1 **Stabilization of recombinant proteins by fusion with heat-resistant obscure**
2 **(Hero) proteins**

3

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14

15 **Abstract**

16 Although recombinant proteins are widely used in biotechnology and pharmaceutical
 17 industries, improving their solubility and stability is often a challenging issue. We
 18 recently discovered a class of highly unstructured heat-resistant obscure (Hero)
 19 proteins, which function to protect other “client” proteins *in trans* from various stresses
 20 *in vitro* and *in vivo*. Here, we show that Hero proteins can enhance the stability of
 21 recombinant proteins when fused *in cis*. Fusion with Hero proteins improved the
 22 otherwise challenging production of TAR DNA-binding protein of 43 kDa (TDP-43) in
 23 *Escherichia coli*. Moreover, fusing with Hero proteins strongly protected the activity of
 24 firefly luciferase bearing destabilizing mutations under various stress conditions. These
 25 data suggest that Hero proteins can be used as versatile stabilization tags for
 26 recombinant protein production.

27

28 **Introduction**

29 Recombinant proteins have been widely used in biotechnology and pharmaceutical
30 industries (1). *Escherichia coli* (*E. coli*) is one of the most common hosts to produce
31 recombinant proteins with high yield and low cost. However, overexpressed proteins in
32 *E. coli* often accumulate in inclusion bodies due to improper folding (2,3). To overcome
33 this limitation, fusion tags such as glutathione-S-transferase (GST) and maltose binding
34 protein (MBP) are frequently used. While helpful in increasing the solubility, GST forms
35 a homodimer in solution, which makes it unsuitable for oligomeric proteins (4,5). MBP
36 can also improve the solubility of tagged proteins, but MBP itself is a protein of ~42.5
37 kDa and this large size may increase the complexity in protein production and
38 downstream processes (6,7).

39 Stability of recombinant proteins after purification is also crucial for their applications.
40 Proteins are generally prone to denaturation especially under stress conditions such as
41 heat and freeze-thaw cycles. Maltodextrin-binding protein from *Pyrococcus furiosus* m
42 (pfMBP) and RNase HI from *Sulfolobus tokodaii* (Sto-RNase HI) are known to increase
43 not only the solubility but also the thermostability of recombinant proteins (8,9).
44 However, while “stabilization tags” are in high demand, they remain poorly explored.

45 We have previously reported that heat-resistant obscure (Hero) proteins, which are

46 heat-soluble, hydrophilic, highly charged, and widespread in animals, including humans.
47 Hero proteins can stabilize various “client” proteins *in vitro* and *in vivo* (10). For
48 example, Hero proteins can protect the enzymatic activity of lactate dehydrogenase
49 (LDH) from desiccation *in vitro* or that of firefly luciferase (Fluc) from heat shock in
50 HEK293T cells. Moreover, Hero proteins can prevent amyotrophic lateral sclerosis
51 (ALS)-associated pathogenic protein aggregations of TAR DNA-binding protein of 43
52 kDa (TDP-43) in cultured motor neurons and in *Drosophila* models for
53 neurodegenerative diseases (10). In light of these stabilization effects previously
54 demonstrated *in trans*, we hypothesized that Hero proteins may help protecting other
55 proteins of interest *in cis*.

56 Here we show that, indeed, recombinant proteins can be significantly stabilized by
57 fusion with Hero proteins. Fusing with Hero proteins improved the otherwise
58 challenging production of recombinant TDP-43 in *E. coli*. Moreover, fusion with Hero
59 strongly protected the enzymatic activity of Fluc bearing destabilizing mutations under
60 stress conditions such as heat, freeze-thaw cycles, and protease treatment. These
61 data suggest that Hero proteins have the potential as stabilization tags for recombinant
62 proteins.

63

64 **Results**

65

66 **Hero tags can improve the protein expression of TDP-43 in *E. coli*.**

67 TDP-43 is intrinsically aggregation-prone, and it is generally difficult to produce TDP-43
68 as a recombinant protein in *E. coli* (11). Based on our previous observation that Hero
69 proteins can suppress aggregation of TDP-43 *in trans* in human cells (10), we
70 wondered if Hero proteins can be used as fusion tags to increase the protein solubility
71 *in cis* in the *E. coli* expression system. We constructed a series of expression vectors,
72 in which TDP-43 was N-terminally tagged with His-FLAG and each of 6 representative
73 human Hero proteins or GST as a control, or His-FLAG alone (Fig. 1A). After protein
74 expression, we separated the soluble and insoluble fractions and analyzed the soluble
75 fraction by capillary-based quantitative Western blotting using anti-FLAG antibody. As
76 previously reported (11), TDP-43 was mostly found in the insoluble fraction (Fig. 1B),
77 and many incomplete peptides and/or degradation products were detected in the
78 soluble fraction (Fig. 1C), highlighting the difficulty of recombinant TDP-43 production in
79 *E. coli*. Compared to the His-FLAG alone (no tag), fusion with GST decreased the
80 protein yield of full-length TDP-43 in the soluble fraction, whereas fusion with Hero9, 11
81 and 20 did not compromise or slightly increased the yield (Fig. 1D). Importantly,

82 Hero11-tagging significantly improved the integrity of soluble TDP-43, with much less
83 degradation products compared to other tags (Fig. 1E). We concluded that fusion with
84 some Hero proteins has the potential to improve the otherwise challenging protein
85 expression of TDP-43 in *E. coli*.

86

87 **Hero tags mitigate the loss of Fluc activity by heat.**

88 We have previously demonstrated that co-expression of Hero proteins in HEK293T
89 cells mitigate the loss of Fluc activity by heat shock (10). To evaluate the protective
90 effect *in cis in vitro*, we expressed Hero or GST-tagged Fluc in *E. coli* using the same
91 expression constructs as our TDP-43 experiment above. Fluc is widely used as a
92 bioluminescent reporter in various species including *E. coli*, and as expected it was
93 expressed in the soluble fraction even without Hero or GST tag (Fig. 2A). Because
94 protein yields with Hero13 and 45 were extremely low, we excluded them from further
95 experiments. We purified the series of recombinant tagged Fluc proteins and adjusted
96 their concentrations (Fig. 2B). After confirming that the luminescence activities are
97 roughly comparable among all the samples (Fig. S1A), we exposed them to heat (Fig.
98 2C) by incubating them at 33 °C (Fig. 2D) or 37 °C (Fig. 2E) for 20 min. We then
99 measured the luminescence and calculated the loss of the enzymatic activity by heat

100 incubation. Except for Hero11, all tags protected the Fluc activity from heat but only
101 modestly (Fig. 2D and E).

102 Because wild-type (WT) Fluc is an intrinsically stable protein, fusion tags may have
103 only little space to improve its stability. It is known that the R188Q single-mutation (SM)
104 and the R188Q/R261Q double-mutation (DM) can strongly destabilize Fluc at high
105 temperatures (≥ 25 °C), without severely compromising the enzymatic activity at 20 °C
106 (12). Therefore, we repeated the heat stress test using Fluc-SM (Fig. 3A–D) and DM
107 (Fig. 3A and E–G). Fusion with Hero9 or Hero20 significantly protected the activity of
108 Fluc-SM at both 33 °C and 37 °C (Fig. 3C and D), whereas GST tag did not show any
109 apparent protection. For Fluc-DM, Hero9 was particularly effective in protecting the
110 enzymatic activity even at 37 °C (Fig. 3F and G). We concluded that tagging with some
111 Hero proteins can mitigate the destabilization of proteins by heat, especially for
112 intrinsically unstable ones.

113

114 **Hero tags protect Fluc activity from freeze-thaw cycles.**

115 In general, proteins tend to be denatured by freezing and thawing (13). Indeed, 2
116 cycles of freezing and thawing strongly compromised the Fluc-WT, SM and DM activity
117 (Fig. 4). Although GST mildly protected the Fluc activity from the freeze-thaw cycles,

118 Hero9 and Hero20 showed superior protection activity for Fluc-SM and Fluc-DM (Fig.
119 4B–D). These data suggest that Hero tags can be used to prevent the loss of function
120 via freeze-thaw cycles (Fig. 4B–D).

121

122 **Hero tags protect Fluc activity from Proteinase K.**

123 Since proteins are generally prone to degradation by proteases both *in vivo* and *in vitro*,
124 we tested if Hero tags can protect client proteins from Proteinase K (PK), a
125 representative serine protease. We incubated the series of Fluc-WT, SM, and DM
126 proteins with PK for 30 min and measured their luminescence activity (Fig. 5). While
127 GST showed no shielding effect, Hero9 strongly protected the activity of Fluc-SM and
128 DM from PK-mediated proteolysis (Fig. 5C and D). Thus, fusion with Hero proteins is a
129 useful strategy to confer increased protease resistance on client proteins (14).

130

131 **Hero proteins protect Fluc activity better in *cis* than in *trans*.**

132 Finally, we compared the protective effect of Hero proteins *in cis* and *in trans*. We
133 purified recombinant GST, Hero9 and Hero11 proteins (which were most effective *in*
134 *cis*), added each of them to the “no tag” Fluc protein in equimolar concentrations, and
135 challenged the mixture with heat, freeze-thaw cycles, or PK. As shown in Fig. S2A–I,

136 the protective effect was only minimum in all the stress conditions. We concluded that
137 fusing with Hero proteins *in cis* works better than mixing with them *in trans*, at least to
138 protect the Fluc activity *in vitro*.

139 **Discussion**

140

141 In this study, we demonstrated that Hero proteins can be used as useful fusion tags
 142 that can protect recombinant proteins under various stress conditions. We envision that
 143 Hero tags act as a physical shield that prevents collisions of molecules leading to
 144 denaturation. Hero tags may be also helpful in promoting and maintaining the proper
 145 folding of client proteins by improving the molecular environment. In this sense, Hero
 146 may be reminiscent of polyethylene glycol (PEG), a post-production modification
 147 commonly used to increase the solubility and stability for biopharmaceuticals (15,16). It
 148 is known that PEG itself can show immunogenicity albeit rarely, and the long-term
 149 toxicity of PEG-modified products has recently been cautioned (17,18). To overcome
 150 this problem, researchers have developed “PAS,” an artificial polypeptide of defined
 151 sequence containing the 3 small amino acids Pro, Ala, and Ser. PAS is biodegradable
 152 and non-immunogenic, yet improves solubility in *E. coli* and protein half-lives *in vivo*
 153 (19). Hero proteins may resemble PAS, except that they are from natural sources. In
 154 our current study, we tested only 6 representative Hero proteins, but the human
 155 genome encodes hundreds of Hero protein candidates, many of which remain to be
 156 characterized (10). Thus, it is possible that there are Hero proteins that act as better

157 stabilization tags than those tested in this study. Moreover, it will be interesting to
158 examine in the future if tandem repeats of Hero proteins may increase the stabilization
159 effect, as was demonstrated for PAS (20).

160 Toward the application of Hero tags, it is important to note that different Hero
161 proteins have different preferences for their client proteins. For example, in our
162 previous research, Hero7 and 11 showed strong resistance to heat shock when co-
163 expressed with Fluc *in trans* in cultured human cells (10). However, the *in-trans*
164 protective effect of Hero11 was only minimum *in vitro* (Fig. 2S). When fused *in cis*,
165 Hero11 rather abolished the Fluc activity *in vitro*, while Hero9 showed the strongest
166 protection (Fig. 3–4). On the other hand, Hero11 strongly improved the integrity of
167 soluble TDP-43 in the *E. coli* expression system (Fig. 1C–E). Those data suggest that
168 even the same Hero protein can behave differently depending on the client protein and
169 condition. Unfortunately, it is currently difficult to predict the best combination between
170 Hero proteins and clients, and it will be important to test multiple Hero proteins to
171 identify one that best protects the protein of interest.

172 In summary, our current study provides the potential of Hero proteins as versatile
173 stabilization tags for recombinant proteins and serve as a starting point for further
174 optimization and engineering.

176 **Figure legends**

177

178 **Figure 1. Hero proteins improve solubility and stability of TDP-43 in *E. coli*.**

179 (A) Schematic representation of Hero-tagged protein expression constructs.

180 (B) Expression of tagged TDP-43 proteins. TDP-43 fused with GST, each Hero protein
181 (Hero7, 9, 11, 13, 20, or 45) or no tag was expressed in *E. coli*, and the soluble and
182 insoluble fractions were analyzed by SDS-PAGE. Expected sizes are 50, 62, 57, 59,
183 60, 62, 70, and 92 kDa for no tag, GST, Hero7, Hero9, Hero11, Hero13, Hero20,
184 and Hero 45, respectively. A representative image from 3 independent experiments
185 is shown. Note that extreme biases in amino acid composition of Hero proteins can
186 affect the mobility of protein bands on SDS-PAGE (10).

187 (C) Capillary-based quantitative Western blotting of the soluble fractions in (B). A
188 representative image from 3 independent experiments is shown. The bands marked
189 with red arrows represent the full-length proteins.

190 (D) Relative quantification of the full-length proteins compared to no tag in (C). Mean $\pm$
191 SD from 3 independent experiments are shown. *P*-values were calculated by the
192 Steel-Dwass test against no tag. None showed $p < 0.05$.

193 (E) Quantification of the fraction of the full-length proteins out of the total proteins in (C).

194 Mean $\pm$ SD from 3 independent experiments are shown. Fusion with Hero11
195 resulted in the highest purity. *P*-values were calculated by Tukey HSD against no
196 tag. *Hero11: $p = 0.01$.

197

198 **Figure 2. Hero tags slightly mitigate the loss of Fluc activity by heat.**

199 (A) Expression of tagged Fluc proteins. Fluc fused with GST, each Hero protein (Hero7,
200 9, 11, or 20) or no tag was expressed in *E. coli*, and the soluble and insoluble
201 fractions were analyzed by SDS-PAGE.

202 (B) SDS-PAGE of His-purified Fluc proteins.

203 (C) Schematic representation of the heat treatment.

204 (D) Heat treatment of Fluc at 33°C for 20 min. Fractions of the remaining Fluc activities
205 after the heat incubation were calculated. Mean $\pm$ SD from 4 independent
206 experiments are shown. *P*-values were calculated by Tukey HSD against no tag.
207 None showed $p < 0.05$.

208 (E) Heat treatment of Fluc at 37°C for 20 min. Fractions of the remaining Fluc activities
209 after the heat incubation were calculated. Mean $\pm$ SD from 4 independent
210 experiments are shown. *P*-values were calculated by Tukey HSD against no tag.
211 *Hero20: $p = 0.0148$.

212

213 **Figure 3. Hero tags markedly mitigate the loss of mutated Fluc activity by heat.**

214 (A) Schematic representation of the heat treatment.

215 (B) SDS-PAGE of His-purified Fluc-SM proteins.

216 (C) Heat treatment of Fluc-SM at 33 °C for 20 min. Fractions of remaining Fluc

217 activities after the heat incubation were calculated. Mean $\pm$ SD from 6 independent

218 experiments are shown. *P*-values were calculated by Tukey HSD against no tag.

219 *Hero9: *p* = 0.0038, Hero11: *p* = 0.0455.

220 (D) Heat treatment of Fluc-SM at 37 °C for 20 min. Fractions of remaining Fluc

221 activities after the heat incubation were calculated. Mean $\pm$ SD from 6 independent

222 experiments are shown. *P*-values were calculated by Tukey HSD against no tag.

223 *Hero9: *p* = 0.0017, Hero11: *p* = 0.007, Hero20: *p* = 0.001.

224 (E) SDS-PAGE of His-purified Fluc-DM proteins.

225 (F) Heat treatment of Fluc-DM at 33 °C for 20 min. Fractions of remaining Fluc

226 activities after the heat incubation were calculated. Mean $\pm$ SD from 5 independent

227 experiments are shown. *P*-values were calculated by Tukey HSD against no tag.

228 *Hero9: *p* = 0.0073.

229 (G) Heat treatment of Fluc-DM at 37 °C for 10 min. Fractions of remaining Fluc

activities after the heat incubation were calculated. Mean $\pm$ SD from 5 independent experiments are shown. *P*-values were calculated by Tukey HSD against no tag. *Hero9: $p = 0.001$.

233

Figure 4. Hero tags protect Fluc activity from freeze-thaw cycles.

(A) Schematic representation of the freeze-thaw cycles.

(B) Freeze-thaw cycles of Fluc-WT. Fractions of remaining Fluc activities after the second cycle were calculated. Mean $\pm$ SD from 5 independent experiments are shown. *P*-values were calculated by Tukey HSD against no tag. *Hero11: $p = 0.045$, Hero20: $p = 0.0344$.

(C) Freeze-thaw cycles of Fluc-SM. Fractions of remaining Fluc activities after the second cycle were calculated. Mean $\pm$ SD from 6 independent experiments are shown. *P*-values were calculated by Tukey HSD against no tag. *GST: $p = 0.014$, Hero7: $p = 0.0309$, Hero9: $p = 0.001$, Hero20: $p = 0.0018$.

(D) Freeze-thaw cycles of Fluc-DM. Fractions of remaining Fluc activities after the second cycle were calculated. Mean $\pm$ SD from 6 independent experiments are shown. *P*-values were calculated by Tukey HSD against no tag. *Hero9: $p = 0.0022$, Hero11: $p = 0.0074$, Hero20: $p = 0.001$.

248

249 **Figure 5. Hero tags protect Fluc activity from Proteinase K.**

250 (A) Schematic representation of the PK treatment.

251 (B) PK treatment of Fluc-WT for 30 min. Fractions of remaining Fluc activities after the

252 PK treatment were calculated. Mean $\pm$ SD from 4 independent experiments. *P*-

253 values were calculated by Tukey HSD against no tag. None showed $p < 0.05$.

254 (C) PK treatment of Fluc-SM for 30 min. Fractions of remaining Fluc activities after the

255 PK treatment were calculated. Mean $\pm$ SD from 6 independent experiments. *P*-

256 values were calculated by Tukey HSD against no tag. *Hero9: $p = 0.0051$.

257 (D) PK treatment of Fluc-WT for 30 min. Fractions of remaining Fluc activities after the

258 PK treatment were calculated. Mean $\pm$ SD from 6 independent experiments. *P*-

259 values were calculated by Tukey HSD against no tag. *Hero9: $p = 0.001$.

260

261 **Figure S1. Basal Fluc activities are comparable with and without fusion tags.**

262 (A) Basal Fluc activities before the stress tests in Figure 2. *P*-values were calculated by

263 the Steel-Dwass test against no tag. None showed $p < 0.05$.

264

265 **Figure S2. Fluc activities are only minimally protected by Hero proteins *in trans*.**

266 (A) Heat treatment of Fluc-WT mixed with buffer alone, GST, Hero9 or Hero11 at 37 °C

267 for 20 min.

268 (B) Heat treatment of Fluc-SM mixed with buffer alone, GST, Hero9 or Hero11 at 37 °C

269 for 20 min.

270 (C) Heat treatment of Fluc-DM mixed with buffer alone, GST, Hero9 or Hero11 at 37 °C

271 for 10 min.

272 (D) Freeze-thaw cycles of Fluc-WT mixed with buffer alone, GST, Hero9 or Hero11.

273 (E) Freeze-thaw cycles of Fluc-SM mixed with buffer alone, GST, Hero9 or Hero11.

274 (F) Freeze-thaw cycles of Fluc-DM mixed with buffer alone, GST, Hero9 or Hero11.

275 (G) PK treatment of Fluc-WT mixed with buffer alone, GST, Hero9 or Hero11 for 30 min.

276 (H) PK treatment of Fluc-SM mixed with buffer alone, GST, Hero9 or Hero11 for 30 min.

277 (I) PK treatment of Fluc-DM mixed with buffer alone, GST, Hero9 or Hero11 for 30 min.

278 For all the data, p-values were calculated by Tukey HSD against no tag. None showed

279 $p < 0.05$.

280

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285

286 **Competing interests**

287 Y.T. and K.T. have a patent application related to this work. E.M. declares no competing
288 interests.

289

290 **Author Contributions**

291 E.M., K.T., and Y.T. designed the project. E.M. performed all the experiments. K.T. and
292 Y.T. supervised the project. E.M., K.T., and Y.T. wrote the manuscript.

293

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- 357

358 **Experimental Procedures**

359 **Plasmid construction**

360 A DNA fragment containing FLAG-tag and GST, Hero7, 9, 11, 13, 20, or 45 (SERF2,
361 C9orf16, C19orf53, C11orf58, BEX3, or SERBP1 respectively) was inserted into pCold
362 I (Takara), together with the client protein, TDP-43, Fluc (WT), Fluc (SM; R188Q) or
363 Fluc (DM; R188Q, R261Q) (12).

364

365 **Protein purification**

366 Recombinant tagged TDP-43 and Fluc proteins were expressed in *E. coli* BL21 strain.
367 The cells were cultivated in 6 or 13 mL for TDP-43 and in 250 mL for Fluc to an OD600
368 of 0.4–0.6 at 37 °C, and then grown at 15 °C overnight with 1 mM isopropyl-β-D-
369 thiogalactoside (IPTG) following cold-shock on ice for 20 min. For TDP-43, the cells
370 were resuspended in lysis buffer [200 mM HEPES-KOH pH7.4, 200 mM KOAc, and
371 200 mM Mg(OAc)₂] supplemented with 0.2 mM TCEP, EDTA-free protease inhibitor
372 cocktail (Roche), and DNase I, sonicated, and centrifuged at 10,000 x g for 10 min. The
373 pellets were resuspended and sonicated again and the soluble and insoluble fractions
374 were analyzed by SDS-PAGE and capillary-based Western blotting. For Fluc, the cells
375 were resuspended in His A buffer [30 mM HEPES-KOH (pH 7.4), 200 mM KOAc, 5%

glycerol] supplemented with EDTA-free protease inhibitor cocktail (Roche), sonicated, and centrifuged at 10,000 x g for 5 min. The supernatant was added to a slurry of cOmplete His-Tag Purification Resin (Roche) or Ni Sepharose High Performance (Merck) and eluted with His B buffer (His A buffer containing 400 mM imidazole). The eluates were mixed with 20 % Glycerol and 1 mM DTT, snap-frozen by liquid-N₂ and stored at –80 °C.

Capillary-based Western blotting

Samples were prepared and analyzed by Jess according to the manufacturer's instruction (Protein Simple). Anti-DDDDK antibody was used as the primary antibody at 1:100 (M185, MBL). Anti-mouse antibody was used as the secondary antibody at 1:100 (Protein Simple).

Stress conditions

High temperature

For the *in-cis* experiments (Fig. 2 and 3), 40 µL of tagged Fluc proteins (~40 nM) were incubated at 33 °C and 37 °C for 20 min, except that the 37 °C incubation for Fluc-DM was for 10 min. For the *in-trans* experiments (Fig. S2A–C), Fluc and GST, Hero9,

394 Hero11, or lysis buffer were mixed (final concentrations ~40 nM) in 40 uL and incubated
395 at 37 °C for 20 min (WT and SM) or 10 min (DM).

396

397 *Freeze and thaw cycles*

398 For the *in-cis* experiments, 80 µL of tagged Fluc proteins (~40 nM) were frozen at –
399 80 °C for 30 min and thawed at room temperature for 10 min. This cycle was repeated
400 twice. For the *in-trans* experiments, Fluc and GST, Hero9, Hero11, or lysis buffer were
401 mixed (final concentrations ~40 nM) in 80 uL, frozen, and thawed twice.

402

403 *Proteinase K treatment*

404 For the *in-cis* experiments, 40 µL of tagged Fluc proteins (~40 nM) were incubated with
405 10 uL of Proteinase K (0.06 U/mL) for 30 min on ice. For the *in-trans* experiments, each
406 Fluc and GST, Hero9, Hero11, or lysis buffer were mixed (final concentrations ~40 nM)
407 in 40 uL and incubated with 10 uL of Proteinase K (0.06 U/mL).

408

409 **Luciferase assay**

410 The luciferase activities of Fluc were measured before and after the stress treatment,
411 using sensilite Enhanced Flash Luminescence (Perkin Elmer) and SPARK 10 M plate

412 reader (TECAN). The fractions of the remaining activity were then calculated.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Supplemental Figure 1

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A

Supplemental Figure 2

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