellow) to form the platform subunit. (D) The D2 symmetry of the
356 DARPin-aldolase fusion demonstrates ample space for target binding. (E) Spheres (radius=60 Å)
357 were drawn in the position where each DARPin binds its target. A globular protein of up to 740
358 kDa could be accommodated on the DARPin-aldolase platform without steric clash. (F) The
359 model of the DARPin-aldolase platform in complex with GFP (green) is shown.

360

361 **Fig 2. The cryoEM structure of the DARPin-aldolase platform in complex with the target**
362 **GFP**

363 (A) Surface of the 3 Å C1 reconstruction of DARPin-aldolase platform in complex with GFP.
364 The crystal structures of GFP (green), the DARPin (orange) and aldolase (yellow) were docked
365 into the cryoEM density. Shown on the left is the overall structure and on the right is the
366 expanded view of GFP and the DARPin fit into the density of the best resolved subunit. The
367 expanded view in the dashed line box is shown from the top (left) and halfway down the
368 DARPin with clipping (right) to indicate the quality of the fit. (B) Ribbon diagram (left) and
369 cryoEM density (right, blue mesh, zoned 1.8 Å within atoms) of an internal aldolase helix
370 (residues Arg369 to Asp387). (C) Ribbon diagram (left) and cryoEM density (right, blue mesh,
371 zoned 1.8 Å within atoms) of the helical linker (residues Ala176 to Ile191) between the DARPin
372 (orange, residues Ala176 to Lys181) and aldolase (yellow, residues Leu182 to Ile191). (D)
373 ResMap local resolution estimate of the final DARPin-aldolase platform in complex with GFP
374 (left) and of the best subunit (right). The expanded view in the dashed line box is shown from the
375 side (left) and halfway into the GFP:DARPin density with clipping (right).

376

377 **Fig 3. Symmetry expanded 3D classification of the GFP:DARPin region of the density.**

378 (A) CryoEM density of the D2 reconstruction of the DARPin-aldolase platform in complex with
379 GFP (grey) is shown with a mask (pink) around one GFP:DARPin region. Symmetry expansion
380 was applied to this particle set so that each GFP:DARPin region in each tetramer could be
381 considered independently in 3D classification. (B) 3D classification without alignment of the
382 symmetry expanded particles yielded five reasonable classes. The number of particles per class is
383 indicated above each class. Each density was viewed at the same threshold in Chimera to
384 facilitate direct comparisons. (C) The classes in Fig 3B were each compared with Class 4 (grey)
385 to show the displacement between classes. The XZ plane is shown and the Y axis is
386 perpendicular to the page. Class 4 was clearly displaced relative to the other classes. (D) The
387 comparison from Fig 3B is now viewed looking down the Z axis. (E) The view from Fig 3D was
388 adjusted to look down the helical linker. Residue Arg190, the C-cap helix 1, and the binding
389 module 3 are labeled with arrows to orient the reader relative to Fig 1.

390

391

392

Supporting information

393

394 **S1 Fig. Attempted platform designs and outcomes**

395 (A) Models of the seven platform base proteins tested here. Different subunits of the platform
396 were drawn by different colors. The fused DARPin (in green) and target GFP (in cyan) were
397 shown for only one subunit for clarity. (B) Table summarizing the progress and problems related
398 to our designs

399

400 **S2 Fig. Sequence of DARPin-aldolase fusion and the purification of GFP-DARPin-aldolase** 401 **complex**

402 (A) The amino acid sequence of DARPin-aldolase fusion is colored with the DARPin sequence
403 in brown and the aldolase sequence in green. The residues that were randomized in the phage
404 display library are colored in red. The secondary structures are indicated on top of the sequence
405 with α -helix in magenta tubes and β -strand in green arrows. The rigid helical linker is
406 represented by a blue tube. (B) Gel filtration chromatography of the purified DARPin-aldolase
407 platform in complex with GFP on Superdex 200 column. The black arrows mark the molecular
408 weight calibration and void volume. Fractions 1 to 5 are labeled. (C) SDS-Page gel stained with
409 Coomassie Blue of fractions 1 to 5 from the gel filtration chromatography. The bands
410 representing the DARPin aldolase platform subunit and the GFP are labeled.

411

412 **S3 Fig. Major steps of the last cycle of cryoEM data processing**

413

414 **S4 Fig. Raw micrographs with CTF correction at 0° and 26° tilt**

415 (A)-(C) Motion-corrected, dose weighted micrograph of DARPin-aldolase platform in complex
416 with GFP in vitreous ice is acquired at a nominal magnification of 165,000 \times (left) with the
417 Fourier transformation (inset) and the CTFFind4 plot result (right). Micrographs were collected
418 at 0° tilt in the first session (A), at 0° tilt in the second session (B) and at 26° tilt in the third
419 session (C). Each micrograph has been low-pass filtered to 10 Å to enhance the contrast. The
420 power spectrum of this micrographs is shown as an inset. CTF estimation fit (orange line) to

421 experimental power spectrum (green line) and quality of fit (blue line) are plotted against spatial
422 frequency ($1/\text{\AA}$). Scale bar, 20 nm.

423
424 **S5 Fig. 2D classes and FSC from the DARPIn-aldolase platform in complex with GFP**
425 **refinement**

426 (A) Representative 2D classification results from Relion. (B) Relion Post Processing Fourier
427 Shell Correlation (FSC) plot for the C1 refinement of the DARPIn-aldolase platform in complex
428 with GFP. A B factor of -75\AA^2 was used to sharpen the map. FSC for the phase randomized
429 masked map (red), the unmasked map (green), the masked map (blue) and the corrected map
430 (black) are plotted. The FSC=0.143 cutoff is annotated with a black horizontal line.

431
432 **S6 Fig. 3D classification without alignment of the symmetry expanded class 2.**
433 Class 2 (yellow, top row) from the 3D classification discussed in Fig 3 contained over 50% of
434 the particles and appeared to lack a DARPIn, so it was selected for an additional round of 3D
435 classification without alignment. The resulting five classes (bottom row) show low resolution
436 density in the GFP:DARPIn region of the map.

437
438 **S1 Movie.**
439 Model of the DARPIn-aldolase platform in complex with four spheres (radius=60 \AA) anchored at
440 the DARPIn binding sites.

441
442 **S2 Movie.**
443 ResMap local resolution estimation of the DARPIn-aldolase platform in complex with GFP.

444

445 Reference

446

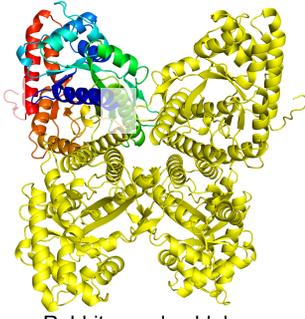
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Figure 1

A



Rabbit muscle aldolase

Platform base

B

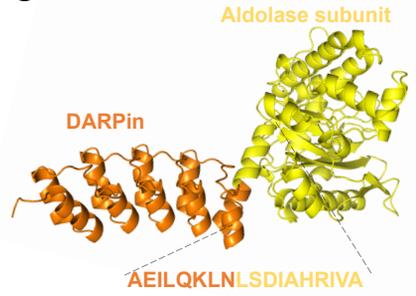


XDXXGXTPHLAAXXG
HLEIVEVLLKZGADVNA

Designed ankyrin repeat protein
(DARPin)

Selectable adaptor

C



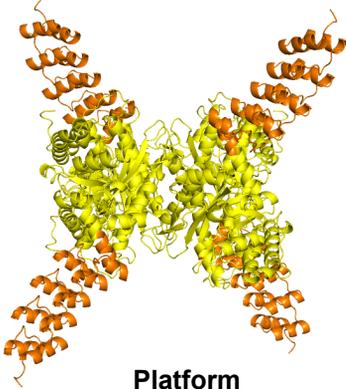
Aldolase subunit

DARPin

AEILQKLNLSDIAHRIVA

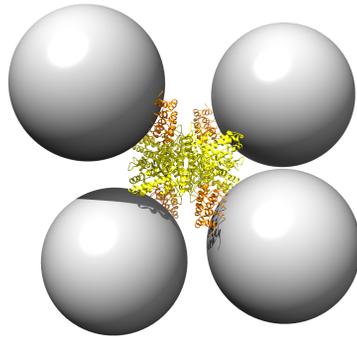
Rigid helical linker

D



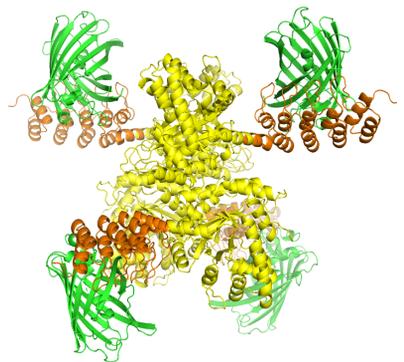
Platform

E



Sphere radius=60 Å

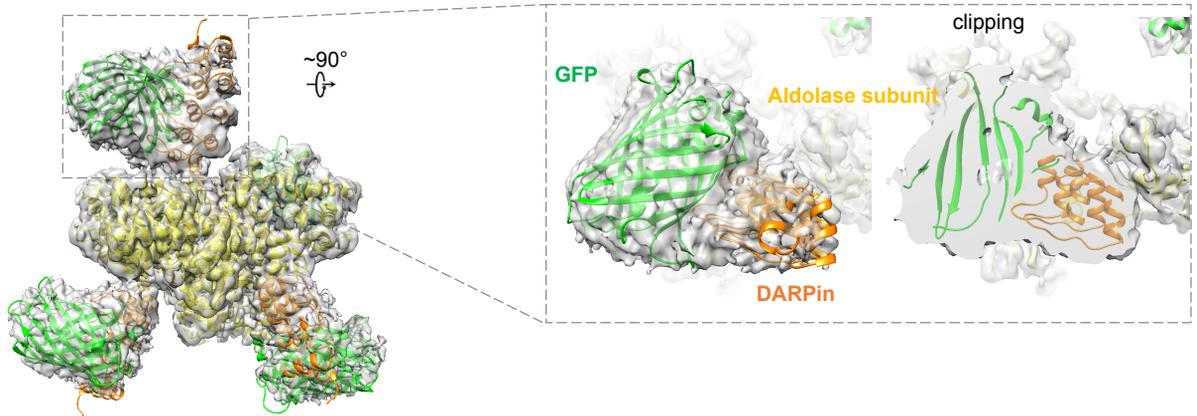
F



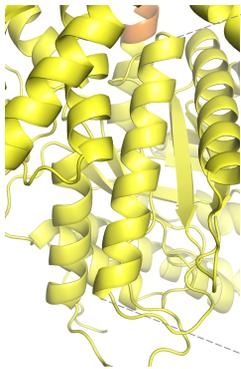
Platform with target

Figure 2

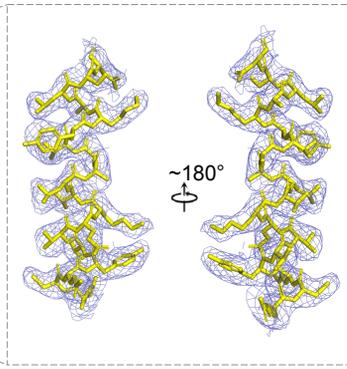
A



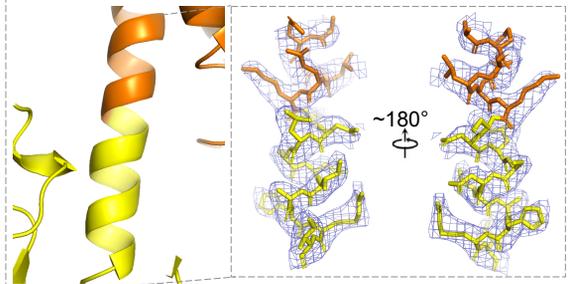
B



Aldolase inner helix



C



Rigid α -helical linker

D

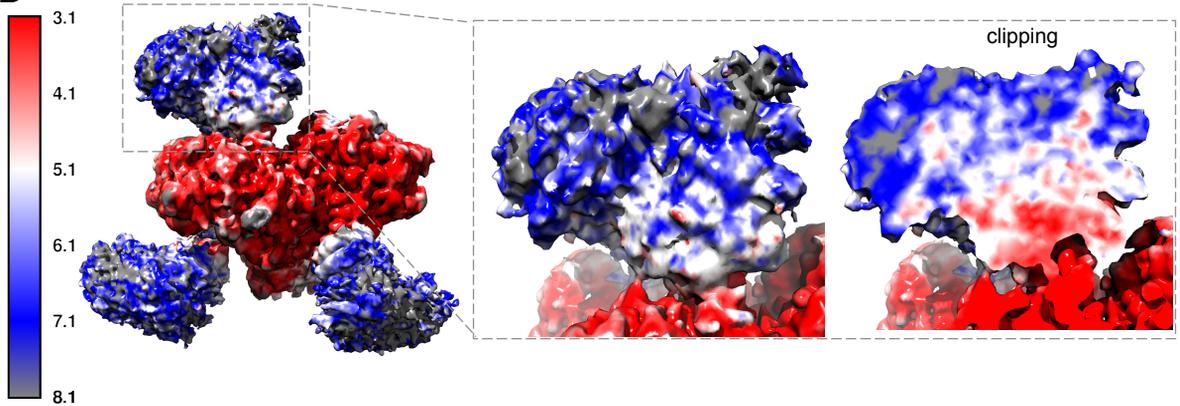
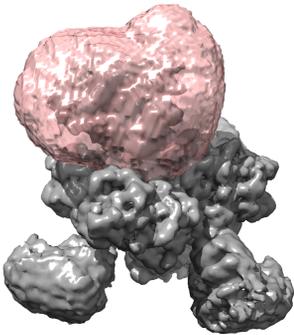


Figure 3

A



B

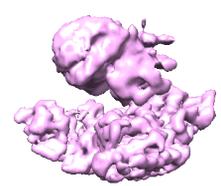
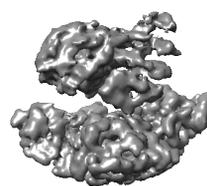
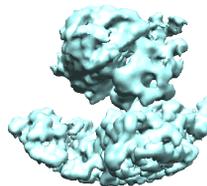
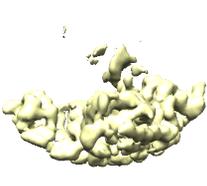
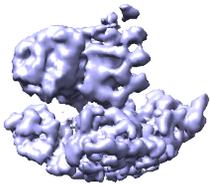
Class 1
58,625 particles

Class 2
507,265 particles

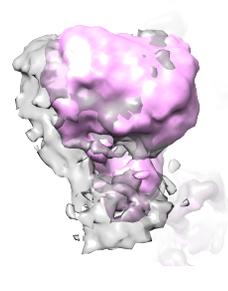
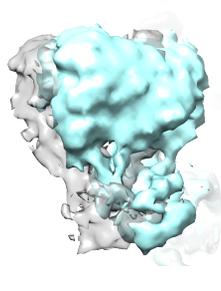
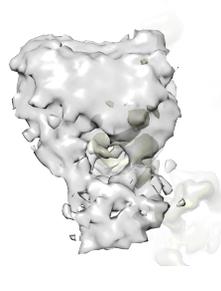
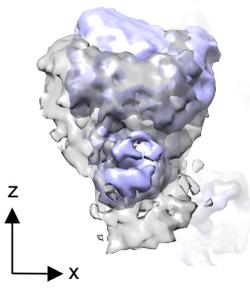
Class 3
39,780 particles

Class 4
26,963 particles

Class 5
312,692 particles



C



D

