



**HAL**  
open science

## Kinetic and catalytic features of N-myristoyltransferases

Frédéric Rivière, Paul Monassa, Carmela Giglione, Thierry Meinzel

► **To cite this version:**

Frédéric Rivière, Paul Monassa, Carmela Giglione, Thierry Meinzel. Kinetic and catalytic features of N-myristoyltransferases. *Methods in Enzymology*, 2023, Modifications and Targeting of Protein Termini: Part A, 684, pp.167-190. 10.1016/bs.mie.2023.02.018 . hal-04234985

**HAL Id: hal-04234985**

**<https://hal.science/hal-04234985v1>**

Submitted on 10 Oct 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 CHAPTER SIX

2

3 **Kinetic and catalytic features of N-myristoyltransferases**

4

5 Frédéric Rivière<sup>iD,a,1,£</sup>, Paul Monassa<sup>iD,a,£</sup>, Carmela Giglione<sup>iD,@,a</sup>, Thierry Meinnel<sup>iD,@,a,\*</sup>

6

7 <sup>a</sup> Université Paris Saclay, CEA, CNRS, Institute for Integrative Biology of the Cell (I2BC),

8 91198 Gif-sur-Yvette cedex, France

9

10 **Running title:** N-myristoyltransferase catalysis

11 **Correspondence:** [carmela.giglione@i2bc.paris-saclay.fr](mailto:carmela.giglione@i2bc.paris-saclay.fr) (C. Giglione) or

12 [thierry.meinnet@i2bc.paris-saclay.fr](mailto:thierry.meinnet@i2bc.paris-saclay.fr) (T. Meinnet)

13 <sup>iD</sup> ORCID iD 0001-6366-1904 (F. Rivière); ORCID iD 0003-4713-8069 (P. Monassa); ORCID

14 iD 0002-7475-1558 (C. Giglione); ORCID iD 0001-5642-8637 (T. Meinnet)

15 <sup>@twitter</sup> @giglionelab (C. Giglione); @meinnet (T. Meinnet)

16 <sup>£</sup> These authors contributed equally

17

18 **Present address**

19 <sup>1</sup> Karolinska Institutet, Department of Biosciences and Nutrition, 14183 Huddinge, Sweden

20

21 **Keywords:** Protein acylation; lysine; myristoylation; acetylation; N-myristoyltransferase; N-  
22 terminus; glycine; enzyme kinetics.

23

24	Contents	
25		
26	1. Introduction.....	3
27	2. Materials, reagents, and buffers .....	4
28	2.1 Equipment .....	4
29	2.2 Enzymes .....	4
30	2.3 Reagents .....	5
31	2.3.1 Fatty acyl derivatives .....	5
32	2.3.2 Peptides .....	6
33	2.3.3 Notes .....	7
34	2.4 Buffer components .....	7
35	2.4.1 For storage at room temperature .....	7
36	2.4.2 For storage at 4°C .....	7
37	2.4.3 For storage at -20°C .....	7
38	3. Kinetic analysis.....	8
39	3.1 GNAT-specific kinetic properties and considerations .....	8
40	3.2 Discontinuous assays.....	8
41	3.2.1 Radioactive labeling.....	8
42	3.2.2 HPLC assays .....	9
43	3.3 Continuous assays .....	11
44	3.3.1 NMT activity coupling conditions .....	11
45	3.3.2 Acylation kinetics .....	12
46	3.3.3 Notes .....	13
47	3.4 Catalytic efficiencies of various substrates: ranges and meanings .....	16
48	3.4.1 CoA donors .....	16
49	3.4.2 Polypeptide acceptor donor.....	18
50	4. Conclusions.....	19
51	Acknowledgements.....	19
52	Funding .....	20
53	References.....	20
54	Figure Legends.....	26
55	Supplementary Figure Legends .....	28
56		
57		

## 58 Abstract

59 N-myristoyltransferases (NMTs) are members of the large family of GCN5-related N-  
60 acetyltransferases (GNATs). NMTs mainly catalyze eukaryotic protein myristoylation, an  
61 essential modification tagging protein N-termini and allowing successive subcellular  
62 membrane targeting. NMTs use myristoyl-CoA (C14:0) as major acyl donor. NMTs were  
63 recently found to react with unexpected substrates including lysine side-chains and acetyl-CoA.  
64 This chapter details the kinetic approaches that have allowed the characterization of the unique  
65 catalytic features of NMTs *in vitro*.

66

## 67 **1. Introduction**

68 Myristoylation describes a protein acylation that adds a fatty acid to the N-terminal  
69 glycines of ~2% of proteins in all eukaryotes (Castrec et al., 2018; Giglione & Meinnel, 2022;  
70 Lodge, Johnson, Weinberg, & Gordon, 1994; Meinnel, 2022; Meinnel, Dian, & Giglione, 2020;  
71 Pierre et al., 2007; Price et al., 2003). The lipid moiety anchors soluble proteins to internal  
72 membrane networks, where they interact with partners to initiate signal transduction  
73 (Bhatnagar, Ashrafi, Futterer, Waksman, & Gordon, 2001; Renna et al., 2013; Resh, 2006;  
74 Traverso et al., 2013b). N-myristoyltransferases (initially named glycylopeptide N-  
75 tetradecanoyltransferases, myristoyl CoA:protein N-myristoyltransferase, NMT; EC 2.3.1.97)  
76 are the only enzyme class known to catalyze myristoylation in eukaryotes and, given their  
77 central role in pathobiology, are promising therapeutic targets (Beauchamp et al., 2020;  
78 Frearson et al., 2010; Mousnier et al., 2018; Wright et al., 2014; Wright, Paape, Price, Smith,  
79 & Tate, 2016). NMTs were long thought to exclusively target proteins with an N-terminal  
80 glycine (Gly-myristoylation), usually through co-translational methionine excision (Kosciuk  
81 & Lin, 2020; Meinnel et al., 2020) or, less frequently, from post-translational cleavage

82 exposing new N-terminal glycines (Martin, Beauchamp, & Berthiaume, 2011; Thinon et al.,  
83 2014). More recently, several studies have indicated that both acetyl-CoA and lysine side  
84 chains can be used as CoA donors and targets, respectively.

85 A general scheme of classic NMT reactivity is provided in **Fig. 1A**. NMTs from  
86 *Arabidopsis thaliana* (AtNMT1), *Saccharomyces cerevisiae* (ScNMT), *Plasmodium*  
87 *falciparum* (PfNMT), or *Homo sapiens* (HsNMT1 and HsNMT2) have been studied *in vitro*  
88 using one or several such approaches (see references in (Giglione & Meinel, 2022)). NMTs  
89 are members of the large GCN5-related N-acetyltransferase family, or GNATs (Bhatnagar et  
90 al., 1998). GNATs include many N-acetyltransferases modifying not only free amine groups  
91 on proteins (N-terminus and/or lysine side-chains) but also small metabolites including, for  
92 example, antibiotics (Salah Ud-Din, Tikhomirova, & Roujeinikova, 2016).

93

## 94 **2. Materials, reagents, and buffers**

### 95 **2.1 Equipment**

96 • 96-well plate reader, if possible equipped with two-way micro-injectors (Infinite M Nano+  
97 plate reader, Tecan).

98 • Microplates for reading absorption: 96-well polystyrene clear flat-bottomed plates (Grenier  
99 Bio One, 655101; the optical path for 0.2 mL is 0.55 cm). It is possible to work with larger  
100 volumes up to 382  $\mu$ l with these plates to increase signal, as the optical path increases  
101 proportionally (1 cm for 350  $\mu$ L). Working with smaller amounts down to 100  $\mu$ L may also be  
102 achieved (Grenier Bio One, 675101; the optical path for 0.1 mL is 0.58 cm).

103 • Microplates for fluorescence measurements, 96-well black solid-bottomed plates (Grenier  
104 Bio, 675076); working volume of 100  $\mu$ L.

### 105 **2.2 Enzymes**

106 • Pyruvate dehydrogenase (PDH; 3.1.3.43 from porcine heart (Sigma-Aldrich P7032; 0.33  
107 U/mg; >4 mg/ml), stored at -20°C. PDH catalyzes the reaction shown in **Fig. 1B**.

108 • HsNMT1, see details for preparation in Chapter 22 of this volume (Monassa et al., 2023).

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

112 • **Fig. S1** shows examples of purified NMT analysis by SDS-PAGE, where the purity is  
113 sufficient for kinetic studies.

## 114 **2.3 Reagents**

### 115 *2.3.1 Fatty acyl derivatives*

116 • Myristoyl-CoA lithium salt (Sigma-Aldrich, M4414) MM = 977.9 g/mol; 5 mg diluted in  
117 25.565 mL. Stock solutions (0.2 mM) are prepared in 10 mM sodium acetate, pH 5.6, and 1%  
118 Triton X-100, except for MALDI analysis, where cholate is used instead of Triton to reduce  
119 background (see below). The low pH prevents slow CoA hydrolysis.

120 • Myristoyl-CoA lithium salt, 80% pure (Sigma-Aldrich, M4414) MM = 977.9 g/mol; 5 mg  
121 diluted in 0.511 mL in 50% DMSO (final concentration 10 mM).

122 • Acetyl-CoA, >93% pure (Sigma-Aldrich, A2056).

123 • Myristic acid (Sigma-Aldrich, M3128).

124 • Radioactive myristic acid, [1-<sup>14</sup>C] or [9,10-<sup>3</sup>H] (Moravek Inc., MC118 or MT918).

125 • Radioactive myristoyl-CoA is no longer available off-the-shelf from commercial suppliers.  
126 Unlike palmitate or stearate, which may be bought from Perkin-Elmer, for instance, radioactive  
127 myristate (<sup>3</sup>H or <sup>14</sup>C) now needs to be ordered as a custom synthesis, e.g., from Selcia, Moravek  
128 Biochemicals Inc., Quotient Sciences, or ViTrax. It can also be prepared from radioactive

129 myristic acid and CoA according to the approach detailed in §3.1, Chapter 22, this volume.  
130 (Monassa et al., 2023)

### 131 2.3.2 Peptides

132 Polypeptides longer than six residues can be used for acylation reactions in the presence  
133 of an acyl donor (Kaplan, Mardon, Bishop, & Varmus, 1988; Towler et al., 1987). Octapeptides  
134 are optimal (see §3.4.2). Octapeptides bury all dedicated pockets of the binding site of NMTs  
135 (Meinzel et al., 2020).

136 • We order our custom octapeptides of any sequence from GenScript Biotech (Rijswijk,  
137 Netherlands), but peptides can also be ordered from many other companies worldwide. We  
138 typically order peptides (>5 mg net weight) with >95% purity delivered in three separate vials  
139 to allow for solubility testing and quality controlling prior to experimental use, usually by  
140 MALDI-TOF/TOF mass spectrometry (see details in Chapter 22, this volume (Monassa et al.,  
141 2023) and (Rivière et al., 2022)). An example with the ARF6-derived octapeptide is given in  
142 **Fig. 2A,B.**

143 • Cys-containing peptides are prone to oxidation. Storing in oxygen-free conditions at -20°C  
144 is recommended if storing for months.

145 • Stock solutions (10-100 mM depending on the solubility) are solubilized in water and stored  
146 at -20°C. Very acidic or basic peptides can require buffering with 50 mM Tris-HCl, pH=7.5.  
147 DMSO solubilization of the dry powder is sometimes useful for hydrophobic peptides.  
148 However, DMSO has a negative impact on NMT rates and should not exceed 1%.

149 • Our reference peptides are (i) *Arabidopsis thaliana* SOS3-derived for Gly-myristoylation or  
150 Gly-acetylation (G(C or S)SVSKKK, SOS3) or GKSFSKPR (GK) and human ARF6-derived  
151 (GKSVLSKIF, ARF6); (ii) the N-acetylated versions of ARF6 (*ac*-GKSVLSKIF; *ac*-ARF6)  
152 or *ac*-KSFSKPR (*ac*K) for Lys-myristoylation.

153 2.3.3 *Notes*

154 Any acyl-CoA variant different from myristoyl-CoA can be synthesized *in vitro* from the  
155 corresponding fatty acid derivatives as detailed in §3.1, Chapter 22 of this volume (Monassa  
156 et al., 2023).

157 **2.4 Buffer components**

158 2.4.1 *For storage at room temperature*

159 • Tris-HCl stock 0.5 M pH 8 q.s. 0.5 L; MM = 121.14 g/mol; 30.285 g Tris base in 500 ml  
160 H<sub>2</sub>O; HCl 37 %  $\approx$  5mL to be added to reach pH 8.0; check pH with a pH meter and again once  
161 back to room temperature.

162 • MgCl<sub>2</sub> stock 100 mM q.s. 50 mL; MM = 203.3 g/mol (6H<sub>2</sub>O); 1.0165 g in 50 mL.

163 • Triton X-100 10% q.s. 15 mL; dilute use 1.5 mL Triton X-100 solution (Sigma-Aldrich,  
164 93443)

165 • EGTA 19.3 mM q.s. 100 mL (MM = 380.35 g/mol). Add 0.734 g to 100 mL with 2 NaOH  
166 chips to reach pH 8 and allow complete solubilization.

167 • Enzyme dilution buffer is Tris 50 mM pH 8 1 mL, BSA (0.1 mg/mL) 100  $\mu$ L, H<sub>2</sub>O 8.9 mL.

168 2.4.2 *For storage at 4°C*

169 • Sodium pyruvate (Sigma-Aldrich, S-8636, 100 mM).

170 2.4.3 *For storage at -20°C*

171 • Bovine serum albumin (BSA, Sigma-Aldrich) 10 mg/mL q.s. 10 mL in H<sub>2</sub>O; aliquots of 200  
172  $\mu$ L.

173 • Dithiothreitol (DTT) 6.4 mM q.s. 20 mL; MM = 154.25 g/mol, 19.74 mg in 20 mL H<sub>2</sub>O;  
174 prepare aliquots of 500  $\mu$ L.



175 • Sodium acetate 10 mM/TritonX-100 1% solution: add sodium acetate 10 mM q.s. 100 mL  
176 (82.03 mg acetate-Na (82.03 g/mol) in 100 mL H<sub>2</sub>O, 1 mL Triton X-100; set to pH 5.6 with a  
177 few drops of acetic acid and NaOH. Aliquots of 2 mL can be prepared.

178 • Thiamine pyrophosphate (TPP) 20 mM q.s. 5 mL (Sigma-Aldrich, C8754); MM = 460.8  
179 g/mol; 46.08 mg in H<sub>2</sub>O. Aliquots of 200 µL are convenient.

180 • NAD 25 mM q.s. 20 mL, MM = 663.4 g/mol; NAD-free acid grade 1 (Roche, 10127973001),  
181 331.7 mg in 20 mL H<sub>2</sub>O. Aliquots of 2 mL are sampled.

182

### 183 **3. Kinetic analysis**

#### 184 **3.1 GNAT-specific kinetic properties and considerations**

185 NMT was long thought to catalyze fatty acylation of only N-terminal glycine residues  
186 (Meinzel et al., 2020). Recent data have shown that NMTs - as most GNATs - may also use  
187 acetyl-CoA as an effective CoA donor (Meinzel, 2022; Su, Kosciuk, Yang, Price, & Lin, 2021)  
188 and lysine side chains for possible protein anchoring (Dian et al., 2020; Kosciuk et al., 2020;  
189 Rivière et al., 2022). The following methods allow kinetic characterization of all these  
190 reactions. Examples and ranges are provided in §3.4 for each reaction type.

#### 191 **3.2 Discontinuous assays**

##### 192 *3.2.1 Radioactive labeling*

193 NMT activity can be assayed in a discontinuous manner at 30°C mainly according to  
194 (Boisson & Meinzel, 2003) based on well-established protocols (King & Sharma, 1991; Raju  
195 & Sharma, 1999). In this assay, the peptide substrate needs to display a strong basic character  
196 with at least three Lys or Arg residues to tightly retain the peptide on anionic bead phospho-  
197 cellulose filter papers. A 3-Arg tail C-terminal tag is sufficient; note that the SOS3 derivative

198 is fine without any alteration due to its native 3-Lys tail. This is a limitation of this assay,  
199 however, as the actual sequence of a putative peptide target can only rarely be assessed.

200 • The assay is performed in Eppendorf tubes (25  $\mu$ l final volume) containing 0.2  $\mu$ Ci (i.e.,  
201  $\sim$ 0.14  $\mu$ M) [ $^3$ H]myristoyl-CoA (61 Ci/mmol), 5-1000  $\mu$ M peptide, 40 mM Tris-HCl (pH 7.4),  
202 0.5 mM EGTA, 4.5 mM 2-mercaptoethanol, and 0.5% Triton X-100.

203 • The reaction is initiated by the addition of NMT diluted in 50 mM Tris-HCl, pH 8.0, 0.1%  
204 Triton X-100, 0.1 mg/mL BSA.

205 • Various incubation times are performed (i.e., for 3, 6, and 9 min) to check and deduce initial  
206 velocities.

207 • 0 min time points together with samples performed in the absence of peptide or enzyme are  
208 mandatory to assess background signal.

209 • Reactions are stopped by filtration through P81 phospho-cellulose (Whatman, no longer  
210 available) or LSA-50 paper (Appelmans et al., 2021). The filters are then dried using a hair  
211 drier for 30 s, washed three times with 25 mM Tris-HCl, pH 7.3, and finally further dried.  
212 Radioactivity is detected by scintillation counting after addition of the scintillation solution  
213 (OptiScint HiSafe; Perkin-Elmer).

214 • Radioactivity-based kinetic assays are no longer recommended for various reasons including  
215 product availability (radioactive myristoyl-CoA is no longer commercially available to our  
216 knowledge and must be prepared; see §2.3.2), time consumption (due, for instance, to  
217 discontinuous monitoring), cost, radioprotection/safety rules, and waste management of long-  
218 lasting radiolabels such as  $^3$ H and  $^{14}$ C.

### 219 3.2.2 HPLC assays

220 Discontinuous assays based on high-performance liquid chromatography (HPLC) analysis  
221 of the reaction of NMT with its substrates are available. Reaction product analysis and

222 quantification are based on the differential retention times of the acylated and unmodified  
223 peptides. The reaction is usually performed in 100  $\mu\text{L}$  volumes and then quenched with one  
224 volume of acetonitrile. After centrifugation (20,000  $\times$  g, 10 min, 4°C), 190  $\mu\text{L}$  of the  
225 supernatant is mixed to 10  $\mu\text{L}$  trifluoroacetic acid (TFA) prior to HPLC loading. Analysis is  
226 achieved on C18 reverse-phase columns (100  $\text{\AA}$ ; 150  $\times$  24.6 mm) with acetonitrile inverse (100  
227 to 0%) gradient elution in 0.1% TFA.

228 The assay was originally coupled with radioactive quantification of the collected fractions  
229 (Rudnick, Duronio, & Gordon, 1992). One main advantage of HPLC analysis over the assay  
230 based on paper filtration is that the peptide sequence does not need to be modified by adding a  
231 basic tag and that actual sequences can be directly challenged without any bias.

232 The HPLC assay can also be used in a non-radioactive manner by monitoring absorbance  
233 intensities at 280 nm if a tryptophan tag is added to the sequence. Tryptophan has a very high  
234 molar absorption coefficient at 280 nm ( $\epsilon_{280}=5690 \text{ M}^{-1}\cdot\text{cm}^{-1}$ ). For instance, the  
235 GKVLSKIFWW sequence was used for ARF6 kinetic analysis with HsNMTs (Kosciuk et al.,  
236 2020; Su et al., 2021). Tryptophan tagging obviously mitigates the main advantage of the  
237 HPLC assay. It also significantly adds hydrophobicity to the peptide, increasing problems with  
238 solubilization. Monitoring absorbance at 210-220 nm – a range arising from the peptide bonds  
239 - along the HPLC profile should reveal the reaction products. Although less sensitive ( $\epsilon_{210}\sim 100$   
240  $\text{M}^{-1}\cdot\text{cm}^{-1}$ ), such detection is in keeping with the analysis of genuine peptides. Finally, product  
241 and substrate detection and characterization of the unmodified peptides can also be monitored  
242 by coupling liquid chromatography with mass spectrometry. Quantification of both substrates  
243 and products may be achieved by taking advantage of the total ion chromatograms associated  
244 with each species (Kosciuk et al., 2020).

245

### 246 3.3 Continuous assays

247 Continuous assays have many advantages (speed, reliability, sensitivity, automatization,  
248 high-throughput) but need to be conducted with great care and mindful of several caveats (see  
249 §4.3.3). For instance, an NMT assay based on 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's  
250 reagent, DTNB) thiol reactivity with CoA – the second product of the reaction – is  
251 accompanied by enzyme inhibition (Rudnick et al., 1992). Some multi-coupled assays aiming  
252 to detect CoA by following NADPH-coupled formation have been established (Farazi,  
253 Manchester, & Gordon, 2000). Here we describe a robust assay based on a unique coupling  
254 enzyme, pyruvate dehydrogenase (PDH), and NADH detection (Boisson & Meinnel, 2003;  
255 Traverso et al., 2013a).

#### 256 3.3.1 NMT activity coupling conditions

257 NMT is assayed by continuously monitoring the formation of NADH by either absorbance  
258 at 340 nm or fluorescence ( $\lambda_{exc}=340$  nm,  $\lambda_{em}=465$  nm) in a coupled assay exploiting PDH  
259 activity (**Fig. 1B**). Coupling with PDH takes advantage of the second product of the reaction  
260 (CoA) to transform NAD into NADH. Signal detection originates from the high  
261 hyperchromicity of NADH at 340 nm with respect to NAD. Given the coupling scheme, one  
262 NADH molecule produced corresponds to one polypeptide acylation. The coupling does not  
263 provide direct information on the actual location of the modification on the polypeptide. MS  
264 approaches involving MS/MS modes are required for this purpose (**Fig. 2**, see §4.2, Chapter  
265 22, this volume (Monassa et al., 2023)).

266 • NMT activity is assayed at 30°C in a reaction mixture containing 50 mM Tris-HCl (pH 8.0),  
267 1 mM MgCl<sub>2</sub>, 0.193 mM EGTA, 0.32 mM DTT, 0.2 mM TPP, 2 mM pyruvate, 0.1 mg/mL  
268 BSA, 0.1% Triton X-100, 2.5 mM NAD<sup>+</sup>, 0.125 units/mL porcine heart PDH (0.33 units/mg),  
269 40 μM myristoyl-CoA, and 1-2000 μM peptide. A final volume of 100-200 μL is used in 96-

270 well plates. The reaction mixture is pre-incubated for 3 min at 30°C before starting the reaction  
271 by adding myristoyl-CoA (20 µL in a final volume of 100 µL from the 200 µM stock). These  
272 parameters avoid problems arising from the solubility of the acyl-CoA derivative.

273 • The following parameters are given to the plate reader: temperature,  $30 \pm 0.5^\circ\text{C}$ ; excitation  
274 wavelength, 340 nm (bandwidth 9 nm); emission wavelength, 465 nm (bandwidth 20 nm);  
275 reading mode, top; integration time, 20 µs; gain value, 100; sampling, 30 points per min  
276 (sequential mode), 3.3 points per min (parallel mode); number of reads, 25 (number of  
277 measures per well per cycle) for parallel mode, 3 for sequential mode, shaking before  
278 measurements, 3 sec (orbital) at 57 rpm.

### 279 3.3.2 Acylation kinetics

280 • NMT acylation reaction kinetics are monitored for 5-60 min, and the data are fitted to obtain  
281 the initial velocities associated with each peptide concentration. Initial velocities need to be  
282 carefully determined; due to establishing the coupling conditions over the very first time points,  
283 they can be underestimated and should not be taken into account before the coupling setting is  
284 properly balanced. Initial velocities are then measured with dozens of linear points. A value of  
285  $6300 \text{ M}^{-1}\cdot\text{s}^{-1}$  is used as the molecular extinction coefficient of NADH at 340 nm for the  
286 absorbance assay. Initial rate values ( $v_i$ ) expressed in  $\text{s}^{-1}$  can be calculated as follows:  $v_i =$   
287  $\text{slope}/(60*261*[\text{NMT}])$ ; the slope is in RFU/min and [NMT] is the final concentration of NMT  
288 in µM.

289 • Curve fits to obtain kinetic parameters are achieved by non-linear regression with GraphPad  
290 Prism 9.5.0 (GraphPad Software). Parameters with standard errors are computed for all  
291 parameters using the complete dataset including replicates. Both  $k_{\text{cat}}$  and  $K_{\text{m}}$  kinetic parameters  
292 are obtained by fitting to the Michaelis-Menten equation. A typical fit with three replicates is  
293 given in **Fig. 3A**. Fitting may use all values taken together or separately (**Fig. 3B**, out- and in-

294 sets). Averages and standard deviations (SDs) of the kinetic parameters are either the result of  
295 the fit with all values or from classic averaging approaches from different fits.  $k_{cat}/K_m$  values  
296 and the associated SDs can also be obtained by taking advantage of the  $k_{SP}$  approach (Johnson,  
297 2019) with  $k_{SP}=k_{cat}/K_m$  and  $v_0/[E]=k_{SP}[S]/(1+[S]/K_m)$ , where  $v_0$  is the reaction rate measured  
298 at NMT concentration  $[E_0]$ .  $k_{cat}/K_m$  SD can also be calculated as follows from mean values of  
299  $k_{cat}$  and  $K_m$  and their associated SDs:  $SD(k_{cat}/K_m)=k_{cat}/K_m[(SD(K_m)/K_m)^2+(SD(k_{cat})/k_{cat})^2]^{1/2}$ .

### 300 3.3.3 Notes

301 ● NMTs use a sequential ordered Bi-Bi mechanism involving binding to the CoA derivative  
302 first and then to the peptidyl target (Rocque, Mcwherter, Wood, & Gordon, 1993; Rudnick et  
303 al., 1991; Salah Ud-Din et al., 2016).

304 ● PDH-specific activity is sensitive to pH and only effective for coupling in the pH 7.5-9.0  
305 range. The optimum pH of ScNMTs is 7.5-8.5, and HsNMT1 is only very active in the pH 7.0-  
306 9.0 range (Rocque et al., 1993). pH 8.0 is optimal for both PDH and NMT activities (**Fig. 4A**).

307 ● Compared with Triton X-100, cholate reduces the NMT rate (**Fig. 4B**). Triton X-100 is  
308 therefore preferred for kinetic studies, while cholate is better suitable to MALDI approaches  
309 (see Chapter 22, this volume (Monassa et al., 2023)).

310 ● Triton X-100 can be avoided when myristoyl-CoA is added to trigger the reaction if dissolved  
311 first in 50% DMSO as a 10 mM solution. The final DMSO concentration (0.2%) is low enough  
312 to not alter kinetics. This avoids the production of small bubbles that perturb signal monitoring,  
313 especially when using microinjectors.

314 ● NADH fluorescence is often considered to be one magnitude more sensitive than absorbance  
315 (Rodriguez-Aparicio, Reglero, Martinez-Blanco, & Luengo, 1991). A main advantage of  
316 fluorescence assays is that they allow the determination of low  $K_m$  values, i.e., when their  
317 values are less than 10  $\mu$ M. However, we have observed with the most recent PDH batches that

318 the signal-to-noise ratio is better with the absorbance-based assay. Unless the  $K_m$  value is lower  
319 than 10  $\mu\text{M}$ , we now prefer to use the absorbance assay.

320 • **Fig. 3A** shows a representative fluorescence measurement series with the velocity in  
321 RFU/min as a function of peptide concentration for one enzyme concentration. Curve fitting to  
322 the Michaelis-Menten equation needs background velocity subtraction from each measurement  
323 (see below). **Fig. 3B** shows a representative initial velocity relative to the peptide concentration  
324 (expressed in  $\text{s}^{-1}$ ).

325 • Due to the coupling reaction, the initial velocity of NMT needs to be rate-limiting. This is  
326 achieved if PDH is in sufficient excess (i.e., its steady-state rate is significantly higher than that  
327 of NMT). For absorbance, we determined a 20  $\mu\text{M}/\text{min}$  limit to the initial velocity measurement  
328 (i.e., 0.13  $A_{340}/\text{min}$  if the optical path is 1 cm). For fluorescence, we determined a similar limit  
329 to the initial velocity measurement (i.e., 5,000 RFU/min, where RFU is relative fluorescence  
330 unit). Exceeding this value, PDH is rate-limiting under our standard assay conditions, and NMT  
331 must be diluted. The PDH concentration can be increased, but it is not recommended. In any  
332 case, this needs to be experimentally verified in any new setting or with any new PDH batch.

333 • In the fluorescence assay, concentrations of produced NADH higher than 30,000 RFU (115  
334  $\mu\text{M}$ ) promote a non-linear response due to the inner filter effect of NADH at 340 nm.  
335 Considering that 40  $\mu\text{M}$  myristoyl-CoA and 100  $\mu\text{M}$  acetyl-CoA correspond to standard  
336 conditions, this issue only becomes relevant if these concentrations are increased above 115  
337  $\mu\text{M}$ .

338 • The continuous assay is suited to high-throughput measurements in 96-well plates by  
339 equipping the plate reader with microinjectors. Each well must first be filled with, for example,  
340 a peptide sample with a multichannel pipette, which needs to be different or at different  
341 concentrations. Next, microinjector A is used to fill each well with the reaction mixture but not  
342 myristoyl-CoA. Microinjector B is used for robotic injection of myristoyl-CoA to start the

343 reaction. This allows multiple parallel kinetic acquisitions in short timeframes (**Fig. 3C**). Note  
344 that the dead volume of microinjectors is significant and it is therefore not recommended to  
345 use microinjectors to inject NMT directly if used at high concentrations and only available in  
346 limited amounts.

347 ● The final concentration of myristoyl-CoA in the assay is 40  $\mu\text{M}$ , which is the upper limit of  
348 solubility in the reaction mixture. This explains why, unusually, it is added to start the reaction,  
349 as it avoids intermediate higher concentrations. In its absence, because of the ordered Bi-Bi  
350 mechanism, the complex is not formed with the peptide. This myristoyl-CoA concentration is  
351 saturating for both ScNMT and AtNMT1 (Boisson & Meinel, 2003) but not for HsNMTs,  
352 where the  $K_m$  values are one order of magnitude higher, i.e., 15  $\mu\text{M}$  (Kishore et al., 1993).

353 ● The 40  $\mu\text{M}$  concentration of myristoyl-CoA limits the signal range to a maximum of 0.25  
354  $A_{340}$  per cm optical path when the peptide concentration is over 40  $\mu\text{M}$ . When the peptide  
355 concentration is lower, the maximum range is directed by the peptide concentration itself. This  
356 needs to be taken into account in any analysis of the initial velocity slopes. With acetyl-CoA  
357 as the CoA donor, we use a 100  $\mu\text{M}$  concentration, which can be extended to 150  $\mu\text{M}$  (Asensio  
358 et al., 2022).

359 ● Examples of kinetics and initial velocity curves are given in **Fig. 5**.

360 ● When the NMT rate is low, the steady state is not immediately established and requires some  
361 time. Deriving the initial velocity therefore sometimes requires removing the first data points  
362 when the reaction is still being established (see **Fig. 5A,B**). This is classic in coupling reactions.

363 ● In any case, the initial velocity ( $v_i$ ) measured in the absence of peptide substrate is not zero  
364 (usually  $<50 \text{ RFU}\cdot\text{min}^{-1}$  or  $0.001 A_{340}/\text{min}$ ) and needs to be subtracted from the measured rate  
365 value (see **Fig. 5A**). This background partly comes from PDH, and its amplitude varies from  
366 one batch to another, eventually promoting slow NAD conversion into NADH and the signal



367 increases with incubation time. A reliable kinetic rate therefore needs to be significantly higher  
368 than this value. This can be achieved by increasing the enzyme concentration.

369 • When using microplates, as zeroing is not feasible for each well (see **Fig. 5A**), we  
370 recommend monitoring the kinetics in the absence of one component (e.g., NMT) for 15-30  
371 minutes to determine both the intrinsic signal of each well and the associated inner drift. This  
372 likely involves slow CoA release due to chemical myristoyl- or acetyl-CoA hydrolysis in the  
373 slightly basic reaction buffer. The reader is then put on hold. After starting the kinetics with  
374 the missing component, the kinetics are then started again and monitored. This also allows the  
375 linking of the blank value determination with the kinetic signal and avoids any wrong  
376 interpretation if the kinetics are too fast, for instance. A typical procedure is shown in **Fig. 5A**.

377 • When the reaction is started with myristoyl-CoA, the slope of the reaction measured without  
378 the peptide is subtracted from those achieved in the presence of increasing peptide  
379 concentrations (**Fig. 5A**).

380 • We have noticed that initial velocities smaller than  $0.001 \mu\text{M}\cdot\text{s}^{-1}$  are difficult to precisely  
381 assess in routine practice. The  $K_m$  value range of most peptides measured to date is 5-200  $\mu\text{M}$ .

### 382 **3.4 Catalytic efficiencies of various substrates: ranges and meanings**

#### 383 *3.4.1 CoA donors*

384 Myristoyl-CoA is the best acyl donor. Other fatty acids can be used *in vitro* by NMT  
385 but with lesser efficiency. Myristate (C14:0) is a rather short fatty acid, and the dedicated fatty  
386 acyl binding site (pocket 1; (Meinzel et al., 2020)) acts as a sieve to prevent longer chains from  
387 efficiently binding to HsNMT1 (see Figure 5A in (Kishore et al., 1993)). Shorter fatty acids  
388 like C12:0 may bind, but the tension of the reactive thiolate between CoA and the fatty acid is  
389 important for achieving the reactive state. It has been reported that fatty acid derivatives shorter  
390 than C10 are not transferred onto protein targets.

391           Recent data have nevertheless shown that acetyl-CoA (a C2 derivative) can be a human  
392 NMT substrate (Su et al., 2021) with efficiency *in vitro* almost as strong as myristoyl-CoA.  
393 Specifically, the authors found that the catalytic efficiency ( $k_{cat}/K_m$ ) for the CoA derivatives  
394 using ARF6 as the acylated peptide measured using an HPLC end-point assay were almost  
395 similar (See Table 1 in (Su et al., 2021)). The kinetic assay we report here in §3.3 was recently  
396 used for the assessment of GNAT activities using acetyl-CoA as the donor (Asensio et al.,  
397 2022). Using this kinetic assay to examine this reaction, we also observe this unexpected  
398 behavior of NMT, but with a catalytic efficiency two orders of magnitude lower than that  
399 observed in the previous report (**Fig. 6A-C**). This discrepancy comes from both the  $K_m$  and the  
400  $k_{cat}$  values, which can be challenged by comparing the  $k_{cat}/K_m$  values relative to each substrate  
401 with both HsNMT1 and HsNMT2 (**Fig. 6D**). We also observed that the kinetic values with  
402 various peptides including ARF6 were again significantly different, and the reason for these  
403 inconsistencies is unknown. It might be due to the presence of the di-tryptophan tag or the  
404 different temperatures used, i.e., 30°C vs 37°C. Regardless, MALDI MS/MS mass  
405 spectrometry unambiguously demonstrates full conversion and unambiguous acetylation of the  
406 N-terminus of the peptide substrate (**Fig. 2C,D**). Note that the MS/MS spectrum does not  
407 demonstrate in this case whether it involves Gly- or Lys-acetylation. An indirect proof of Gly-  
408 over Lys-acetylation may come from the study of the N-terminal acetylated version of the same  
409 peptide and demonstration that it cannot be acetylated further. This is the case for ARF6  
410 acetylation, as the *ac*-ARF6 peptide - a peptide known to be acylated on the Lys amino group  
411 by HsNMT1 (Rivière et al., 2022) - is not acetylated by HsNMT1.

412           Finally, the physiological significance of the NMT-associated acetylation reaction *in*  
413 *cellulo* needs to be cautiously interpreted, as recently discussed (Meinzel, 2022). This is all the  
414 more true if kinetic data also contribute to disfavor - as we observe - acetyl-CoA over  
415 myristoyl-CoA.

### 416 3.4.2 Polypeptide acceptor donor

417 • Octapeptides are optimal and fully recapitulate not only the selectivity of NMT for Gly-  
418 myristoylation but also the kinetic parameters of a full-length protein (Boisson, Giglione, &  
419 Meinnel, 2003; Boisson & Meinnel, 2003; Rivière et al., 2022). In all cases, the kinetic  
420 parameters of Gly-myristoylation are remarkably similar to those of the full-length protein.

421 • Gly-myristoylation turnover number is low ( $\sim 0.1 \text{ s}^{-1}$ ); this is the “price to pay” for the high  
422 selectivity of NMTs.  $K_m$  values of polypeptide substrates are in turn low (10  $\mu\text{M}$  range) and  
423 are representative of the binding constant of the peptide (Meinnel, 2022).

424 • The catalytic efficiencies of Gly-myristoylation are on average of the order of  $10^3\text{-}10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$ .  
425 Nevertheless, the overall values obtained with octapeptides derived from genuine protein  
426 substrates which myristoylation was observed *in cellulo* is large, ranging from 10 to  $10^5 \text{ M}^{-1}\cdot\text{s}^{-1}$   
427 (Castrec et al., 2018).

428 • Lys-myristoylation by NMT was recently shown to occur with a number of sequences  
429 provided they display the XKyySKy motif (Rivière et al., 2022), where X corresponds to 0-3  
430 amino acids and y is any amino acid; the first Lys side chain is the recipient of the  
431 myristoylation (Dian et al., 2020; Kosciuk et al., 2020). To date, only ARF6 has been shown  
432 *in cellulo* to undergo secondary Lys-myristoylation on Lys3, but it needs first to undergo Gly-  
433 myristoylation on Gly2. The authors proposed that the Gly-myristoyl side chain can be  
434 embedded within the water channel to allow the second myristoyl-CoA to react (Kosciuk et  
435 al., 2020). A typical fit of Lys-myristoylation reactivity is shown in **Fig. 5D** with an N-  
436 acetylated version of ARF6. Catalytic performances of all tested Lys-myristoylation substrates  
437 are poor compared with Gly-myristoylation, mostly due to the very slow turnover number ( $k_{cat}$ )  
438 value (Rivière et al., 2022), which is two orders of magnitude lower and explains the 100-fold  
439 lower catalytic efficiencies of the two reactions. In fact, when the two reactions are feasible,

440 like for ARF6, Gly-myristoylation is always favored. A search for further substrates in humans  
441 failed (Rivière et al., 2022). For instance, peptide MKLRSKAA, derived from the N-terminus  
442 of human glucoside xylosyltransferase 2 (GXLT2), is not an HsNMT1 substrate *in vitro*.

443

#### 444 **4. Conclusions**

445 In addition to the steady-state kinetic analysis presented in this chapter, pre-steady-state  
446 studies of NMTs using fast kinetic approaches requiring fluorescence-based stopped- or  
447 quenched-flow apparatus are available to derive finer details of the chemical mechanism and  
448 constants of NMTs (Farazi et al., 2000). This has provided the kinetic mechanism of ScNMT  
449 (see Figure 8 in (Farazi et al., 2000)). HsNMT and ScNMT share a similar mechanism (Rocque  
450 et al., 1993). A synthetic picture of the HsNMT catalytic mechanism is available (see Figure 1  
451 in (Meinzel, 2022)). A unique feature of NMTs is that the rate limiting step of the reaction  
452 corresponds to the dissociation of the acylated peptide product ( $0.1 \text{ s}^{-1}$ ), whereas the chemical  
453 transformation is fast ( $>10 \text{ s}^{-1}$ ). This involves a conformational change (Dian et al., 2020).

454 Recent kinetic studies of NMTs have provided evidence that NMTs are very tightly linked  
455 to the GNAT family, not only through structural similarities of the common active site fold but  
456 also by their unique capacity to use acyl-CoA (including acetyl-CoA derivatives) on various  
457 amine reactive acceptor groups. This definitely integrates NMT - a previously unique outlier -  
458 within the GNAT family at the biochemical level. Another close example of similar  
459 performance is provided by a new family of plastid GNATs (Bienvenut et al., 2020; Giglione  
460 & Meinzel, 2021).

461

#### 462 **Acknowledgements**

463 We acknowledge Benoit Castrec, Sara Ciccone, Rémy F. Dutheil, and José A. Traverso  
464 for their respective inputs in the setting of the various approaches presented in this manuscript.

465

#### 466 **Funding**

467 This work was supported by French National Research Agency (ANR) DynaMYT (ANR-  
468 20-CE44-0013) and Fondation ARC (ARCPJA32020060002137) grants to TM. This work has  
469 benefited from the support of a French State grant (ANR-17-EUR-0007, EUR SPS-GSR)  
470 managed by the ANR under an Investments for the Future program (ANR-11-IDEX-0003-02).  
471 FR was supported by grants from Région Ile-de-France (17012695) and Fondation pour la  
472 Recherche Médicale (FDT202001010779).

473

#### 474 **References**

- 475 Appelmans, O., Kashyap, R. S., Gilles, P., De Borggraeve, W. M., Voet, A., & Van Lint, J.  
476 (2021). LSA-50 paper: An alternative to P81 phosphocellulose paper for radiometric  
477 protein kinase assays. *Analytical Biochemistry*, *630*, 114313.
- 478 Asensio, T., Dian, C., Boyer, J. B., Rivière, F., Meinnel, T., & Giglione, C. (2022). A  
479 continuous assay set to screen and characterize novel protein N-acetyltransferases  
480 unveils rice General Control Non-repressible 5-Related N-Acetyltransferase 2 activity.  
481 *Frontiers in Plant Science*, *13*, 832144.
- 482 Beauchamp, E., Yap, M. C., Iyer, A., Perinpanayagam, M. A., Gamma, J. M., Vincent, K. M.,  
483 et al. (2020). Targeting N-myristoylation for therapy of B-cell lymphomas. *Nature*  
484 *Communications*, *11*, 5348.
- 485 Bhatnagar, R. S., Ashrafi, K., Futterer, K., Waksman, G., & Gordon, J. I. (2001). Biology and  
486 enzymology of protein N-myristoylation. In F. Tamanoi & D. S. Sigman (Eds.), *The*  
487 *Enzymes* (Vol. XXI (Protein lipidation), pp. 241-286). San Diego: Academic Press.

488 Bhatnagar, R. S., Futterer, K., Farazi, T. A., Korolev, S., Murray, C. L., Jackson-Machelski,  
489 E., et al. (1998). Structure of N-myristoyltransferase with bound myristoylCoA and  
490 peptide substrate analogs. *Nature Structural Biology*, *5*, 1091-1097.

491 Bienvenut, W. V., Brünje, A., Boyer, J.-B., Mühlenbeck, J. S., Bernal, G., Lassowskat, I., et  
492 al. (2020). Dual lysine and N-terminal acetyltransferases reveal the complexity  
493 underpinning protein acetylation. *Molecular Systems Biology*, *16*, e9464.

494 Boisson, B., Giglione, C., & Meinnel, T. (2003). Unexpected protein families including cell  
495 defense components feature in the N-myristoylome of a higher eukaryote. *Journal of*  
496 *Biological Chemistry*, *278*, 43418-43429.

497 Boisson, B., & Meinnel, T. (2003). A continuous assay of myristoyl-CoA:protein N-  
498 myristoyltransferase for proteomic analysis. *Analytical Biochemistry*, *322*, 116-123.

499 Castrec, B., Dian, C., Ciccone, S., Ebert, C. L., Bienvenut, W. V., Le Caer, J.-P., et al. (2018).  
500 Structural and genomic decoding of human and plant myristoylomes reveals a  
501 definitive recognition pattern. *Nature Chemical Biology*, *14*, 671-679.

502 Dian, C., Pérez-Dorado, I., Rivière, F., Asensio, T., Legrand, P., Ritzefeld, M., et al. (2020).  
503 High-resolution snapshots of human N-myristoyltransferase in action illuminate a  
504 mechanism promoting N-terminal Lys and Gly myristoylation. *Nature*  
505 *Communications*, *11*, 1132.

506 Farazi, T. A., Manchester, J. K., & Gordon, J. I. (2000). Transient-state kinetic analysis of  
507 *Saccharomyces cerevisiae* myristoylCoA:protein N-myristoyltransferase reveals that a  
508 step after chemical transformation is rate limiting. *Biochemistry*, *39*, 15807-15816.

509 Frearson, J. A., Brand, S., McElroy, S. P., Cleghorn, L. A., Smid, O., Stojanovski, L., et al.  
510 (2010). N-myristoyltransferase inhibitors as new leads to treat sleeping sickness.  
511 *Nature*, *464*, 728-732.

512 Giglione, C., & Meinnel, T. (2021). Evolution-driven versatility of N terminal acetylation in  
513 photoautotrophs. *Trends in Plant Science*, 26, 375-391.

514 Giglione, C., & Meinnel, T. (2022). Mapping the myristoylome through a complete  
515 understanding of protein myristoylation biochemistry. *Progress in Lipid Research*, 85,  
516 101139.

517 Johnson, K. A. (2019). New standards for collecting and fitting steady state kinetic data.  
518 *Beilstein J Org Chem*, 15, 16-29.

519 Kaplan, J. M., Mardon, G., Bishop, J. M., & Varmus, H. E. (1988). The first seven amino acids  
520 encoded by the v-src oncogene act as a myristylation signal: lysine 7 is a critical  
521 determinant. *Molecular and Cellular Biology*, 8, 2435-2441.

522 King, M. J., & Sharma, R. K. (1991). N-myristoyl transferase assay using phosphocellulose  
523 paper binding. *Analytical Biochemistry*, 199, 149-153.

524 Kishore, N. S., Wood, D. C., Mehta, P. P., Wade, A. C., Lu, T., Gokel, G. W., et al. (1993).  
525 Comparison of the acyl chain specificities of human myristoyl-CoA synthetase and  
526 human myristoyl-CoA:protein N-myristoyltransferase. *Journal of Biological*  
527 *Chemistry*, 268, 4889-4902.

528 Kosciuk, T., & Lin, H. (2020). N-Myristoyltransferase as a glycine and lysine  
529 myristoyltransferase in cancer, immunity, and infections. *ACS Chem Biol*, 15, 1747-  
530 1758.

531 Kosciuk, T., Price, I. R., Zhang, X., Zhu, C., Johnson, K. N., Zhang, S., et al. (2020). NMT1  
532 and NMT2 are lysine myristoyltransferases regulating the ARF6 GTPase cycle. *Nature*  
533 *Communications*, 11, 1067.

534 Lodge, J. K., Johnson, R. L., Weinberg, R. A., & Gordon, J. I. (1994). Comparison of  
535 myristoyl-CoA:protein N-myristoyltransferases from three pathogenic fungi:

536 Cryptococcus neoformans, Histoplasma capsulatum, and Candida albicans. *Journal of*  
537 *Biological Chemistry*, 269, 2996-3009.

538 Martin, D. D., Beauchamp, E., & Berthiaume, L. G. (2011). Post-translational myristoylation:  
539 Fat matters in cellular life and death. *Biochimie*, 93, 18-31.

540 Meinnel, T. (2022). Comment on “Binding affinity determines substrate specificity and enables  
541 discovery of substrates for N-Myristoyltransferases”. *ACS Catalysis*, 12, 8195-8201.

542 Meinnel, T., Dian, C., & Giglione, C. (2020). Myristoylation, an ancient protein modification  
543 mirroring eukaryogenesis and evolution. *Trends in Biochemical Sciences*, 45, 619-632.

544 Monassa, P., Rivière, F., Dian, C., Frottin, F., Giglione, C., & Meinnel, T. (2023). Biochemical  
545 and structural analysis of N-myristoyltransferase-mediated protein tagging. *Methods in*  
546 *Enzymology*, 686, Chapter 22.

547 Mousnier, A., Bell, A. S., Swieboda, D. P., Morales-Sanfrutos, J., Perez-Dorado, I., Brannigan,  
548 J. A., et al. (2018). Fragment-derived inhibitors of human N-myristoyltransferase block  
549 capsid assembly and replication of the common cold virus. *Nature Chemistry*, 10, 599-  
550 606.

551 Pierre, M., Traverso, J. A., Boisson, B., Domenichini, S., Bouchez, D., Giglione, C., et al.  
552 (2007). N-Myristoylation regulates the SnRK1 pathway in Arabidopsis. *The Plant Cell*,  
553 19, 2804-2821.

554 Price, H. P., Menon, M. R., Panethymitaki, C., Goulding, D., McKean, P. G., & Smith, D. F.  
555 (2003). Myristoyl-CoA:protein N-myristoyltransferase, an essential enzyme and  
556 potential drug target in kinetoplastid parasites. *Journal of Biological Chemistry*, 278,  
557 7206-7214.

558 Raju, R. V., & Sharma, R. K. (1999). Preparation and assay of myristoyl-CoA:protein N-  
559 myristoyltransferase. *Methods in Molecular Biology*, 116, 193-211.



560 Renna, L., Stefano, G., Majeran, W., Micaella, C., Meinel, T., Giglione, C., et al. (2013).  
561 Golgi traffic and integrity depend on N-myristoyl transferase-1 in Arabidopsis. *The*  
562 *Plant Cell*, 25, 1756-1773.

563 Resh, M. D. (2006). Trafficking and signaling by fatty-acylated and prenylated proteins.  
564 *Nature Chemical Biology*, 2, 584-590.

565 Rivière, F., Dian, C., Dutheil, R. F., Monassa, P., Giglione, C., & Meinel, T. (2022). Structural  
566 and large-scale analysis unveil the intertwined paths promoting NMT-catalyzed lysine  
567 and glycine myristoylation. *Journal of Molecular Biology*, 434, 167843.

568 Rocque, W. J., McWherter, C. A., Wood, D. C., & Gordon, J. I. (1993). A comparative analysis  
569 of the kinetic mechanism and peptide substrate specificity of human and  
570 *Saccharomyces cerevisiae* myristoyl-CoA:protein N-myristoyltransferase. *Journal of*  
571 *Biological Chemistry*, 268, 9964-9971.

572 Rodriguez-Aparicio, L. B., Reglero, A., Martinez-Blanco, H., & Luengo, J. M. (1991).  
573 Fluorometric determination of phenylacetyl-CoA ligase from *Pseudomonas putida*: a  
574 very sensitive assay for a newly described enzyme. *Biochimica et Biophysica Acta*,  
575 1073, 431-433.

576 Rudnick, D. A., Duronio, R. J., & Gordon, J. I. (1992). Methods for studying myristoyl-  
577 coenzyme A: protein N-myristoyltransferase. In N. M. Hooper & A. J. Turner (Eds.),  
578 *Lipid modification by proteins. A practical approach* (pp. 37-61). Oxford: IRL Press.

579 Rudnick, D. A., McWherter, C. A., Rocque, W. J., Lennon, P. J., Getman, D. P., & Gordon, J.  
580 I. (1991). Kinetic and structural evidence for a sequential ordered Bi Bi mechanism of  
581 catalysis by *Saccharomyces cerevisiae* myristoyl-CoA:protein N-myristoyltransferase.  
582 *Journal of Biological Chemistry*, 266, 9732-9739.

583 Salah Ud-Din, A. I., Tikhomirova, A., & Roujeinikova, A. (2016). Structure and functional  
584 diversity of GCN5-related N-acetyltransferases (GNAT). *International Journal of*  
585 *Molecular Sciences*, *17*, E1018.

586 Su, D., Kosciuk, T., Yang, M., Price, I. R., & Lin, H. (2021). Binding affinity determines  
587 substrate specificity and enables discovery of substrates for N-myristoyltransferases.  
588 *ACS Catalysis*, *11*, 14877-14883.

589 Thinon, E., Serwa, R. A., Broncel, M., Brannigan, J. A., Brassat, U., Wright, M. H., et al.  
590 (2014). Global profiling of co- and post-translationally N-myristoylated proteomes in  
591 human cells. *Nature Communications*, *5*, 4919.

592 Towler, D. A., Adams, S. P., Eubanks, S. R., Towery, D. S., Jackson-Machelski, E., Glaser,  
593 L., et al. (1987). Purification and characterization of yeast myristoyl CoA:protein N-  
594 myristoyltransferase. *Proceedings of the National Academy of Sciences*, *84*, 2708-2712.

595 Traverso, J. A., Giglione, C., & Meinnel, T. (2013a). High-throughput profiling of N-  
596 myristoylation substrate specificity across species including pathogens. *Proteomics*, *13*,  
597 25-36.

598 Traverso, J. A., Micalella, C., Martinez, A., Brown, S. C., Satiat-Jeunemaitre, B., Meinnel, T.,  
599 et al. (2013b). Roles of N-Terminal fatty acid acylations in membrane compartment  
600 partitioning: Arabidopsis h-type thioredoxins as a case study. *The Plant Cell*, *25*, 1056-  
601 1077.

602 Wright, M. H., Clough, B., Rackham, M. D., Rangachari, K., Brannigan, J. A., Grainger, M.,  
603 et al. (2014). Validation of N-myristoyltransferase as an antimalarial drug target using  
604 an integrated chemical biology approach. *Nature Chemistry*, *6*, 112-121.

605 Wright, M. H., Paape, D., Price, H. P., Smith, D. F., & Tate, E. W. (2016). Global profiling  
606 and inhibition of protein lipidation in vector and host stages of the sleeping sickness  
607 parasite *Trypanosoma brucei*. *ACS Infectious Diseases*, *2*, 427-441.

608

## 609 **Figure Legends**

610 Fig. 1. Reactions catalyzed by the main enzymes useful for NMT studies. (A) NMT. (B) PDH.

611

612 Fig. 2. MALDI MS characterization of the modification state of an NMT substrate. MALDI-

613 TOF/TOF analysis of the GK peptide upon NMT acetylation. (A) MS analysis of the GK

614 peptide. (B) MS analysis of the HsNMT1-acetylated GK peptide. The 42 Da increment

615 compared with panel A is noticeable. (C) MS/MS analysis of the non-modified GK peptide.

616 (D) MS/MS analysis of the NMT-acetylated GK peptide.

617

618 Fig. 3. Continuous fluorescent kinetic analysis of Gly- and Lys-myristoylation by NMT. (A)

619 Rough initial velocity of myristoylation by AtNMT1 (expressed as RFU/min) as a function of

620 SOS3 peptide concentration. Three replicates are displayed. The background velocity in the

621 absence of peptide substrate (or of NMT) as shown here is 50 RFU/min. After subtracting the

622 background velocity kinetic, which cannot be neglected, parameters can be calculated. Here,

623  $V_{max} = 689 \pm 26$  RFU/min;  $K_m = 18.0 \pm 2.6$   $\mu$ M. (B) Example of a classic kinetic analysis - here

624 of ARF6 Gly-myristoylation with HsNMT1 - showing the different modes of fitting, either

625 with all three replicates together or with distinct fits with each replicate (inset). Here, the values

626 are  $K_m = 7.8 \pm 2.2$   $\mu$ M,  $k_{cat} = 0.032 \pm 0.002$   $s^{-1}$ ,  $k_{cat}/K_m = 4121 \pm 948$   $M^{-1} \cdot s^{-1}$ . (C) Example of a high-

627 throughput analysis of a 96-sample with a plate reader. The injectors allow robotic starts and

628 precise timing. The inset shows the overlay of all kinetics. Initial velocities need to be measured

629 at the early, linear stages. (D) Kinetics of Lys-myristoylation of *ac*-ARF6 showing the 2-order

630 of magnitude decrease in turnover number compared with Gly-myristoylation. Here,

631  $K_m = 17.0 \pm 4.8$   $\mu$ M,  $k_{cat} = 0.00078 \pm 0.00004$   $s^{-1}$ ,  $k_{cat}/K_m = 43 \pm 7$   $M^{-1} \cdot s^{-1}$ .

632

633 Fig. 4. Impact of various biochemical features on PDH and NMT activities. Gly-myristoylation  
634 of NMT was studied with peptide SOS3. (A) Impact of pH on PDH activity (grey) and ScNMT  
635 activity (data from Table 1 in (Towler et al., 1987)). (B) Impact of cholate on AtNMT1 activity.  
636

637 Fig. 5. Examples of kinetics, initial velocity slopes, and relative fit to the Michaelis-Menten  
638 equations. Measurements are made on a plate reader. The sampling is 10 seconds. (A) SOS3  
639 peptide (0 or 100  $\mu\text{M}$ ) Gly-myristoylation kinetics in the presence or absence of HsNMT1 (0.5  
640  $\mu\text{M}$ ): a typical pipeline with various parts of the analysis is detailed. (B) Influence of GK  
641 peptide concentration on HsNMT2 acetylation with acetyl-CoA (100  $\mu\text{M}$ ). (C) Influence of  
642 acetyl-CoA peptide concentration on HsNMT1 acetylation with ARF6 (500  $\mu\text{M}$ ). (D) Fit to the  
643 Michaelis-Menten equation of the slopes of panel (C).  
644

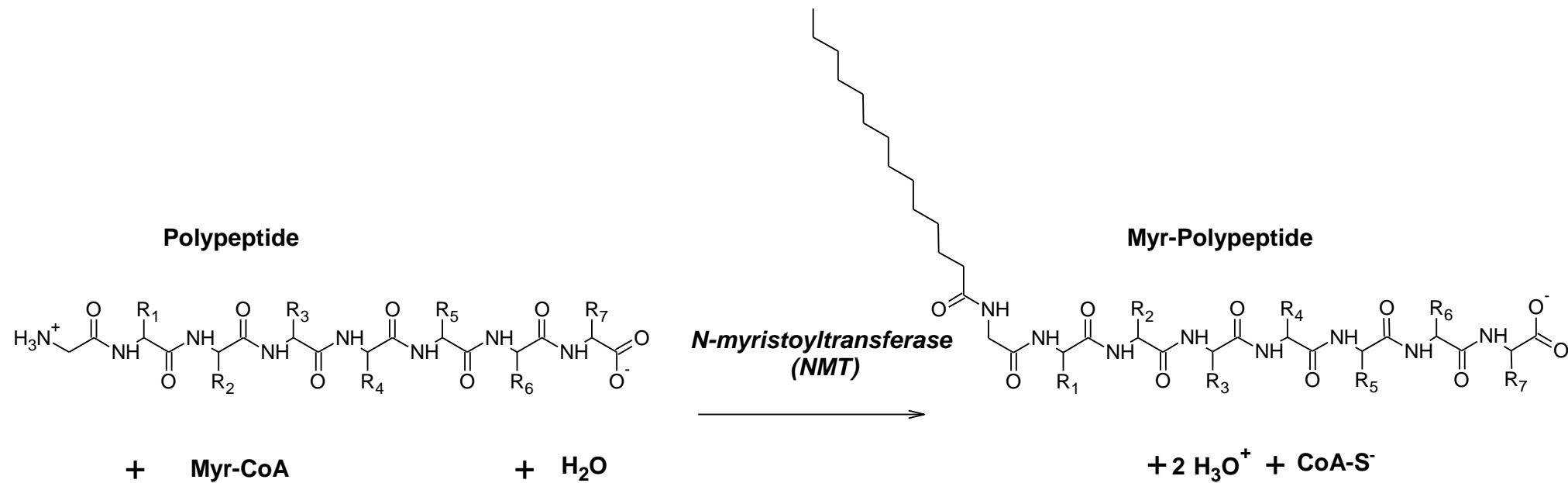
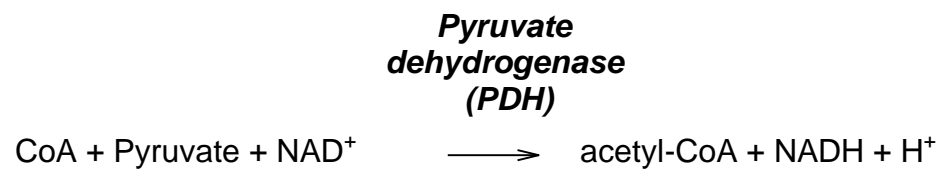
645 Fig. 6. NMT as an N-acetyl transferase *in vitro*. (A) Kinetics of Gly-acetylation of ARF6 by  
646 HsNMT1. The fit was made with the  $k_{SP}$  approach to yield  $K_m=106\pm 22 \mu\text{M}$ ,  $k_{cat}/K_m=27.5\pm 4.3$   
647  $\text{M}^{-1}\cdot\text{s}^{-1}$ . (B) Michaelis-Menten assessment of the parameters of Acetyl-CoA. ARF6 peptide  
648 concentration was set at 500  $\mu\text{M}$ , which corresponds to 82% of the maximum velocity. Higher  
649 concentrations are difficult to reach without bias. (C) Table of all parameters of Gly-acetylation  
650 by HsNMT1 and HsNMT2 with two peptides assayed at 100  $\mu\text{M}$  acetyl-CoA, ARF6, and GK  
651 as determined with the continuous assay described in this manuscript. (D) Comparison of the  
652 acetylation performances (Catalytic efficiency,  $k_{cat}/K_m$ ) obtained by two independent teams and  
653 two different methods to determine the kinetic parameters. Data in black are associated to those  
654 of the other panel of this figure. The data in grey are from (Su et al., 2021); NA, data not  
655 available.  
656

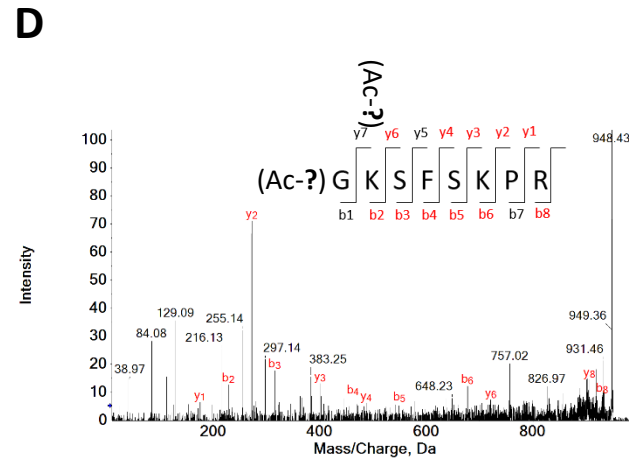
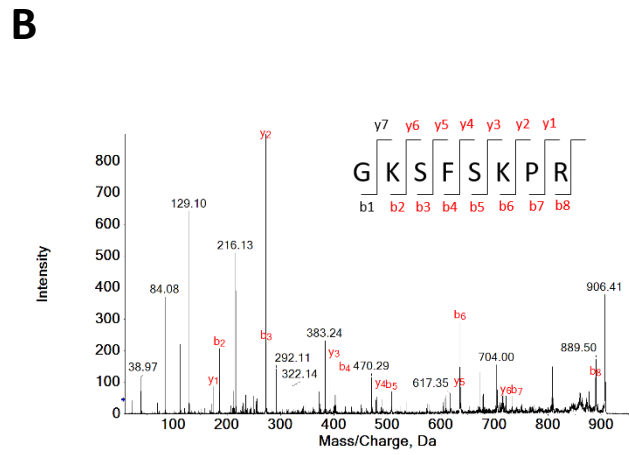
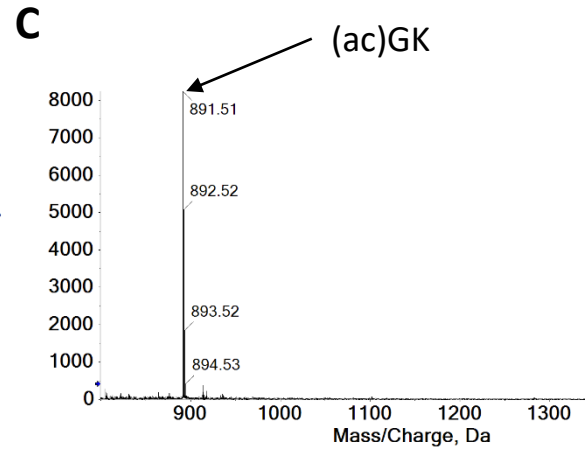
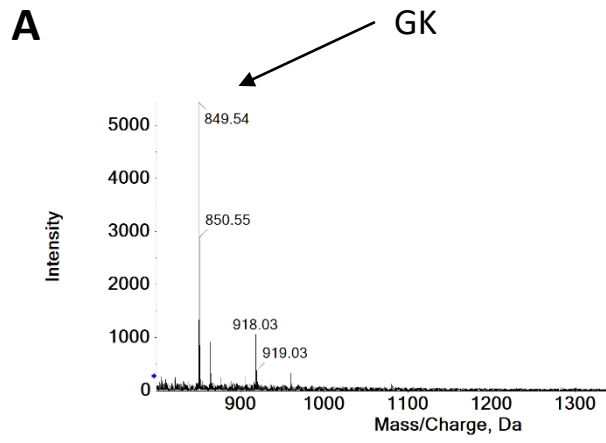
657 **Supplementary Figure Legends**

658

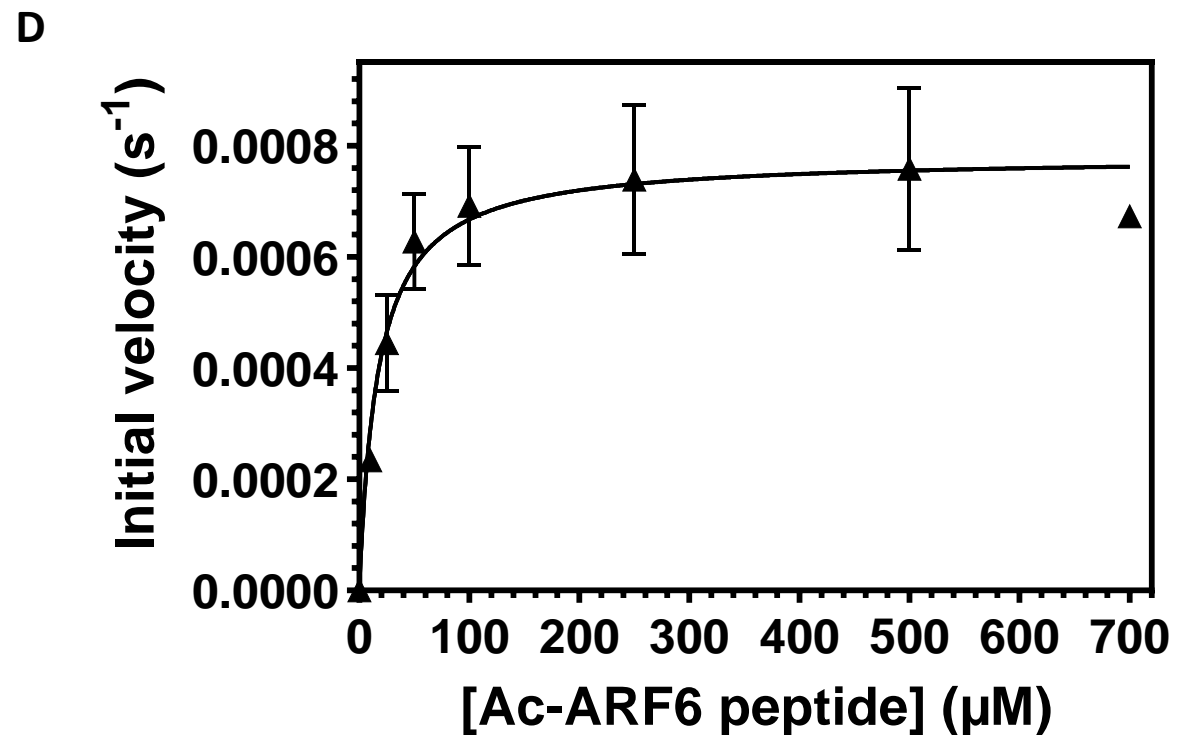
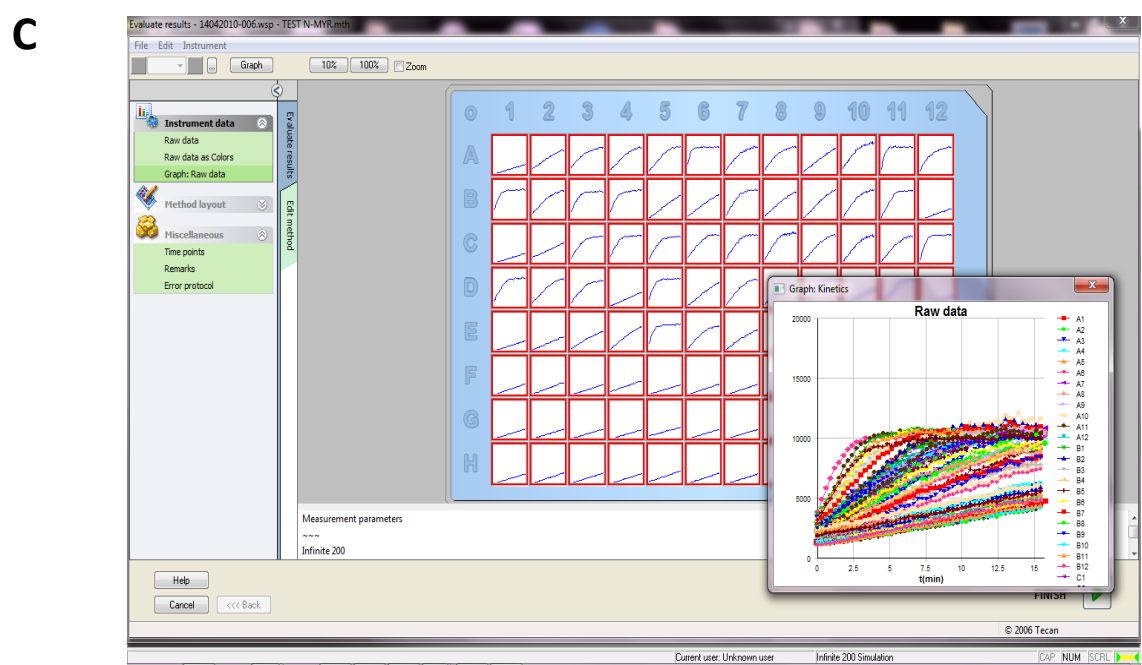
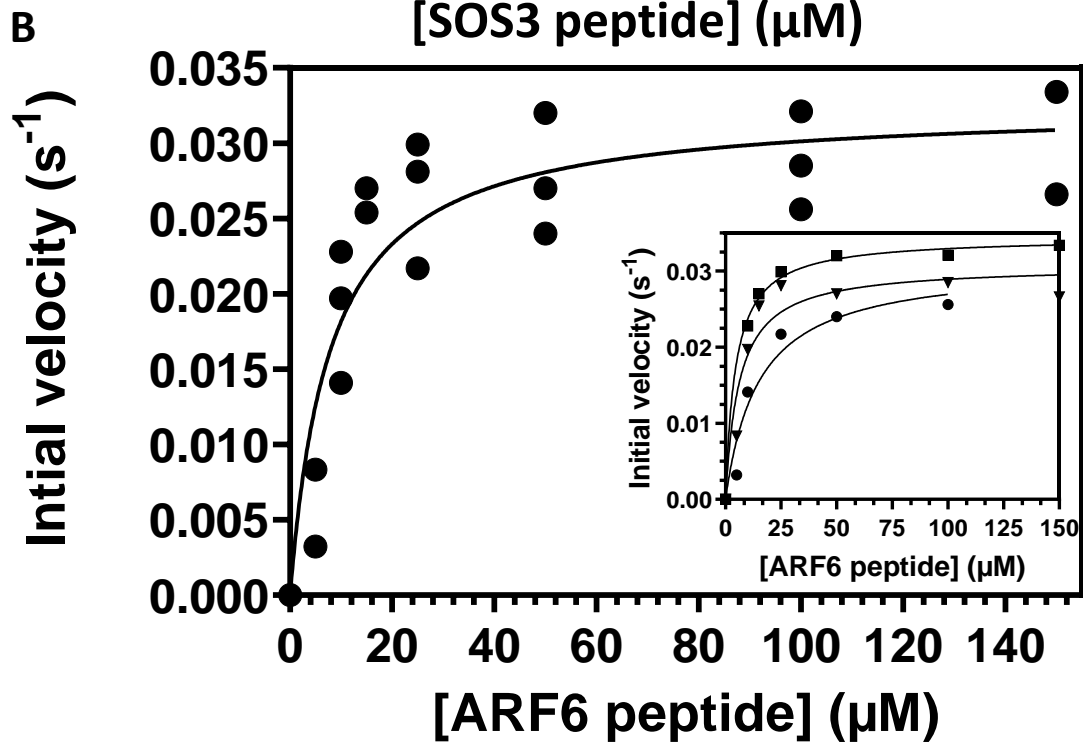
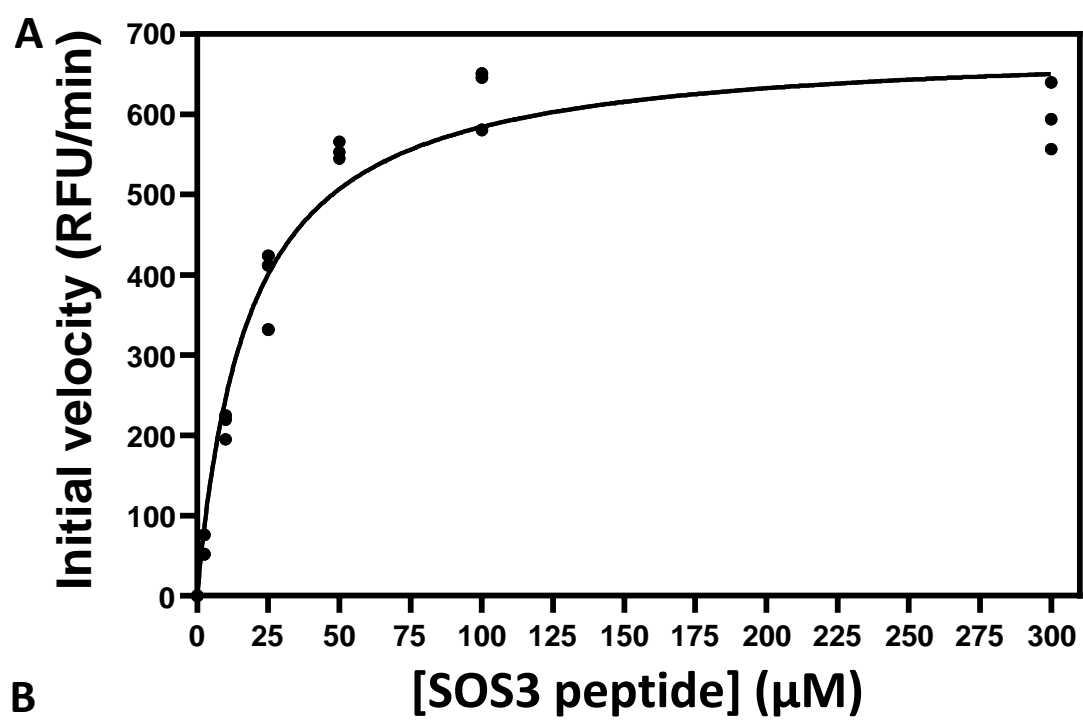
659 Fig. S1. Various purified versions of NMTs.

660

**A****B****Fig. 1**

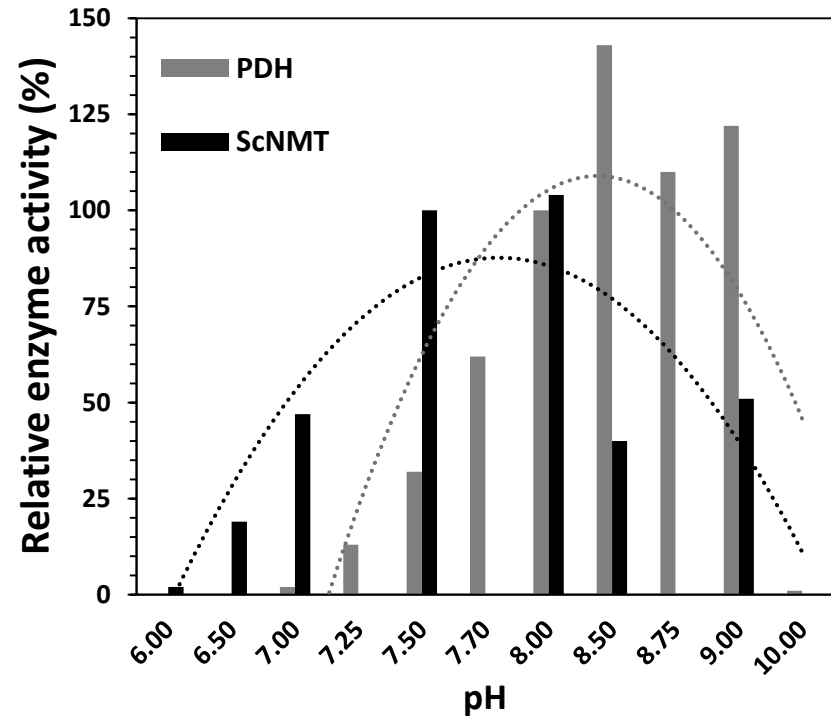
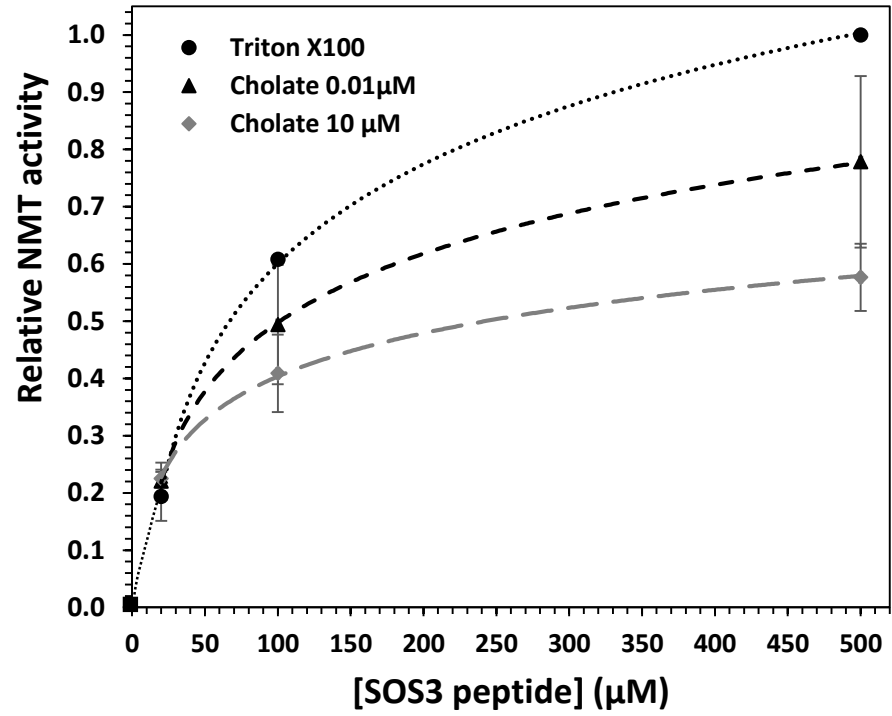


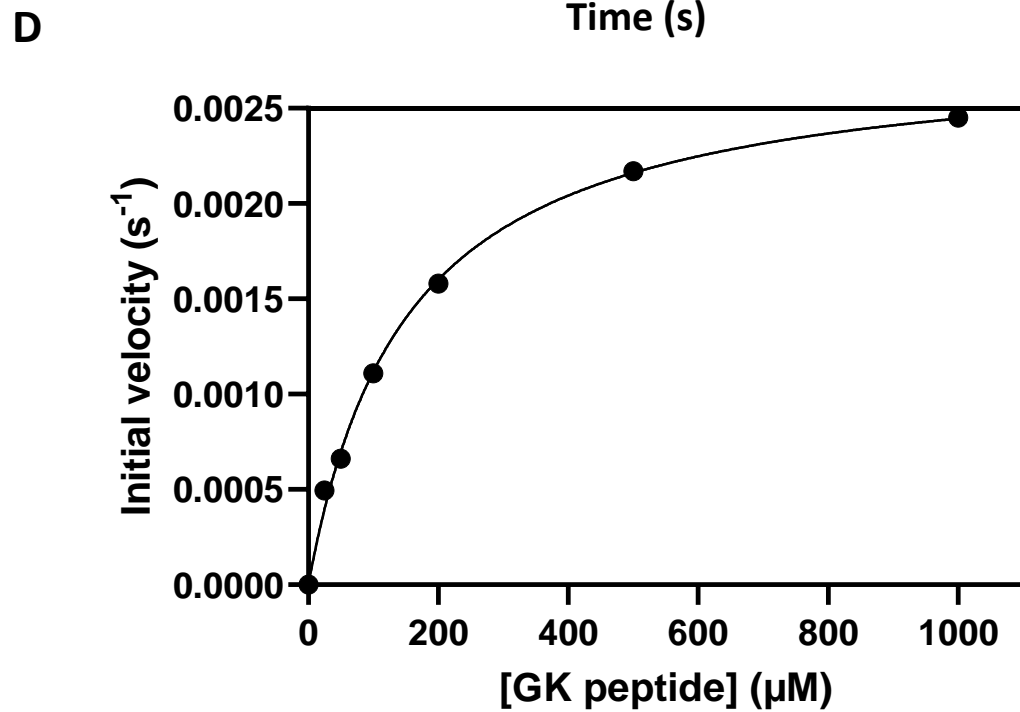
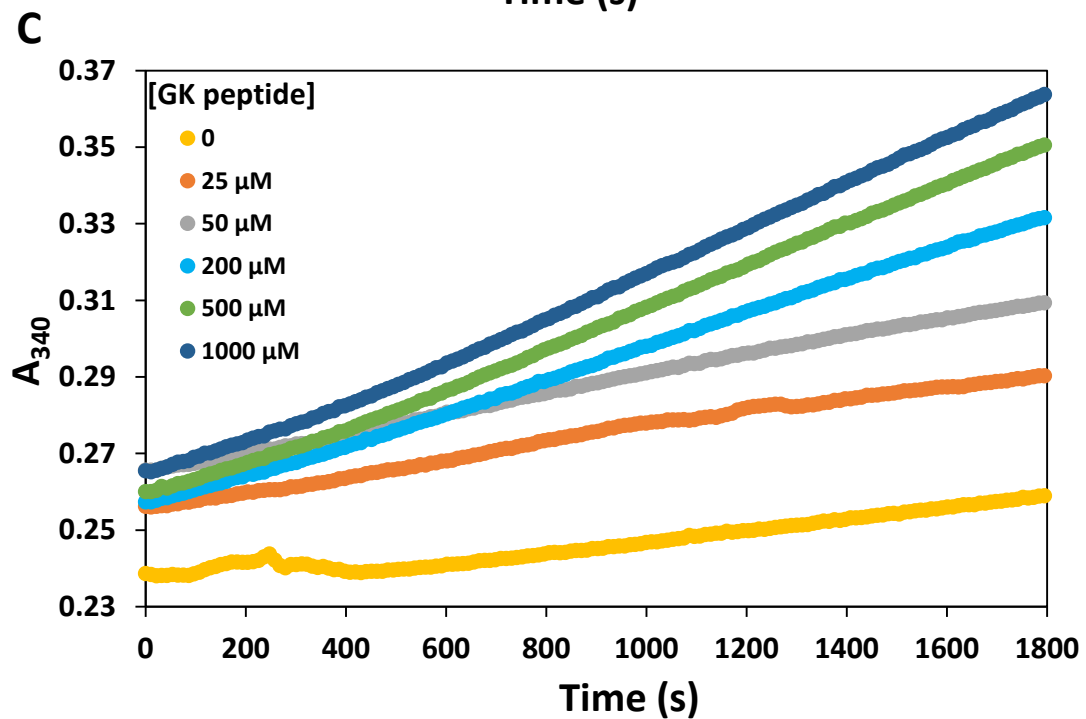
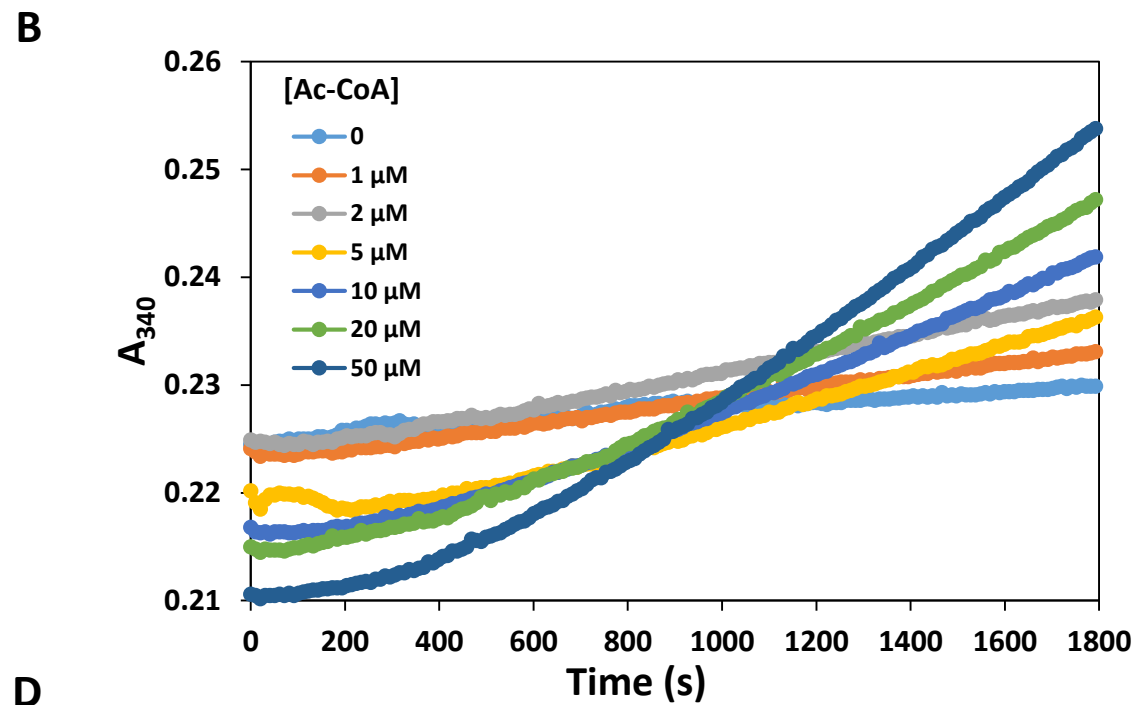
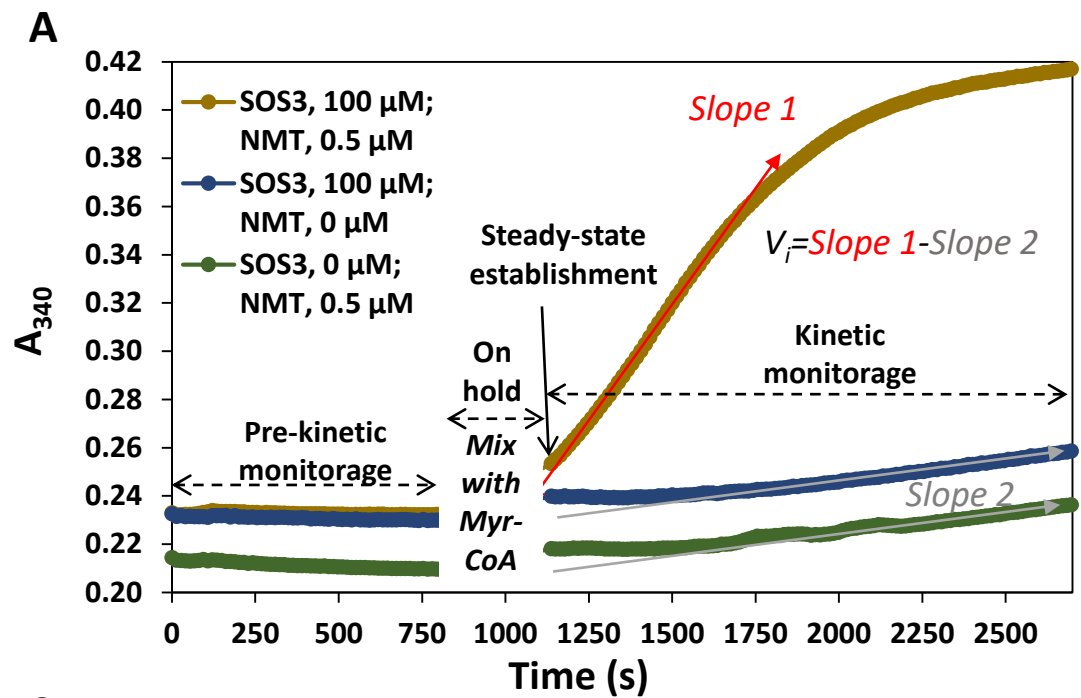
**Fig. 2**



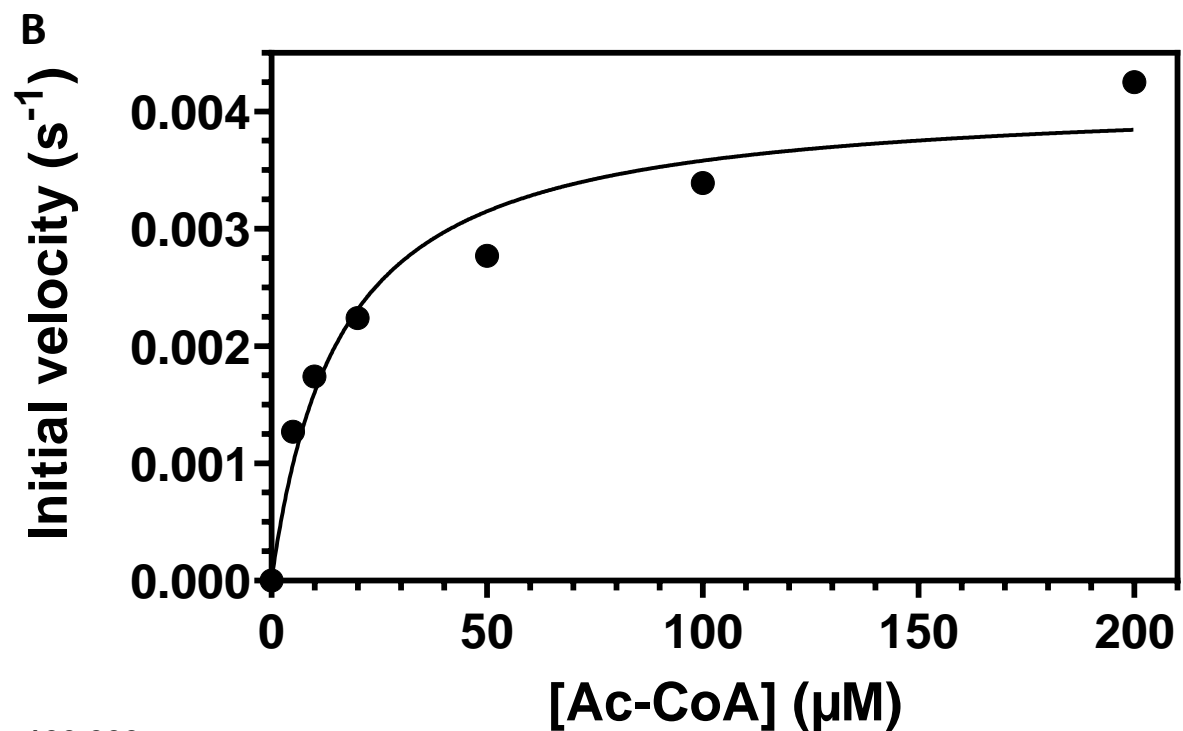
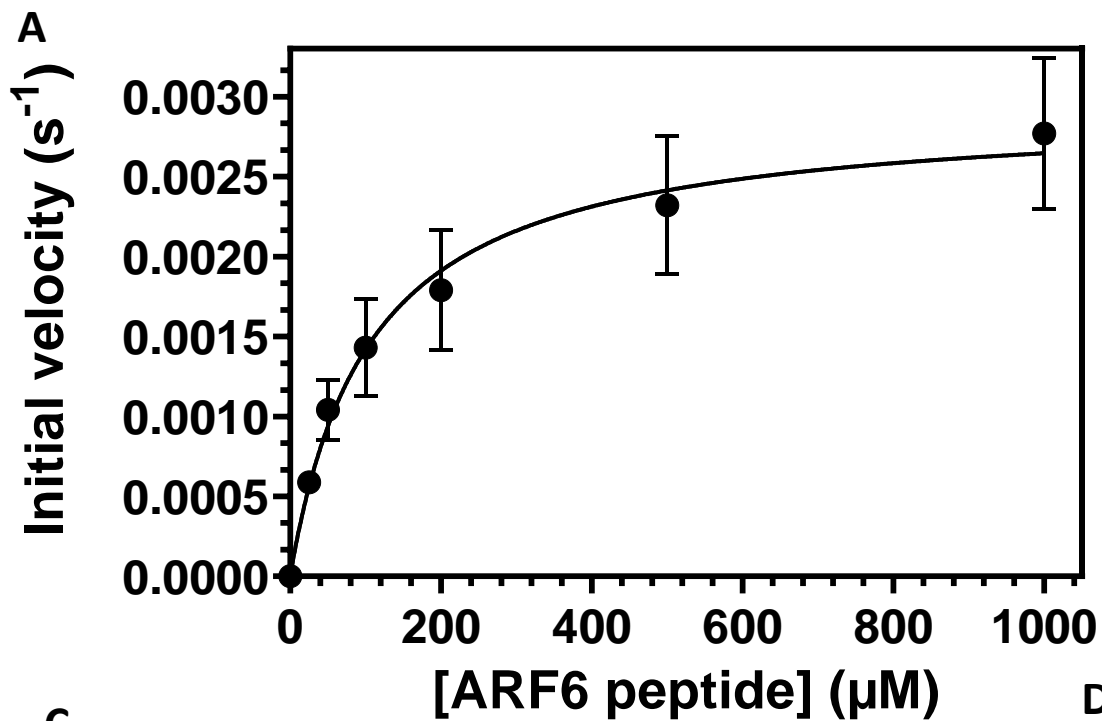
**Fig. 3**



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

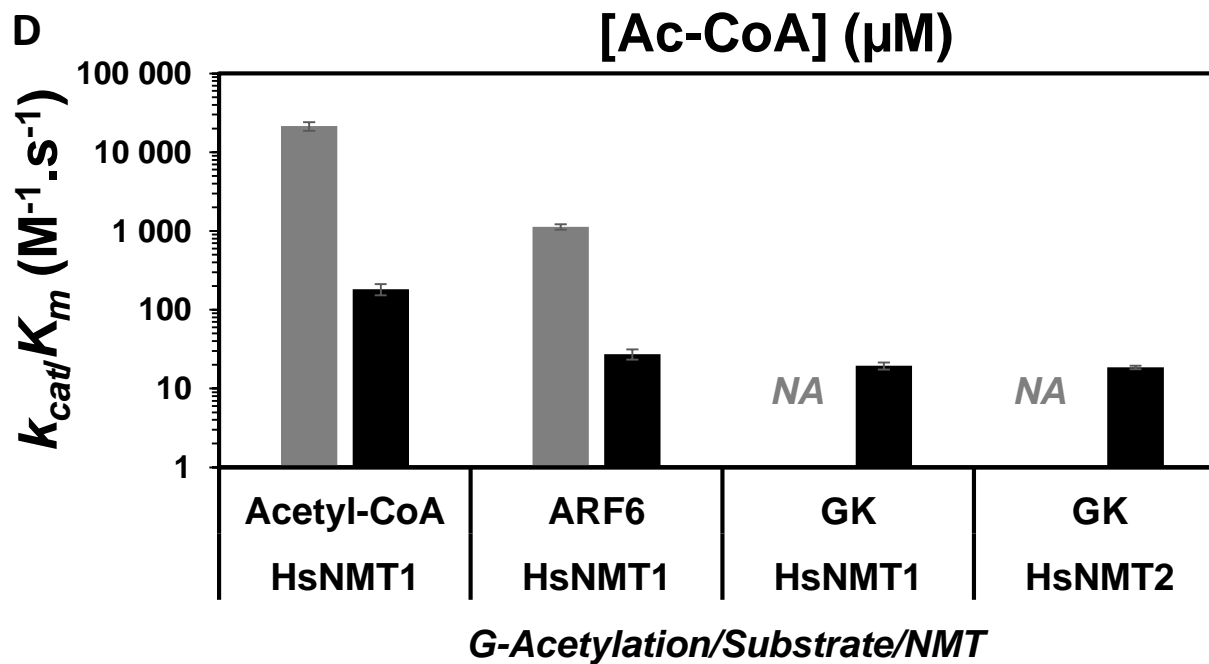


**Fig. 5**

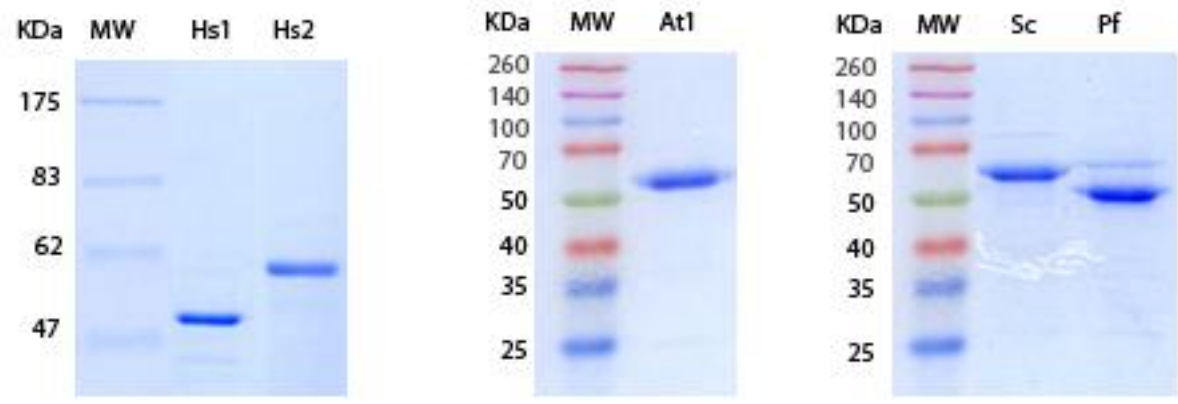


**C**

NMT	Kinetic parameter	Substrate	Value
HsNMT1	$K_m$ ( $\mu M$ )	Ac-CoA	$16 \pm 4$
		ARF6	$106 \pm 22$
		GK	$149 \pm 22$
HsNMT2		GK	$156 \pm 18$
HsNMT1	$k_{cat}$ ( $s^{-1}$ )	ARF6	$0.0029 \pm 0.0003$
HsNMT1 & HsNMT2		GK	$0.0029 \pm 0.0004$



**Fig. 6**



**Fig. S1**