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**Biochemical and structural analysis of
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1 CHAPTER FIVE

2

3 **Biochemical and structural analysis of N-myristoyltransferase-mediated**
4 **protein tagging**

5

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80 Abstract

81 N-terminal myristoylation is an essential eukaryotic modification crucial for cellular
82 homeostasis in the context of many physiological processes. Myristoylation is a lipid
83 modification resulting in a C14 saturated fatty acid addition. This modification is challenging
84 to capture due to its hydrophilicity, low abundance of target substrates, and the recent discovery
85 of unexpected NMT reactivity including myristoylation of lysine side chains and N-acetylation
86 in addition to classical N-terminal Gly-myristoylation. This chapter details the high-end
87 approaches developed to characterize the different features of N-myristoylation and its targets
88 through *in vitro* and *in vivo* labeling.

89

90 1. Introduction

91 N-myristoyltransferases (glycylpeptide N-tetradecanoyltransferases, myristoyl
92 CoA:protein N-myristoyltransferases, NMT; EC 2.3.1.97) are a class of essential enzyme
93 catalysts of protein myristoylation. NMTs target N-terminal glycines (Gly-myristoylation)
94 usually unmasked by co-translational methionine excision (Giglione & Meinel, 2022;
95 Meinel, Dian, & Giglione, 2020). A general scheme of classical NMT reactivity is provided
96 in **Fig. 1A**. The initiator methionine is removed by methionine aminopeptidases (MetAPs,
97 EC:3.4.11.18). Most eukaryotes express two MetAPs, MetAP1 and MetAP2, both of which
98 display high cleavage efficiency of Met-Gly peptide bonds (Frottin et al., 2006; Giglione,
99 Boularot, & Meinel, 2004). Post-translational proteolytic cleavages induced by caspases
100 (EC:3.4.22.xx) in the context of apoptosis in metazoans may also expose new N-terminal
101 glycines previously embedded within the polypeptide sequence that may similarly undergo
102 Gly-myristoylation (Martin, Beauchamp, & Berthiaume, 2011; Thinon et al., 2014). As caspase
103 3 (EC:3.4.22.56) restricts new N-termini to only small residues (i.e., Ala, Gly, Asn, and Ser),

104 its cleavage specificity is well suited for this purpose and is the major post-translational
105 provider of post-translational Gly-myristoylation (Meinzel, 2022; Rivière et al., 2022). Two
106 percent of multicellular eukaryote proteins are predicted to undergo Gly-myristoylation, mostly
107 of co-translational origin (Castrec et al., 2018; Meinzel, 2022; Meinzel et al., 2020).

108 However, experimental proof of myristoylation only exists for a minority – most from
109 human – of protein targets. Evidence of myristoylation of a novel target protein is often
110 indirect, for instance by establishing protein localization with or without the N-terminal Gly,
111 which is usually substituted with an Ala (Traverso et al., 2013b). This so-called G2A
112 substitution is known to inhibit myristoylation (Gordon, Duronio, Rudnick, Adams, & Gokel,
113 1991). Robust biochemical approaches for investigating NMT targeting were initially reported
114 in the 1990s (see, for instance, (Rudnick, Duronio, & Gordon, 1992) and this series, volume
115 250, chapters 29-37, (Casey & Buss, 1995)). However, in the early 2010s, new technologies
116 emerged to study myristoylation including state-of-the-art mass spectrometry, click and
117 bioorthogonal chemistry, and new structural approaches. This chapter is dedicated to the main
118 approaches available for deciphering the modification state of NMT targets.

119

120 **2. Materials, reagents, and buffers**

121 **2.1 Equipment**

122 *2.1.1 Instruments*

- 123 • Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer
124 for mass spectrometry (MS) and MS/MS spectra acquisition (AB Sciex LLC; MALDI-
125 TOF/TOF 5800 instrument).

126 • Nano liquid chromatography-coupled electrospray mass spectrometer (Dionex UltiMate
127 3000; AB Sciex LLC, TripleTOF 4600) for exact mass determination of entire proteins larger
128 than 30 kDa.

129 • Multicolor fluorescence imager (Typhoon RGB Biomolecular Imager, GE Healthcare)
130 equipped with 532 nm-excited fluorescence and 580 nm bandpass (580 BP 30) filter for
131 wavelength emission range of 565–595 nm suited to Cy3 or TAMRA fluorochromes. The 633
132 nm-excited fluorescence with 670 nm bandpass (670 BP 30) for wavelength emission detection
133 range of 655–685 nm is suited to Cy5-type probe detection. Note that Typhoon Trio imagers
134 offer additional chemiluminescence detection.

135 • Chemiluminescence and staining imaging system equipped with Chemi/UV/Stain-Free tray
136 (ChemDoc Touch, Bio-Rad).

137 • Ultrasonic sonicator (Q700, Qsonica) equipped with a 12 mm Macrotip (amplitude 120 μm).

138 2.1.2 *Small items*

139 • Precast gels (12%) for electrophoretic separation in standard Tris-glycine buffer (Mini-
140 PROTEAN TGX, Bio-Rad, 4561043).

141 • Polyvinylidene fluoride (PVDF) immunoblot membranes (Immun-Blot Low Fluorescence
142 membranes, Bio-Rad, 1620260-4).

143 • Bradford assay kit (Bio-Rad, 500001) for protein quantification determined against a
144 standard bovine serum albumin (BSA) calibration curve (Bradford, 1976).

145 • Lipofectamine 3000 (Thermo Fisher Scientific, L3000015) for cell transfection.

146 • Polyethylene glycol 6000 (Sigma-Aldrich, 81260) for protein crystallization.

147 2.2 **Plasmids, strains, and growth medium for protein expression**

148 2.2.1 *Plasmids*

- 149 • pET28b tobacco etch virus (TEV) vector (LifeScience Market, PVT10578) featuring a 6-His
150 tag. We use a similar but in-lab-engineered version of pET28a TEV featuring a 10-His tag as
151 described below. Expression requires kanamycin (50 µg/mL) in the growth medium.
- 152 • pET16b (Novagen, 69662). Expression requires 50 µg/mL ampicillin in the growth medium.
- 153 • pETM30 is a pET28b derivative featuring an N-terminal-GST fusion (Dümmler, Lawrence,
154 & De Marco, 2005). Expression requires kanamycin (50 µg/mL) in the growth medium.
- 155 • pET21-ARF6-His6, a kind gift from M. Franco (IPMC, Nice, France) for ARF6-His
156 overexpression and purification in *E. coli*. ARF6-His6 is 21.0 kDa (ARF6 is 19.9 kDa).
- 157 • pJAF211 (Addgene, 52407) for ARF6-Flag overexpression in mammalian cells. The cDNA
158 sequence originates from mice and encodes the same peptide chain as in humans. The Flag-tag
159 is at the C-terminal. ARF6-Flag is 23.8 kDa.
- 160 • pETDuet-1 (Novagen) for dual overexpression of both NMT and target protein of interest
161 cDNAs.

162 2.2.2 Bacterial growth

- 163 • Rosetta 2-DE3pLys bacterial strain (Novagen). Genotype is $F^- ompT gal dcm lon hsdS_B(r_B^-$
164 $m_B^-) \lambda(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB^+]_{K-12}(\lambda^S)$). pLysSRARE produces T7
165 lysozyme and tRNA genes to supplement *E. coli* rare codons AGG, AGA, AUA, CUA, CCC,
166 and GGA. Add chloramphenicol (34 µg/mL) to ensure plasmid propagation in the growth
167 medium.
- 168 • 2xTY growth medium. Mix 16 g Bacto Tryptone, 10 g Bacto Yeast Extract, and 5 g NaCl in
169 900 mL distilled H₂O. Adjust the pH to 7.0 with NaOH and finally adjust to 1 L with distilled
170 H₂O. Sterilize by autoclaving.

171 2.3 Enzymes

172 2.3.1 Commercial

173 • Acyl-CoA synthetase (ACS; EC 6.2.1.3) from *Pseudomonas sp.* (Creative Enzymes, NATE-
174 1712; 15 U/mg; 1000 U). Dilute the sample to 1 U/ μ L in 5 mM Tris-HCl pH 7.4, 1 mM DTT,
175 55% glycerol for storage at -20°C. ACS catalyzes the reaction shown in **Fig. 1B**.

176 • Recombinant TEV (rTEV, EC 3.4. 22.44) protease (Genscript, Z03030, 10 U/ μ l); rTEV
177 contains the 231 amino acid sequence of TEV fused to an N-terminal His tag. It cleaves the
178 Glu-Asn-Leu-Tyr-Phe-Gln/[Gly/Ser] sequence (Kapust et al., 2001) and can be removed by
179 nickel cation affinity assays. To reduce purchase costs, TEV and rTEV can also be
180 overexpressed and easily purified from recombinant plasmid vectors (Enríquez-Flores et al.,
181 2022; Nautiyal & Kuroda, 2018).

182 2.3.2 NMTs

183 In this section, we describe the NMT preparations required for kinetic or structural
184 analysis. Protein concentrations are measured with the aforementioned Bio-Rad kit. Purity
185 needs to be >95% for kinetic analysis and >99.9% for crystallization purposes. We usually
186 clone into a pET16b featuring a Factor Xa protease cleavage or a pET28b derivative vector
187 featuring an N-terminal 10x His tag fused with a TEV protease cleavage site. Expression is
188 performed at 22°C in Rosetta2-DE3pLysS bacterial cells. The protocol reported below relates
189 to HsNMT1[*I*] (Dian et al., 2020) but may be used for any NMT (see below).

190 • Like Factor Xa, the TEV protease-cleavable linker of the inter-domain
191 (CATCACCATCACCATCACCATCACCATCACGAGAATCTTTATTTTCAGGGC;
192 encoding here Glu-Asn-Leu-Tyr-Phe-Gln/Gly fused in-frame downstream of the His-tag and
193 upstream of the start of the sequence of interest) permits *in vitro* cleavage of the His-tag from
194 the expressed NMT if the pET28-derived vector is used for protein purification. Upon
195 cleavage, the His-tag is removed from the NMT sample of interest by taking advantage of a
196 second affinity column on the same resin. The NMT is easily recovered, as it is not retained

197 and elutes with the flow-through. Unlike TEV or Factor Xa, the rTEV protease is equipped
198 with a His-tag and can also be retained on the second affinity column, allowing its removal and
199 fine purification of an NMT version devoid of protease contaminants, especially useful for
200 crystallization purposes.

201 • NMTs are easy to overexpress in *Escherichia coli* and purify in two steps, taking advantage
202 of the N-terminal tags. C-terminal tags need to be avoided, as the C-terminal carboxylate of
203 NMTs acts as the catalytic base and cannot be engaged in another peptide bond (Dian et al.,
204 2020). N-terminal deletions of NMT sequences up to the B'A' domain can be achieved without
205 modification of the catalytic parameters. Trimming of this domain modifies the catalytic
206 mechanism and tends to increase the turnover number (Dian et al., 2020).

207 • After growth at 37°C in 2 L 2x TY medium supplemented with the *ad hoc* antibiotics until
208 0.4-0.5 OD₆₀₀, the temperature is reduced to 22°C for 30 min to reach 0.7-0.9 OD₆₀₀. Bacteria
209 are induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and grown for
210 another 20 h at 22°C under vigorous shaking. Cells are harvested by centrifugation and lysed
211 by sonication (amplitude 50%, 10 s on and 30 s off) for 90 s at 4°C after resuspension in 10
212 mL/per g of cell paste in 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 5 mM 2-mercaptoethanol,
213 and 5 mM imidazole (buffer C) supplemented with 1 mg/mL lysozyme and 5 μL benzonase.
214 After centrifugation at 40,000 x g, the clarified lysate is loaded at 3 mL/min onto an
215 immobilized nickel ion affinity chromatography column (HisTrap Crude FF, 5 mL, GE
216 Healthcare) and washed with buffer C with 25 mM imidazole. Elution is carried out at 2
217 mL/min with buffer C plus 0.25 M imidazole with a linear gradient.

218 The pool of purified protein is next dialyzed overnight against 20 mM Tris-HCl (pH 8.9),
219 150 mM NaCl, 25 mM imidazole, and 5 mM 2-mercaptoethanol in the presence of TEV
220 protease (1 mg per 15 mg purified protein). His-tag-free HsNMT1 is purified with a HisTrap
221 Crude FF column. The flow-through with HsNMT1 is diluted five times in 20 mM Tris-HCl

222 (pH 8.9) and 1 mM DTT (buffer B) and applied to an anion exchange chromatography column
223 (HiTrap Q FF, 5 mL, GE Healthcare). HsNMT1 is eluted with buffer B supplemented with 0.2
224 M NaCl. Finer purification of some NMTs might require a final gel filtration step, but we have
225 not needed this for our crystallization of HsNMT1.

226 • Highly purified NMT fractions are pooled, concentrated to 15–30 mg/ mL with an Amicon
227 centrifugal filtration device (Merck Millipore) in 20 mM Tris–HCl (pH 8.0), 0.2 M NaCl, 1
228 mM DTT, and stored at -80°C before crystallization trials. Protein samples for kinetic analysis
229 are stored at -20°C in the same buffer plus 55% glycerol.

230 • Human NMT (HsNMT1). The HsNMT1(*l*) isoform containing residues 81-496 corresponds
231 to the major isoform (Pierre et al., 2007). We cloned HsNMT1(*l*) into pET16b as an N-terminal
232 His-tag fusion (Pierre et al., 2007), and the recombinant protein was expressed and purified as
233 described (Castrec et al., 2018). Isoform HsNMT1(*s*) containing residues 99-496 was cloned
234 into pET28b and purified as described (Dian et al., 2020).

235 • Full-length HsNMT2, AtNMT, PfNMT, and ScNMT can be expressed and purified
236 according to similar protocols as previously described (Castrec et al., 2018; Traverso, Giglione,
237 & Meinnel, 2013a).

238 2.3.3 *IpaJ* protease

239 *IpaJ* (EC:3.4.22) is a cysteine protease displaying tight cleavage specificity for myristoyl-
240 glycine-starting peptides that hydrolyzes the peptide bond immediately following the glycine
241 residue (Burnaevskiy et al., 2013; Burnaevskiy, Peng, Reddick, Hang, & Alto, 2015; Rivière
242 et al., 2022). A scheme of the reaction catalyzed by *IpaJ* is provided in **Fig. 1C**.

243 A truncated (i.e., devoid of the N-terminal secretion peptide) and more soluble (3M)
244 variant of the open reading frame of *Shigella flexneri* protease *IpaJ* is cloned into pETM30 as
245 a fusion with a N-terminal His-tag and glutathione S-transferase (GST) as described (Rivière

246 et al., 2022). IpaJ is produced from Rosetta2-DE3pLysS cells. Bacteria are grown in 1 L of 2x
247 TY medium supplemented with kanamycin (50 µg/mL) and chloramphenicol (34 µg/mL) at
248 37°C under vigorous shaking. Protein overexpression is induced with 0.5 mM IPTG at
249 $OD_{600}=0.8$. Cells are transferred at 20°C and grown for 20 h. Cells are centrifuged at 5,000 x
250 g and the pellet resuspended (10 mL/g) in lysis buffer (20 mM Tris pH 8.0, 0.2 M NaCl, 5 mM
251 2-mercaptoethanol, 5 mM imidazole, 5% glycerol). Cells are lysed with a sonicator (amplitude
252 50%, 10 s on and 30 s off) for 3 min at 4°C. The sample is then centrifuged at 40,000 x g (Rotor
253 JA25-50, Beckman Coulter) for 20 min and the pellet discarded. The supernatant is loaded on
254 a Ni-IMAC-HisTrapTM FF 5 mL column (GE Healthcare) at 3 mL/min; elution is achieved
255 with a linear 0-0.5 M imidazole gradient run over 100 ml. Purified proteins are dialyzed using
256 Spectra-Por7 semi-permeable dialysis membranes (8 kDa cut-off; Thermo Fisher Scientific)
257 for 48 h against conservation buffer (20 mM Tris pH 8.0, 0.2 M NaCl, 5 mM DTT, 55%
258 glycerol) at 4°C. The sample is stored at -20°C.

259 **2.4 Reagents**

260 *2.4.1 Fatty acyl derivatives*

261 ● Myristoyl-CoA lithium salt (Sigma-Aldrich, M4414) MM = 977.9 g/mol; 5 mg diluted in
262 25.565 mL. Stock solutions (0.2 mM) are prepared in 10 mM sodium acetate, pH 5.6, and 1%
263 Triton X-100, except for MALDI analysis, where cholate is used instead of Triton to reduce
264 background (see below). The low pH prevents slow CoA hydrolysis.

265 ● 80% pure myristoyl-CoA lithium salt (Sigma-Aldrich, M4414) MM = 977.9 g/mol; 5 mg
266 diluted in 0.511 mL in 50% DMSO (final concentration 10 mM), e.g., for use in crystallization
267 trials.

268 ● Acetyl-CoA, >93% pure (Sigma-Aldrich, A2056).

269 ● Myristic acid (Sigma-Aldrich, M3128).

- 270 ● Alkynyl myristic acid (Myr-Alk; Click Chemistry Tools, 1164-100).
271 ● Azido myristic acid (Myr-Az; Click Chemistry Tools, 1345-100).
272 ● Radioactive myristoyl-CoA (^3H or ^{14}C), which must be ordered as a custom synthesis, e.g.,
273 from Selcia, Moravek Biochemicals Inc., Quotient Sciences, or ViTrax or prepared as
274 described in §3.1 from radiolabeled myristic acid (Moravek Inc, MC118 or MT918).

275 2.4.2 Peptides

276 Custom peptides octapeptides of any sequence are all from GenScript Biotech (Rijswijk,
277 Netherlands).

278 2.4.3 Reactants for gel imaging

- 279 ● Pre-stained protein ladder 10-250 kDa (PageRuler Plus, Thermo Fisher, 26620).
280 ● TAMRA-PEG3-azide (BroadPharm, BP-22479) for in-gel imaging alkyne-tagged proteins
281 by click chemistry. The TAMRA excitation peak corresponds to the Cy3 laser line and is fully
282 compatible with parallel Cy5 detection.
283 ● TAMRA DBCO (Click Chemistry Tools, A131-5) is a reactive probe used for imaging
284 alkyne-tagged biomolecules via a copper-free click reaction.
285 ● Biotin-PEG3-Azide (Thermo Fisher Scientific, J64996.MB; Sigma-Aldrich, 762024).
286 ● TAMRA-biotin-PEG3-azide (Click Chemistry Tools, 1048).
287 ● AlexaFluor Streptavidin 635 (Thermo Fisher Scientific, S32364) for fluorescence detection
288 of proteins clicked with a biotin-PEG3-azide probe after blotting on PVDF membranes.
289 ● ARF6-specific rabbit polyclonal antibody (Bethyl Laboratories, Inc; A305-238A).
290 ● Beads coupled to Flag-tag antibody from the $\mu\text{MACS}^{\text{TM}}$ DYKDDDDK Isolation Kit (Miltenyi
291 Biotec).
292 ● NMT inhibitor IMP-1088 (Cayman Chemical Company, 25366) or DDD85646 (Cayman
293 Chemical Company, 13839).

294 **2.5 Buffer components**

295 *2.5.1 For storage at room temperature*

296 • MgCl₂ stock 100 mM q.s. 50 mL; MM = 292.24 g/mol; 1.0165 g in 50 mL.

297 • Triton X-100 10% q.s. 15 mL; dilute use 1.5 mL Triton X-100 solution (Sigma-Aldrich,
298 93443).

299 • Ethylenediaminetetraacetic acid 0.5 M (EDTA) q.s. 100 mL; MM = 203.3 g/mol (Sigma-
300 Aldrich, 03609). Add 101.6 g in H₂O and dissolve, taking advantage of NaOH addition (high
301 concentration or chips) to reach pH 8 and allow complete solubilization in 100 mL final
302 volume.

303 • 4x Laemmli buffer is 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol
304 blue, and 0.125 M Tris HCl, pH 6.8.

305 • Phosphate Buffer Saline (PBS) buffer is 137 mM NaCl, 27 mM KCl, 10 mM Na₂PO₄, 18
306 mM, K₂PO₄ and is adjusted to pH 7.4.

307 *2.5.2 For storage at -20°C*

308 • Bovine serum albumin (BSA, Sigma-Aldrich) 10 mg/mL q.s. 10 mL in H₂O; aliquots of 200
309 µL.

310 • Dithiothreitol (DTT) 6.4 mM q.s. 20 mL; MM = 154.25 g/mol, 19.74 mg in 20 mL H₂O;
311 prepare aliquots of 500 µL. Storage at -80°C is even better.

312 • Sodium acetate 10 mM/Triton X-100 1% solution: add sodium acetate 10 mM q.s. 100 mL
313 (82.03 mg acetate-Na (82.03 g/mol) in 100 mL H₂O, 1 mL Triton X-100; adjust to pH 5.6 with
314 a few drops of acetic acid and NaOH. Aliquots of 2 mL can be prepared.

315

316 **3. Preparation of noncommercial N-myristoyl protein or CoA derivatives**

317 **3.1 Myristoyl-CoA and any unusual CoA acyl derivatives**

318 This method was first established in the early 1990s (Devadas et al., 1992; Kishore et al.,
319 1991). It involves acyl-CoA synthetase (ACS, **Fig. 1B0.03** U/ μ L final concentration in a 100
320 μ L final volume consisting of 160 μ M fatty acid or a myristate derivative (radiolabeled or
321 clickable), 1 mM CoA (from stock 5 mM), 5 mM ATP (from stock 20 mM), 3 mM DTT, 0.05
322 % Triton X-100, 5 mM Tris pH 7.4, 2.5 mM MgCl₂, and 50 μ M EGTA.

323 The enzyme catalyst (ACS, 0.03 U/ μ L final) is added to start the reaction for 1 h at 37°C.
324 After 25 min, more than 50% of the myristate is already converted into myristoyl-CoA. This
325 is sufficient for further labeling of protein targets. The reaction product can be used as is and
326 without further purification in protein labeling experiments (see below).

327 • Radioactive labeling may also be useful for imaging and band quantification of cell extracts
328 following pulse labeling with myristate. Radioactive imaging needs very long exposure times
329 (Berthiaume, Peseckis, & Resh, 1995). Click chemistry-based imaging using reactive
330 precursors may provide similar but faster results, although absolute quantification is less
331 convenient and reliable (see §4.4).

332 • Chemical synthesis of the azido derivative of Myristoyl-CoA (ω -Azido undecanoic acid CoA
333 thioester) has been described (Heal, Wickramasinghe, Leatherbarrow, & Tate, 2008). The
334 protocol can be applied to a variety of myristate analogs which organic synthesis methods are
335 also available (Devadas et al., 1992).

336

337 **3.2 Myristoylated protein substrates**

338 *3.2.1 Fatty acid tagging of target proteins with NMT in vitro*

339 The protocol originates from that previously set up for the myristoylation of two small
340 GTPases, *A. thaliana* ARA6 (Boisson & Meinel, 2003) and human ARF6 (Padovani,

341 Zeghouf, Traverso, Giglione, & Cherfils, 2013). The purification of ARF6 from pET21-Arf6-
342 His6 to homogeneity is detailed in (Padovani et al., 2013).

343 • The final reaction volume is 1 mL with 25 μ M of protein of interest, 1 μ M HsNMT1, and 80
344 μ M myristoyl-CoA. The CoA donor can correspond to any fatty acyl derivative, such as those
345 reported in §3.1 and originating from the labeling reaction described in this section. The
346 reaction is run for 30 min at 30°C in 50 mM Tris pH 8.0, 1 mM MgCl₂, 0.2 mM EGTA, and
347 0.3 mM DTT. In the case of small G-proteins, addition of GTP (0.2 mM) has been shown to
348 stimulate acylation rates comparable to GDP. ScNMT is less efficient than HsNMT1 or
349 AtNMT.

350 • The final product can be characterized by denaturing PAGE or mass spectrometry (see below,
351 **Fig. 2** and **Fig. 3**)

352 • Use of click-reactive myristoyl-CoA can be a way to tag the protein and characterize it on
353 gels using, for instance, DBCO- or azido-TAMRA (see below).

354 3.2.2 *Myristoyl tagging in cell free translation systems*

355 Insect cell-free protein synthesis from extracts of the *Spodoptera frugiperda* Sf21 cell
356 line is an alternative to produce and tag the target protein considered *in vitro* (Suzuki et al.,
357 2010). Coupled transcription and translation of any cDNA of interest cloned in a plasmid under
358 the control of the T₇ promoter can be achieved with the TnT T7 Insect Cell Extract Protein
359 Expression System (Promega, L1101). Radiolabeled or clickable myristate can be added to the
360 reaction to follow protein myristoylation of the target by SfNMT (see §4.4).

361 Rabbit reticulocyte lysates (Promega, L4970) or wheat germ extracts (Promega, L4380)
362 may be used to take advantage of mammalian or plant NMT tagging (Deichaite, Casson, Ling,
363 & Resh, 1988; Yamauchi et al., 2010). *In vitro* transcribed mRNAs need to be produced first
364 and provided as translation templates (Utsumi et al., 2004).

365 3.2.3 *Bacteria-driven target labeling*

366 Like eukaryotes, bacteria express a methionine aminopeptidase (Giglione et al., 2004;
367 Giglione, Fieulaine, & Meinnel, 2009). Nevertheless, bacteria do not possess a myristoylation
368 system equivalent to NMT, preventing genuine Gly-myristoylation (Deichaite et al., 1988;
369 Giglione & Meinnel, 2022; Knoll, Johnson, Bryant, & Gordon, 1995; Rudnick, Mcwherter,
370 Gokel, & Gordon, 1993). To allow target acylation in bacteria, one needs to (i) rely on
371 heterologous NMT expression and (ii) take advantage of the fact that prokaryotes possess both
372 a fatty acid import system (*fadL*) and ACS (*fadD* in *E. coli*, **Fig. 1B**). This allows bacteria to
373 import fatty acids from the growth medium and to transform them into acyl-CoA derivatives
374 (Giglione & Meinnel, 2022). Provided non-minimal cell extracts are added to the growth
375 medium - usually yeast extracts - myristoyl-CoA can therefore be made available in bacterial
376 cells for protein N-myristoylation by NMT. Nevertheless, without further supplementation,
377 bioavailable concentrations of free myristoyl-CoA are extremely low in bacteria, because C14
378 derivatives are very poorly represented in yeast and because acyl compounds are weakly
379 soluble in the absence of albumin, the natural scavenger of fatty acids in animal fluids (Giglione
380 & Meinnel, 2022). To promote significant myristoyl-CoA concentrations, myristate - diluted
381 from a 100-fold stock solution - can be supplied to bacteria prior to induction with IPTG at a
382 final concentration of 50 μ M bound to 6 μ M BSA (Franco, Chardin, Chabre, & Paris, 1995).
383 BSA has an average of seven myristate-binding sites (Giglione & Meinnel, 2022).

384 Myristoylation of any favorite target in bacteria is therefore feasible provided (i) the
385 recombinant target gene is available and can be retrieved in the soluble fraction in, for example,
386 *E. coli* cells; (ii) the NMT gene can be co-expressed with the target, for instance through either
387 a compatible or a dual expression plasmid such as pETDuet-1 (Glück, Hoffmann, Koenig, &
388 Willbold, 2010); and (iii) myristate or any derivative - including radioactive or clickable

389 compounds - is provided in the bacterial growth medium. This system was described early on
390 using ScNMT and a recombinant murine protein kinase (Duronio et al., 1990). ScNMT is
391 usually provided – for instance through the pBB131 plasmid – but appears less efficient than
392 mammalian or plant NMTs. Complete Gly-myristoylation of HIV-1 Nef protein can be
393 obtained with HsNMT1 (Glück et al., 2010).

394 Finally, having in hand a target gene easily purifiable by, for example, taking advantage
395 of a C-terminal tag - such as ARF6-His in section 2.2.1 - is of benefit to further studies but it
396 is not mandatory when studying issues related to acylation occurrence. The availability of
397 specific antibodies and/or metabolic labeling with radioactive or clickable precursors is
398 sufficient to conclude using approaches such as those described in the next section. Such
399 investigations with ARF1 and ARF6 are described in volume 404 of this series (Ha, Thomas,
400 Stauffer, & Randazzo, 2005).

401 3.2.4 *Quality controls*

402 • Denaturing gel electrophoresis is one first gross approach. It was noted early on that protein
403 acylation often promotes faster migration of the acylated product assessed by denaturing gel
404 electrophoresis (SDS-PAGE), for example for the yeast ADP-ribosylation factors ARF1/2
405 (Lodge et al., 1997) or human ARF6 (**Fig. 2A**). Therefore, comparison of the relative intensities
406 produced data on the acylation reaction yield. However, such faster migration is not universal
407 but only occurs more frequently than average, and it is not specific since it can sometimes be
408 reversed or absent for other proteins. Longer migration times on high-resolution gels may
409 improve the migration shift between the two species (**Fig. 2B**, lane 1). If a radioactive
410 derivative is used as the CoA donor, an autoradiogram is used to directly visualize the labeling.
411 If using a clickable fatty acyl donor (reaction conditions and details are provided in §4.4),
412 reactivity with any convenient fluorophore such as TAMRA coupled to an alkyl or azide

413 reactive group for Cu(I)-catalyzed azide-alkyne click chemistry reactions (CuAAC) or strain-
414 promoted azide-alkyne click chemistry reactions (SPAAC) is most useful, and fluorescence
415 overlapping with the blue staining may be a convenient tool for checking acylation (see **Fig.**
416 **2C**). The sample is separated by SDS-PAGE and the gel directly imaged on a fluorescence
417 imager. However, this approach is qualitative.

418 • MALDI-TOF/TOF characterization of the purified product is a finer assessment of protein
419 myristoylation (see **Fig. 3A-B**) but it may be unsuitable for very large proteins (see §4.2).

420 **4. Characterization of NMT-induced modifications on protein targets**

421 **4.1 General considerations**

422 The rules of NMT-mediated Gly-myristoylation specificity have been investigated for
423 decades, and it occurs in about 2% of the human and *A. thaliana* (a dicot model plant)
424 proteomes (Castrec et al., 2018; Meinnel, 2022; Meinnel et al., 2020). Specificity was first
425 studied in detail for the yeast enzyme ScNMT (Bhatnagar, Ashrafi, Futterer, Waksman, &
426 Gordon, 2001; Towler, Gordon, Adams, & Glaser, 1988), which has similar specificity to other
427 NMTs but appears to disfavor some proteins with negatively charged residues located at
428 positions 8-9. Whether NMTs follow different rules in other organisms, including unicellular
429 organisms, is unknown. Nevertheless, studies on organisms such *Toxoplasma gondii*,
430 *Plasmodium falciparum*, or *Leishmania donovani* indicate similar overall rules (Brannigan et
431 al., 2010; Broncel et al., 2020; Traverso et al., 2013a). Lys-myristoylation catalyzed by NMT
432 appears to act only as a post-translational modification of proteins starting with Gly-Lys
433 residues, including ARF6 (Rivière et al., 2022).

434 Studies aiming to define the full set of proteins targeted by NMT led to the application
435 of proteome-wide approaches with click chemistry tools together with state-of-the-art
436 electrospray ionization (ESI) mass spectrometry instruments. Detailed protocols and methods

437 have recently become available (Kallemeijn et al., 2021). Bioinformatics analyses based on
438 machine learning approaches on partial datasets, such as using support vector machines, are
439 also helping to complete the picture (Castrec et al., 2018; Madeo, Savojardo, Martelli, &
440 Casadio, 2022).

441 This section describes several methods focusing on *in vitro* approaches with selected
442 targets of interest, including full-length proteins or N-terminal-derived short peptides. 80% of
443 target proteins in the complete human proteome (Meinzel, 2022), plants, and probably any
444 organism are soluble cytosolic proteins of average length less than 40 kDa. This makes
445 overexpression and further purification from bacterial cells to homogeneity in sufficient
446 quantities for further studies including MS easy. With short peptides, the MS approach is often
447 most useful for deciphering the actual modification state, including the type of modification.
448 This can be difficult to establish, especially for proteins starting with Gly-Lys residues – both
449 amino groups are potentially acylated - such as for ARF6. In some cases, and with longer
450 peptides or full-length putative target proteins, additional approaches are needed. We also
451 introduce in this context the IpaJ pipeline (**Fig. 3**; (Rivière et al., 2022)) and click chemistry-
452 based approaches.

453 **4.2 MALDI mass spectrometry**

454 *4.2.1 Main outlines*

455 • 300 μ L of a mixture containing 50 mM Tris (pH 8.0), 0.193 mM EGTA, 1 mM $MgCl_2$, 1
456 mM DTT, 5 μ M sodium cholate, 40 μ M Myr-CoA solution (stock solution 0.2 mM Myr-CoA,
457 10 mM sodium acetate, 2.5 μ M sodium cholate), 0.5 μ M NMT, and 100 μ M of synthetic
458 peptide or full-length protein are pre-incubated at 30°C. The myristoylation reaction is
459 followed over time by collecting 10 μ L samples further diluted in 90 μ L of water/acetonitrile
460 (90/10) solution.

- 461 • The different samples are then diluted five times in the matrix solution of 5 mg/mL (i) α -
462 cyano-4-hydroxycinnamic acid solubilized in water/acetonitrile/formic acid (50/50/0.1%) for
463 peptides or (ii) 3,5-dimethoxy-4-hydroxycinnamic acid in water/acetonitrile/formic acid
464 (50/50/0.1%) for whole proteins.
- 465 • 1 μ L of each dilution is spotted on a metal target and dried.
- 466 • MS and MS/MS spectra from each sample are acquired with a MALDI-TOF/TOF instrument
467 in positive ion mode.
- 468 • Survey scans are performed using delayed extraction (390 ns) in reflector mode for a total of
469 15,000 shots. MS/MS scans are operated with a collision energy of 1 kV. Peptide and fragment
470 mass tolerances are set at 10 ppm and 0.8 Da, respectively.
- 471 • Mass spectra are analyzed with PeakView 1.2 software (AB Sciex LLC). The default
472 threshold in MS/MS peak labeling and finding is 5% and centroid height percentage is 50%,
473 as recommended by the manufacturer. MS/MS deviations from theoretical values are on
474 average less than 0.03 Da.

475 4.2.2 Notes

- 476 • For MALDI mass spectrometry, Triton X-100 needs to be avoided as a detergent. The
477 steroid-based anionic detergent sodium cholate substitutes for the Triton X-100 used in enzyme
478 kinetics studies.
- 479 • MS/MS analysis allowing accurate analysis of the modification state can be achieved on
480 short peptides. Assignments of peptides with non-natural amino acids can also be performed
481 using ProteinProspector v6.2.2 (<https://prospector.ucsf.edu/prospector/mshome.htm>)
482 (Chalkley, Baker, Medzihradzky, Lynn, & Burlingame, 2008). Theoretical mass values of all
483 fragments are double-checked at https://web.expasy.org/peptide_mass/.

- 484 ● Demonstration of Gly-myristoylation analysis of tryptic fragments from full-length proteins
485 is feasible by MALDI MS/MS (Suzuki et al., 2006; Suzuki et al., 2010).
- 486 ● MALDI MS on proteins larger than 30 kDa is challenging. In this case, electrospray
487 ionization (ESI) MS is better suited.
- 488 ● MALDI promotes one singly charged ion, usually H^+ ($[M+H]^+$), but also sometimes doubly
489 charged ions, triply charged ions, and Na^+ adducts (+23 Da). Due to multiple charge values for
490 each molecular mass induced by ESI, mass spectra must be deconvoluted (Xu et al., 2018).
- 491 ● MALDI MS also works fine with short peptides (see an example in Figure 2, Chapter 26,
492 this volume (Rivière, Monassa, Giglione, & Meinel, 2023) and (Rivière et al., 2022)).
- 493 ● Controls in the absence of myristoyl-CoA or catalysts such as NMT need to be run and
494 analyzed by MS in parallel.
- 495 ● The monoisotopic mass is used as a mass reference with highly pure, chemically-synthesized
496 peptides. With purified proteins, the average mass is taken into account. The mass at the top of
497 the mass peak is always considered.
- 498 ● With high molecular weight proteins such as ARF6-His, determination of the average mass
499 by MALDI is more accurate by taking the $m/z=2$ state charge into account, even though less
500 intense and not always available (**Fig. 3**).
- 501 ● Standard deviation values are obtained after several independent measurements of the mass
502 of the same sample.

503 **4.3 The NMT/IpaJ pipeline**

504 This semi-quantitative assay was established to decipher the type of NMT-catalyzed
505 modification, e.g., Gly- or Lys-myristoylation (Rivière et al., 2022). The complete pipeline -
506 based on the high selectivity of IpaJ for myristoyl-glycine-starting proteins - is described in

507 detail in **Fig. 3** for a full-length protein (ARF6) but can be equally applied to short peptides, as
508 reported (Rivière et al., 2022).

509 • The peptide (100 μ M) is first incubated for 1 h in the presence of 0.5-1 μ M HsNMT1 or
510 HsNMT2 (T1). The buffer is the same as for NMT activity measurements but contains 5 μ M
511 cholate instead of Triton X-100.

512 • Full IpaJ cleavage conditions involve further incubation at 20°C for 1 h in the presence of
513 10 μ M IpaJ-3M and 0.1 mM DTT (T2).

514 • 1-5 μ M HsNMT1 is finally added for another 1 h at 30°C to ensure full acylation of all
515 possible substrates (T3). T0 corresponds to the T3 timepoint (3 h) with the peptide diluted in
516 the incubation buffer but in the absence of any enzyme.

517 **4.4 Click chemistry-based protein imaging**

518 *4.4.1 Overview*

519 Click chemistry approaches can be achieved if the target protein has previously been
520 tagged with a reactive acyl myristate *in vitro*. This is detailed in §3.2.

521 An alternative to such *in vitro* analysis is often required to obtain *in cellulo*-relevant
522 information on the actual myristoylation state of the target protein. This requires prior cell
523 labeling with a myristate precursor and applies to proteins expressed in cell-free (see §3.2.2),
524 bacterial (see §3.2.3) or mammalian cells systems (see §4.4.2). This precursor is metabolized
525 into a CoA derivative, which NMT can then use to acylate proteins (see details in (Giglione &
526 Meinel, 2022)). To identify the tagged protein originating from *in cellulo* studies, it can be
527 either immunoprecipitated with another C-terminal tag if a transgene is used or with specific -
528 preferentially polyclonal - antibodies. In any case, PAGE analysis followed by specific imaging
529 is often used to detect the target protein. This requires a click or biorthogonal compatible
530 fluorophore such as alkyne- or DBCO-TAMRA derivatives, depending on the myristate label

531 chosen as the precursor for protein labeling. One major challenge is to ensure that the
532 fluorescent signal arises from the selected target if not purified beforehand by
533 immunoprecipitation. Assessing the correlation between migration and labeling is one
534 approach to ensure this but is only indicative of the actual modification state.

535 If myristate reactivity is first challenged with a biotin derivative (see §2.4.3),
536 identification of the merged fluorescence originating from a mixture of (i) a fluorescent-
537 streptavidin probe (e.g., labeled with a Cy3 fluorophore) and (ii) the specific antibody revealed
538 with a secondary antibody conjugated to a compatible fluorophore (see §2.4, e.g., Cy5) on the
539 same immunoblot may be more accurate (see Figure 2 in (Neopane et al., 2022)). However,
540 the associated data, even with unambiguous merging of fluorescent signals, does not
541 definitively prove the myristoylation state. Therefore, to assess this *in vivo* myristoylation,
542 specific NMT inhibitors such as IMP-1088 or DDD85646 can be used (2 μ M) to demonstrate
543 block of the modification (Kosciuk et al., 2020; Mousnier et al., 2018). An additional very
544 strong proof may be possible by comparing overexpressing and control samples, i.e., increased
545 intensity of the signal observed upon overexpression of the target protein. **Fig. 2D** illustrates
546 this approach with ARF6 in human cells.

547 4.4.2 *In cellulo labeling procedures with acyl precursors*

548 • HEK293 cells are maintained at 37°C in 5% CO₂ in Dulbecco's modified Eagle medium
549 (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin
550 (Gibco), 100 U/mL streptomycin sulfate, 2 mM L-glutamine, and a cocktail of non-essential
551 amino acids.

552 • Cells are transfected with the pJAF211 plasmid in Lipofectamine 3000 (Thermo Fisher
553 Scientific) according to the manufacturer's instructions to express ARF6-Flag. After 3 hours
554 of transfection, the medium is renewed with fresh culture medium, to which 20-40 μ M alkyne

555 myristate (Alk-myristate) or unmodified myristate is added. After 18 to 24 h of culture, the
556 cells are harvested and lysed with a lysis buffer (PBS pH 7.4, 0.1% SDS, and 1% Triton X-
557 100) and a protease inhibitor cocktail (cOmplete ULTRA Tablets, Mini, EDTA-free,
558 EASYpack). Protein concentration is determined with the standard Bradford assay (Bio-Rad).

559 4.4.3 *In-gel imaging*

560 • Cell lysates are diluted to 2 $\mu\text{g}/\mu\text{L}$ total protein in lysis buffer. A mix of click reagent is
561 prepared and added to the lysates to obtain the following final concentrations: 40 μg total
562 protein, 100 μM azide probe (TAMRA and/or biotin); 1 mM CuSO_4 ; 1 mM TCEP (tris(2-
563 carboxyethyl)phosphine); and 100 μM THPTA (tris(3-hydroxypropyltriazolylmethyl)amine).
564 After 1 h of incubation at room temperature (21°C) with shaking at 12 Hz, the reaction is
565 stopped by adding EDTA to 5 mM final concentration.

566 • For direct fluorescence analysis following SDS-PAGE when the reactive probe is coupled to
567 a TAMRA fluorophore, the products of the click reaction are denatured and diluted with 1/4
568 volume of 4x Laemmli buffer and then migrated on 12% SDS-PAGE. The metabolically
569 labeled proteins are visualized on the gels with an imager using Cy3 filters ($\lambda_{\text{ex}}=532$ nm;
570 $\lambda_{\text{em}}=610$ nm).

571 • N-terminal protein lipidations in cell extracts mostly lead to two types of acylations,
572 myristoylation (C14) and palmitoylation (C16). These modification differ by the nature of their
573 linkage to the protein, amide or ester, respectively. Distinction between the two types of
574 modifications may be challenging because of metabolic interconversions of fatty acid
575 precursors (Giglione & Meinnel, 2022). The high susceptibility and effective cleavage of the
576 ester bond in alkaline conditions - obtained for instance with KOH (0.1 M in methanol) for 4
577 h at room temperature (Bizzozero, 1995) - is a relevant approach to check that the detected
578 acylation does arise from N-terminal amidation and is most likely catalyzed by NMT.

579 4.4.4 Immunoprecipitation

580 • One milligram of total protein from cell lysates is combined with 5 µg of target antibody and
581 adjusted to 500 µL in IP-MS Cell Lysis Buffer (Pierce MS-compatible Magnetic IP Kit,
582 Thermo Fisher Scientific) and then incubated overnight with shaking. The remaining procedure
583 is carried out according to the manufacturer's protocol. In brief, 0.25 mg of magnetic beads
584 coated with A/G proteins are washed in the lysis buffer and then incubated with the
585 antibody/antigen mixture for 1 h at room temperature. The mixture is then washed 5 times and
586 the antigens eluted, and the supernatant recovered and dried in a concentrator under vacuum
587 (SpeedVac).

588 • Immunoprecipitation is carried out using the µMACS™ DYKDDDDK Isolation Kit
589 (Miltenyi Biotec) according to the manufacturer's protocol.

590 • The tagged protein is visualized with specific antibodies. An example of ARF6 detection
591 with Flag-tag antibodies is shown in **Fig. 2E**.

592 • To increase the concentration, protein samples can be subjected to methanol:chloroform (4:1)
593 precipitation and centrifuged for 5 min at 13,000 x g. The upper aqueous phase is discarded to
594 leave the interface layer containing intact protein precipitate. 450 µL methanol is added to the
595 tube, vortexed, and centrifuged for 5 min at 13,000 x g to pellet the protein. The pellet is washed
596 with 450 µL methanol to remove residual reaction components. The pellets are allowed to air
597 dry. The dried protein precipitates can be resolubilized in Laemmli sample buffer for further
598 SDS-PAGE analysis.

599 4.4.5 Fluorescence imaging after immunoblotting

600 • Proteins separated by 12-15% SDS-PAGE are transferred (25 mM Tris; 190 mM glycine;
601 20% MetOH) onto a polyvinylidene fluoride (PVDF) membrane at 4°C 30 V overnight. The

602 PVDF membrane is incubated and blocked in 3% TBST BSA (20 mM Tris pH 7.5; 150 mM
603 NaCl; 0.1% Tween 20; 3% BSA) for 90 min at room temperature.

604 • The membrane is incubated with the primary – here anti-Flag or anti-ARF6 - antibody diluted
605 1:1000 according to the manufacturer's recommendations, before being washed 5 times in
606 TBST BSA 1%.

607 • The membrane is incubated with a secondary anti-IgG conjugate antibody tagged with a Cy5
608 fluorophore and the streptavidin–Cy3 derivative diluted at 1:10,000 in the blocking buffer at
609 4°C overnight.

610 • Imaging is performed with both the Cy3 and Cy5 channels and the fluorescence merged as
611 required.

612 **5. Structural studies of NMT and its complexes with substrates and** 613 **products**

614 **5.1 Structural overview**

615 NMTs are made up of a two-GNAT core (Meinzel et al., 2020). The N-terminal domain
616 is of varying length and is not folded. High resolution crystal structures are available for NMTs
617 from *Aspergillus fumigatus* (Shimada, Suzuki, & Katakura, 2015), *Candida albicans*
618 (Bhatnagar et al., 1998), *Homo sapiens* (both forms and various isoforms (Castrec et al., 2018;
619 Thinon et al., 2014)), *Leishmania donovani* and *major* (Brannigan et al., 2010; Frearson et al.,
620 2010), *Plasmodium vivax* (Goncalves et al., 2012) and *Saccharomyces cerevisiae* (Wu et al.,
621 2007). HsNMT1's longest structures start at residue 99, which includes a crucial B'A' helix
622 (Castrec et al., 2018). Many HsNMT1 structures start at residue 109 or 116. HsNMT2 crystal
623 structures start at residues 112 (4C2X, (Thinon et al., 2014)) and 116 (6PAU (Kosciuk et al.,
624 2020)) and miss the important element involved in a major conformational change contributing
625 to product release (Dian et al., 2020).

626 AlphaFold software predictions (Jumper et al., 2021) of the structure of several NMTs
627 are available at UniProt (<https://www.uniprot.org/>). Interestingly, the AlphaFold predictions of
628 HsNMT, PvNMT and AtNMT structures suggest that there is an additional alpha helix at the
629 beginning of the folded GNAT domain (residues Asn82 in HsNMT1, Met88 in HsNMT2,
630 Asp27 in PvNMT, Leu31 in AtNMT1), a structure hitherto not identified by crystallographic
631 approaches (**Fig. 4A** for both HsNMTA and HsNMT2; see also AF-Q9LTR9-F1 and AF-
632 A0A1G4HIY1-F1 for AtNMT1 and PvNMT at <https://alphafold.com/>).

633 Recent investigations have provided details of NMTs in action with their two substrates.
634 Below are the approaches used to capture such short-lived complexes.

635 **5.2 Crystallization and structure determination**

636 *5.2.1 General crystallization conditions*

637 In recent studies, HsNMT1(s) was used to solve the structures of the complexes with
638 various substrate peptides (Castrec et al., 2018; Dian et al., 2020; Kosciuk et al., 2020; Rivière
639 et al., 2022). The associated PDB entries are indicated in the published papers.

640 Suitable crystals of HsNMT1:MyrCoA:peptide substrates were obtained by co-
641 crystallization using the hanging-drop vapor diffusion method at 20°C in the crystallization
642 conditions previously described (Castrec et al., 2018; Dian et al., 2020). Briefly, crystallization
643 droplets are formed by mixing 2 µL of the of HsNMT1:MyrCoA:peptide complex (ratio
644 1:1.5:1.5) at 6-9 mg/mL with 2 µL of the precipitant solution containing either 0.1 M MgCl₂,
645 0.2 M NaCl, 0.1 M sodium citrate pH 5.6, and 18-24% (w/v) PEG 6K or 0.1 M sodium acetate
646 pH 4.6 and 18-24% (w/v) PEG 6K. Crystals are cryoprotected in the reservoir solution
647 supplemented with 15% (v/v) glycerol and flash cooled in liquid nitrogen.

648 *5.2.2 Obtaining crystals of NMT in complex with reactants or reaction intermediates*

649 GNATs use a sequential ordered Bi-Bi mechanism involving binding first to the CoA
650 derivative and then to the peptidyl target (Salah Ud-Din, Tikhomirova, & Roujeinikova, 2016).
651 This also applies to NMTs and has been demonstrated for ScNMT and HsNMT1 (Rocque,
652 Mcwherter, Wood, & Gordon, 1993; Rudnick et al., 1991). This feature needs to be taken into
653 account in crystallographic trials.

654 ● CoA derivative complexes. NMT crystal structures – as is the case for most GNATs – are
655 most often determined in complex with their cognate CoA derivative. Crystals devoid of the
656 CoA derivative are rarely obtained. This probably originates from the structural remodeling of
657 the CoA binding site upon binding. When overexpressed in *E. coli*, the acetyl-CoA derivative
658 tightly binds to GNATs and it appears unnecessary to add it. Nevertheless, myristoyl-CoA is
659 not available, as fatty acyl CoA derivatives do not accumulate in *E. coli* (Giglione & Meinnel,
660 2022; Meinnel, 2022). The growth medium can be supplemented with myristate, as it can be
661 converted *in cellulo* by ACS in bacteria (**Fig. 1B**). Myristoyl-CoA can also be added in
662 crystallization trials.

663 ● Peptidyl complexes. Multiple factors have significant impact on the success in obtaining
664 crystals of intermediates. From our experience gained while generating the data reported in
665 (Dian et al., 2020) with peptides X (GNCSFSKRRA), Y (GSNKSKPK), U (*ac*-GKSFSKPR),
666 and V (*ac*-KSFSKPR), these include:

667 - Soaking (peptide Y) instead of co-crystallization (peptide X) to reduce reaction time. It is
668 tempting to say that a 15 min soak used to solve the complex with peptide Y seems to have
669 been sufficient to pick up the intermediate, while co-crystallization with peptide X gave time
670 for the reaction to occur;

671 - Both low and different pHs of the precipitant solutions (pH 4.6 with peptide Y and pH 5.6
672 with peptide X, (Dian et al., 2020)) significantly contributed to slowing down the NMT
673 reaction (optimum pH is 8.0);

674 - Nature of the two peptides for which NMT displays different catalytic efficiencies;
675 - Crystal trials must be performed under many different conditions (i.e., different peptides,
676 different peptide concentrations, different soaking times), and most of them will remain
677 inconclusive (e.g., no crystal, poor resolution, no intermediate). Only successful datasets are
678 presented in publications, probably representing the outcome of many trials. We also observed
679 that several crystals need to be collected from the same batch to finally observe a few of them
680 showing reaction intermediates.

681 *5.2.3 Dataset collection, structure resolution, and refinement*

682 Complete X-ray datasets of complexes are collected at 100K, a single wavelength from
683 a single crystal, at synchrotron facilities such as the French National Synchrotron Facility
684 (SOLEIL) PROXIMA-1 or PROXIMA-2 beamlines or at the European Radiation Synchrotron
685 Facility (ESRF) with the ID30A1 and ID30A3 beamlines.

686 • Datasets are integrated with XDS (Kabsch, 1993) and scaled and reduced using AIMLESS
687 from the CCP4 package (Evans & Murshudov, 2013). For crystals suffering from anisotropic
688 diffraction, data are processed with STARANISO on unmerged data (Tickle et al., 2018) before
689 AIMLESS data reduction.

690 • Crystals of HsNMT1 complexes belong essentially to the space group $P2_12_12$ with similar
691 unit cell parameters, except for some belonging to $C2$ with distinct unit cell parameters. In both
692 space groups identified, unit cells contain two NMT molecules per asymmetric unit. Structure
693 resolution was accomplished in all cases using the molecular replacement method and solved
694 using PHASER (Mccoy et al., 2007) and the HsNMT1:MyrCoA:peptide ternary complex
695 (PDB entry 5O9T or 6SK2) as a search model. The structure of AN was solved using MOLREP
696 (Vagin & Teplyakov, 1997) and protein coordinates of HsNMT1:MyrCoA (PDB entry 5O9T)
697 as a search model.

- 698 • Structures are subjected to alternating refinement cycles using PHENIX and manual model
699 building using COOT (Adams et al., 2010; Emsley, Lohkamp, Scott, & Cowtan, 2010;
700 Murshudov et al., 2011). Good quality electron density maps enable the refinement of substrate
701 peptide, reaction intermediate, and reaction product molecules bound to HsNMT1 in each
702 complex. Chemical compound libraries are generated using the PRODRG server (Schuttelkopf
703 & Van Aalten, 2004) in combination with eLBOW from the PHENIX suite. The geometry of
704 the final models is validated using MOLPROBITY (Chen et al., 2010). Figures are generated
705 using PYMOL (DeLano Scientific LLC, <http://pymol.sourceforge.net/>).
- 706 • Omit electron density maps can be calculated using PolderMap (Liebschner et al., 2017)
707 from the Phenix suite after omitting the peptide of each active site and excluding the bulk
708 solvent around the omitted region (**Fig. 4B**).
- 709 • Ball and stick representations in 2D are a good way to challenge and compare active site
710 interactions (**Fig. 4C**)

711 **6. Conclusions and future prospects**

712 Here we report a series of multiscale biochemical analyses that allow the capture and fine
713 definition of NMT catalysis and the validation of putative targets. This permits not only time
714 and space investigation but also challenging substrate selectivity experiments based on the
715 chemical choice and specificity provided by this unique family of catalysts. This exemplifies
716 the remarkable fluidity of the reaction catalyzed *in vitro* by NMTs. Other - mostly click
717 chemistry-based - methods are available for fine characterization of myristoylation at the
718 cellular proteome level (see Chapter 24 by H. Lin et al. in this volume or (Kallemeijn et al.,
719 2021)). Kinetic analysis of myristoylation by NMTs of octapeptides derived from actual
720 protein N-termini have been validated as a relevant and robust approach to contribute to
721 completing this effort at proteome scales (Boisson, Giglione, & Meinnel, 2003; Castrec et al.,

722 2018; Meinnel, 2022; Traverso et al., 2013a). Such methods are detailed in Chapter 26 in this
723 volume (Rivière et al., 2023).

724 Future progress in biochemical characterization will need to better address the acylation
725 yield (i.e., the ratio of each N-terminal acylation state to the total amount of a given protein) of
726 each target, as it likely varies from one physiological condition to another. With differences in
727 myristoylation, the overall subcellular localization of the same target changes and most likely
728 favors different functions of the target. Therefore, investigation of the myristoylation yield on
729 a maximum number of targets - preferentially not overproduced *in cellulo* – will be required in
730 the future. Although such approaches exist in the case of N-terminal acetylation (Bienvenut,
731 Giglione, & Meinnel, 2015, 2017; Bogaert & Gevaert, 2020), this is not yet available for NMT
732 targets. This approach will probably need to enrich the N-terminal peptides without loss of the
733 most hydrophobic ones, including N-myristoylated peptides. Unfortunately, currently available
734 click-based approaches are not directly suited to this purpose, as they lead to the selection of
735 only the myristoylated state and the loss of all other isoforms.

736

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756

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991

992

993 **Figure Legends**

994

995 Fig. 1. Reactions catalyzed by the main enzymes useful for NMT studies. (A) NMT. (B) ACS,
996 X is any acyl chain and may possibly end with an azide or an alkyne reactive group. (C) IpaJ.

997

998 Fig. 2. Electrophoresis analysis of the myristoylation state of a protein target. ARF6-His-tag
999 reactivity with myristate derivatives was probed by denaturing electrophoresis and revealed
1000 using different approaches. (A) 12% SDS-PAGE analysis of ARF6 after Coomassie blue
1001 staining; Lane 1, molecular weight marker; Lane 2, unmodified ARF6; Lane 3 myristoylated
1002 ARF6. Panels B & C. 15% SDS-PAGE ARF6 analysis after labeling with Myr-Alk or myristate
1003 or mixed with plant cell extracts (lanes 3-7) or not (lane 1). The PEG3-azide-TAMRA probe
1004 was reacted at increasing concentrations of 1, 10, 25, 50 and 100 μ M (lanes 3-7). Lane 8
1005 corresponds to ARF6 labeled with the unclickable physiological myristate. Lane 2 is the
1006 molecular marker. (B) Coomassie blue staining (C) TAMRA fluorescence imaging of the same
1007 gel after azide-TAMRA click reaction. The two arrows indicate the unmodified (top) and
1008 modified (bottom) versions of ARF6. Only the modified, faster migrating version reacts with
1009 the TAMRA click probe. (D) Coomassie blue (left panel) and TAMRA fluorescence after
1010 azide-TAMRA-biotin click reaction (right panel) signals of protein samples from HEK293
1011 protein lysates (40 μ g/lane) analyzed by SDS-PAGE. Cells were fed with Myr-Alk or not and
1012 ARF6-Flag was overexpressed or not, as indicated. (E) HEK293 protein lysates overproducing
1013 samples ARF6-Flag (INP, input) were immunoprecipitated (IP) and analyzed by SDS-PAGE
1014 and revealed by blue staining (left panel) or by immunoblotting with anti-Flag antibody (right
1015 panel).

1016

1017 Fig. 3. MALDI MS IpaJ pipeline applied to myristoylation of full-length protein ARF6. The
1018 monocharged ($z=1$) and doubly-charged ($z=2$) masses are visible on the m/z MALDI MS
1019 spectrum. The spectra displayed in the figure were obtained with HsNMT2. Similar data were
1020 obtained with HsNMT1. Masses are deduced from the more resolute doubly charged masses.
1021 (A) T0, ARF6 protein alone. (B) T1, myristoylated ARF6 (Myr-ARF6) on its N-terminal Gly.
1022 (C) T3, ARF6 devoid of N-terminal Gly as a result of IpaJ action (Δ G-ARF6) and
1023 remyristoylation of the Lys2 side chain by NMT (Δ G-Myr(K)-ARF6). (D) Recapitulation of
1024 the complete IpaJ pipeline with expected masses at each step and comparison between
1025 theoretical and measured masses.

1026

1027 Fig. 4. Main structural components of NMTs. (A) AlphaFold-predicted 3D structures of
1028 HsNMT1 and HsNMT2 (UniProt entries P30419 and O60551, AlphaFold IDs AF-P30419-F1
1029 and AF-O60551-F1). Overlap of the two structures each displayed in green and pink,
1030 respectively. The additional N-terminal helix (17 residues long) is displayed on top of the
1031 panel. The structures correspond to residues 85-496 and 88-498, respectively. The disordered
1032 N-terminus is not displayed. Both Myristoyl-CoA and peptide ligands at their binding sites are
1033 figured in orange and yellow, respectively. (B) Detailed “fofc omit” sigmaA-weighted electron
1034 density maps ($mF_{obs} - DF_{calc}$, PHI_{calc}) are shown in grey for each of the structures with
1035 peptide ligand *ac*-K undergoing Lys-myristoylation., Myr, MyrCoA, and CoA carbon atoms
1036 are in orange when unlinked to the peptide moiety and in green when covalently bound to the
1037 peptide. (C) Overlap of GK (HPCA) with *ac*K (A subunit).

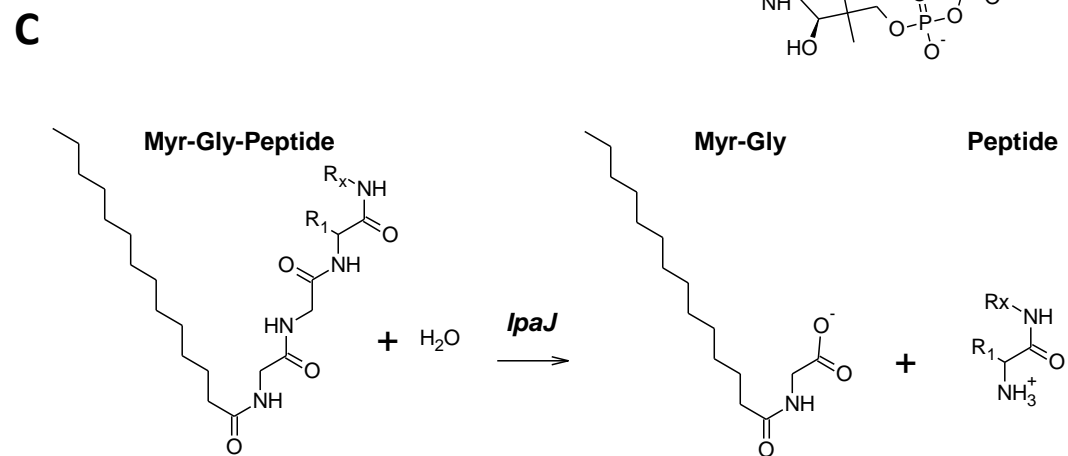
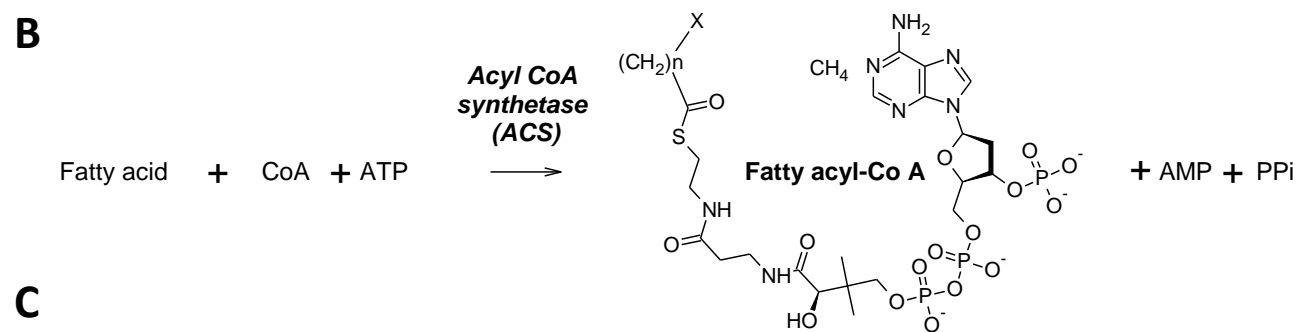
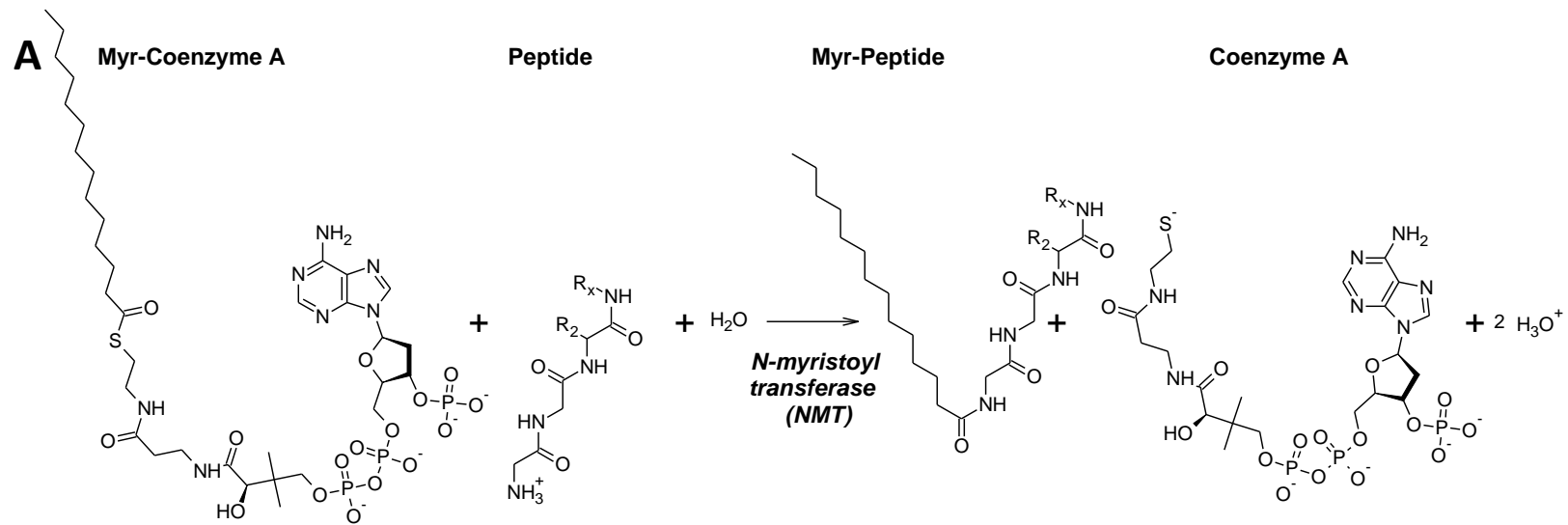


Fig. 1

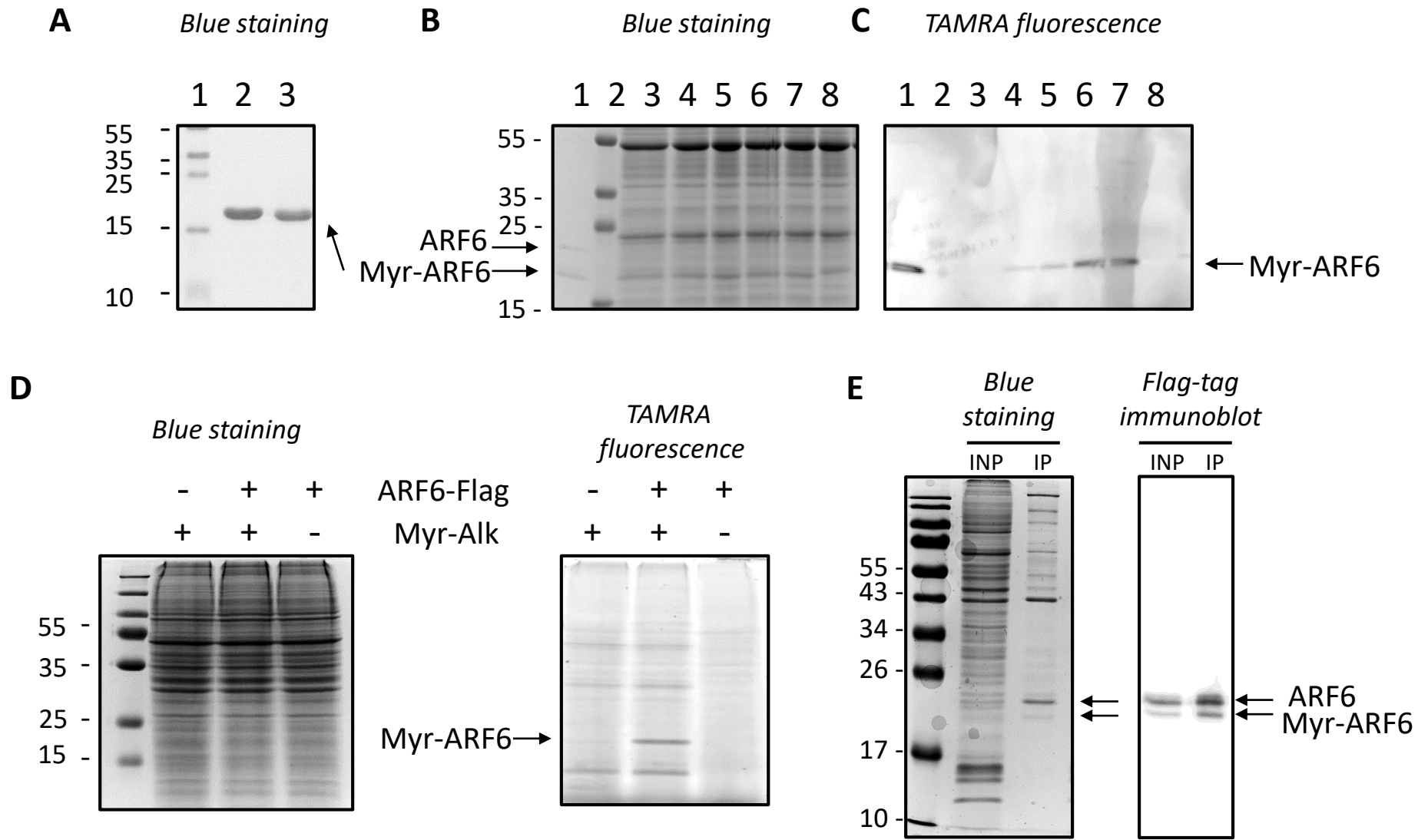
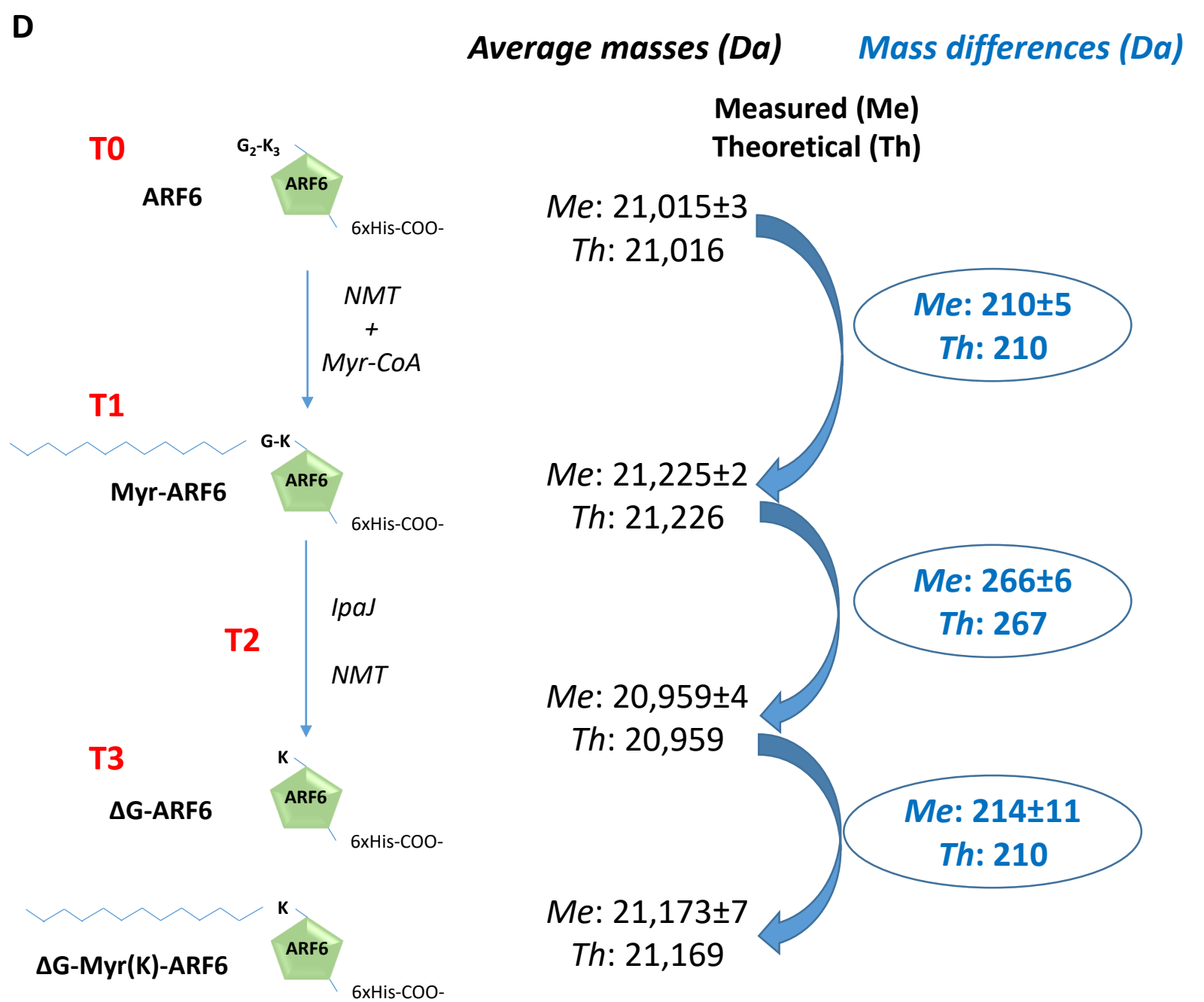
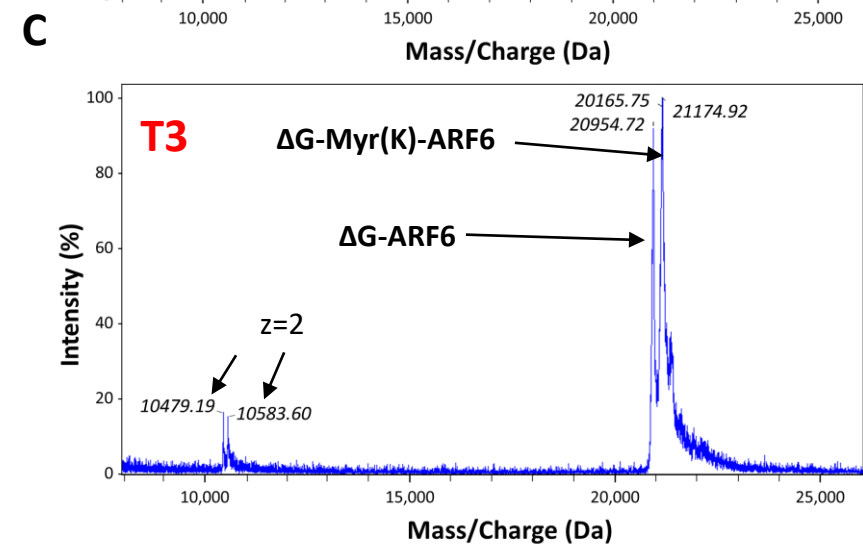
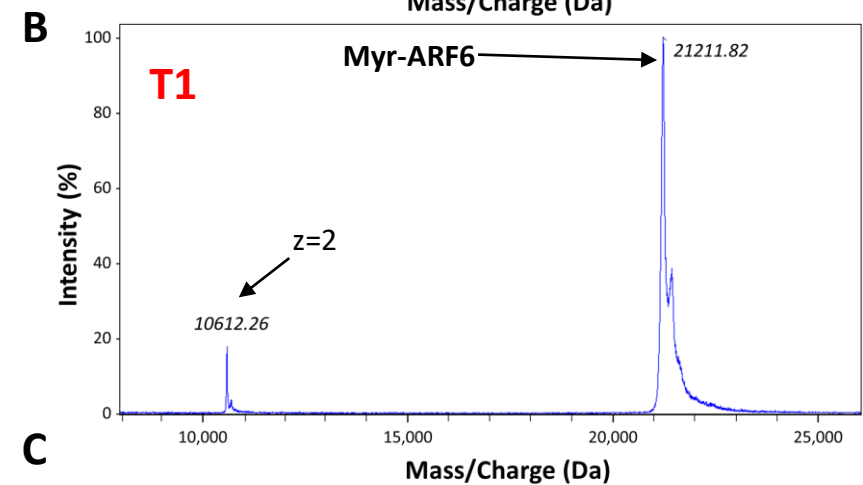
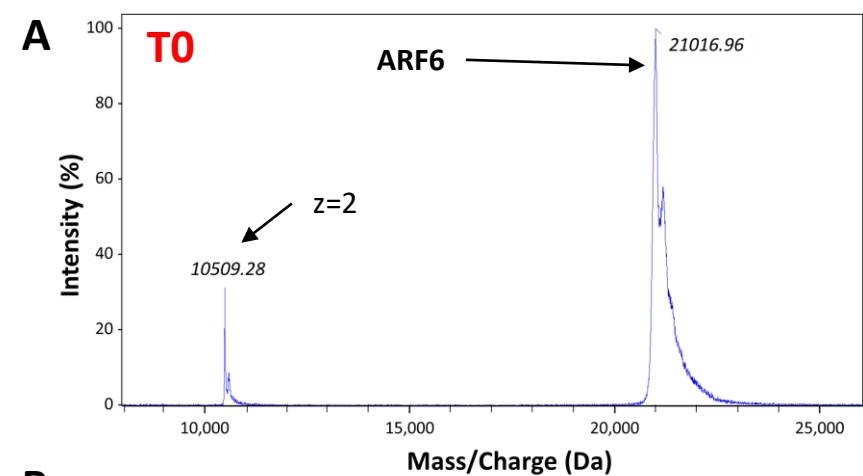
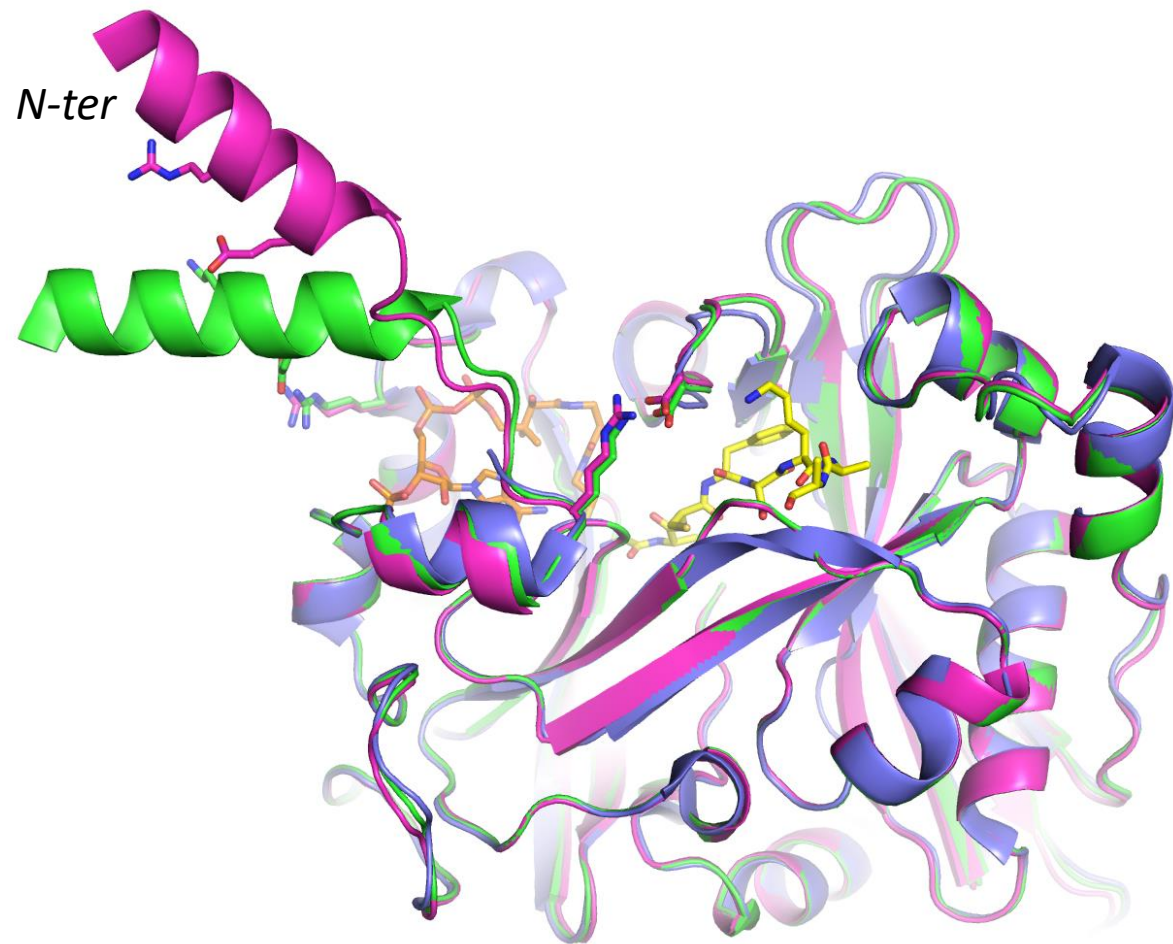
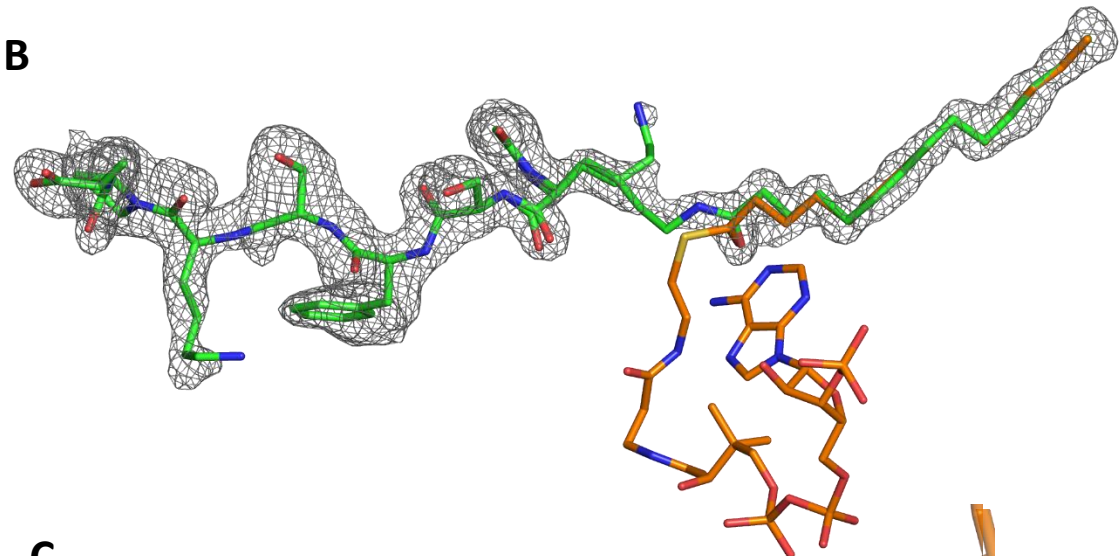
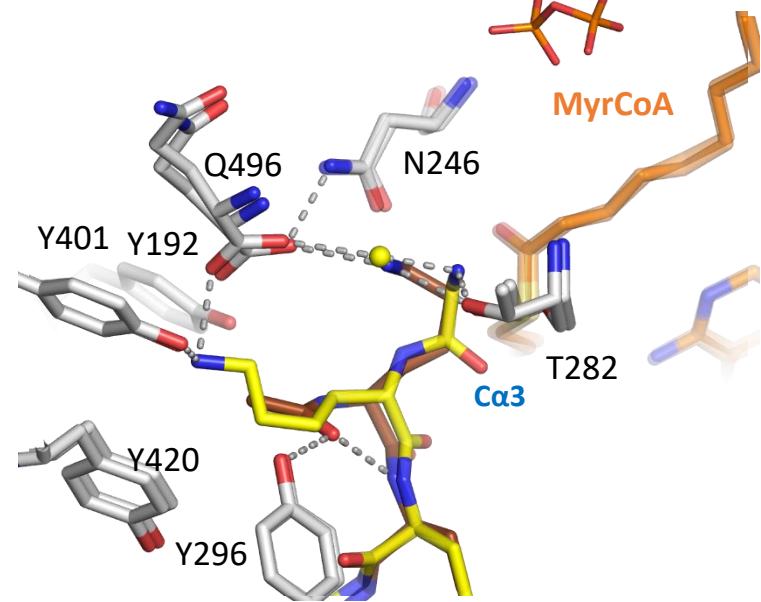


Fig. 2



A**B****C****Fig. 4**