

Supplementary Information

CUL4A-DDB1-DCAF10 is an N-recognin for N-terminally acetylated Src kinases

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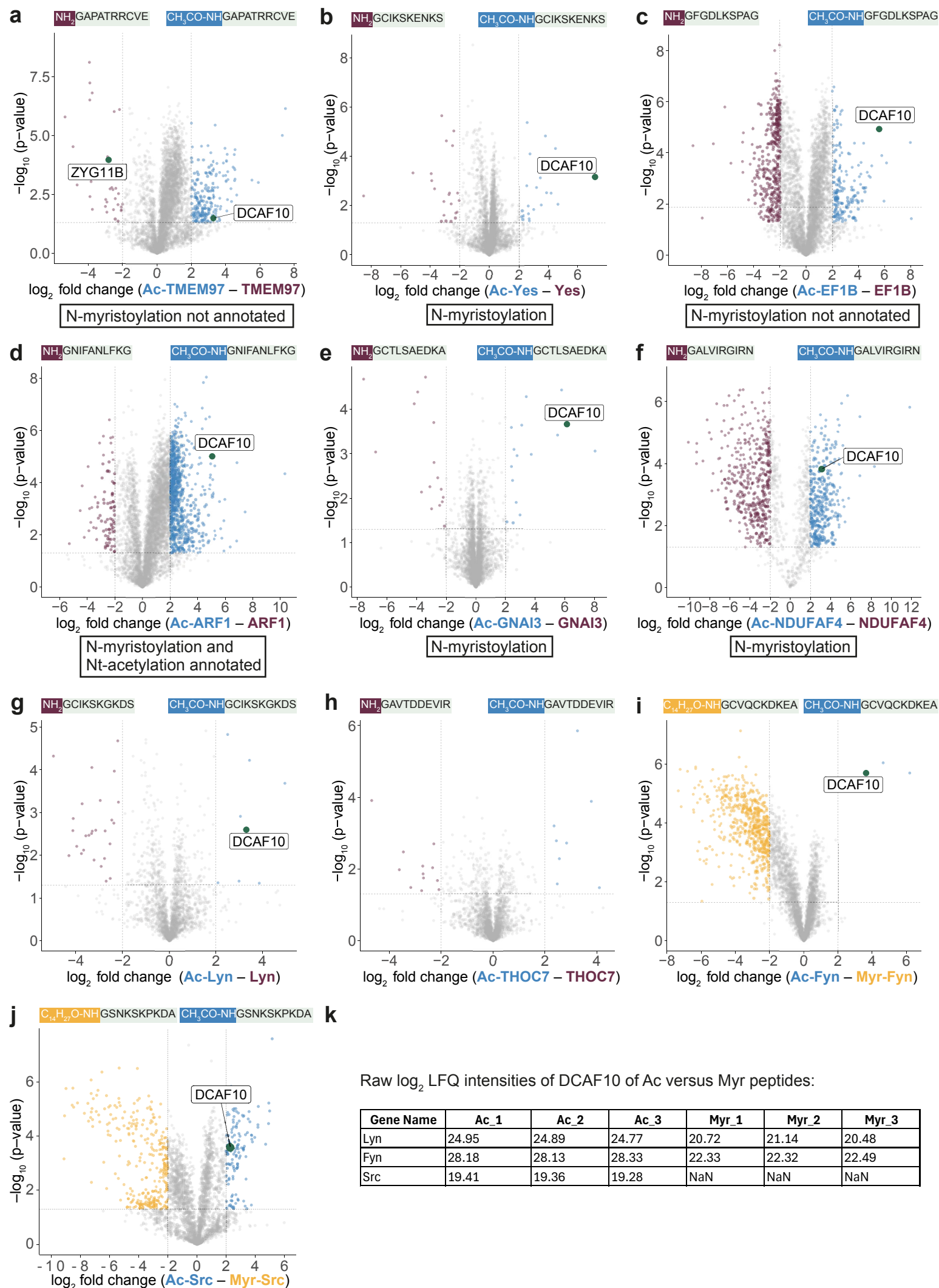
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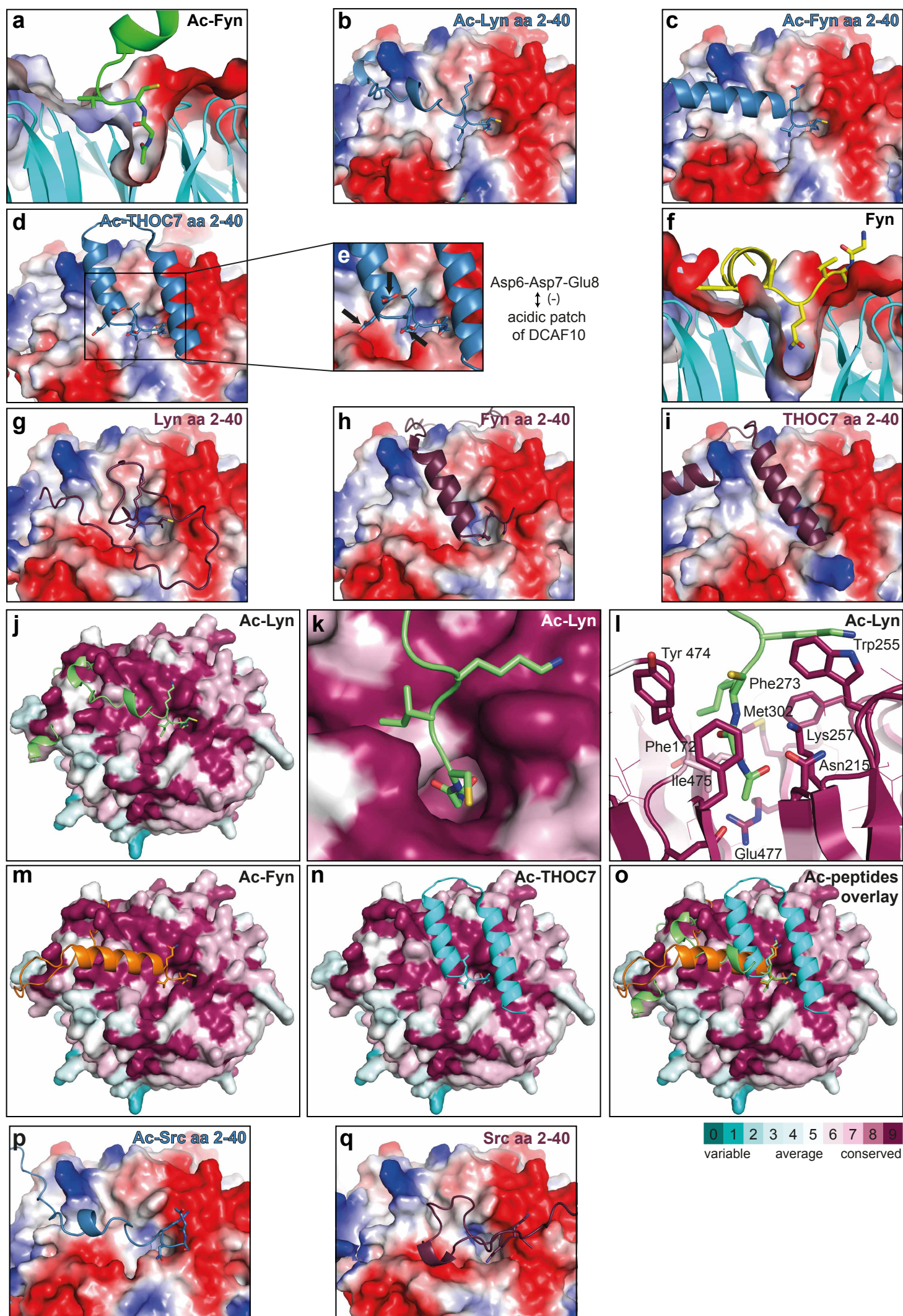
Supplementary Figure 1 Kremer et al.

58 **Supplementary Figures and Legends**

59 **Supplementary Fig. 1. DCAF10 binds acetylated N-terminal glycine residues.**

60 **a-f)** Volcano plots comparing binding partners of free (Nt-free) and acetylated (Nt-Ac) N-
61 termini of peptides derived from the indicated proteins (aa 1–10 + KKK-biotin;
62 Supplementary Table S1). The analyzed peptides are from **(a)** TMEM97, **(b)** Yes, **(c)** EF1B,
63 **(d)** ARF1, **(e)** GNAI3, **(f)** NDUFAF4. The $-\log_{10}$ adjusted p-value (two-sided Student's t-
64 test with permutation-based multiple-testing correction; y-axis) is plotted against the $\log_2$
65 fold change (x-axis). Threshold for significance: $-\log_{10}$ p-value ≥ 1.3 (p-value ≤ 0.05),
66 $\log_2$ fold change ≤ -2 or ≥ 2 . DCAF10 and ZYG11B are marked in green, significant Nt-
67 free binders in purple, and significant Nt-Ac binders in blue ($n = 3$ independent biological
68 replicates). **g-h)** Volcano plots as in **(a-f)** using homogenized liver tissue from mice. **(g)**
69 Lyn, **(h)** THOC7. **i-j)** Volcano plots as in **(a-f)** comparing binding partners of Nt-Ac and
70 Nt-Myr **(i)** Fyn and **(j)** Src. In addition to **(a-f)**, significant Nt-Myr binders are marked in
71 yellow. **k)** Overview table showing raw $\log_2$ LFQ intensities before data imputation
72 comparing the abundance of DCAF10 in Nt-Ac and Nt-Myr forms of Lyn, Fyn, and Src.

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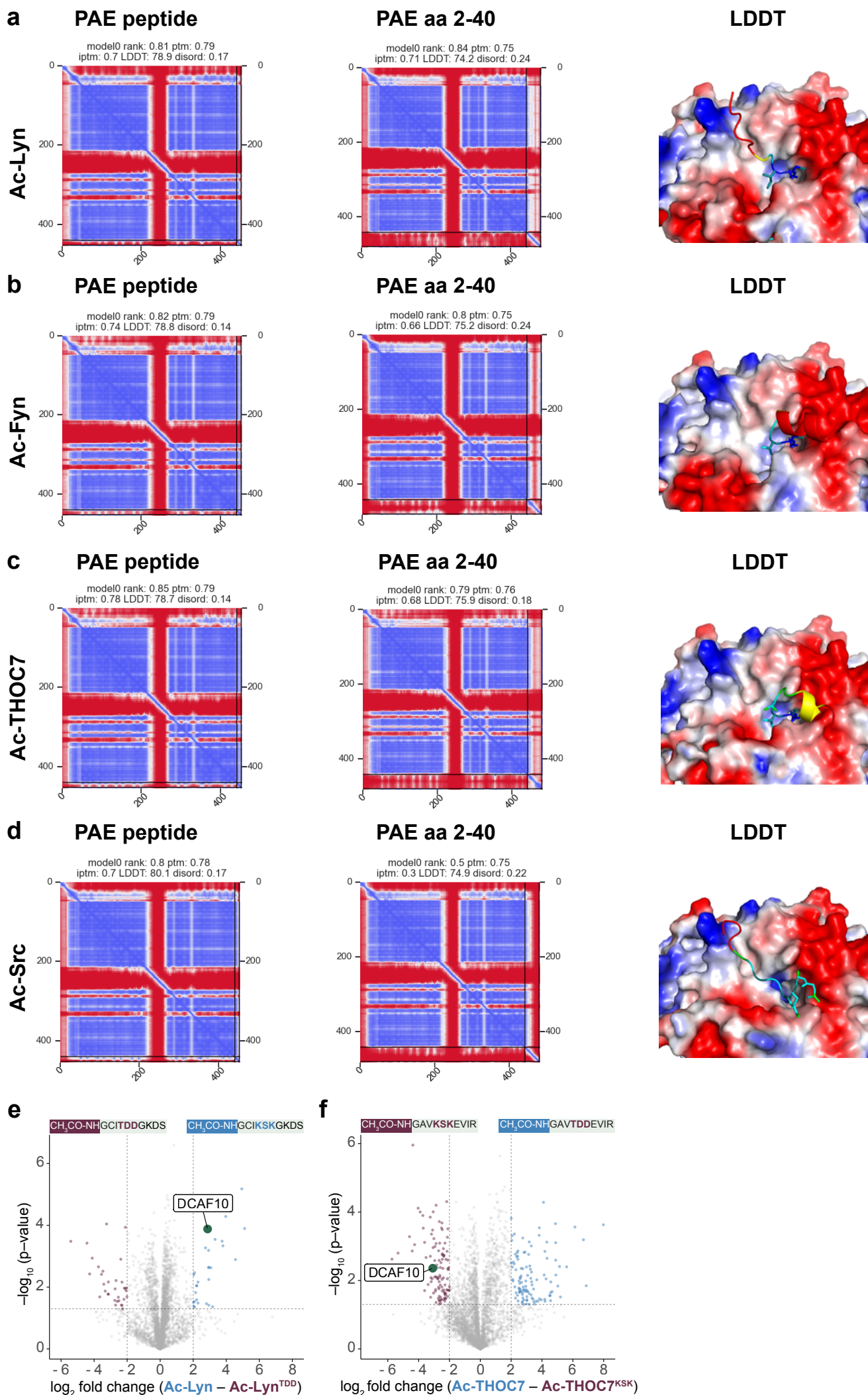


Supplementary Figure 2 Kremer et al.

74 **Supplementary Fig. 2. AF3 predictions.**

75 **a-f)** Cross-sections of the DCAF10 β -propeller (aa 120–559, UniprotID: Q5QP82),
76 highlighting the deep tunnel bound to Nt-Fyn peptides. Shown are **a)** Nt-Ac Fyn (green),
77 **f)** Nt-free Fyn (yellow). **b-e)** Top views of the DCAF10 β -propeller bound to the first 39
78 aa (aa 2–40) of various proteins. Nt-Ac aa 2–40: **b)** Nt-Ac Lyn, **c)** Nt-Ac Fyn, **d)** Nt-Ac
79 THOC7 (black box indicates region shown in **e)**). **e)** Close-up view of **d)**: interaction of
80 Asp6-Asp7-Glu8 (shown as sticks) of THOC7 with the acidic DCAF10 surface. Nt-free aa
81 2–40: **g)** Nt-free Lyn, **h)** Nt-free Fyn, **i)** Nt-free THOC7. DCAF10's electrostatic surface
82 potential is displayed (red = negative, blue = positive, white = neutral or hydrophobic), as
83 calculated by PyMOL. Acetylated fragments insert deeply into the central DCAF10 tunnel,
84 whereas non-acetylated peptides are consistently excluded. **j-o)** Conservation analysis of
85 the DCAF10 β -propeller. Color code: cyan: variable, white: moderate conservation, purple:
86 highly conserved. **j)** Full β -propeller view with Nt-Ac Lyn, **k)** Close-up of the tunnel
87 entrance with Nt-Ac Lyn; **l)** Interacting residues in DCAF10. **m-o)** Additional Nt-Ac
88 fragments aa 2–40: **m)** Nt-Ac Fyn (orange), **n)** Nt-Ac THOC7 (cyan), **o)** overlay of Nt-Ac
89 Lyn, Fyn, and THOC7 models. **p-q)** Cross-sections of the DCAF10 β -propeller with **(p)**
90 Nt-Ac Src; **(q)** Nt-free Src. Panels **(p-q)** use the same color code as panels **(a-f)**.

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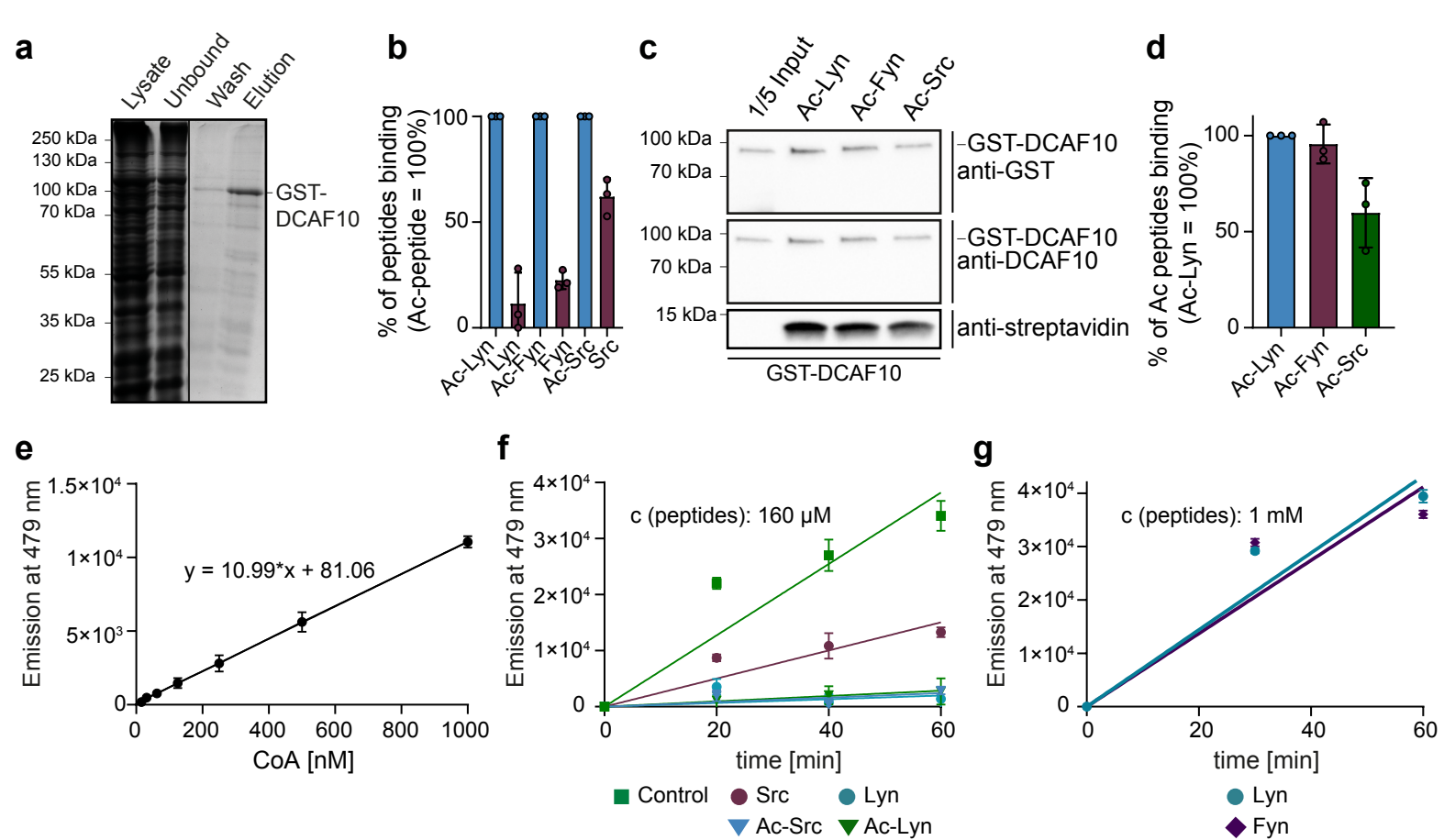
Supplementary Figure 3 Kremer et al.

92 **Supplementary Fig. 3. PAE and LDDT maps of AF3 predictions, and volcano plots of**
93 **N-terminal peptide variants.**

94 **a-d)** PAE (Predicted Aligned Error) plots for the best models and LDDT (Local Distance
95 Difference Test) maps of AF3 models for DCAF10 binding to Ac-peptides and fragments
96 (aa 2–40). Left: PAE plots of Nt-Ac peptides (aa 1–10 + KKK-biotin). Middle: PAE plots
97 of aa 2–40. Right: LDDT maps of Nt-Ac peptides. Analyzed peptides **(a)** Nt-Ac Lyn, **(b)**
98 Nt-Ac Fyn, **(c)** Nt-Ac THOC7, **(d)** Nt-Ac Src. PAE plots are color-coded: blue (highly
99 significant scores), red (insignificant). LDDT maps are color-coded: blue (very high
100 confidence, LDDT > 90), cyan (high confidence, LDDT 70–90), yellow (medium
101 confidence, LDDT 50–70), orange (low confidence, LDDT 30–50), and red (very low
102 confidence, LDDT < 30). The DCAF10 surface is colored by electrostatic potential as in

103 **Supplementary Fig. 2. a-f). e-f)** Volcano plots comparing binding partners of **(e)** Ac-Lyn
104 vs. Ac-Lyn^{TDD} and **(f)** Ac-THOC7 vs. Ac-THOC7^{KSK}. The -log₁₀ adjusted p-value (two-
105 sided Student's t-test with permutation-based multiple-testing correction; y-axis) is plotted
106 against the log₂ fold change (x-axis). Threshold for significance: -log₁₀ p-value ≥ 1.3 (p-
107 value ≤ 0.05), log₂ fold change ≤ -2 or ≥ 2. DCAF10 is marked in green, significant binding
108 partners in purple and blue (*n* = 3 independent biological replicates).

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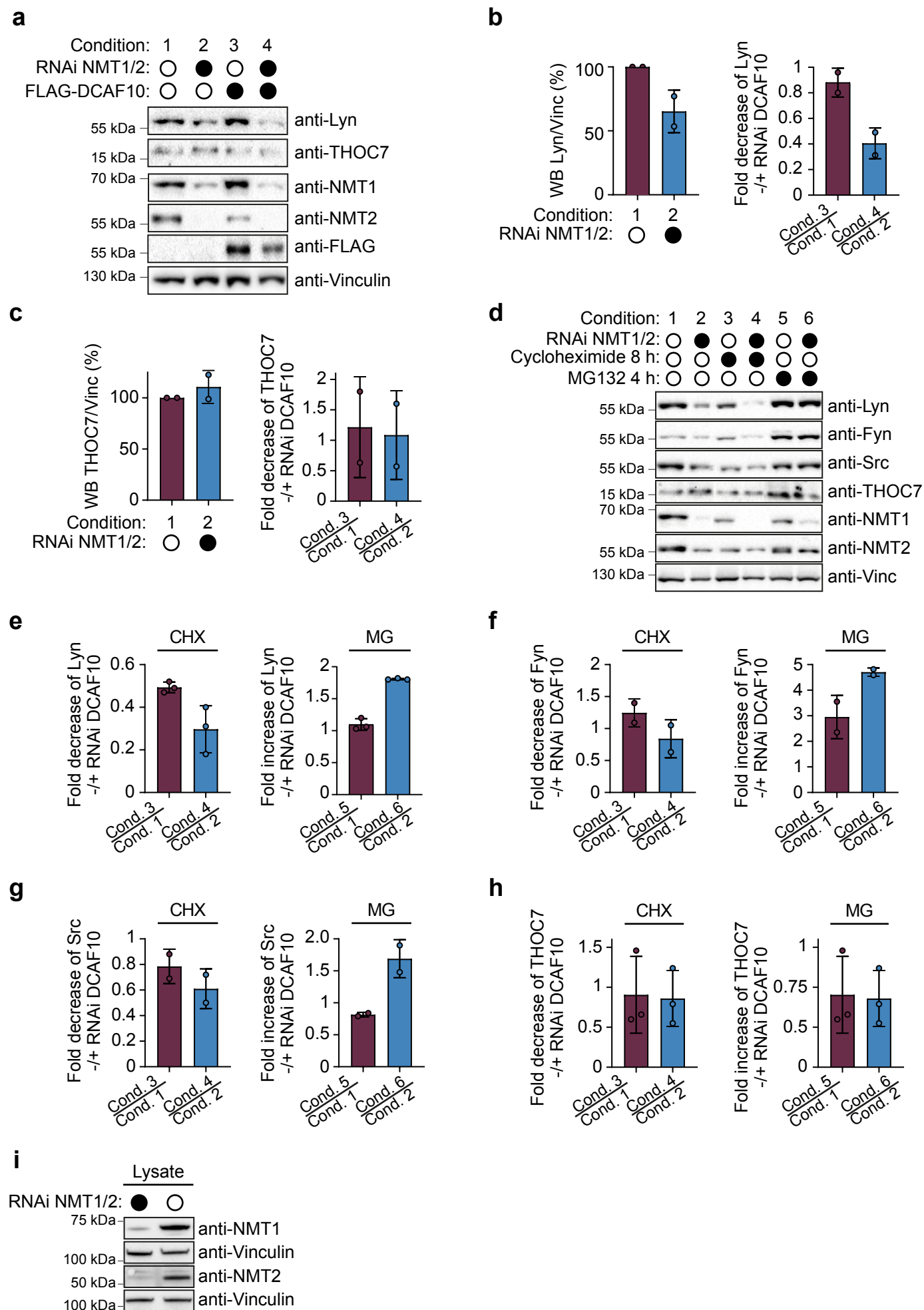


Supplementary Figure 4 Kremer et al.

110 **Supplementary Fig. 4. *In vitro* binding of DCAF10 to Nt-Ac peptides and *in vitro* Nt-**
111 **acetylation assay setup.**

112 **a)** Coomassie-stained SDS-PAGE gel showing GST-DCAF10 purification. **b)** WB
113 quantification of **Fig. 2m-p**. Mean $\pm$ s.d. with individual data points are shown ($n = 3$
114 independent binding assays). **c)** Peptide pull-downs with Ac-peptides of Lyn, Fyn and Src
115 and GST-DCAF10 as bait. **d)** Quantification of WBs; Lyn binding set to 100%; mean
116 $\pm$ s.d. with individual data points are shown ($n = 3$ independent binding assays). **e)**
117 Fluorescent CPM signal as a function of CoA-SH concentration. The linear regression of
118 this graph was used to calculate Nt-acetylation levels and Michaelis-Menten kinetics. **f-g)**
119 Time-courses of *in vitro* Nt-acetylation assays for Lyn, Ac-Lyn, Src, Ac-Src, Fyn, and a
120 control peptide. 160 μ M peptides (**f**) or 1mM (**g**) were incubated with 20 nM NatA and 80
121 μ M acetyl-CoA for 20, 40 and 60 min to assess the linear range of the reaction. Data
122 represent the mean $\pm$ standard deviation ($n = 4$ independent enzyme assays).

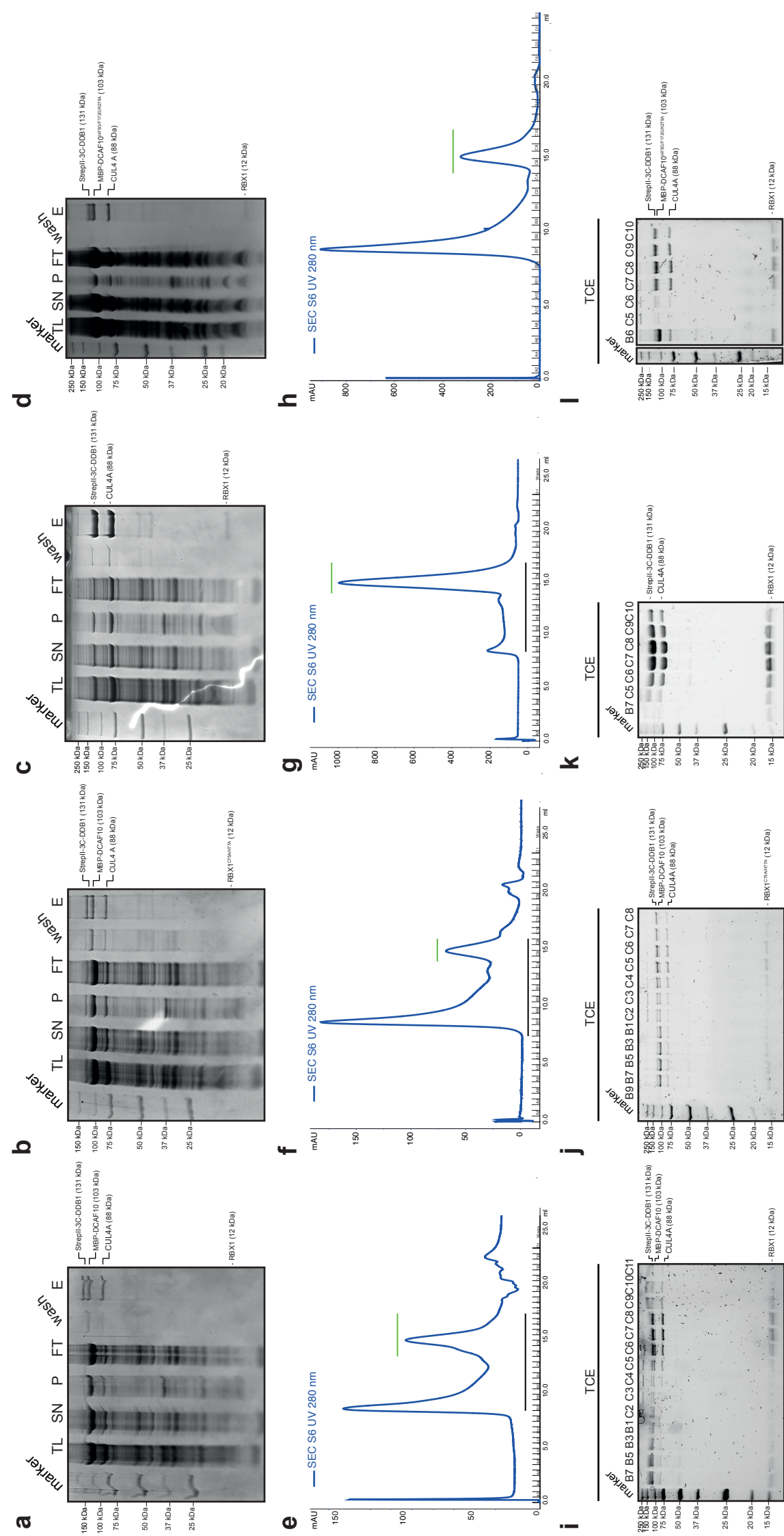
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Supplementary Fig. 5 Kremer et al.

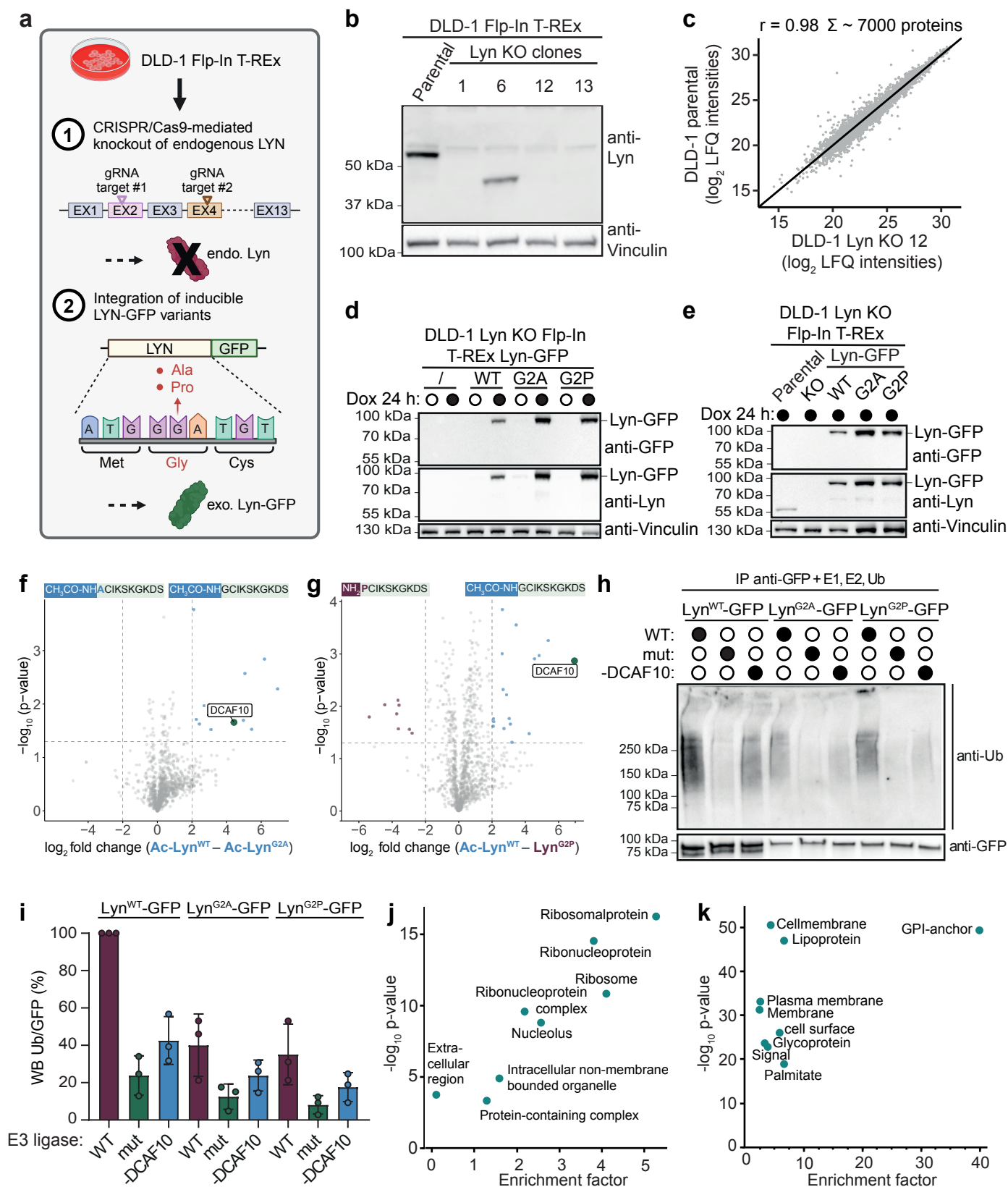
124 **Supplementary Fig. 5. NMT1/2 knockdown leads to an increased turnover of SFKs**
125 **and overexpression of DCAF10 reduces SFK levels after NMT1/2 knockdown.**

126 **a)** HeLa cells were treated with NMT1/2 siRNA for 72 h or left untreated in combination
127 with FLAG-DCAF10 overexpression for 24 h. Representative WBs are shown. **b-c)** Left:
128 WB quantifications with and without NMT1/2 siRNA of Lyn (**b**) and THOC7 (**c**)
129 normalized to vinculin. Right: Fold change of Lyn (**b**) and THOC7 (**c**) after DCAF10
130 overexpression. Untreated: purple; with NMT1/2 RNAi: blue. Individual data points are
131 indicated ($n = 2$ or 3 independent biological replicates). MG132 = proteasome inhibitor;
132 CHX = cycloheximide translational inhibitor. **d)** HeLa cells were treated for 72 h with and
133 without NMT1/2 siRNA and treated with cycloheximide or MG132 for the indicated
134 periods of time. Representative WBs are shown. Filled circles indicate treatment; empty
135 circles indicate no treatment. **e-h)** Fold-change analysis of protein expression levels under
136 the indicated conditions. Left: fold change after 8 h cycloheximide treatment of (**e**) Lyn,
137 (**f**) Fyn, (**g**) Src and (**h**) THOC7. Right: fold change after 4 h MG132 treatment of (**e**) Lyn,
138 (**f**) Fyn, (**g**) Src and (**h**) THOC7. Untreated: purple; with NMT1/2 siRNA: blue ($n = 3$
139 independent biological replicates). **i)** WB of cellular lysates used to immunoprecipitate
140 Lyn for *in vitro* ubiquitination assays in **Figure 5**. Source data are provided as a Source
141 Data file.



142 **Supplementary Fig. 6. Purification of CUL4A, RBX1, StrepII-DDB1, 6xHIS-MBP-**
143 **DCAF10 complexes.**

144 **a-d)** Affinity purifications of the E3 ligase full-length wild-type complex (StrepII-DDB1,
145 CUL4A, RBX1, 6xHIS-MBP-DCAF10), RBX1^{mut} complex (StrepII-DDB1, CUL4A,
146 RBX1^{C75A/H77A}, 6xHIS-MBP-DCAF10), -DCAF10 complex (StrepII-DDB1, CUL4A,
147 RBX1) and DCAF10^{mut} complex (StrepII-DDB1, CUL4A^{I475G/F172G/K257A}, RBX1,
148 6xHISMBP-DCAF10) using Strep-Tactin®XT 4Flow® beads to purify the whole complex
149 via the Strep-II tag fused to DDB1 from insect cells. Coomassie brilliant blue staining of
150 SDS-PAGE gels are shown total lysate (TL), soluble supernatant (SN), pellet (P) and
151 unbound flowthrough (FT) after incubation with beads, the wash of beads (wash), and the
152 eluate (E). **e-h)** Strep-II tag purifications were run on a size-exclusion (SEC) column.
153 Chromatograms of absorbance at A280 are shown. Black lines correspond to fractions
154 visualized on SDS gels below (**i-h**), green lines to the pooled fractions taken for the in vitro
155 ubiquitination assay. **i-h)** Fractions from (**e-h**) (black line) loaded on SDS gels and
156 visualized via TCE (2,2,2-trichloroethanol) gels. Source data are provided as a Source Data
157 file.

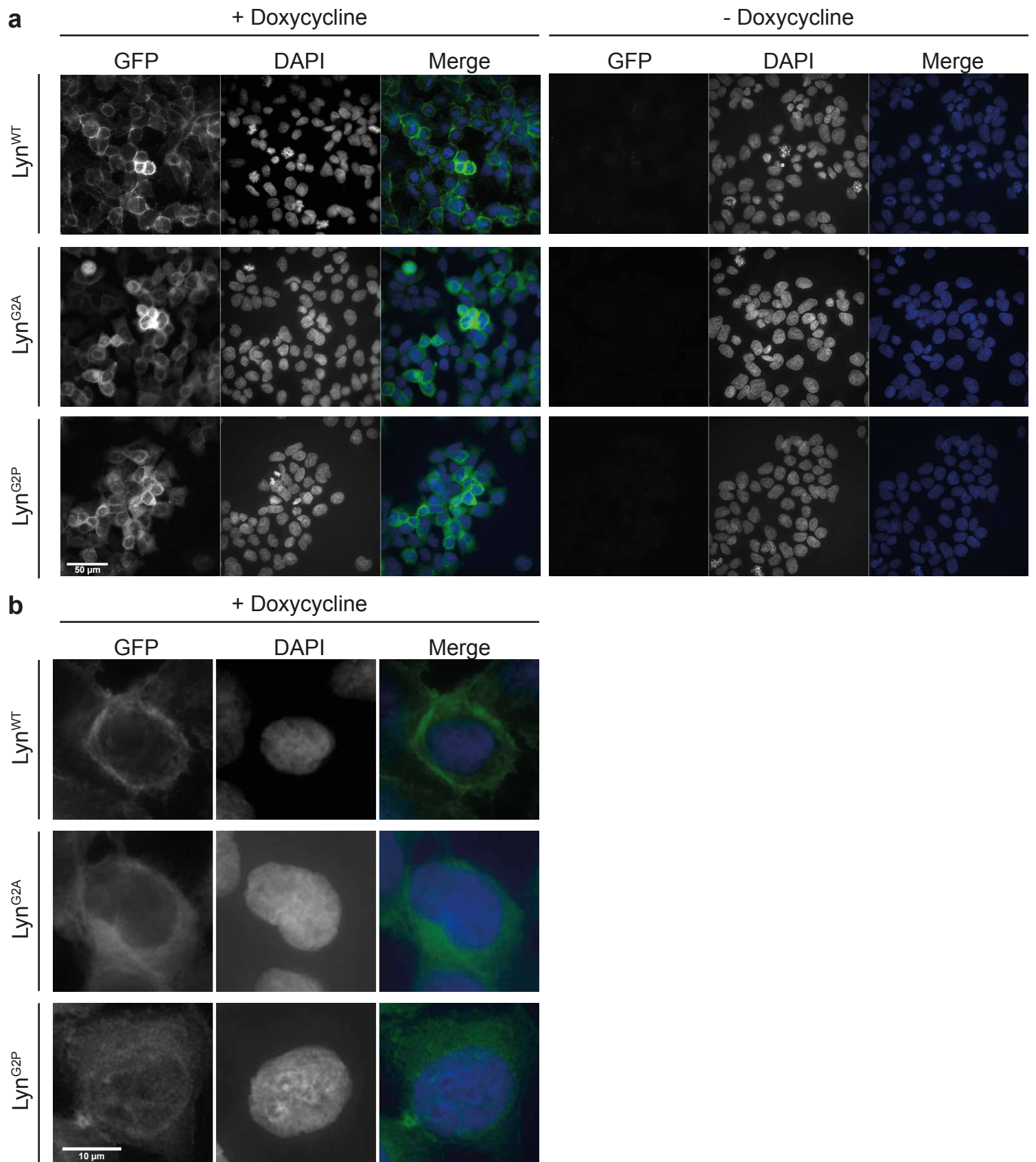


158 **Supplementary Fig. 7. Generation of DLD-1 Lyn KO cell lines expressing doxycycline**
159 **inducible Lyn-GFP variants (WT, G2A or G2P) and supporting experiments.**

160 **a)** Strategy for cell line generation: (1) Endogenous Lyn in parental DLD-1 Flp-In T-REx
161 cells was knocked out (KO) using the CRISPR/Cas9. (2) Lyn-GFP WT and Nt-variants
162 (G2A, G2P) were stably integrated into the Lyn KO genome. *Created in BioRender: Bange,*
163 *T. (2025) <https://BioRender.com/z58tr5a>.* **b)** Validation of Lyn KO. WBs against Lyn and
164 vinculin confirm disruption of endogenous Lyn in 75% of selected clones; clone 12 was
165 used for stable integration. **c)** MS-based proteome comparison of parental and Lyn KO
166 clone 12 cells. Log2 intensity values show comparable proteomes ($r = 0.98$; ~7000
167 quantified proteins). $N = 3$ independent biological replicates **d)** Induction of Lyn-GFP
168 variants with 100 ng/mL doxycycline (24 h) in Lyn KO cells. WBs confirm absence of
169 endogenous Lyn and doxycycline-dependent expression of Lyn-GFP variants. **e)**
170 Comparison of endogenous versus induced Lyn-GFP signals in parental and Lyn-GFP cell
171 lines after 24 h doxycycline treatment. ($n = 3$ independent biological replicates for **d-e**). **f-**
172 **g)** Volcano plots comparing binding partners of mutant Nt-peptides of Lyn (aa 1–10 +
173 KKK-biotin; **Supplementary Table 1**). **f)** Ac-Ala vs. AcGly, **g)** Pro vs. Ac-Gly. Volcano
174 plots were generated using log2 fold changes (x-axis) and $-\log_{10}$ adjusted p-values (y-
175 axis) from a two-sided Student's *t*-test with permutation-based multiple-testing correction
176 (significance thresholds: $-\log_{10} p \geq 1.3$ and $\log_2$ fold change ≤ -2 or ≥ 2); DCAF10
177 marked in green, significant Nt-free binders in purple, Nt-Ac binders in blue ($n = 3$
178 independent biological replicates). **h)** DLD-1 Lyn^{WT}-GFP, Lyn^{G2A}-GFP and Lyn^{G2P}-GFP
179 cells were induced with doxycycline for 24 h. Lyn-GFP variants were immunoprecipitated
180 using anti-GFP beads and subjected to *in vitro* ubiquitination assays with WT, RBX1^{mut},
181 and -DCAF10 complexes. **i)** Quantification of Ub signals normalized to GFP; mean $\pm$ s.d.
182 with individual data points are shown ($n = 3$ independent enzymatic assays). **j-k)** Fisher's
183 exact test of significant interaction partners of Lyn^{WT}-GFP IPs (**Fig. 6j-l**) (adjusted p-value

184 with Benjamini-Hochberg correction < 0.01). The $-\log_{10}$ adjusted p-value of the test (y-
185 axis) is blotted against the enrichment factor (x-axis). **(j)** NMT1/2 RNAi treated lysates $\pm$
186 doxycycline (Dox); **(k)** control lysates $\pm$ doxycycline (Dox).

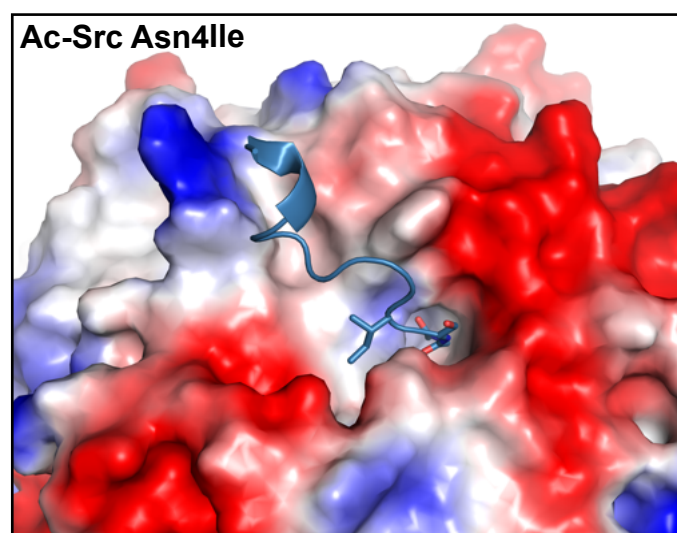
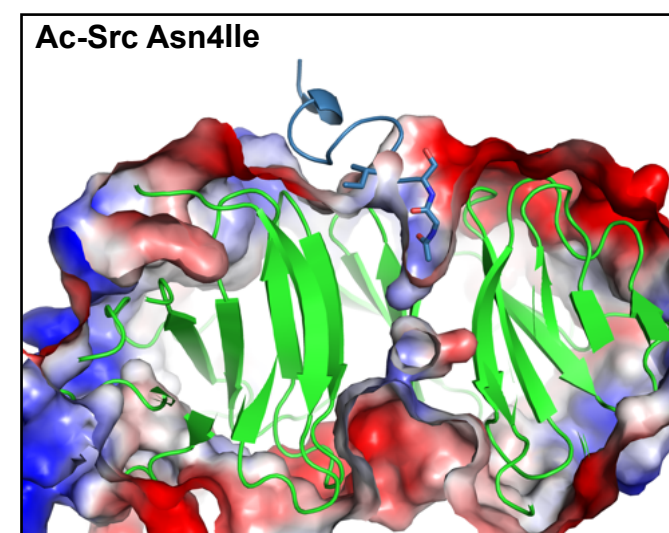
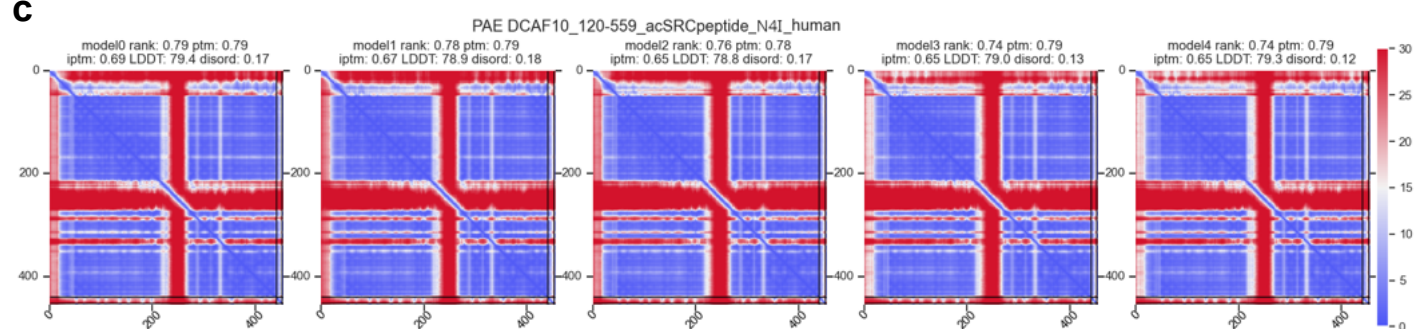
187



188 **Supplementary Fig. 8. Fluorescence of DLD-1 Lyn KO Flp-In T-REx cells expressing**
189 **Lyn^{WT}-GFP, Lyn^{G2A}-GFP or Lyn^{G2P}-GFP.**

190 **a)** Lyn (WT, G2A or G2P)-GFP expression was induced for 24 h with 100, 10 and 10 ng/mL
191 doxycycline, respectively (left panel), or no doxycycline was added as control (right panel).
192 GFP fluorescence and DAPI signals, used to visualize nuclei of fixed cells, were imaged
193 with a 60x objective, confirming that all cells express Lyn-GFP upon doxycycline
194 treatment. **b)** Cropped image of a representative cell for each Nt-variant from the images
195 in **(a)**, showing Lyn-GFP localization.

196

a**b****c**

197 **Supplementary Fig. 9. AF3 predictions of Src Asn4Ile mutant.**

198 **a)** Top view of DCAF10 (aa 120–559, UniprotID: Q5QP82) with Nt-Ac Src Asn4Ile (aa2-
199 40). DCAF10's electrostatic surface potential is displayed (red = negative, blue = positive,
200 white = neutral or hydrophobic), as calculated by PyMOL **b)** Cross section of DCAF10
201 (aa 120-559) with Src Asn4Ile (blue). **c)** PAE (Predicted Aligned Error) plots for the best
202 AF3 model for DCAF10 binding to Nt-Ac Src Asn4Ile (aa 2–40).

203 **Supplementary Methods**

204 **Peptide pull-downs**

205 Peptide sequences are listed in **Supplementary Table 1**. Peptide pull-downs were
206 performed as previously described¹. Briefly, magnetic streptavidin beads (Dynabeads™
207 MyOne™ Streptavidin T1; Thermo Fisher Scientific, Waltham, MA, USA) were
208 incubated with biotinylated peptides (4 µg of peptide/50 µL of bead slurry) for 30 min at
209 room temperature (RT) in PBS. Beads were then washed three times with PBS. After
210 washing, 1 mg of HeLa lysates or 2 mg of liver lysates was taken, and lysis buffer (75
211 mM HEPES pH 7.5, 150 mM KCl, 1.5 mM EGTA pH 8.0, 1.5 mM MgCl₂, 10% (v/v)
212 glycerol, 0.075% NP-40, supplemented with 1mM DTT, PhosSTOP, and Protease
213 Inhibitor Cocktail) was added to each sample. Lysates were incubated with streptavidin
214 beads for 16 h at 4°C. HeLa pull-downs were washed twice with lysis buffer and twice
215 with wash buffer (lysis buffer without glycerol, NP-40, DTT, PhosSTOP, and Protease
216 Inhibitor Cocktail). Liver pulldowns were washed twice with cold low salt buffer (50 mM
217 HEPES pH 7.5, 140 mM NaCl, 1% Triton X-100), once with cold high salt buffer (50
218 mM HEPES pH 7.5, 500 mM NaCl, 1% Triton X-100), and once with TBS. Organs were
219 isolated from male C57BL/6N mice of 8–10 weeks of age that were maintained in
220 individually ventilated cages under a 12-h light/dark cycle and were provided with sterile
221 food and water under specific-pathogen-free conditions. All procedures were performed
222 in accordance with European law regarding the protection of animal welfare and with
223 approval by the local government authorities (animal facility registered for breeding and
224 use of animals for scientific purpose KVR-I/221-TA166/22_03-06). Mouse liver tissue
225 used in this study consisted of surplus material from animals euthanized for unrelated
226 experiments in this laboratory. No animal procedures were performed specifically for this
227 study, and no animals were handled by the authors. After withdrawing the supernatant,
228 samples were directly digested on beads and processed for MS analysis. Samples were

229 reduced, alkylated, digested directly on beads with LysC/Trypsin (protein:enzyme ratio
230 of 1:100), and desalted/concentrated on C18 reversed phase stage tips as previously
231 described^{24,56}. MS measurements settings are stated in **MS measurements and raw file**
232 **processing**.

233

234 **Purification of GST-DCAF10**

235 DCAF10 was amplified from pcDNA3.1+/C-(K)-DYK (GenScript; Piscataway, New
236 Jersey, USA) and, via Gibson cloning, integrated into the first cassette of pGEX-6P-2rbs
237 (GenBank accession code KM817768)⁵⁷. BL21(DE3) cells (Thermo Fisher Scientific,
238 Waltham, MA, USA) transformed with pGEX-6P-2rbs-DCAF10 were inoculated from a
239 single colony picked from a Lysogeny Broth (LB) agar (LB medium with 1.5% (w/v)
240 agar) plate containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol, then
241 cultured overnight in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl,
242 pH7.4) with the appropriate antibiotics. Terrific broth (TB) medium (24 g/L yeast extract,
243 20 g/L tryptone, glycerol 4 mL/L, 100 mL/L phosphate buffer containing 0.17 M KH₂PO₄
244 and 0.72 M K₂HPO₄) was used to prepare a 1:100 dilution of the starter culture, and OD₆₀₀
245 (600 nm) was measured. At OD₆₀₀ = 1, protein induction was initiated by adding 1 mM
246 IPTG for overnight expression at 16°C for 18 h with shaking (120 rpm). The following
247 day, pellets were homogenized and lysed in glycerol lysis buffer (20 mM Tris-HCl pH
248 6.8, 300 mM NaCl, 10% (v/v) glycerol, 5 mM 2-mercaptoethanol, supplemented with
249 Protease Inhibitor Cocktail). Lysis was performed on ice for 30 min, followed by
250 sonication (7 × 20 sec pulses with 20 sec breaks on ice) using a Bandelin sonoplus
251 sonicator (Sonotrode MS1.5, 70% output, 20W). Lysates were centrifuged at 4°C for 1 h
252 at 14.000 × g, and the supernatant was transferred in a new tube. 1 L of TB growth
253 medium culture was lysed in 20 mL of supernatant (collected in 20 × 1 mL tubes). GST
254 beads were used according to manufacturer's instructions (PierceTM Glutathione magnetic

255 agarose bead; Thermo Fisher Scientific, Waltham, MA, USA). For each 1 mL of
256 supernatant, 100 μ L of GST magnetic beads were used. 100 μ L of GST magnetic beads
257 were washed twice with 500 μ L of wash buffer (125 mM Tris-HCl pH 7.4, 150 mM NaCl,
258 1 mM DTT, 1 mM EDTA) in a 2 mL tube. The lysis supernatant was diluted 1:1 with
259 wash buffer, and incubated with the beads on an end-over-end rotator for 1 h at 25°C.
260 After incubation, the supernatant was removed, and the beads were washed twice with
261 wash buffer lacking DTT and EDTA. Protein elution was then performed by incubating
262 the beads with glutathione elution buffer (10 mM reduced glutathione, 125 mM Tris-HCl
263 pH 7.4, 250 mM NaCl, 0.3% Triton X-100) for 30 min at 25°C on an end-over-end rotator.
264 This process was repeated with glutathione elution buffer containing 0.1% Triton X-100
265 under the same conditions. Elution fractions were pooled and concentrated by
266 centrifugation (4°C, 25 min, 4500 rpm) using a 30 kDa cut-off concentrator (Merck
267 Millipore, #UFC803008, Darmstadt, Germany). Purified proteins were analyzed by
268 colloidal Coomassie stain, WB and MS.

269

270 **Streptavidin pull-downs with GST-DCAF10 and biotinylated peptides**

271 Streptavidin magnetic beads (DynabeadsTM MyOneTM Streptavidin T1; Thermo Fisher
272 Scientific, Waltham, MA, USA) were washed twice with 500 μ L of wash buffer (125 mM
273 Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Triton X-100). An excess of peptides was added
274 (to ensure full occupancy of the beads) and incubated for 30 min at RT. After peptide
275 binding, beads were again washed three times with 500 μ L of wash buffer. The pull-down
276 was then performed in a 100 μ L reaction volume with final concentrations of 0.5 μ M
277 GST-DCAF10 and 5 μ M peptide. GST-DCAF10 or GST alone (as a control) was added
278 to the peptide-streptavidin beads and incubated on a rotator for 1 h at RT. After incubation,
279 the beads were washed three times with 500 μ L of wash buffer and resuspended in 4x

280 Laemmli buffer. 1/5 of the input (GST-DCAF10 and GST) and 9/10 of the sample were
281 loaded on a 10% SDS gel and blotted with anti-GST and anti-DCAF10 antibodies, while
282 1/10 was loaded on a 15% SDS gel and blotted with anti-streptavidin antibody to assess
283 equal peptide loading.

284 For direct comparison of binding to acetylated peptides (Ac-Lyn, Ac-Fyn and Ac-Src),
285 Streptavidin Sepharose High Performance, GE Healthcare, Chicago, IL, USA) were used.
286 After washing with a high salt wash buffer without detergents (125 mM Tris-HCl pH 7.4,
287 400 mM NaCl), an excess of peptides was added and incubated for 30 min at RT. For each
288 reaction, 10 μ L of slurry beads were used. After peptide-binding, beads were again
289 washed once with a high salt wash buffer without detergents and twice with binding buffer
290 without detergents (125 mM Tris-HCl pH 7.4, 150 mM NaCl). The pull-downs were
291 performed in a 100 μ L reaction volume in binding buffer without detergents with a final
292 concentration of 0.5 μ M GST-DCAF10 and incubated rotating for 1 h at RT. After
293 incubation, the beads were washed five times with 500 μ L of high salt wash buffer without
294 detergents (125 mM Tris-HCl pH 7.4, 300 mM NaCl) and resuspended in 2x Laemmli
295 buffer. 1/5 of the input (GST-DCAF10) and 9/10 of each sample were loaded on a 4–15%
296 Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad, Hercules, CA, USA) and
297 blotted with anti-GST and anti-DCAF10 antibodies, while 1/10 was loaded on a different
298 a 4–15% Mini-PROTEAN® TGX™ Precast Protein Gel and blotted with anti-
299 streptavidin antibody to assess equal peptide loading.

300

301 **Cloning, expression, and purification of human NatA**

302 Human NatA (hNatA) full-length (FL) was cloned in a pFL vector expressing both
303 genes, NAA15 with a 6 $\times$ C-term histidine tag (NAA15-6xHIS) and NAA10². The
304 expression was carried out in insect cells (Tnao38) for 72 h infected 1:20 with

305 baculovirus. Washed cell pellets were lysed by sonication in a lysis buffer containing 200
306 mM NaCl, 50 mM HEPES pH 8.0, 20 mM Imidazole, and 2 mM TCEP. The protein
307 purification was performed as a three-step purification, starting with an affinity nickel
308 column, followed by an ion-exchange column with a gradient of 50–300 mM NaCl, and
309 finished with size exclusion chromatography (SEC) using an S200 10/300 column. The
310 main peaks were collected, and the protein was concentrated in the SEC buffer (150 mM
311 NaCl, 50 mM HEPES pH 8.0, 1 mM EDTA). The purified hNatA was flash-frozen in
312 liquid nitrogen and stored at -80°C.

313

314 ***In vitro* Nt-acetylation assay**

315 The method has been described in detail previously². Briefly, different peptides
316 (**Supplementary Table 1**) and concentrations (an eight-step, 1:1 dilution series for
317 Michaelis-Menten curves) as indicated were incubated with 20 nM hNatA and 80 μM Ac-
318 CoA in reaction buffer (50 mM HEPES pH 8.0, 0.5 mM EDTA, 0.005% CHAPS) for 60
319 (Src) or 120 min (Lyn), at RT in a 384-well microplate (Corning 4514) ($n = 4$).
320 Afterwards, the enzymatic reaction was stopped by adding 1:1 DMSO with 10 μM (final
321 concentration 5 μM) of the thiol reactive CPM (7-Diethylamino-3-
322 (4'-Maleimidylphenyl)4-Methylcoumarin). After 1 h at RT, the readout was performed
323 with a Tecan Spark® Multimode Microplate Reader (excitation 390 nm, emission 479
324 nm). Background values at 0 μM peptide were subtracted, and control reactions omitting
325 NatA or Ac-CoA were performed. Dilution series of CoA incubated with 5 μM CPM were
326 used to generate a linear equation ($y = 10.99 \cdot x + 81.06$) to calculate acetylation velocities
327 for NatA-mediated peptide acetylation. Experiments with different time points and
328 concentrations were performed to ensure that the reaction remained within a linear range.

329

330 **Cloning and expression of MBP-DCAF10, StrepII-DDB1 CUL4A, and RBX1**

331 The cDNA sequences encoding for the human proteins DDB1 (FL), CUL4A (FL),
332 RBX1 (FL), and DCAF10 (FL) were first cloned in a pLIB vector separately. DDB1 with
333 an N-term cleavable (HRV 3C protease) StrepII-tag and DCAF10 were cloned into a
334 vector containing an Nt-6xHIS-MBP fusion with a 3C protease cleavage site. Gibson
335 cloning was performed by using pre-defined primer pairs (listed in **Supplementary Table**
336 **4**) corresponding to cassettes including each gene and combining them to one expression
337 vector (pbiG1a)³. The WT construct contained cassettes I, II, and III with the genes coding
338 for StrepII-3C-DDB1, CUL4A, and RBX1. This vector was used as a template to perform
339 a site-directed mutagenesis to introduce two point mutations in the RBX1 gene, resulting
340 in the change of amino acid residues cysteine 75 and histidine 77 to alanine
341 (RBX1^{C75A/H77A}). Accordingly, the pLIB-Nt-6xHIS-MBP vector containing the DCAF10
342 WT gene was used to introduce mutations resulting in F172G, K257A and I475G. For
343 expression of the four different constructs, the baculovirus/insect cell system was used.
344 Therefore, pLIB and pbig1a plasmids were recombined with bacmid DNA to generate
345 bacmids, which were then used to transfect Sf9 (*Spodoptera frugiperda*) cells and to
346 produce high-titer V2 virus^{4,5}. The virus containing the DNA for StrepII-DDB1, CUL4A,
347 and RBX1 was co-infected with the virus containing the bacmid coding for MBP-
348 DCAF10 at a 1:3 ratio (3× excess of DCAF10). V2 was used at a 1:50 ratio for StrepII-
349 DDB1, CUL4A and RBX1 (WT or C75A/H77A), and at a 1:16.6 ratio for MBPDCAF10
350 (WT or F172G/K257A/I475G). For the expression culture, Tnao38^{6,7} insect cells were
351 used and incubated at 27°C for 72 h in a shaker.

352

353 **Purification of MBP-DCAF10, StrepII-DDB1, CUL4A, and RBX1**

354 Insect cells were harvested by centrifugation and a wash with PBS, before cell pellets
355 were flash-frozen in liquid nitrogen and stored at -80°C. For protein purification, cell
356 pellets were resuspended in lysis buffer (50 mM HEPES pH 7.5, 250 mM NaCl, 10%

(v/v) glycerol, 2 mM TCEP, supplemented with Protease Inhibitor Cocktail), and lysed by sonication. Lysates were cleared by centrifugation at 28,000 rpm for 45 min at 4°C using a JA-30.50 rotor (Beckman Coulter, Brea, CA, USA). The cleared and filtered (0.8 µm) lysate was applied to 1 mL of Strep-Tactin®XT 4Flow® resin (iba), pre-equilibrated once with wash buffer (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA) and lysis buffer. The lysate was incubated on a tube roller for 16 h at 4°C. After incubation, beads were washed with 50 mL of lysis buffer. Bound proteins were eluted with 10 mL of lysis buffer supplemented with 50 mM biotin, after 15 min incubation on a tube roller at 4°C. The eluate was concentrated using 50 kDa cut-off concentrators and loaded onto a Superdex 200 10/300 or 16/60 SEC column (GE Healthcare, Chicago, IL, USA), pre-equilibrated in SEC buffer (250 mM Tris pH 7.5, 250 mM NaCl, 10% (v/v) glycerol, 2 mM TCEP). Fractions containing the E3 ligase complex (DCAF10, DDB1, CUL4A, and RBX1) were concentrated, flash-frozen in liquid nitrogen, and stored at -80°C. Purified proteins were visualized during and after the purification by SDS-gels containing TCE (2,2,2-trichloroethanol)⁶². The identity of proteins was confirmed by WB and MS.

372

373 ***In vitro* ubiquitination assays**

374 The *in vitro* ubiquitination assays were performed as previously described⁸. In brief,
375 E1 (50 nM), UBCH5B (250 nM), ubiquitin (1.5 µg), Mg-ATP (3 mM), ligation buffer,
376 and water were pre-mixed. The premix was divided, and either CUL4A-RBX1-
377 DDB1DCAF10 (WT), DDB1-CUL4A-RBX1^{C75A/H77A}-DCAF10 (mut) or CUL4A-
378 RBX1DDB1 (-DCAF10) (250 nM each) was added. 15 µL of each premix were incubated
379 with substrates (endogenous Lyn or Lyn-GFP constructs) bound to beads for 1 h at 30°C
380 with shaking. Samples were then washed four to five times with a buffer containing 50
381 mM HEPES pH 7.5, 10% (v/v) glycerol, 150 mM NaCl, 1% Triton X-100 and 1 mM
382 EDTA, and loaded on a gradient gel (4–15%). Ubiquitination of substrates was assessed

383 by WB using an anti-ubiquitin antibody. Equal substrate loading was evaluated by anti-
384 Lyn or anti-GFP antibodies. The WBs were incubated with an anti-DCAF10 antibody
385 (and/or anti-DDB1 or anti-CUL4A antibodies) to ensure that the E3 ligases were washed
386 away after the reaction. Endogenous, unmodified Lyn as the substrate was prepared as
387 follows: Dynabeads™ Protein A (10006D, Thermo Fisher Scientific, Waltham, MA,
388 United States) were used for binding 5 µL of anti-Lyn antibody to 40 µL of bead slurry in
389 Ab Binding & Washing Buffer (0.1 M sodium phosphate pH 8.2, 0.01% (v/v) Tween-20)
390 for 1 h at RT. After washing once with Ab Binding & Washing Buffer (0.1 M sodium
391 phosphate pH 8.2, 0.01% (v/v) Tween-20), beads were incubated for ca. 16 h at 4°C with
392 250 µg of HeLa lysate, either treated for 48 h with siRNA against NMT1/2 or control.
393 After two washes using 500 µL low salt buffer (50 mM HEPES pH 7.5, 140 mM NaCl,
394 1% Triton X-100), two washes using 500 µL high salt buffer (50 mM HEPES pH 7.5, 500
395 mM NaCl, 1% Triton X-100), and one wash using TBS (50 mM Tris pH 7.4, 150 mM
396 NaCl), immunoprecipitated Lyn was used as the substrate for *in vitro* ubiquitination
397 assays.

398 Different versions of C-terminally GFP-fused Lyn (WT, G2A or G2P) as substrates
399 were prepared as follows: GFP-Trap magnetic agarose beads (ChromoTek, Proteintech,
400 Munich, Germany) were incubated with lysates from DLD-1 Lyn KO cells stably
401 expressing inducible Lyn^{WT}-GFP, Lyn^{G2A}-GFP or Lyn^{G2P}-GFP. Expression was induced
402 by adding 10–100 ng/mL doxycycline (dox) for 24 h. To obtain similar amounts of Lyn,
403 beads were incubated with different amounts of lysates (1000, 100 or 200 µg of Lyn-GFP
404 lysates, respectively) for ca. 16 h at 4°C. The following day, after two washes using 500
405 µL of low salt buffer, two washes using 500 µL of high salt buffer, and one wash using
406 TBS, immunoprecipitated Lyn was used as the substrate for *in vitro* ubiquitination assays.

407

408 **SiRNA knockdown and overexpression experiments in HeLa, DLD-1, and RPE-1**

409 **cell lines**

410 HeLa, DLD-1 and all thereof derived stable cell lines, and RPE-1 cells were grown in
411 Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine
412 serum (FBS). Cells were counted and diluted to 100,000 cells/mL. For siRNA
413 knockdown, the transfection reagent Lipofectamine RNAiMAX (Invitrogen, Thermo
414 Fisher Scientific, Waltham, MA, USA) was used at a final dilution of 1:333 (3 μ L/mL of
415 medium). Pools of siRNAs, including siNMT1, siNMT2, siDCAF10, siZYG11B, and
416 siZER1, or combinations of different siRNAs, were reverse transfected at a concentration
417 of 25 nM for each siRNA and incubated as indicated (48–72 h). As control, cells were
418 treated only with Lipofectamine RNAiMAX. Both siRNAs and RNAiMAX were diluted
419 in Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA). For overexpression of
420 DCAF10, the transfection reagent Lipofectamine 2000 (Thermo Fisher Scientific,
421 Waltham, MA, USA) was used at a final dilution of 1:200. For one 6-well of a 6-well
422 plate, 4 μ g of DNA were combined with Lipofectamine 2000 and diluted in Opti-MEM.
423 The medium was changed 4–6 h post-transfection. The transfection was performed one
424 day after plating, and the cells were incubated for an additional 24 h. For proteasomal or
425 translational inhibition, the cells were treated with 10 μ M MG132 or 100 μ g/mL
426 cycloheximide for 4 and 8 h before lysis, respectively.

427

428 **High-pH fractionation for DCAF10 and ZYG11B**

429 10 μ g of cellular lysates were reduced, alkylated, and digested with LysC/Trypsin
430 (protein:enzyme ratio of 1:100) according to MS standard in-solution digest⁹. Peptides
431 were then separated with a high-pH fractionation kit (eight fractions for DCAF10 and
432 four fractions for ZYG11B; Thermo Fisher Scientific, Waltham, MA, USA). Each
433 fraction was measured by MS (see **Mass spectrometry measurements and raw file**
434 **processing** for settings).

435

436 **Site-directed mutagenesis**

437 To generate Lyn variants with mutated N-terminal amino acid (G2A and G2P), site-
438 directed mutagenesis was performed on the pcDNA5/FRT/TO-Lyn^{WT}-EGFP-IRES
439 construct using primers (listed in **Supplementary Table 4**) that carry single (for G2A) or
440 double (for G2P) mismatches to wild-type Lyn in PCR reactions. The resulting PCR
441 products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega;
442 Madison, WI, USA) according to the manufacturer's instructions, followed by DpnI
443 digestion, phosphorylation, and ligation. Subsequently, half of the ligation reaction was
444 transformed into DH5α competent cells. After isolation, the plasmid DNA from single
445 colonies was sent for Sanger sequencing (Eurofins Genomics, Munich, Germany) to
446 confirm successful mutagenesis.

447

448 **Generation of DLD-1 Lyn KO Flp-In T-REx cell lines**

449 DLD-1 Lyn KO Flp-In T-REx cells were generated using two guide RNAs (gRNAs),
450 each containing a specific crRNA (listed in **Supplementary Table 4**) and a tracrRNA. To
451 form each Cas9-gRNA ribonucleoprotein (RNP), a gRNA was mixed with the homemade
452 wild-type Cas9 (prepared following the procedure described in¹⁰). The electroporation
453 method using SF Cell Line 4D-Nucleofector X Kit S (Lonza Bioscience, Basel,
454 Switzerland) was applied to transfect the Cas9-gRNA RNP into cells. Briefly, 250,000
455 cells were resuspended in 20 µL of SF Full Electroporation Buffer containing 16.4 µL of
456 SF Nucleofector Solution and 3.6 µL of Supplement. Next, 1.25 µL of Cas9
457 Electroporation Enhancer (IDT) and 3.75 µL of Nuclease Free Duplex Buffer were added
458 to the cell suspension, followed by addition of 5 µL of the RNP. The mixture was then
459 transferred to a free well of a 16-well Nucleocuvette strip. The strip was placed into the
460 4DNucleofector System (Lonza Bioscience, Basel, Switzerland), and cells were

461 electroporated using the CM137 program. Following electroporation, the cells were kept
462 in the strip at RT for 10 min before being transferred to a 6-well plate containing
463 prewarmed DMEM supplemented with 10% FBS and 100 U/mL penicillin/streptomycin.
464 After a day, all cells from the 6-well plate were transferred to a 10-cm dish. After two
465 days, single cells were diluted into each well of a 96-well plate using a single cell
466 dispenser (Dispencell; SEED Biosciences, Épalinges, Vaud, Switzerland) following the
467 manufacturer's instructions. The 96-well plate was incubated for two weeks, after which
468 cells were expanded in a 24-well plate and a 6-well plate until clones reached 80%
469 confluency. Once clones reached 80% confluency in 6-well plates, they were harvested.
470 One third of the cells from each clone were frozen, and the remaining cells were analyzed
471 by genomic sequencing (primers in **Supplementary Table 4**), WB and MS. Positive
472 clones were then expanded for cell line generation.

473

474 **Integration of Lyn^{WT}-GFP and N-terminal mutants, Lyn^{G2A}-GFP and Lyn^{G2P}-GFP,**
475 **in DLD-1 Lyn KO Flp-In T-REx cell lines**

476 Stable cell lines expressing C-terminally GFP-fused Lyn, either WT, G2A or G2P
477 mutants, were generated in DLD-1 Lyn KO Flp-In T-REx cells using FRT/Flp-mediated
478 recombination technique⁵². One day prior to transfection, cells were seeded in 10-cm
479 dishes to reach a confluency of 50% on the day of transfection. For each transfection
480 reaction, 30 µL of X-tremeGENE transfection reagent (Roche, Basel, Switzerland) were
481 mixed with 500 µL of pre-warmed Opti-MEM and incubated for 5 min at RT. In the
482 meantime, 1 µg of the pCDNA5/FRT/TO-EGFP-IRES vector containing the respective
483 Lyn cDNA (WT, G2A or G2P) was mixed with 9 µg of pOG44 and added to the X-
484 tremeGENE mix. After 20 min incubation at RT, the transfection mix was added dropwise
485 to the cells, and cells were cultured for 48 h. Next, cells were subjected to selection in
486 DMEM supplemented with 10% FBS, 300 µg/mL hygromycin B, and 8 µg/mL blasticidin

487 for two weeks. Resulting foci were pooled, expanded, and validated for expression of the
488 transgenes by WB and fluorescence microscopy. The inducible gene expression was
489 tested by addition of 10–100 ng/mL dox for 8–24 h.

490

491 **Immunoprecipitation of Lyn^{WT}-GFP**

492 Lyn^{WT}-GFP was immunoprecipitated from lysates of DLD-1 Lyn KO Flp-In T-REx
493 cells expressing inducible Lyn^{WT}-GFP, following 72 h NMT1/2 knockdown and 24 h dox
494 induction using GFP-Trap magnetic agarose beads. Briefly, 10 µL of bead slurry per
495 reaction was equilibrated three times with ice-cold lysis buffer (75 mM HEPES pH 7.5,
496 150 mM KCl, 1.5 mM EGTA pH 8.0, 1.5 mM MgCl₂, 10% (v/v) glycerol, 0.075% NP40,
497 supplemented with 1mM DTT, PhosSTOP, and Protease Inhibitor Cocktail). Then, 500
498 µL of each lysate sample (control, dox induction, and NMT1/2 knockdown with or
499 without dox induction) containing approximately 300 µg total protein, were added to
500 tubes containing the equilibrated beads. Samples were rotated end-over-end for 1.5–2 h
501 at 4°C. After incubation, the beads were washed twice with 500 µL of cold lysis buffer
502 and twice with 500 µL of cold lysis buffer lacking NP-40, glycerol, DTT, phosSTOP, and
503 Protease Inhibitor Cocktail. Finally, the immunoprecipitates were resuspended in 50 µL
504 of 8 M urea, incubated at 25°C for 30 min, and stored at -20°C prior to on-bead digestion
505 and stage tip clean-up for MS analysis. Each experimental condition was performed in
506 triplicates.

507

508 **Fluorescence microscopy**

509 Cells were seeded on coverslips to be 90% confluent on the day of fixation. Lyn-GFP
510 expression was induced by 24 h dox treatment (100 ng/mL for Lyn^{WT}, and 10 ng/mL for
511 Lyn^{G2A} and Lyn^{G2P}), or media was just replaced for the controls (without dox). Cells were
512 washed once with PBS before fixation with 4% PFA in PBS for 10 min at RT. After three

513 washes with PBS, cells were permeabilized with PBS-T (PBS with 0.1% (v/v) Tween20)
514 for 10 min at RT. Afterwards, cover slips were incubated in 0.5 µg/mL DAPI diluted in
515 PBS-T for 5 min at RT. After a final wash in ddH₂O, the coverslips were dried on the air
516 and mounted with Mowiol as a mounting medium on glass slides. Image acquisition of
517 cells was performed at RT using a Deltavision Elite System (GE Healthcare, Chicago, IL,
518 USA) equipped with an IX-71 inverted microscope (Olympus, Tokyo, Japan), a 60x/1.42
519 Plan Apo N objective and a pco.edge sCMOS camera (PCO-TECH Inc., Kelheim,
520 Germany). Images were acquired as z-stacks containing 16 sections with a distance of
521 200 nm using the software softWoRx (GE Healthcare, Chicago, IL, USA). Raw data were
522 then deconvolved and converted into average intensity projections, exported, and saved
523 as 16-bit TIFF files. Figures were edited and arranged using ImageJ. Acquisition settings:
524 Green channel: 0.1 sec, 50% transmission; DAPI channel: 0.01 sec, 32% transmission.

525

526 **Mass spectrometry measurements and raw file processing**

527 Samples (peptide pull-downs, IPs and high-pH fractionations) were separated on an
528 EASY-nLC 1200 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) using
529 in-house-packed 50-cm reversed-phase columns (75 cm diameter, 1.9 mm C18 ReproSil
530 particles, Dr. Maisch GmbH) (60°C at 300 nL/min flow rate with an 83 min gradient from
531 5–60% acetonitrile with 0.1% formic acid). Peptides were directly sprayed via a nano
532 electrospray source in an Orbitrap Exploris™ 480 (Thermo Fisher Scientific, Waltham,
533 MA, USA). Data were acquired in a data-independent mode (DIA), acquiring one survey
534 scan (MS scan) with 120 k resolution and subsequently 85 windows with an isolation
535 width of 7.7 Th with 1 Dalton overlap from 350 to 1000 m/z at a resolution of 15 k (3.2
536 sec cycle time) (data points per peak = 6). Cycle times were calculated based on the
537 median elution time of HeLa QC runs to obtain 6 data points. The target value was set to

538 300% for the MS scan and 1000% for the MS/MS scans. The maximum injection time
539 was 45 msec for the MS scans. HCD Collision energy was set to 30%.

540 **Data Analysis and statistics**

541 Resulting raw files from MS measurements were processed with DIA-NN (version
542 1.8.2 beta 22) using a UniProt human database (reference January 2023) or UniProt
543 mouse database (reference January 2023)⁵³. Oxidation (M), M excision, Nt-acetylation,
544 and carbamidomethylation (C) were given as modifications. One miscleavage and two
545 modifications per peptide were allowed. A false discovery rate cutoff of 1% was applied.
546 MS measurements were performed in triplicates for peptide pull-downs, whole proteome
547 analysis and IPs. Label-free quantifications were calculated by the integrated DIA-NN
548 algorithm¹¹.

549 Peptide pull-downs from Ac and free N-termini in **Fig. 1** and **Supplementary Fig. 1**
550 were analyzed altogether; Ac and Myr, swap and Ala-Ala-Ala mutants, and Nt-Lyn
551 mutants were analyzed together, respectively. Lyn^{WT}-GFP IPs were analyzed together and
552 high pH fractionations of DCAF10 and Zyg11B. Comparisons for IPs and peptide pull-
553 downs were performed pairwise, filtering for 3 out of 3 replicates in one condition and
554 subsequent data imputation (downshift: 1.8, width: 0.3) in Perseus¹².

555 Intensities of high-pH fractions from the report.pg_matrix.tsv were then summed and
556 log₂-transformed (log₂) for overall protein quantities. DCAF10 and ZYG11B were
557 detected in control cells but not in KD cells. To not overestimate the KD, we considered
558 that the protein amounts of ZYG11B and ZER in the siRNA treated cells must be below
559 the detection limit of these MS runs. Therefore, we used for our knock-down estimation
560 the log₂ intensity of DCAF10 or ZYG11B, respectively, in the control cells and subtracted
561 it from the lowest quantified protein. For DCAF10: 22.37 (mean log₂ intensity of
562 DCAF10) - 14.35 (log₂ intensity of the lowest quantified protein) means at least a log₂

563 reduction of 8. For ZYG11B: 19.7 (mean $\log_2$ intensity of Zyg11B) - 15.7 ($\log_2$ intensity
564 of lowest quantified protein) means at least a $\log_2$ reduction of 4.

565

566 **Supplementary Material Tables**

567 Peptides have been purchased from GenScript (Piscataway, NJ, USA).

568 **Supplementary Table 1. Peptides**

Gene name	Peptide sequence	Protein name / UniProt ID	N-terminal modification	C-terminal modification
ARF1	GNIFANLFKGKK	ADP-ribosylation factor 1 / P84077	/	K(biotin)
ARF1	GNIFANLFKGKK	ADP-ribosylation factor 1 / P84077	acetyl-group	K(biotin)
COX17	PGLVDSNPAPKK	Cytochrome c oxidase copper chaperone / Q14061	/	K(biotin)
EF1B	GFGDLKSPAGKK	Elongation factor 1-beta / P24534	/	K(biotin)
EF1B	GFGDLKSPAGKK	Elongation factor 1-beta / P24534	acetyl-group	K(biotin)
FYN	GCVQCKDKEAKK	Tyrosine-protein kinase Fyn / P06241	/	K(biotin)
FYN	GCVQCKDKEAKK	Tyrosine-protein kinase Fyn / P06241	acetyl-group	K(biotin)
FYN	GCVQCKDKEAKK	Tyrosine-protein kinase Fyn / P06241	myristoyl-group	K(biotin)
GNAI3	GCTLSAEDKAKK	Guanine nucleotidebinding protein G(i) subunit alpha-3 / P08754	/	K(biotin)
GNAI3	GCTLSAEDKAKK	Guanine nucleotidebinding protein G(i) subunit alpha-3 / P08754	acetyl-group	K(biotin)
LYN	GCIKSKGKDSKK	Tyrosine-protein kinase Lyn / P07948	/	K(biotin)
LYN	GCIKSKGKDSKK	Tyrosine-protein kinase Lyn / P07948	acetyl-group	K(biotin)
LYN	GCIKSKGKDSKK	Tyrosine-protein kinase Lyn / P07948	myristoyl-group	K(biotin)
LYN G2A	ACIKSKGKDSKK	Tyrosine-protein kinase Lyn / P07948	acetyl-group	K(biotin)
LYN G2P	PCIKSKGKDSKK	Tyrosine-protein kinase Lyn / P07948	/	K(biotin)
LYN AAA	GCIAAAGKDSKK	Tyrosine-protein kinase Lyn / P07948	acetyl-group	K(biotin)

LYN TDD	GCITDDGKDSKK	Tyrosine-protein kinase Lyn / P07948	acetyl-group	K(biotin)
<i>NatA target peptide</i>	SASEAGVRWGRP VGRRRR	/	/	/
NDUFAF4	GALVIRGIRNKK	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 4 / Q9P032	/	K(biotin)
NDUFAF4	GALVIRGIRNKK	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 4 / Q9P032	acetyl-group	K(biotin)
SRC	GSNKS KPKDAKK	Proto-oncogene tyrosineprotein kinase Src / P12931	/	K(biotin)
SRC	GSNKS KPKDAKK	Proto-oncogene tyrosineprotein kinase Src / P12931	acetyl-group	K(biotin)
SRC	GSNKS KPKDAKK	Proto-oncogene tyrosineprotein kinase Src / P12931	myristoyl-group	K(biotin)
THOC7	GA VTDDEVIRKK	THO complex subunit 7 / Q619Y2	/	K(biotin)
THOC7	GA VTDDEVIRKK	THO complex subunit 7 / Q619Y2	acetyl-group	K(biotin)
THOC7 KSK	GA VKSKEVIRKK	THO complex subunit 7 / Q619Y2	acetyl-group	K(biotin)
TMEM97	GAPATRR CVEKK	Sigma intracellular receptor 2 / Q5BJF2	/	K(biotin)
TMEM97	GAPATRR CVEKK	Sigma intracellular receptor 2 / Q5BJF2	acetyl-group	K(biotin)
YES	GCIKSKENKSKK	Tyrosine-protein kinase Yes / P07947	/	K(biotin)
YES	GCIKSKENKSKK	Tyrosine-protein kinase Yes / P07947	acetyl-group	K(biotin)

569

570

Supplementary Table 2. Cell lines

Cell line	Plasmid	Gene	Tag	Reference
DLD-1 Flp-In™ T-REx™ Lyn KO	/	/	/	this study
DLD-1 KO Lyn Flp-In™ T-REx™ Lyn ^{G2A} -GFP	pCDNA5/FRT /TO-EGFP-	LYN- G2A	GFP (C-term)	this study

	IRES			
DLD-1 Flp-In TM T-REx TM	/	/	/	A gift from AG Musacchio, Dr. Stefano Maffini (MPI Dortmund)
DLD-1 Flp-In TM T-REx TM Lyn KO	/	/	/	this study
DLD-1 KO Lyn Flp-In TM T-REx TM Lyn ^{G2A} -GFP	pCDNA5/FRT /TO-EGFP-IRES	LYN- G2A	GFP (C-term)	this study
DLD-1 KO Lyn Flp-In TM T-REx TM Lyn ^{G2P} -GFP	pCDNA5/FRT /TO-EGFP-IRES	LYN- G2P	GFP (C-term)	this study
DLD-1 KO Lyn Flp-In TM T-REx TM Lyn ^{WT} -GFP	pCDNA5/FRT /TO-EGFP-IRES	LYN- WT	GFP (C-term)	this study
Hela Flp-In TM T-REx TM	/			A gift from S. Taylor (University of Manchester)
RPE-1 Flp-In TM T-REx TM	/			A gift from AG Musacchio, Dr. Stefano Maffini (MPI Dortmund)

Supplementary Table 3. siRNAs

siRNA	Company	Identifier
ON-TARGETplus siRNA Human DCAF10	Dharmacon	L-014344-01-0005
ON-TARGETplus siRNA Human NMT1	Dharmacon	L-004316-01-0005
ON-TARGETplus siRNA Human NMT2	Dharmacon	L-004317-01-0005
ON-TARGETplus siRNA Human ZER1	Dharmacon	L-019424-02-0005
ON-TARGETplus siRNA Human ZYG11B	Dharmacon	L-021798-02-0005

Supplementary Table 4. Oligonucleotides and primers (F: Forward, R: Reverse)

Sequence 5' to 3'	Company	Purpose
GTTCTGTTCCAGGGGCCCTGGGATCCAT GTTTCCCTTTGGGCCCCATAG	Metabion	F primer DCAF10 to clone in pGEX6P-2rbs for Gibson cloning
CAGTCAGTCACGATGCGGCCGCTCGAGC TACTAAACTTTGGCTGTACAAAGAAAC CCG	Metabion	R primer DCAF10 to clone in pGEX6P-2rbs for Gibson cloning
TTCTTCCAGTTGCCCCCTCT	Metabion	crRNA for Lyn KO (sequence 1)
GGAGGCCTCATACCCATTACA	Metabion	crRNA for LYN KO (sequence 2)

GCCACCATGGTGAGCAAG	Metabion	pcDNA5-F to linearize pcDNA5/FRT/TO-EGFP-IRES
GGTACCAAGCTTAAGTTTAAACGC	Metabion	pcDNA5-R to linearize pcDNA5/FRT/TO-EGFP-IRES
TTAAACTTAAGCTTGGTACCATGGGATG TATAAAATCAAAAGGG	Metabion	F primer to amplify LYN cDNA from pCR4-TOPO-LYN for Gibson cloning
CCCTTGCTCACCATGGTGGCAGGCTGCT GCTGGTATTG	Metabion	R primer to amplify LYN cDNA from pCR4-TOPO-LYN for Gibson cloning
CTTAAGTTTAAACGCTAGAGTCCG	Metabion	LYN-R Site-directed mutagenesis of LYN in the pcDNA5/FRT/TOLYN ^{WT} -EGFP- IRES construct
CTTGGTACCATGGCATGTATAAAATC	Metabion	LYN G2A-F Site-directed mutagenesis of LYN in the pcDNA5/FRT/TO-LYN ^{WT} -EGFP- IRES construct
CTTGGTACCATGCCATGTATAAAATC	Metabion	LYN G2P-F Site-directed mutagenesis of LYN in the pcDNA5/FRT/TOLYN ^{WT} -EGFP- IRES construct
GCTACCCGGGATGAACATCCGCAATGC C	Sigma	NAA10 F - Amplifying full-length wildtype human NAA10 for pFH vector
TGCAGCTAGCTTAGCTGGCGCTATCGC	Sigma	NAA10 R - Amplifying full-length wildtype human NAA10 for pFH vector
GATCGGATCCATGCCAGCCGTGTCCCTG	Sigma	NAA15 F - Amplifying full-length wildtype human NAA15-6xHIS for pFH vector
CGTAGTCGACCTAGTGATGGTGATGGTG ATGGATCTCGTTGGCCAGCTC	Sigma	NAA15-6xHIS R - Amplifying fulllength wildtype human NAA156xHIS for pFH vector
TTGTTTCAAGGTCCTGGATCCTCGTACA ACTACGTGGTAACGGC	Sigma	DDB1 F StrepII - Gibson cloning in pLIB
TCCTCTAGTACTTCTCGACAAGCTTTTA CTAATGGATCCGAGTTAGCTCCTCC	Sigma	DDB1 R - Gibson cloning in pLIB
CCACCATCGGGCGCGGATCCATGCATCA CCATCACCATCACTTTCCTTTGGGCCC CATAGC	Sigma	DCAF10 6xHIS F - Gibson cloning in pLIB
CTGTTCCAGGGGCCCCGGATCCTTTCCCT TTGGGCCCCATAGC	Sigma	DCAF10 F MBP - Gibson cloning in pLIB
TCCTCTAGTACTTCTCGACAAGCTTTTAT CAAACTTTGGCTGATACAAAGAAACC CG	Sigma	DCAF10 R - Gibson cloning in pLIB
CCACCATCGGGCGCGGATCCATGGCG GACGAGGCC	Sigma	CUL4A F - Gibson cloning in pLIB

TCCTCTAGTACTTCTCGACAAGCTTTTAT CAGGCCACGTAGTGGTACTG	Sigma	CUL4A R - Gibson cloning in pLIB
CCACCATCGGGCGCGGATCCATGGCGG CGGCGATG	Sigma	RBX1 F - Gibson cloning in pbig1a
TCCTCTAGTACTTCTCGACAAGCTTTTA CTAATGCCCATACTTCTGGAAGTCCC	Sigma	RBX1 R - Gibson cloning in pbig1a
AACGCTCTATGGTCTAAAGATTTAAATC GACCTACTCCGGAATATTAATAGATC	Sigma	Cas I F - Gibson cloning in pbig1a
AAACGTGCAATAGTATCCAGTTTATTTA AATGGTTATGATAGTTATTGCTCAGCG	Sigma	Cas I R - Gibson cloning in pbig1a
AAACTGGATACTATTGCACGTTTAAATC GACCTACTCCGGAATATTAATAGATC	Sigma	Cas II F - Gibson cloning in pbig1a
AAACATCAGGCATCATTAGGTTTATTTA AATGGTTATGATAGTTATTGCTCAGCG	Sigma	Cas II R - Gibson cloning in pbig1a
AAACCTAATGATGCCTGATGTTTAAATC GACCTACTCCGGAATATTAATAGATC	Sigma	Cas III F - Gibson cloning in pbig1a
AACCCCGATTGAGATATAGATTTATTTA AATGGTTATGATAGTTATTGCTCAGCG	Sigma	Ω R - Gibson cloning in pbig1a
GTACGGTTGCATGGGGAGTCGCCAACG CTGCTTTTCATTTCCACTGCATC	Sigma	RBX1 mut C75A/H77A - Gibson cloning in pbig1a
ATTGGAGACATTTTGATGGCTTG	Metabion	F - Diagnostic primers for LYN KO exon 2
TTTCTTCACACAAAAGAATGTGACC	Metabion	R - Diagnostic primers for LYN KO exon 2
TTCTTCCAGTTGCCCCCTCT	Metabion	F - Diagnostic primers for LYN KO exon 4
GGAGGCCTCATACCCATTACA	Metabion	R - Diagnostic primers for LYN KO exon 4
CACCCACGGCGCCGTCGGCAACCTCGAG TACTC	Eurofins	DCAF10 mut F172G
CACACTAGCTGGGTGGCGAACATCGAATA TGATAC	Eurofins	DCAF10 mut K257A
GTAGGCAGGGGTTACGGCAAAGAACTTT GCTTC	Eurofins	DCAF10 mut I475G

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Supplementary Table 5. Plasmids

Plasmid backbone	Gene	Reference
pbiG1a	/	A gift from AG Musacchio, (MPI Dortmund)
pbiG1a	DDB1, CUL4A, RBX1 human	this study
pbiG1a	DDB1, CUL4A, RBX1 ^{C75A/H77A} human	this study
pcDNA3.1+/c-(k)-DYK	DCAF10 human	Genscript ORF #OHu10624D
pcDNA5/FRT/TO--EGFP-IRES	LYN ^{G2A} human	this study
pCDNA5/FRT/TO-EGFP-IRES	/	A gift from AG Musacchio, Dr. Stefano Maffini (MPI Dortmund)
pcDNA5/FRT/TO-EGFP-IRES	LYN ^{WT} human	this study
pcDNA5/FRT/TO-EGFP-IRES	LYN ^{G2P} human	this study

pCR4-TOPO-LYN	LYN human	Horizon Discovery/Dharmacon (#MHS6278-202856900)
pFL	/	A gift from AG Musacchio (MPI Dortmund)
pFL	NAA15-6xHIS, NAA10 human	²
pGEX-6P-2rbs	DCAF10 human	this study
pGEX-6P-2rps	/	GenBank accession code KM817768
pLIB	/	A gift from AG Musacchio (MPI Dortmund)
pLIB	CUL4A human	this study
pLIB	RBX1 human	this study
pLIB-6xHis-MBP	DCAF10 human	this study
pLIB-6xHis-MBP	DCAF10 ^{F172G/K257A/I475G} human	this study
pLIB-HIS-MBP	/	A gift from AG Musacchio, Dr. John Weir (MPI Dortmund)
pLIB-StrepII	DDB1 human	this study
pLIB-StrepII-3C	/	A gift from AG Musacchio (MPI Dortmund)
pOG44	/	A gift from AG Musacchio, Dr. Stefano Maffini (MPI Dortmund)

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Supplementary Table 6. Antibodies

Antibody	Company	Identifier	Dilution
anti-CUL4A Rabbit pAb	Cell Signaling	#2699	1:1000
anti-DCAF10/ WDR32 Rabbit pAb	Thermo Fisher Scientific	#PA5-24133	1:1000
anti-DDB1 (D4C8) Rabbit mAb	Cell Signalling	#6998	1:1000
anti-FLAG (DYKDDDK) Mouse mAb	Cell Signaling	#8146S	1:5000
anti-Fyn Rabbit pAb	Cell Signaling	#4023S	1:1000
anti-GFP Rabbit pAb	In-house	AG Musacchio	1:1000
anti-GFP Mouse mAb	Roche	#11814460001	1:10000
anti-GST Mouse mAb	Merck Millipore	#71097	1:1000
anti-Lyn (C13F9) Rabbit mAb	Cell Signaling	#2796S	1:1000
anti-mouse IgG HRP linked antibody	Cell Signaling	#CST 7076S	1:10000
anti-NMT1 Rabbit pAb	abcam	#AB186123	1:1000
anti-NMT2 Rabbit pAb	abcam	#AB230028	1:1000
anti-rabbit IgG HRP-linked Antibody	Cell Signaling	#7074S	1:10000
anti-Src (36D10) Rabbit mAb	Cell Signaling	#2109S	1:1000
anti-Streptavidin Rabbit pAb	Rockland immunochemicals	#100-4195	1:10000

anti-THOC7 Rabbit pAb	abcam	#ab155218	1:500
anti-Ubiquitin-HRP (P4D1) Mouse mAb	Cell Signaling	#14049	1:1000
anti-Vinculin Mouse mAb	Sigma	#V9131	1:10000
anti-ZER1 Rabbit pAb	Proteintech	#16647-1-AP	1:1000
anti-ZYG11B Rabbit pAb	antibodies-online.com	#ABIN4916715	1:500
VeriBlot Detection Reagent (for IP samples)	abcam	#ab131366	1:500

Supplementary Table 7. Antibiotics used in bacterial and cell culture

Reagent	Working concentration	Company
Ampicillin	100 µg/mL	Sigma
Blasticidin	8 µg/mL	Sigma
Chloramphenicol	34 µg/mL	Sigma
Doxycycline	10–100 ng/mL	Sigma
Gentamicin	10 µg/mL	Sigma
Hygromycin B	400 µg/mL	Sigma
Kanamycin	50 µg/mL	Sigma
Penicillin-streptomycin	100 U/mL	Gibco/Thermo Fisher Scientific
Tetracyclin	7 µg/mL	Sigma

Supplementary Table 8. Reagents and Kits

Reagent	Company	Reference number
Acetyl-Coenzyme A	Sigma Aldrich	A2056
Biotin	iba	2-1016-002
BL21(DE3)	Thermo Fisher Scientific	EC0114
CoA	Sigma Aldrich	C4282
CPM (7-Diethylamino-3-(4'-Maleimidylphenyl)-4-Methylcoumarin)	Invitrogen	D346
Cycloheximide	Sigma Aldrich	C4859-1mL
Dulbecco's Modified Eagle Medium (DMEM), high glucose, GlutaMAX Supplement, pyruvate	Gibco/Thermo Fisher Scientific	31966047
Dynabeads™ MyOne™ Streptavidin	Invitrogen/ Thermo Fisher Scientific	65601
Dynabeads™ Protein A Immunoprecipitation Kit	Invitrogen/ThermoFisher Scientific	10006D
Fetal bovine serum (FBS)	Pan Biotech	P13-3001
GeneJET Plasmid Miniprep Kit	Thermo Fisher Scientific	K0502
GFP-Trap magnetic agarose	ChromoTek	gtma
Glutathione	Thermo Fisher Scientific	78259
GST magnetic agarose beads	Pierce/ Thermo Fisher Scientific	#78602

IPTG	Thermo Fisher Scientific	15529019
Lipofectamine RNAiMAX	Invitrogen/ Thermo Fisher Scientific	13778-150
Lipofectamine® 2000	Invitrogen/ Thermo Fisher Scientific	11668-027
LysC	Wako Chemicals	129-02541
MG132	Sigma-Aldrich	474787
Monarch DNA Gel Extraction Kit	New England BioLabs	T1120S
Monarch PCR & DNA Cleanup Kit	New England BioLabs	T1130S/L
Opti-MEM I reduced-Serum medium	Gibco/Thermo Fisher Scientific	31985062
PageRuler Plus Prestained Protein Ladder	Thermo Fisher Scientific	26619
Phosphostop	Roche	04906845001
Pierce TM Glutathione Magnetic Agarose	Thermo Fisher Scientific	78602
Precision Plus Protein Standard Dual color	BIO-RAD	1610374
Protease Inhibitors	Roche	04693132001
PureYield Plasmid Miniprep System	Promega	A1223
Recombinant Human UbcH5a/UBE2D1 Protein (E2)	Bio-Techne GmbH	E2-622
Recombinant Human Ubiquitin Activating Enzyme (UBE1)	Bio-Techne GmbH	E-304-050
Recombinant Human Ubiquitin Protein (Ub)	Bio-Techne GmbH	P0CG47.1
SF Cell Line 4D-Nucleofector X Kit S	Lonza	V4XC-2032
Strep-Tactin®XT 4Flow® beads	iba	2-5010-010
Trypsine	Sigma-Aldrich	T6567
Wizard SV Gel and PCR Clean-Up System	Promega	A9281
X-tremeGENE 360 Transfection reagent	Sigma-Aldrich	XTG360RO

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Supplementary Table 9. List of enzymes

Enzyme	Supplier	Reference Number
<i>Bam</i> HI HF	New England Biolabs GmbH	R3136S
Benzonase® Nuclease	Sigma-Aldrich	9025-65-4
<i>Dpn</i> I restriction enzyme	New England Biolabs GmbH	RO176S
Gibson Cloning Master Mix (containing T5 Exonuclease, Phusion polymerase, Tag ligase)	New England Biolabs GmbH/ <i>inhouse</i> AG Musacchio	E2611S
<i>Hind</i> III HF	New England Biolabs GmbH	R3104S
Phusion® High-Fidelity PCR 2x Master Mix	New England Biolabs GmbH	M0530S
<i>Pme</i> I	New England Biolabs GmbH	R0560S
Q5® High-Fidelity 2x Master Mix	New England Biolabs GmbH	M0494S
<i>Swa</i> I	New England Biolabs GmbH	R0604S
<i>T4</i> DNA Ligase	New England Biolabs GmbH	M0202S
<i>T4</i> Polynucleotide Kinase	New England Biolabs GmbH	M0201S

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Supplementary Table 10. Software and Algorithms

Software	Version	Supplier
BioRender	-	BioRender
DIA-NN	1.8.2. beta 22 and 27	https://github.com/vdemichev/DiaNN ¹¹
GraphPad Prism	Version 8.4.3	GraphPad Software, LLC.
ImageJ	2.0.0-rc-69/1.52p	National Institutes of Health
Image Lab Software	6.0.1	BIO-RAD
Maxquant	1.6.14, 2.0.3.1	https://www.maxquant.org/ ¹²
Perseus	1.6.50, 2.0.11	https://maxquant.net/perseus/ ¹¹
PyMOL: The PyMOL molecular Graphics System	Version 3.0	Schrödinger, LLC
Resolve3D softWoRx-Acquire	Version: 7.2.0	Release RC4
Rstudio	version 2023.06.0	Posit Software, PBC
SnapGene	7.1.2	GSL Biotech LLC
AF3	3.0.0	¹³

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