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A phospho-regulated ensemble signal motif of α -TAT1 drives dynamic microtubule acetylation

Abhijit Deb Roy^{1*}, Evan G. Gross², Gayatri S. Pillai², Shailaja Seetharaman^{3,4}, Sandrine Etienne-Manneville³, Takanari Inoue^{1*}

¹ Department of Cell Biology and Center for Cell Dynamics, Johns Hopkins University School of Medicine, 855 North Wolfe Street, Baltimore, MD 21205, USA

² The Johns Hopkins University, Baltimore, MD 21218, USA

³Cell Polarity, Migration and Cancer Unit, Institut Pasteur, UMR3691 CNRS, Equipe Labellisée Ligue Contre le Cancer, F-75015, Paris, France.

⁴Université Paris Descartes, Sorbonne Paris Cité, 12 Rue de l'École de Médecine, 75006 Paris, France.

* Correspondence:

Abhijit Deb Roy abhijit.debroy@gmail.com

Takanari Inoue ictinoue@jhmi.edu

Abstract:

Spatiotemporal patterns of microtubule modifications such as acetylation underlie diverse cellular functions. While the molecular identity of the acetylating agent, α -tubulin N-acetyltransferase 1 (α -TAT1), as well as the functional consequences of microtubule acetylation have been revealed, the molecular mechanisms that regulate multi-tasking α -TAT1 action for dynamic acetylation remain obscure. Here we identified a signal motif in the intrinsically disordered C-terminus of α -TAT1, which comprises three functional elements - nuclear export, nuclear import and cytosolic retention. Their balance is tuned via phosphorylation by serine-threonine kinases to determine subcellular localization of α -TAT1. While the phosphorylated form binds to 14-3-3 adapters and accumulates in the cytosol for maximal substrate access, the non-phosphorylated form is sequestered inside the nucleus, thus keeping microtubule acetylation minimal. As cancer mutations have been reported to this motif, the unique ensemble regulation of α -TAT1 localization may hint at a role of microtubule acetylation in aberrant physiological conditions.

Introduction:

Acetylation of Lysine-40 of α -tubulin is an evolutionarily conserved post-translational modification observed across eukaryotic species^{1–3}, which is involved in diverse physiological and pathological states^{4–6}. Acetylation is mainly observed in polymerized microtubules^{7–9} and may provide structural flexibility to stabilize microtubules against bending forces^{10–13}. In cultured cells, microtubule acetylation mediates focal adhesion dynamics, adaptation to extracellular matrix rigidity as well as regulation of tissue stiffness^{14–17}. Additionally, acetylated microtubules regulate touch sensation in *M. musculus*, *C. elegans* and *D. melanogaster*, suggesting a role in mechano-response^{18–21}. Microtubule acetylation has been implicated in axonal transport in neurons^{22–24}, migration in cancer cells^{5,25–27}, autophagy^{6,28,29}, podosome stabilization in osteoclasts³⁰ and viral infections^{31–34}. α -TAT1 is the only known acetyltransferase for α -tubulin^{35,36} in mammals. α -TAT1 predominantly catalyzes α -tubulin in stable polymerized microtubules^{19,37}, and may have additional effects on microtubules independent of its catalytic activity³⁸.

Although microtubule acetylation is spatially and temporally regulated downstream of many molecular signaling pathways, little is known about how these pathways converge on α -TAT1 to achieve such dynamic patterns. In the present study, we used computational sequence analyses and live cell microscopy to identify a conserved motif in the intrinsically disordered C-terminus of α -TAT1, consisting of an NES and an NLS, that mediates its spatial distribution. We show that cytosolic localization of α -TAT1 is critical for microtubule acetylation. We further demonstrate that nuclear localization of α -TAT1 is inhibited by the action of serine-threonine kinases, specifically cyclin dependent kinases (CDKs), Protein Kinase A (PKA) and Casein kinase 2 (CK2) and identify 14-3-3 proteins as binding partners of α -TAT1. Our findings establish a novel role of the intrinsically disordered C-terminus in

62 controlling α -TAT1 function by regulating its intracellular localization downstream of kinase
63 and phosphatase activities.
64

Results:

α -TAT1 localization mediates microtubule acetylation: α -TAT1 has an N-terminal catalytic domain that shows homology to other acetyltransferases, while its C-terminus was not resolved in crystal structures³⁹ (Fig. 1a). Based on its amino acid sequence, α -TAT1 C-terminus was predicted to be intrinsically disordered by both IUPred2A⁴⁰ and PrDOS⁴¹ prediction servers (Fig. 1a, Supplementary Fig. S1a, b, c). To explore if the intra-cellular localization of α -TAT1 is regulated, we sought to identify any localization signals present in the α -TAT1 amino acid sequence (Supplementary Fig. S1a). The prediction program NetNES⁴² identified a putative NES in α -TAT1 C-terminus (Fig. 1a, Supplementary Fig. S2). On the other hand, PSORT-II⁴³ subcellular localization program predicted that α -TAT1 should be predominantly localized to the nucleus due to the presence of a putative class 4 NLS⁴⁴ in its C-terminus (Fig. 1a). The region encompassing the putative NES and NLS is conserved across the human α -TAT1 isoforms (Supplementary Fig. S3a), as well as across mammalian α -TAT1 proteins (Supplementary Fig. S3b). To test whether α -TAT1 indeed showed any intracellular distribution pattern, we expressed mVenus- α -TAT1 in HeLa cells, and observed distinct nuclear exclusion in most cells, although a subset of cells showed lack of exclusion (Fig. 1b). Based on our categorization of the distribution patterns (see Methods, Fig. 1c), we determined that approximately 77% of cells showed cytosolic distribution, 22% showed diffused pattern (i.e., both cytosolic and nuclear distribution) and 1% of cells showed nuclear enrichment of mVenus- α -TAT1 (Fig. 1d). These observations were consistent with ratiometric analysis (see Methods, Fig. 1c) of mVenus- α -TAT1 distribution (Fig. 1e). Time lapse microscopy showed temporal changes in fluorescence intensity of mVenus- α -TAT1 in cell nuclei and cytosol (Supplementary Fig. S4a, b), suggesting that α -TAT1 localization is dynamic by nature.

α -TAT1 preferentially acetylates polymerized microtubules, which are typically cytosolic. Based on this, we hypothesized that spatial regulation of α -TAT1 may control its function. Exogenous expression of mVenus- α -TAT1 or its catalytic domain (residues 1-236)^{19,39} (Fig. 1f) was sufficient to significantly increase α -tubulin acetylation in HeLa cells compared to non-transfected cells (Fig. 1g, h). To test whether nuclear localization may sufficiently sequester α -TAT1 from microtubules, we tethered the NLS from cMyc to α -TAT1 catalytic domain and thus localized it to the nucleus (Fig. 1f, g top panel). Exogenous expression of NLS-mVenus- α -TAT1(1-236) did not increase α -tubulin acetylation levels compared to non-transfected cells (Fig. 1 g, h), suggesting that nuclear sequestration of α -TAT1 inhibits its function.

α -TAT1 undergoes Exportin 1 dependent nuclear export: To dissect the molecular mechanisms of α -TAT1 localization in the cytosol, we speculated that α -TAT1 is actively exported out of the nucleus, and/or that α -TAT1 binds to a protein that keeps the complex out of the nucleus. We began by testing the first possibility by assessing involvement of the nuclear export machinery. Exportin 1 (Exp1), also called Chromosome region maintenance 1 protein homolog (CRM1), mediates nuclear export of many proteins^{45,46}. GFP- α -TAT1, but not GFP, co-immunoprecipitated with endogenous Exp1 (Fig. 2a), indicating an interaction between these two proteins. Treatment with 100 nM Leptomycin-B (LMB), an inhibitor of Exp1 mediated nuclear export⁴⁷, significantly decreased the number of cells displaying nuclear exclusion of mVenus- α -TAT1 compared to vehicle (Fig 2b, c, d). Inhibition of nuclear export was initiated within an hour of LMB treatment, although some cells were refractory to the treatment (Supplementary Fig. S5a, b). Decreased LMB concentrations (1 nM and 10 nM) had a comparable impact as 100 nM dosage (Supplementary Fig. S5c, d). Furthermore, LMB treatment induced significant reduction in α -tubulin acetylation levels in HeLa cells within 4

hours, compared to vehicle (Fig. 2e, f). Our data suggest that α -TAT1 is actively exported from the nucleus to the cytosol in an Exp1 dependent manner and that this export facilitates α -TAT1 function. The residual exclusion of α -TAT1 could be due to Exp1-independent nuclear export pathways or association with other cytosolic proteins, which was tested later (see below).

Nuclear export of α -TAT1 is mediated by a C-terminal NES: Our data demonstrate that α -TAT1 function is linked to Exp1 mediated nuclear export. To examine whether nuclear export of α -TAT1 is regulated by its catalytic activity, we expressed a catalytic dead mutant, mVenus- α -TAT1(D157N)³⁹, in HeLa cells. mVenus- α -TAT1(D157N) did not display any loss of nuclear exclusion (Supplementary Fig. S6a, b, c). The catalytic domain, mVenus- α -TAT1(1-236), displayed a complete loss of nuclear exclusion; whereas the C-terminus, mVenus- α -TAT1(236-323), displayed a distribution pattern comparable to that of WT (Fig. 3a, b, c, d). In addition, inhibition of Exp1 by 100 nM LMB significantly reduced the nuclear exclusion of mVenus- α -TAT1 C-terminus (Fig. 3e). Exclusion of α -TAT1 C-terminus (size $\approx$ 38 kDa) further indicates that nuclear exclusion of mVenus- α -TAT1 (size $\approx$ 63 kDa) is not simply due to size exclusion of passive diffusion into nuclei, and demonstrates that nuclear exclusion of α -TAT1 is a transferable property mediated by its C-terminus.

Exp1 dependent nuclear export is typically mediated by binding with short stretches of hydrophobic, often Leucine rich, NES^{42,45} that are often found in disordered regions of the cargo proteins. As previously mentioned, NetNES suggested the presence of a conserved NES between V286 and L297 in α -TAT1 C-terminus (Supplementary Fig S2). Interestingly, this region is also predicted to be a site of protein-protein interactions by ANCHOR2 prediction software⁴⁰ (Supplementary Fig. S1b). Truncation of this putative NES, α -TAT1-delNES, abrogated its nuclear exclusion (Fig. 3a, b, c, d). Although alanine substitution of the

140 hydrophobic residues in the V286-L297 region, α -TAT1(VL/A), decreased its nuclear
 141 exclusion, the loss of exclusion was less than expected from our observations with LMB
 142 treatment (Supplementary Fig. S6a, b, c). On further examination of the NetNES prediction,
 143 we observed that the NES predicted by the Hidden Markov Model (Supplementary Fig. S2)
 144 included the residues between L282 and L297. Additional Alanine substitutions of L282 and
 145 F285, α -TAT1(NES/A) (Supplementary Fig. S6d), further reduced nuclear exclusion of α -TAT1
 146 (Fig. 3a, b, c, d, Supplementary Fig. S6a, b, c), suggesting that these residues contribute to
 147 the nuclear export of α -TAT1. Taken together, our data suggest that α -TAT1 has a
 148 hydrophobic NES in its C-terminus.

149

150 **α -TAT1 interacts with 14-3-3 proteins.** Our observations thus far with nuclear exclusion of
 151 mVenus- α -TAT1 indicate that the putative NLS identified by the PSORT prediction server is
 152 either non-functional or is basally inhibited with occasional activation. 14-3-3 protein binding
 153 has been reported to negatively regulate nuclear import by inhibiting binding of importins to
 154 NLS^{48–50}. Part of the putative NLS sequence “PAQRRRTR” bears similarity to 14-3-3 binding
 155 motif RXX(pS/pT)XP⁵¹, and 14-3-3-Pred⁵², a 14-3-3 interaction prediction server identified
 156 T322 as a potential 14-3-3 binding site. To examine whether α -TAT1 bound to 14-3-3 proteins,
 157 we co-expressed GFP- α -TAT1 with HA-tagged 14-3-3- β or 14-3-3- ζ in HEK-293T cells and
 158 performed a co-immunoprecipitation assay. GFP- α -TAT1, but not GFP alone, co-precipitated
 159 with HA-14-3-3- β and HA-14-3-3- ζ (Fig. 4a). These observations are consistent with our
 160 observations in mass spectrometry analysis of α -TAT1 in HEK cells that identified 14-3-3- β
 161 and 14-3-3- ζ as potential interactors⁵³.

162

163 **Serine/threonine kinase activities mediate cytosolic localization of α -TAT1.** 14-3-3
 164 binding to proteins is mediated by phosphorylated serine and threonine residues⁵¹. NetPhos

165 prediction server identified over 30 putative phosphosites in α -TAT1, of which nine residues
 166 have been reported to be phosphorylated in phospho-proteomic studies (Supplementary Fig.
 167 S7a, b). Treatment with 100 nM Staurosporin, a pan-kinase inhibitor, significantly increased
 168 nuclear localization of α -TAT1 (Fig. 4b, c, Supplementary Fig. S8), suggesting that
 169 phosphorylation negatively regulated nuclear localization of α -TAT1. To identify the specific
 170 kinases that regulate α -TAT1 localization, we treated cells expressing mVenus- α -TAT1 with
 171 several serine-threonine kinase inhibitors (summarized in Supplementary Table T1). Both RO-
 172 3306 (inhibitor for CDK1 and CDK2^{54,55}) and PD0332991 (also called Pablociclib, inhibitor for
 173 CDK4 and CDK6^{56,57}), but not Purvalanol-B (inhibits CDK1, CDK2 and CDK5⁵⁵), increased
 174 nuclear localization of mVenus- α -TAT1 in HeLa cells (Fig. 4b, c, Supplementary Fig. S8).
 175 Silmitasertib (also called CX-4945, inhibitor for CK2⁵⁸) but not D-4476 (inhibitor for CK1⁵⁹)
 176 also had a similar impact (Fig. 4b, c, Supplementary Fig. S8). Although H-89 (inhibitor for
 177 PKA) only had a moderate effect on mVenus- α -TAT1 localization, co-expression of mCFP-
 178 PKI, a more potent inhibitor for PKA⁶⁰, considerably increased nuclear localization of mVenus-
 179 α -TAT1 (Fig 4c, Supplementary Fig. S8, Supplementary Table T1). Finally, treatment with
 180 LJ1308 (inhibitor for Ribosomal S9 kinase) appeared to further increase nuclear exclusion of
 181 mVenus- α -TAT1 (Fig. 4c, Supplementary Table T1). These data demonstrate that nuclear
 182 localization of α -TAT1 is negatively regulated by CDKs, CK2 and PKA.

183
 184 **Nuclear localization of α -TAT1 is phospho-inhibited:** Since α -TAT1(1-284) did not display
 185 nuclear exclusion (Fig. 3b, c, d), we reasoned that the phosphosites which inhibit nuclear
 186 localization of α -TAT1 might be located between F285 and R323. NetPhos prediction server
 187 identified S294, T303, S315 and T322 as potential phosphosites in this region
 188 (Supplementary Fig. S7a, indicated in red box), wherein only S315 and T322 have been
 189 reported to be phosphorylated in phosphoproteomic studies (Supplementary Fig. S7b) and

190 they also flank the putative NLS (Fig. 5a). Importin- α binds with NLS enriched in basic
 191 residues through a charge-based interaction⁴⁴. Phosphorylation of amino acids adjacent to
 192 such an NLS may inhibit the association of Importin- α binding through a disruption of the
 193 charge balance in the NLS region⁶¹. Alanine substitution of T322, but not of S315, significantly
 194 increased nuclear localization of mVenus- α -TAT1 (Fig. 5a, b, c, d, Supplementary Fig. S9a, b,
 195 c). Alanine substitution of both S315 and T322, α -TAT1(ST/A), showed considerably more
 196 nuclear localization of mVenus- α -TAT1 than T322 alone (Fig 5a, b, c, d). These data suggest
 197 that T322 phosphorylation inhibits nuclear localization of α -TAT1, while S315 may play a co-
 198 operative role in such inhibition. Substitution of S315 with acidic residues (S315D) appeared
 199 to boost nuclear exclusion of α -TAT1, whereas substitution of T322 with acidic residues
 200 (T322E) or both (ST/DE) displayed increased diffused pattern, but not increased nuclear
 201 accumulation (Supplementary Fig. S9a, b, c). This may be because these acidic residues,
 202 unlike phosphate moieties, do not sufficiently counter the basic residues in the NLS; or that
 203 phospho-T322, and to a lesser extent, phospho-S315 phosphorylation may be involved in
 204 protein-protein interactions, possibly with 14-3-3 isoforms, that inhibit nuclear localization of α -
 205 TAT1. These observations suggest that the phosphate moiety in phosphorylated T322 and
 206 S315 is critical for inhibition of α -TAT1 nuclear import.

207 One possible explanation of increased nuclear localization of T322A mutant is that
 208 phospho-T322 mediates nuclear export of α -TAT1. Truncation of the putative NLS including
 209 T322, α -TAT1-delNLS, did not increase nuclear localization (Fig. 5a, b, c, d), indicating that
 210 T322 did not mediate nuclear export of α -TAT1. Furthermore, alanine substitution of the
 211 hydrophobic residues in α -TAT1 NES as well as S315 and T322, α -TAT1(NES/A, ST/A) not
 212 only abrogated nuclear exclusion, but considerably increased nuclear accumulation of α -TAT1
 213 (Fig. 5a, b, c, d), suggesting additive effects of NES inhibition and NLS activation. α -
 214 TAT1(NES/A, ST/DE) mutant also showed diffused pattern but not nuclear accumulation

215 (Supplementary Fig. S9a, b, c). Taken together, our observations suggest that phospho-T322
216 inhibits α -TAT1 NLS and that the α -TAT1 NES and NLS act independently of one another.
217

Discussion:

One of the bottlenecks in elucidating the role of microtubule acetylation in biological phenomena is the knowledge gap of how upstream molecular signaling pathways control α -TAT1 function to modulate microtubule acetylation. Our study demonstrates that intracellular α -TAT1 localization is a dynamically regulated process, orchestrated by a balance of nuclear export and import, which modulates microtubule acetylation levels (Fig. 5e). To our knowledge, this is the first study to identify the molecular mechanisms that spatially regulate α -TAT1. We have demonstrated a hitherto unknown role of the inherently disordered α -TAT1 C-terminus and identified novel interactions with 14-3-3 proteins and several kinases. TAK1 dependent phosphorylation of α -TAT1 Serine-237 has been reported to stimulate its catalytic property⁶². In neurons, p27^{kip1} directly binds to α -TAT1 and stabilizes it against proteasomal degradation²⁴, thus enhancing α -tubulin acetylation. Our observation that spatial sequestration of α -TAT1 from microtubules modulates acetylation dynamics suggests a role of the nucleus as a reservoir or sequestration chamber to control protein access of substrates. Regulated spatial sequestration of biomolecules can control their action^{63–67} and aberrant localization of proteins have been reported in many diseases^{68–71}. Our study further highlights the role of spatial signaling processes in controlling protein function.

We have demonstrated active nuclear export of α -TAT1 by Exp1 through an NES rich in hydrophobic residues, which was critical for efficient microtubule acetylation. In addition, we have identified an NLS consistent with non-canonical class IV NLS⁴⁴. Interestingly, position 7 of this NLS, which should not be an acidic residue, is occupied by Threonine-322. Since phosphorylation of threonine can significantly increase its net negative charges, it is ideally situated to act as an ON/OFF switch for the NLS. Although we have identified Threonine-322 to be the critical phospho-residue that inhibits nuclear import, Serine-315 appears to provide

243 additional inhibition. The increased nuclear localization of ST/A mutant over T322A mutant
244 raises the possibility that S315 and T322 may aggregate signals from different signaling
245 pathways to fine-tune α -TAT1 localization.

246 Our data demonstrate that nuclear localization of α -TAT1 is inhibited by kinase action,
247 possibly on Threonine-322 and Serine-315. Specifically, our study shows a role of CDKs, PKA
248 and CK2 in coordinating spatial distribution of α -TAT1. Such phospho-regulation of α -TAT1
249 provides a possible mechanism for the changes in α -TAT1 localization and microtubule
250 acetylation observed at different stages of the cell cycle⁷². We identified Threonine-322 to be
251 a putative binding site for 14-3-3 proteins and demonstrated that α -TAT1 binds to 14-3-3- β
252 and 14-3-3- ζ proteins. 14-3-3s typically interact with phospho-serines or phospho-threonines
253 in intrinsically disordered regions and may mediate nuclear transport of proteins by masking
254 NES or NLS⁷³. Furthermore, 14-3-3 proteins may significantly alter the structure of their
255 binding partners to align along their rigid α -helical backbone, to expose or hide critical binding
256 sites⁷⁴. Comparable kinase-mediated regulation of nuclear export and nuclear import has
257 previously been reported in a few transcription regulators^{75–78}. In particular, regulation of
258 Cdc25 localization by Checkpoint kinase1 (Chk1) mediated phosphorylation of and
259 subsequent recruitment of 14-3-3- β to an NLS-proximal phosphosite is virtually the same as
260 our proposed model (Fig. 5e) of α -TAT1 localization^{78,79}, suggesting that such kinase-
261 mediated balancing of nuclear export and import is a general strategy for protein localization.
262 α -TAT1 is unique in this aspect in that unlike the other proteins that are spatially regulated in
263 this manner, α -TAT1 has no known substrates in the nucleus and that its nuclear localization
264 appears to be primarily to sequester it from microtubules. Of course, it is possible that nuclear
265 import of α -TAT1 facilitates interactions with presently unidentified substrates located in the
266 nucleus. In a similar vein, 14-3-3 proteins and Exp1 are also acetylated^{80,81}, and it is intriguing
267 to speculate that these might be substrates of α -TAT1.

268 It is worthwhile to consider that a significant number of post-translational modifications
 269 of α -TAT1 appear on its intrinsically disordered C-terminus (Supplementary Fig. 7b).
 270 Disordered regions may act as a signaling hub by interacting with multiple proteins, thus
 271 facilitating complex formation and acting as integrators of signaling pathways⁸². We have
 272 demonstrated the presence of an NES, NLS, phosphorylation sites and putative 14-3-3
 273 binding sites within the α -TAT1 C-terminus. That it is well conserved across mammalian
 274 species as well as in all the human isoforms suggests a critical role of the α -TAT1 C-terminus
 275 in its function. Indeed, numerous cancer-associated mutations curated in COSMIC⁸³
 276 (www.cancer.sanger.ac.uk) and TCGA Research Network (www.cancer.gov/tcga) databases,
 277 in the ATAT1 gene are located in the intrinsically disordered C-terminal region. More
 278 specifically, there are a considerable number of deletions, frame shifts and missense
 279 mutations encompassing the NES and the NLS regions, which may be expected to affect the
 280 spatial distribution of α -TAT1. Whether these mutations underlie the pathogenesis in these
 281 cancers remain to be examined. Considering the role of microtubule acetylation in a wide
 282 array of cellular activities, it may be conjectured that loss of spatial regulation of α -TAT1 may
 283 be present in other diseases as well.

284 In conclusion, we propose a new model for regulation of microtubule acetylation
 285 through spatial sequestration of α -TAT1 (Fig. 5e), which include three key aspects: presence
 286 of an NES that facilitates Exp1 mediated nuclear export, presence of an NLS to mediate
 287 nuclear import and finally, modulation of this nuclear import by kinases. Further investigation
 288 into the role of specific kinases on α -TAT1 localization may yield a better understanding of its
 289 function in cellular processes and pathologies and help identify new therapeutic targets.

290 **Materials and Methods**

291

292 **Cell culture and transfection:** HeLa and HEK-293T cells were cultured in DMEM basal
293 media and passaged every third day of culture. For optimal growth, the media were
294 supplemented with 10% (v/v) fetal bovine serum and Penicillin/Streptomycin, and the cells
295 were maintained under standard cell culture conditions (37 °C and 5% CO₂). The cell lines
296 were regularly checked for mycoplasma contamination. FuGENE 6 reagent (Promega,
297 Madison, WI) was used for transient transfection of HeLa cells according to the
298 manufacturer's instructions. For immunoprecipitation assays, HEK cells were transfected
299 using calcium phosphate method.

300 **DNA plasmids:** H2B-mCherry construct was a generous gift from Dr. Sergi Regot. α -TAT1
301 construct was a generous gift from Dr. Antonina Roll-Mecak. The α -TAT1 construct was
302 subcloned into the pTriEx-4 vector (Novagen) using PCR and restriction digestion with
303 mVenus at the N terminus and α -TAT1 at the C terminus. H2B-mCherry and CFP-PKI
304 constructs were respectively subcloned into mCherry-C1 and mCer3-C1 vectors (Clontech).
305 GFP- α TAT1 construct was a gift from from Dr. Philippe Chavrier and Dr. Guillaume
306 Montagnac. HA-14-3-3 plasmids were a generous gift from Dr. Michael Yaffe. As indicated in
307 the results and figure legends, tags of compatible fluorescent proteins including Cerulean,
308 mVenus and mCherry were appended to facilitate detection. Unless specified otherwise, the
309 termini of tagging were positioned as in the orders they were written. Truncations of α -TAT1
310 were generated by PCR. Point mutations of α -TAT1 were generated using overlapping PCR.
311 The open reading frames of all DNA plasmids were verified by Sanger sequencing.

312 **Sequence alignment:** Protein sequence alignment was performed using Clustal-W⁸⁴
313 (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

314 **Nuclear transport and kinase inhibitors:** LMB was purchased from LC Laboratories
 315 (catalog # L6100). SB203580 (Sigma Aldrich, catalog # S8307), Doramapimod (BIRB 796,
 316 Selleck Chemicals, catalog # S1574), CHIR99021 (Sigma Aldrich, catalog # SML1046),
 317 Sostrastaurin (Selleck Chemicals, catalog # S2791), RO-3306 (Selleck Chemicals, catalog #
 318 S7747), Ipatasertib (RG7440, Selleck Chemicals, catalog # S2808), Capivasertib (AZD5363,
 319 Selleck Chemicals, catalog # S8019), Silmitasertib (CX 4945, Selleck Chemicals, catalog #
 320 S2248), KU-55933 (Sigma Aldrich, catalog # SML1109) were generous gifts from Dr. Sergi
 321 Regot. SB239063 (Sigma Aldrich, catalog # S0569) was a generous gift from Dr. Jun Liu. The
 322 rest of the kinase inhibitors were purchased as indicated: Staurosporine (Sigma Aldrich,
 323 catalog # 569397), LJ1308 (Sigma Aldrich, catalog # SML1788), Y-27632 (LC Laboratories,
 324 catalog # Y-5301), Gö 6976 (Sigma Aldrich, catalog # 365250), Gö 6983 (Sigma Aldrich,
 325 catalog # G1918), H-89 (Sigma Aldrich, catalog # B1427), D4476 (BioVision, catalog # 1770),
 326 KN-62 (Selleck Chemicals, catalog # S7422), KU-57788 (MedChemExpress, catalog # HY-
 327 11006), Purvalanol B (AdipoGen Life Sciences, catalog # SYN-1070), KT-5823 (Cayman
 328 Chemicals, catalog # 10010965), PD0332991 (Sigma Aldrich, catalog # PZ0199).

329 **Immunofluorescence assays:** HeLa cells were transiently transfected with mVenus- α -TAT1,
 330 mVenus- α -TAT1 catalytic domain and NLS-mVenus- α -TAT1 catalytic domain. 24 hours post-
 331 transfection, cells were fixed using ice-cold methanol for 10 minutes, washed thrice with cold
 332 PBS, blocked with 1% BSA in PBS for one hour and then incubated overnight at 4°C with
 333 monoclonal antibodies against tubulin (Millipore, catalog # MAB1864) and acetylated α -
 334 Tubulin (Sigma Aldrich, catalog # T7451). Next day, the samples were washed thrice with cold
 335 PBS and incubated with secondary antibodies (Invitrogen) for one hour at room temperature,
 336 after which they were washed thrice with PBS and images were captured by microscopy. For
 337 LMB treatment, HeLa cells were dosed with 100 nM LMB or equal volume of vehicle (EtOH),

338 incubated for 4 hours, after which methanol fixation and immunostaining was performed as
339 described above.

340 **Immunoprecipitation assays:** HEK293T cells were transiently transfected with pEGFP-c1
341 (GFP-Ctl) or GFP- α TAT1 with HA-14-3-3 β or HA-14-3-3 ζ using the calcium phosphate
342 method. Cell lysates were prepared by scraping cells using 1X lysis buffer (10X recipe- 50
343 mM Tris pH 7.5, triton 20%, NP40 10%, 2 M NaCl, mixed with cOmplete protease inhibitor
344 tablet - Roche, Product number 11873580001). Cell lysates rotated on a wheel at 4°C for 15
345 min and centrifuged for 10 min at 13,000 rpm 4°C to pellet the cell debris. A small volume of
346 the supernatant was used as the soluble input. Soluble detergent extracts were incubated
347 with GFP nanobody (NanoTag, N0310) for 1 h at 4°C. Samples were then centrifuged and
348 washed thrice with wash buffer (250 mM NaCl, 0.1% Triton X-100 in PBS). The resin and the
349 soluble input were then mixed with Laemmli buffer (composed of 60 mM Tris-HCl pH 6.8, 10%
350 glycerol, 2% SDS and 50 mM DTT with the addition of protease and phosphatase inhibitors).
351 Samples were boiled 5 min at 95°C before loading in polyacrylamide gels. Gels were
352 transferred for western blot and membranes were blocked with TBST (0.1% Tween) and 5%
353 milk and incubated 1 h with the primary antibody and 1 h with HRP-conjugated secondary
354 antibody. Bands were revealed with ECL chemiluminescent substrate (Biorad). Two different
355 western blots were used to visualize GFP-Input and HA-14-3-3 proteins due to similar
356 molecular weights. Antibodies used: GFP-HRP (NB600-313, Novus Biologicals), anti-HA (rat;
357 Merck; 11867423001), anti-exportin-1 (mouse; BD Transduction Laboratories™; 611832).
358 Secondary HRP antibodies were all purchased from Jackson ImmunoResearch.

359 **Microscopy and image analyses:** All imaging was performed with an Eclipse Ti microscope
360 (Nikon) with a 100X objective (1.0X zoom and 4X4 binning) and Zyla 4.2 sCMOS camera
361 (Andor), driven by NIS Elements software (Nikon). Time lapse imaging was performed at 15
362 min intervals for 10-15 hours. All live cell imaging was conducted at 37°C, 5% CO₂ and 90%

363 humidity with a stage top incubation system (Tokai Hit). Vitamin and phenol red-free media
364 (US Biological) supplemented with 2% fetal bovine serum were used in imaging to reduce
365 background and photobleaching. Inhibitors and vehicles were present in the imaging media
366 during imaging. All image processing and analyses were performed using Metamorph
367 (Molecular Devices, Sunnyvale, CA, USA) and FIJI software (NIH, Bethesda, MD, USA).

368 For categorical analysis of mVenus- α -TAT1 localization, images were visually
369 inspected and classified as displaying either cytosolic, diffused, or nuclear localization of
370 mVenus fluorescence signal. For ratiometric analysis, the ratio of the fluorescence intensity
371 from region of interest ($\approx 10 \mu\text{m}$ diameter) in the nucleus to that in a perinuclear area was
372 used to minimize any volumetric artifacts (Fig. 1c). To determine the baseline Nuc/cyto ratio
373 for cytosolic (<0.8) and nuclear (>1.2), we visually identified WT mVenus- α -TAT1 cells, with or
374 without LMB treatment, that showed distinctly cytosolic or nuclear localization and used the
375 rounded average ratio values from these cells ($n > 100$ cells). All cells showing nuc/cyto ratio
376 in between were classified as diffused. For both categorical and ratiometric analyses, H2B-
377 mCherry signal was used to identify nuclei; in a few cases where H2B-mCherry signal was
378 absent, phase images were used to identify the nuclei. Cells displaying too much (near
379 saturation in 16 bit) or too little (approximately less than 1.5-fold signal over background) of
380 mVenus or mCherry fluorescence signal, or those which appeared to be dying on visual
381 inspection were not included during image acquisition.

382 For immunofluorescence assays with exogenous expression of mVenus- α -TAT1
383 plasmids, transfected cells were identified by the presence of mVenus fluorescence signal.
384 The ratio of acetylated α -Tubulin over α -Tubulin (Ac. α -Tub/ α -Tub) for transfected cells was
385 normalized against that for non-transfected cells averaged over 20 untransfected cells from
386 the same dish. For LMB and vehicle treatment, Ac. α -Tub/ α -Tub ratios are shown.

387 **Statistical analyses:** Microsoft Excel (Microsoft, Redmond, WA, USA) and R (R Foundation
 388 for Statistical Computing, Vienna, Austria) were used for statistical analyses. Exact number of
 389 samples for each data set are specified in the respective figure legends. Data was pooled
 390 from at least three independent experiments (technical replicates) and within each experiment
 391 typically data from at least 40 cells were obtained (biological replicates). In some cases with
 392 kinase inhibitors that induced cell death, we collected data from smaller number of cells per
 393 experiment but increased the number of experiments to ensure sufficient data. Sample sizes
 394 were chosen based on the commonly used range in the field without performing any statistical
 395 power analysis. Normal probability plot (Supplementary Fig. S10) was utilized to confirm nor-
 396 mal distribution of the Nuc/Cyto ratio of mVenus- α TAT1. Extreme outliers ($<Q1 - 3 \times IQR$ or
 397 $>Q3 + 3 \times IQR$) were excluded from plots and statistical analyses. *P*-values were obtained from
 398 two-tailed Students *t*-test assuming unequal variance. Exact *P*-values for kinase inhibitor as-
 399 says are available in Supplementary Table T1.

400 **Data availability:** All relevant data and source codes are included. Plasmid constructs will be
 401 available through Addgene.

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411

412 **Contributions**

413 ADR initiated the project and designed and performed most of the experiments and data
414 analyses. GSP and EGG performed experiments and data analyses under the guidance of
415 ADR and TI. SS performed the immunoprecipitation assays under the guidance of SEM. ADR
416 and TI wrote the final version of the manuscript based on contributions from all the authors.

417

418 **Competing interests**

419 The authors declare no competing interests.

420

421 **Figure legends**

422 **Figure 1. Intracellular distribution of α -TAT1 mediates its function.**

423 a) Cartoon showing predicted NES and NLS in intrinsically disordered C-terminus of α -TAT1,
 424 adapted from PDB: 4GS4, b) intracellular distribution of mVenus- α -TAT1, red dotted lines out-
 425 line nuclei as identified by H2B-mCherry in lower panel, c) cartoon showing criteria for cate-
 426 gorical and ratiometric analyses of α -TAT1 distribution, d) categorical analysis (n = 1032 cells)
 427 and e) ratiometric analysis (n = 304 cells) of mVenus- α -TAT1 localization, f) cartoon showing
 428 α -TAT1 mutants used in g) immunofluorescence assays showing levels of acetylated α -tubulin
 429 and total α -tubulin, transfected cells are indicated with red arrowheads, h) ratio of acetylated
 430 α -tubulin to total tubulin intensities with exogenous expression of α -TAT1 and its mutants,
 431 normalized against that of non-transfected cells, (WT: 50, catalytic domain: 44, NLS-catalytic
 432 domain: 48 cells). Scale bar = 10 μ m. ***: $P < 0.001$ and NS: not significant, Student's *t*-test.

434 **Figure 2. α -TAT1 undergoes Exp1 mediated nuclear export.**

435 a) Co-immunoprecipitation of endogenous Exp1 with GFP- α -TAT1, b) intracellular distribution
 436 of mVenus- α -TAT1 with vehicle (EtOH) and 100 nM LMB treatment, nuclei are indicated in red
 437 dotted lines, c) categorical analysis (WT: 1032, vehicle: 450, LMB: 495 cells) and d) rati-
 438 ometric analysis (WT: 304, vehicle: 210, LMB: 240 cells) of mVenus- α -TAT1 localization with
 439 vehicle and LMB treatment, e) immunofluorescence images showing acetylated and total α -
 440 tubulin in HeLa cells with vehicle or LMB treatment, f) ratio of acetylated to total α -tubulin with
 441 vehicle or LMB treatment (vehicle:120, LMB: 130 cells). Scale bar = 10 μ m. ***: $P < 0.001$ and
 442 NS: not significant, Student's *t*-test.

444 **Figure 3. Intracellular distribution of α -TAT1 is mediated by its C-terminus.**

445 a) Cartoon showing α -TAT1 mutant design, b) categorical analysis (WT: 1032, Cat. Dom: 371,
446 C-term: 290, delNES: 343 and NES/A: 501 cells) and c) ratiometric analysis (WT: 304, Cat.
447 Dom: 213, C-term: 220, delNES: 221 and NES/A: 212 cells) of mVenus- α -TAT1 mutant locali-
448 zation as indicated, d) representative images showing intracellular distribution of mVenus- α -
449 TAT1 mutants as listed, red dotted lines outline nuclei, e) ratiometric analysis of intracellular
450 distribution of mVenus- α -TAT1 C-term with 100 nM LMB (C-term: 220, LMB: 209 cells. Scale
451 bar = 10 μ m. ***: $P < 0.001$ and NS: not significant, Student's t -test.

452
453 **Figure 4. Nuclear localization of α -TAT1 is phospho-inhibited.**

454 a) Co-immunoprecipitation of GFP- α -TAT1 with HA-14-3-3 β and HA-14-3-3 ζ proteins, b) intra-
455 cellular distribution of mVenus- α -TAT1 with Staurosporine, RO-3306, PD0332991 and Sil-
456 mitasertib treatment, nuclei are indicated in red dotted lines, c) ratiometric analysis of mVe-
457 nus- α -TAT1 localization with kinase inhibitors (WT:304, STS: 183, RO-3306: 221,
458 PD0332991: 234, Purvalanol-B: 243, D4476:253, Silmitasertib: 253, H89: 219, PKI: 208, Ipa-
459 tasertib: 259, Capiisertib: 218, Go6976: 297, Go6983: 241, Sostrastaurin: 401, KT-5823: 269,
460 SB203580: 259, Birb796: 262, KU55933: 317, LJ1308: 229, CHIR99021: 227, KN-62:210, KU-
461 57788: 249, Y-27632: 161 cells). Scale bar = 10 μ m. ***: $P < 0.001$ and NS: not significant, Stu-
462 dent's t -test.

463
464 **Figure 5. α -TAT1 has a C-terminal phospho-inhibited NLS.**

465 a) Cartoon showing α -TAT1 putative NLS flanked by potential phosphosites and correspond-
466 ing mutants, b) intracellular distribution of mVenus- α -TAT1 mutants as indicated, nuclei are
467 outlined in red dotted lines, c) ratiometric analysis (WT: 304, T322A: 228, ST/A: 194, delNLS:
468 208, NES/A, ST/A: 225 cells) and d) categorical analysis (WT: 1032, T322A: 346, ST/A: 290,
469 delNLS: 563, NES/A, ST/A: 476 cells) of intracellular localization of mVenus- α -TAT1 mutants,

470 e) proposed model of α -TAT1 localization and its impact on microtubule acetylation. Scale bar
471 = 10 μ m. *** P <0.001 and NS, not significant, Student's t -test.
472

References:

1. L'Hernault, S. W. & Rosenbaum, J. L. Chlamydomonas alpha-tubulin is posttranslationally modified by acetylation on the epsilon-amino group of a lysine. *Biochemistry* **24**, 473–478 (1985).
2. Diggins, M. A. & Dove, W. F. Distribution of acetylated alpha-tubulin in Physarum polycephalum. *J. Cell Biol.* **104**, 303–309 (1987).
3. Janke, C. & Montagnac, G. Causes and Consequences of Microtubule Acetylation. *Curr. Biol.* **27**, R1287–R1292 (2017).
4. Schatten, G. *et al.* Acetylated alpha-tubulin in microtubules during mouse fertilization and early development. *Dev. Biol.* **130**, 74–86 (1988).
5. Boggs, A. E. *et al.* α -Tubulin Acetylation Elevated in Metastatic and Basal-like Breast Cancer Cells Promotes Microtentacle Formation, Adhesion, and Invasive Migration. *Cancer Res.* **75**, 203–215 (2015).
6. Esteves, A. R. *et al.* Acetylation as a major determinant to microtubule-dependent autophagy: Relevance to Alzheimer's and Parkinson disease pathology. *Biochim. Biophys. Acta BBA - Mol. Basis Dis.* **1865**, 2008–2023 (2019).
7. L'Hernault, S. W. & Rosenbaum, J. L. Chlamydomonas alpha-tubulin is posttranslationally modified in the flagella during flagellar assembly. *J. Cell Biol.* **97**, 258–263 (1983).
8. Maruta, H., Greer, K. & Rosenbaum, J. L. The acetylation of alpha-tubulin and its relationship to the assembly and disassembly of microtubules. *J. Cell Biol.* **103**, 571–579 (1986).
9. Piperno, G., LeDizet, M. & Chang, X. J. Microtubules containing acetylated alpha-tubulin in mammalian cells in culture. *J. Cell Biol.* **104**, 289–302 (1987).

- 497 10. Sudo, H. & Baas, P. W. Acetylation of Microtubules Influences Their Sensitivity to Severing
498 by Katanin in Neurons and Fibroblasts. *J. Neurosci.* **30**, 7215–7226 (2010).
- 499 11. Portran, D., Schaedel, L., Xu, Z., Théry, M. & Nachury, M. V. Tubulin acetylation protects
500 long-lived microtubules against mechanical ageing. *Nat. Cell Biol.* **19**, 391–398 (2017).
- 501 12. Xu, Z. *et al.* Microtubules acquire resistance from mechanical breakage through
502 intraluminal acetylation. *Science* **356**, 328–332 (2017).
- 503 13. Eshun-Wilson, L. *et al.* Effects of α -tubulin acetylation on microtubule structure and
504 stability. *Proc. Natl. Acad. Sci.* **116**, 10366–10371 (2019).
- 505 14. Joo, E. E. & Yamada, K. M. MYPT1 regulates contractility and microtubule acetylation to
506 modulate integrin adhesions and matrix assembly. *Nat. Commun.* **5**, 3510 (2014).
- 507 15. Bance, B., Seetharaman, S., Leduc, C., Boëda, B. & Etienne-Manneville, S. Microtubule
508 acetylation but not detyrosination promotes focal adhesion dynamics and astrocyte
509 migration. *J. Cell Sci.* **132**, (2019).
- 510 16. Swiatlowska, P., Sanchez-Alonso, J. L., Wright, P. T., Novak, P. & Gorelik, J. Microtubules
511 regulate cardiomyocyte transversal Young's modulus. *Proc. Natl. Acad. Sci.* **117**, 2764–
512 2766 (2020).
- 513 17. Coleman, A. K., Joca, H. C., Shi, G., Lederer, W. J. & Ward, C. W. Tubulin acetylation
514 increases cytoskeletal stiffness to regulate mechanotransduction in striated muscle.
515 *bioRxiv* 2020.06.10.144931 (2020) doi:10.1101/2020.06.10.144931.
- 516 18. Zhang, Y. *et al.* Identification of genes expressed in *C. elegans* touch receptor neurons.
517 *Nature* **418**, 331–335 (2002).
- 518 19. Shida, T., Cueva, J. G., Xu, Z., Goodman, M. B. & Nachury, M. V. The major α -tubulin K40
519 acetyltransferase α TAT1 promotes rapid ciliogenesis and efficient mechanosensation.
520 *Proc. Natl. Acad. Sci. U. S. A.* **107**, 21517–21522 (2010).

- 521 20. Morley, S. J. *et al.* Acetylated tubulin is essential for touch sensation in mice. *eLife* **5**,
522 e20813 (2016).
- 523 21. Yan, C. *et al.* Microtubule Acetylation Is Required for Mechanosensation in *Drosophila*.
524 *Cell Rep.* **25**, 1051-1065.e6 (2018).
- 525 22. Lin, S., Sterling, N. A., Junker, I. P., Helm, C. T. & Smith, G. M. Effects of α TAT1 and
526 HDAC5 on axonal regeneration in adult neurons. *PloS One* **12**, e0177496 (2017).
- 527 23. Fourcade, S. *et al.* Loss of SIRT2 leads to axonal degeneration and locomotor disability
528 associated with redox and energy imbalance. *Aging Cell* **16**, 1404–1413 (2017).
- 529 24. Morelli, G. *et al.* p27Kip1 Modulates Axonal Transport by Regulating α -Tubulin
530 Acetyltransferase 1 Stability. *Cell Rep.* **23**, 2429–2442 (2018).
- 531 25. Lee, C.-C., Cheng, Y.-C., Chang, C.-Y., Lin, C.-M. & Chang, J.-Y. Alpha-tubulin
532 acetyltransferase/MEC-17 regulates cancer cell migration and invasion through epithelial–
533 mesenchymal transition suppression and cell polarity disruption. *Sci. Rep.* **8**, 17477
534 (2018).
- 535 26. Castro-Castro, A., Janke, C., Montagnac, G., Paul-Gilloteaux, P. & Chavrier, P.
536 ATAT1/MEC-17 acetyltransferase and HDAC6 deacetylase control a balance of
537 acetylation of alpha-tubulin and cortactin and regulate MT1-MMP trafficking and breast
538 tumor cell invasion. *Eur. J. Cell Biol.* **91**, 950–960 (2012).
- 539 27. Oh, S. *et al.* Genetic disruption of tubulin acetyltransferase, α TAT1, inhibits proliferation
540 and invasion of colon cancer cells through decreases in Wnt1/ β -catenin signaling.
541 *Biochem. Biophys. Res. Commun.* **482**, 8–14 (2017).
- 542 28. Geeraert, C. *et al.* Starvation-induced Hyperacetylation of Tubulin Is Required for the
543 Stimulation of Autophagy by Nutrient Deprivation. *J. Biol. Chem.* **285**, 24184–24194
544 (2010).

- 545 29. McLendon, P. M. *et al.* Tubulin hyperacetylation is adaptive in cardiac proteotoxicity by
546 promoting autophagy. *Proc. Natl. Acad. Sci.* **111**, E5178–E5186 (2014).
- 547 30. Destaing, O. *et al.* A novel Rho-mDia2-HDAC6 pathway controls podosome patterning
548 through microtubule acetylation in osteoclasts. *J. Cell Sci.* **118**, 2901–2911 (2005).
- 549 31. Elliott, G. & O'Hare, P. Herpes Simplex Virus Type 1 Tegument Protein VP22 Induces the
550 Stabilization and Hyperacetylation of Microtubules. *J. Virol.* **72**, 6448–6455 (1998).
- 551 32. Husain, M. & Harrod, K. S. Enhanced acetylation of alpha-tubulin in influenza A virus
552 infected epithelial cells. *FEBS Lett.* **585**, 128–132 (2011).
- 553 33. Sabo, Y. *et al.* HIV-1 induces the formation of stable microtubules to enhance early
554 infection. *Cell Host Microbe* **14**, (2013).
- 555 34. Zan, J. *et al.* Rabies Virus Infection Induces Microtubule Depolymerization to Facilitate
556 Viral RNA Synthesis by Upregulating HDAC6. *Front. Cell. Infect. Microbiol.* **7**, (2017).
- 557 35. Kalebic, N. *et al.* α TAT1 is the major α -tubulin acetyltransferase in mice. *Nat. Commun.* **4**,
558 1–10 (2013).
- 559 36. Kim, G.-W., Li, L., Ghorbani, M., You, L. & Yang, X.-J. Mice lacking α -tubulin
560 acetyltransferase 1 are viable but display α -tubulin acetylation deficiency and dentate
561 gyrus distortion. *J. Biol. Chem.* **288**, 20334–20350 (2013).
- 562 37. Szyk, A. *et al.* Molecular Basis for Age-Dependent Microtubule Acetylation by Tubulin
563 Acetyltransferase. *Cell* **157**, 1405–1415 (2014).
- 564 38. Kalebic, N. *et al.* Tubulin Acetyltransferase α TAT1 Destabilizes Microtubules
565 Independently of Its Acetylation Activity. *Mol. Cell. Biol.* **33**, 1114–1123 (2013).
- 566 39. Friedmann, D. R., Aguilar, A., Fan, J., Nachury, M. V. & Marmorstein, R. Structure of the α -
567 tubulin acetyltransferase, α TAT1, and implications for tubulin-specific acetylation. *Proc.*
568 *Natl. Acad. Sci. U. S. A.* **109**, 19655–19660 (2012).

569 40. Mészáros, B., Erdős, G. & Dosztányi, Z. IUPred2A: context-dependent prediction of
570 protein disorder as a function of redox state and protein binding. *Nucleic Acids Res.* **46**,
571 W329–W337 (2018).

572 41. Ishida, T. & Kinoshita, K. PrDOS: prediction of disordered protein regions from amino acid
573 sequence. *Nucleic Acids Res.* **35**, W460–464 (2007).

574 42. la Cour, T. *et al.* Analysis and prediction of leucine-rich nuclear export signals. *Protein*
575 *Eng. Des. Sel.* **17**, 527–536 (2004).

576 43. Horton, P. *et al.* WoLF PSORT: protein localization predictor. *Nucleic Acids Res.* **35**,
577 W585–587 (2007).

578 44. Kosugi, S. *et al.* Six Classes of Nuclear Localization Signals Specific to Different Binding
579 Grooves of Importin α . *J. Biol. Chem.* **284**, 478–485 (2009).

580 45. Kutay, U. & Güttinger, S. Leucine-rich nuclear-export signals: born to be weak. *Trends Cell*
581 *Biol.* **15**, 121–124 (2005).

582 46. Hutten, S. & Kehlenbach, R. H. CRM1-mediated nuclear export: to the pore and beyond.
583 *Trends Cell Biol.* **17**, 193–201 (2007).

584 47. Sun, Q. *et al.* Nuclear export inhibition through covalent conjugation and hydrolysis of
585 Leptomycin B by CRM1. *Proc. Natl. Acad. Sci.* **110**, 1303–1308 (2013).

586 48. Sekimoto, T., Fukumoto, M. & Yoneda, Y. 14-3-3 suppresses the nuclear localization of
587 threonine 157-phosphorylated p27Kip1. *EMBO J.* **23**, 1934–1942 (2004).

588 49. Mancini, M. *et al.* 14-3-3 ligand prevents nuclear import of c-ABL protein in chronic
589 myeloid leukemia. *Traffic Cph. Den.* **10**, 637–647 (2009).

590 50. Huang, X. *et al.* Shade-induced nuclear localization of PIF7 is regulated by
591 phosphorylation and 14-3-3 proteins in Arabidopsis. *eLife* **7**, (2018).

592 51. Yaffe, M. B. *et al.* The Structural Basis for 14-3-3:Phosphopeptide Binding Specificity. *Cell*
593 **91**, 961–971 (1997).

594 52. Madeira, F. *et al.* 14-3-3-Pred: improved methods to predict 14-3-3-binding
595 phosphopeptides. *Bioinforma. Oxf. Engl.* **31**, 2276–2283 (2015).

596 53. Seetharaman, S. *et al.* Microtubules tune mechanosensitive cell responses. *bioRxiv*
597 2020.07.22.205203 (2020) doi:10.1101/2020.07.22.205203.

598 54. Vassilev, L. T. *et al.* Selective small-molecule inhibitor reveals critical mitotic functions of
599 human CDK1. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 10660–10665 (2006).

600 55. Jorda, R. *et al.* How Selective Are Pharmacological Inhibitors of Cell-Cycle-Regulating
601 Cyclin-Dependent Kinases? *J. Med. Chem.* **61**, 9105–9120 (2018).

602 56. Fry, D. W. *et al.* Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and
603 associated antitumor activity in human tumor xenografts. *Mol. Cancer Ther.* **3**, 1427–1438
604 (2004).

605 57. Toogood, P. L. *et al.* Discovery of a Potent and Selective Inhibitor of Cyclin-Dependent
606 Kinase 4/6. *J. Med. Chem.* **48**, 2388–2406 (2005).

607 58. Ferguson, A. D. *et al.* Structural basis of CX-4945 binding to human protein kinase CK2.
608 *FEBS Lett.* **585**, 104–110 (2011).

609 59. Rena, G., Bain, J., Elliott, M. & Cohen, P. D4476, a cell-permeant inhibitor of CK1,
610 suppresses the site-specific phosphorylation and nuclear exclusion of FOXO1a. *EMBO*
611 *Rep.* **5**, 60–65 (2004).

612 60. Kim, A. K., Wu, H. D. & Inoue, T. Rational Design of a Protein Kinase A Nuclear-cytosol
613 Translocation Reporter. *Sci. Rep.* **10**, 9365 (2020).

614 61. Harreman, M. T. *et al.* Regulation of nuclear import by phosphorylation adjacent to nuclear
615 localization signals. *J. Biol. Chem.* **279**, 20613–20621 (2004).

616 62. Shah, N. *et al.* TAK1 activation of alpha-TAT1 and microtubule hyperacetylation control
617 AKT signaling and cell growth. *Nat. Commun.* **9**, 1–12 (2018).

618 63. Shimada, Y., Gulli, M. P. & Peter, M. Nuclear sequestration of the exchange factor Cdc24
619 by Far1 regulates cell polarity during yeast mating. *Nat. Cell Biol.* **2**, 117–124 (2000).

620 64. Volmat, V., Camps, M., Arkinstall, S., Pouysségur, J. & Lenormand, P. The nucleus, a site
621 for signal termination by sequestration and inactivation of p42/p44 MAP kinases. *J. Cell*
622 *Sci.* **114**, 3433–3443 (2001).

623 65. Formstecher, E. *et al.* PEA-15 mediates cytoplasmic sequestration of ERK MAP kinase.
624 *Dev. Cell* **1**, 239–250 (2001).

625 66. Yang, W., Wightman, R. & Meyerowitz, E. M. Cell Cycle Control by Nuclear Sequestration
626 of CDC20 and CDH1 mRNA in Plant Stem Cells. *Mol. Cell* **68**, 1108-1119.e3 (2017).

627 67. Lasker, K. *et al.* Selective sequestration of signalling proteins in a membraneless
628 organelle reinforces the spatial regulation of asymmetry in *Caulobacter crescentus*. *Nat.*
629 *Microbiol.* **5**, 418–429 (2020).

630 68. Chen, Y. *et al.* Aberrant subcellular localization of BRCA1 in breast cancer. *Science* **270**,
631 789–791 (1995).

632 69. Jiao, W. *et al.* Aberrant nucleocytoplasmic localization of the retinoblastoma tumor
633 suppressor protein in human cancer correlates with moderate/poor tumor differentiation.
634 *Oncogene* **27**, 3156–3164 (2008).

635 70. Hung, M.-C. & Link, W. Protein localization in disease and therapy. *J. Cell Sci.* **124**, 3381–
636 3392 (2011).

637 71. Wang, X. & Li, S. Protein mislocalization: mechanisms, functions and clinical applications
638 in cancer. *Biochim. Biophys. Acta* **1846**, 13–25 (2014).

639 72. Nekooki-Machida, Y. *et al.* Dynamic localization of α -tubulin acetyltransferase ATAT1
640 through the cell cycle in human fibroblastic KD cells. *Med. Mol. Morphol.* **51**, 217–226
641 (2018).

642 73. Pennington, K. L., Chan, T. Y., Torres, M. P. & Andersen, J. L. The dynamic and stress-
643 adaptive signaling hub of 14-3-3: emerging mechanisms of regulation and context-
644 dependent protein–protein interactions. *Oncogene* **37**, 5587–5604 (2018).

645 74. Yaffe, M. B. How do 14-3-3 proteins work?-- Gatekeeper phosphorylation and the
646 molecular anvil hypothesis. *FEBS Lett.* **513**, 53–57 (2002).

647 75. Oeckinghaus, A. & Ghosh, S. The NF-κB Family of Transcription Factors and Its
648 Regulation. *Cold Spring Harb. Perspect. Biol.* **1**, (2009).

649 76. Spencer, S. L. *et al.* The Proliferation-Quiescence Decision Is Controlled by a Bifurcation
650 in CDK2 Activity at Mitotic Exit. *Cell* **155**, 369–383 (2013).

651 77. Regot, S., Hughey, J. J., Bajar, B. T., Carrasco, S. & Covert, M. W. High-Sensitivity
652 Measurements of Multiple Kinase Activities in Live Single Cells. *Cell* **157**, 1724–1734
653 (2014).

654 78. Deneke, V. E., Melbinger, A., Vergassola, M. & Di Talia, S. Waves of Cdk1 activity in S-
655 phase synchronize the cell cycle in Drosophila embryos. *Dev. Cell* **38**, 399–412 (2016).

656 79. Uchida, S. *et al.* Binding of 14-3-3β but not 14-3-3σ controls the cytoplasmic localization of
657 CDC25B: binding site preferences of 14-3-3 subtypes and the subcellular localization of
658 CDC25B. *J. Cell Sci.* **117**, 3011–3020 (2004).

659 80. Choudhary, C. *et al.* Lysine acetylation targets protein complexes and co-regulates major
660 cellular functions. *Science* **325**, 834–840 (2009).

661 81. Andersen, J. L. *et al.* A biotin switch-based proteomics approach identifies 14-3-3ζ as a
662 target of Sirt1 in the metabolic regulation of caspase-2. *Mol. Cell* **43**, 834–842 (2011).

663 82. Wright, P. E. & Dyson, H. J. Intrinsically Disordered Proteins in Cellular Signaling and
664 Regulation. *Nat. Rev. Mol. Cell Biol.* **16**, 18–29 (2015).

665 83. Tate, J. G. *et al.* COSMIC: the Catalogue Of Somatic Mutations In Cancer. *Nucleic Acids*
666 *Res.* **47**, D941–D947 (2019).

667 84. F, M. *et al.* The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic*
668 *Acids Res.* **47**, W636–W641 (2019).
669
670

Figure 1

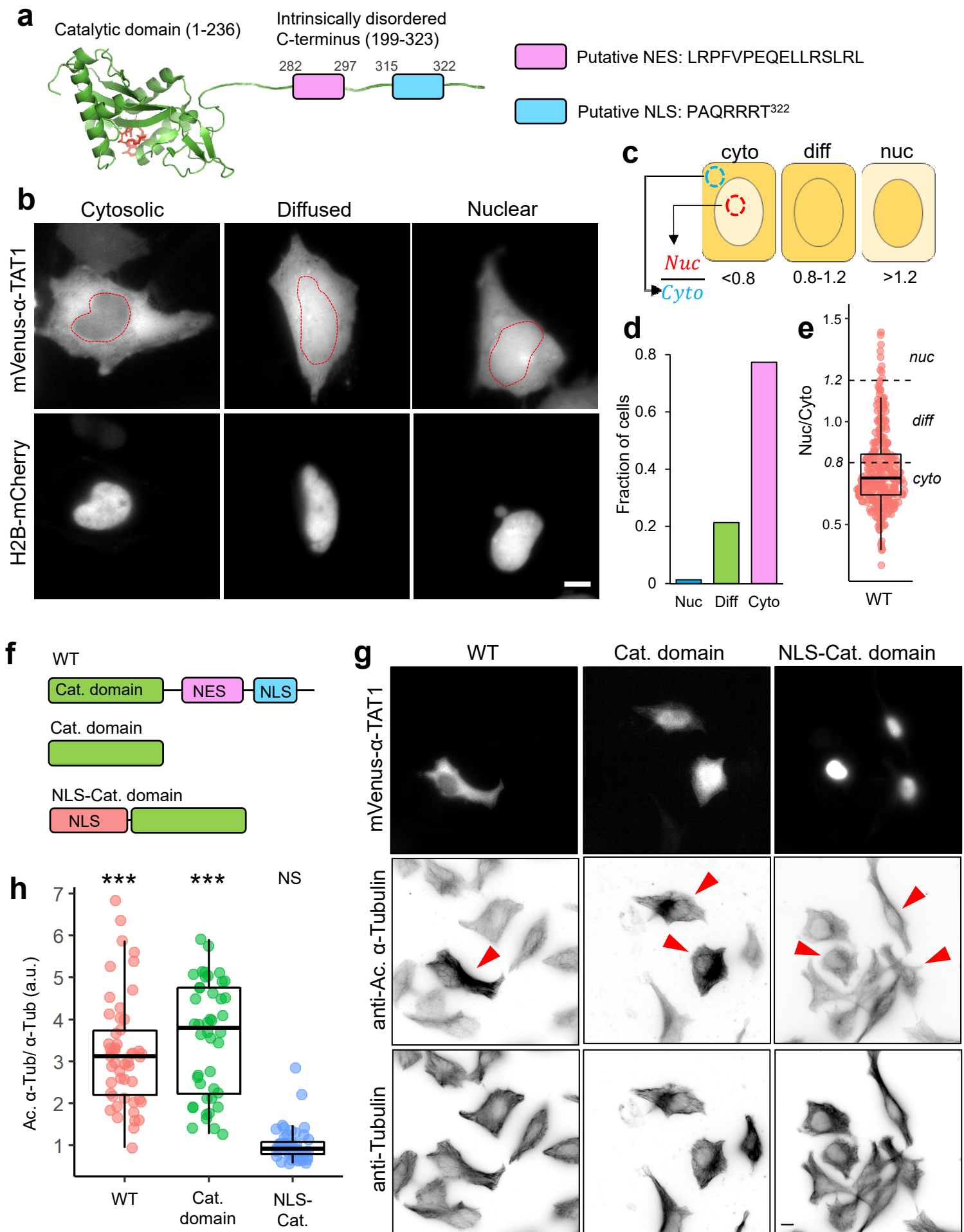


Figure 2

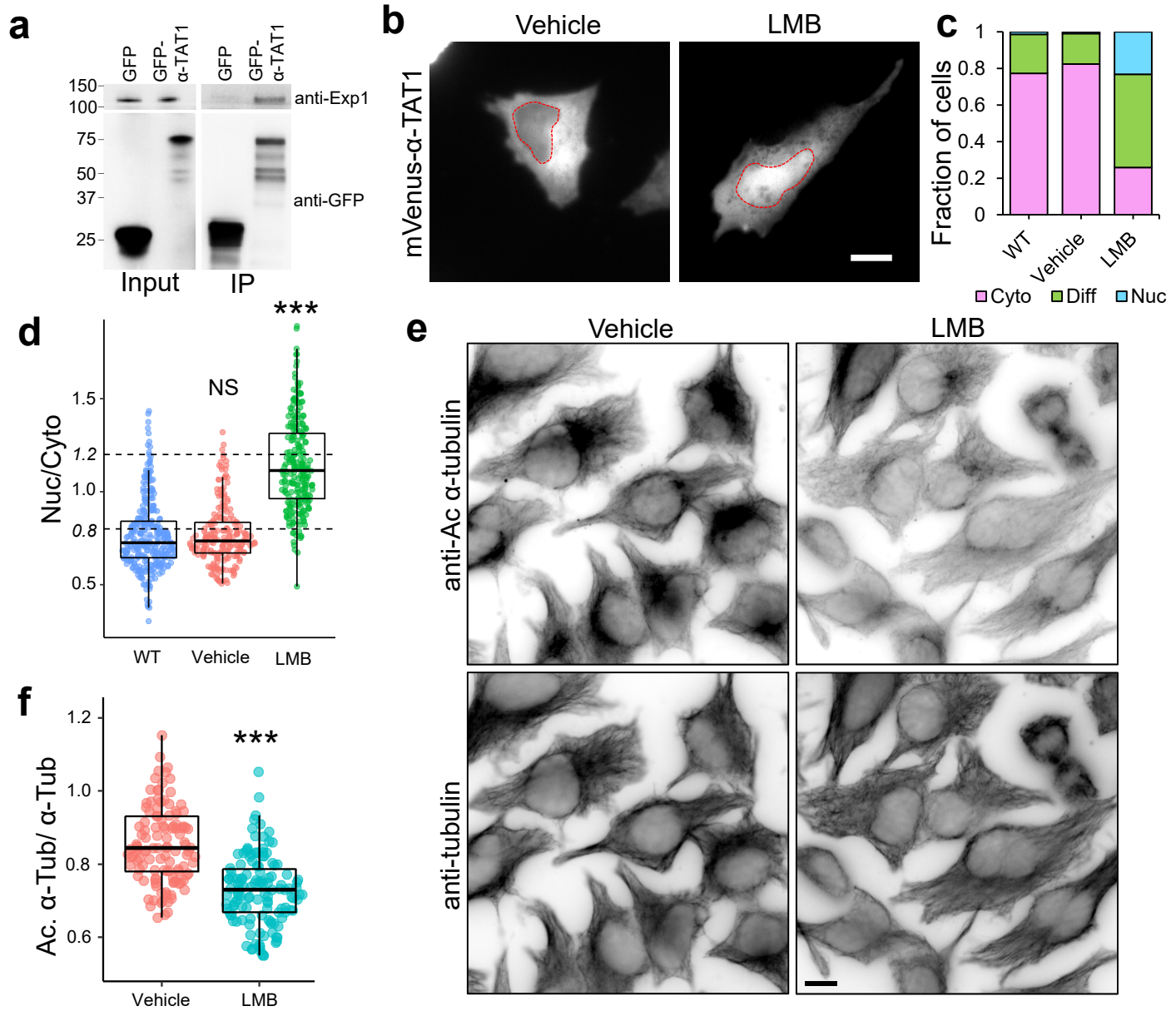


Figure 3

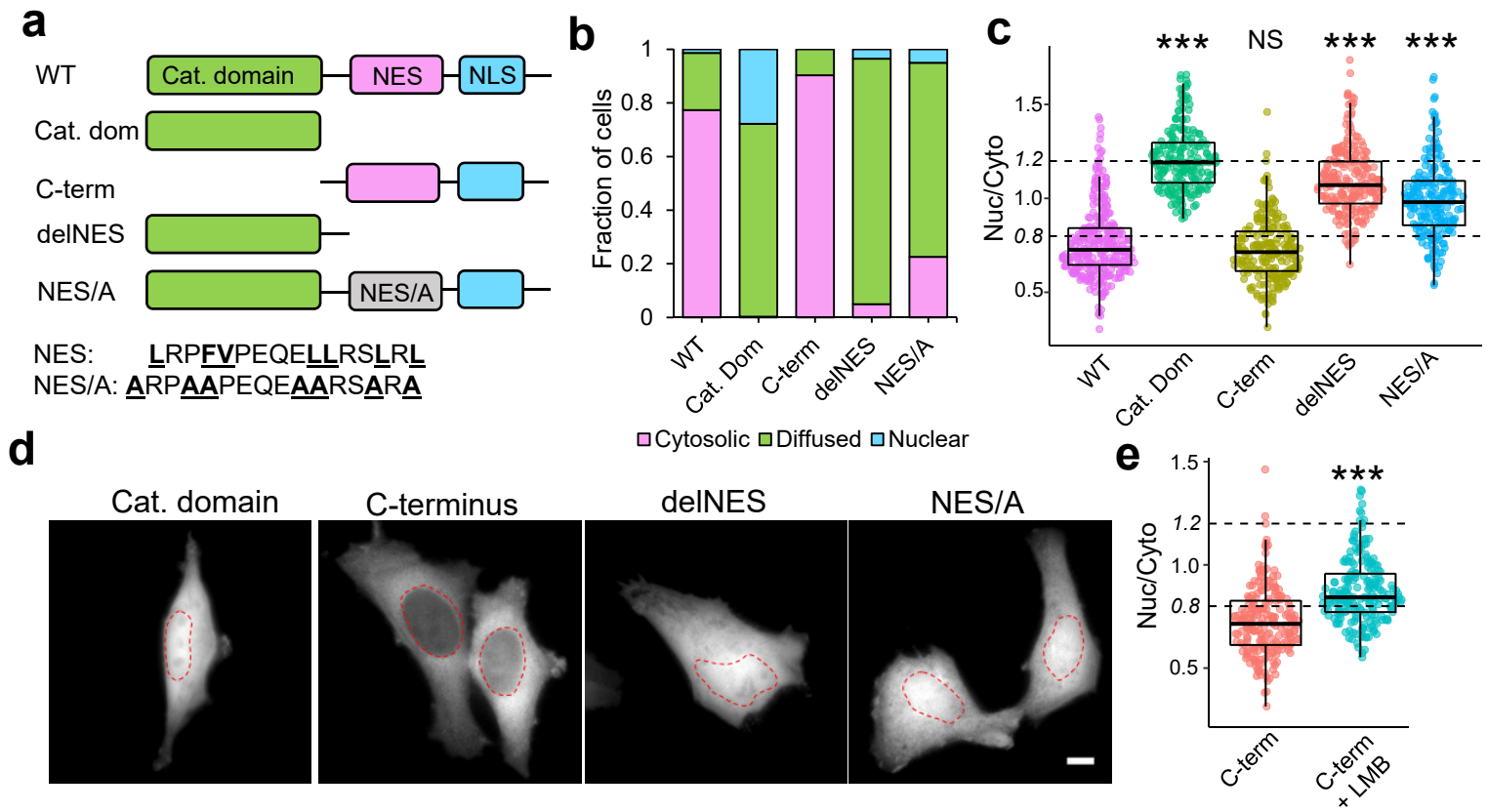


Figure 4

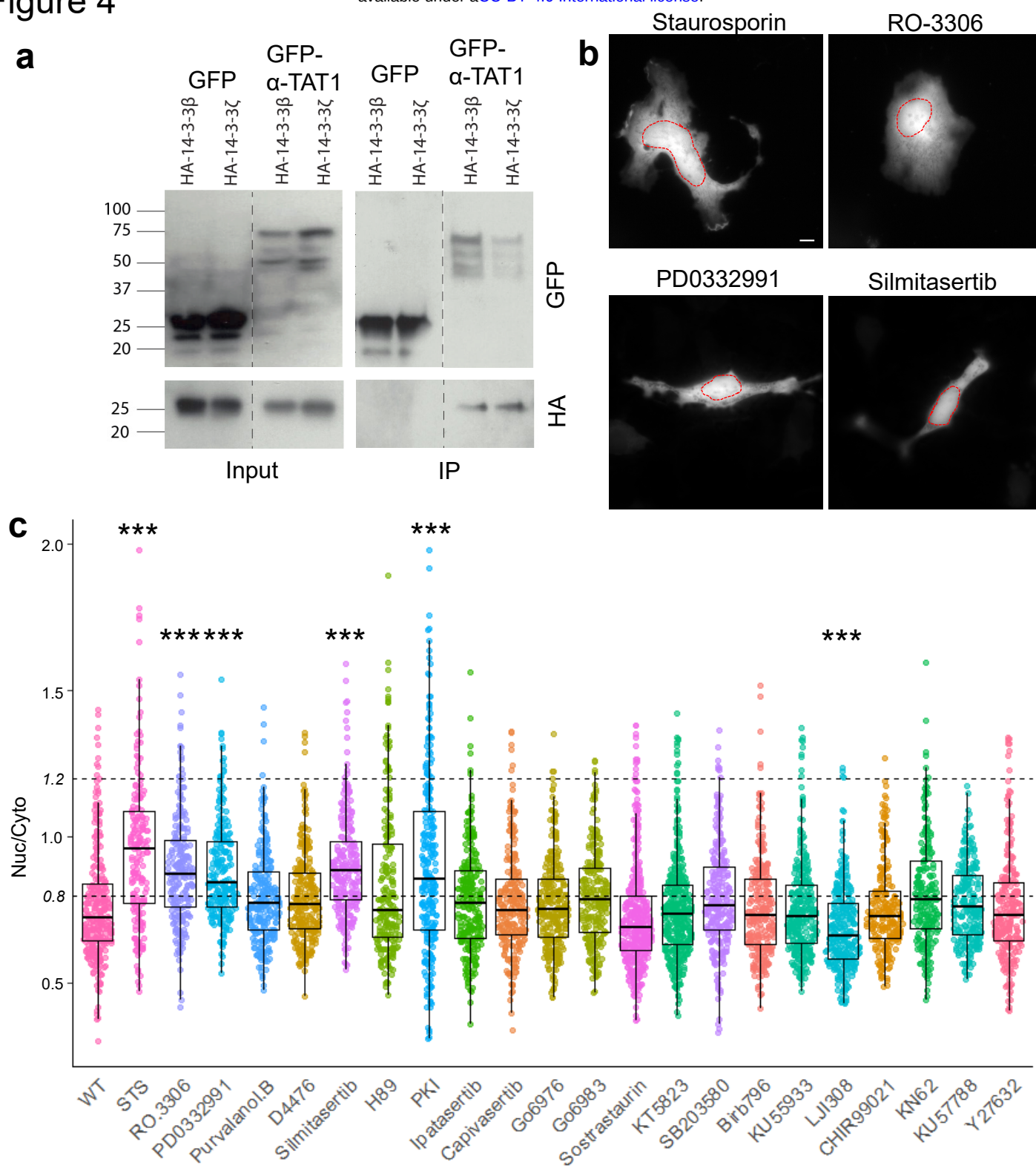
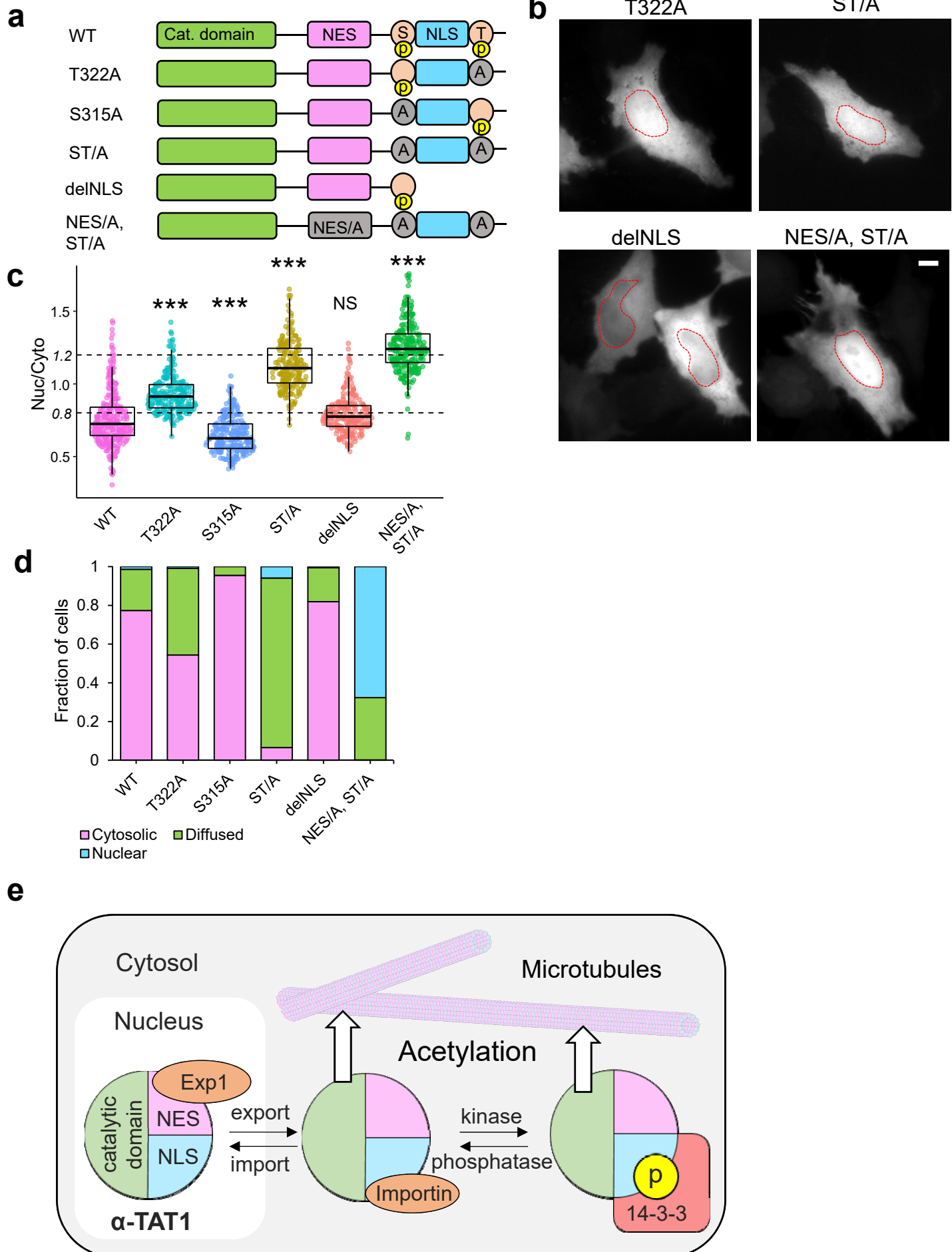


Figure 5



Supplementary Information

A phospho-regulated signal motif of α -TAT1 drives dynamic microtubule acetylation

Abhijit Deb Roy^{1*}, Evan G. Gross², Gayatri S. Pillai², Shailaja Seetharaman^{3,4}, Sandrine Etienne-Manneville³, Takanari Inoue^{1*}

¹ Department of Cell Biology and Center for Cell Dynamics, Johns Hopkins University School of Medicine, Baltimore, 855 North Wolfe Street, MD 21205, USA

² The Johns Hopkins University, Baltimore, MD 21218, USA

³Cell Polarity, Migration and Cancer Unit, Institut Pasteur, UMR3691 CNRS, Equipe Labellisée Ligue Contre le Cancer, F-75015, Paris, France.

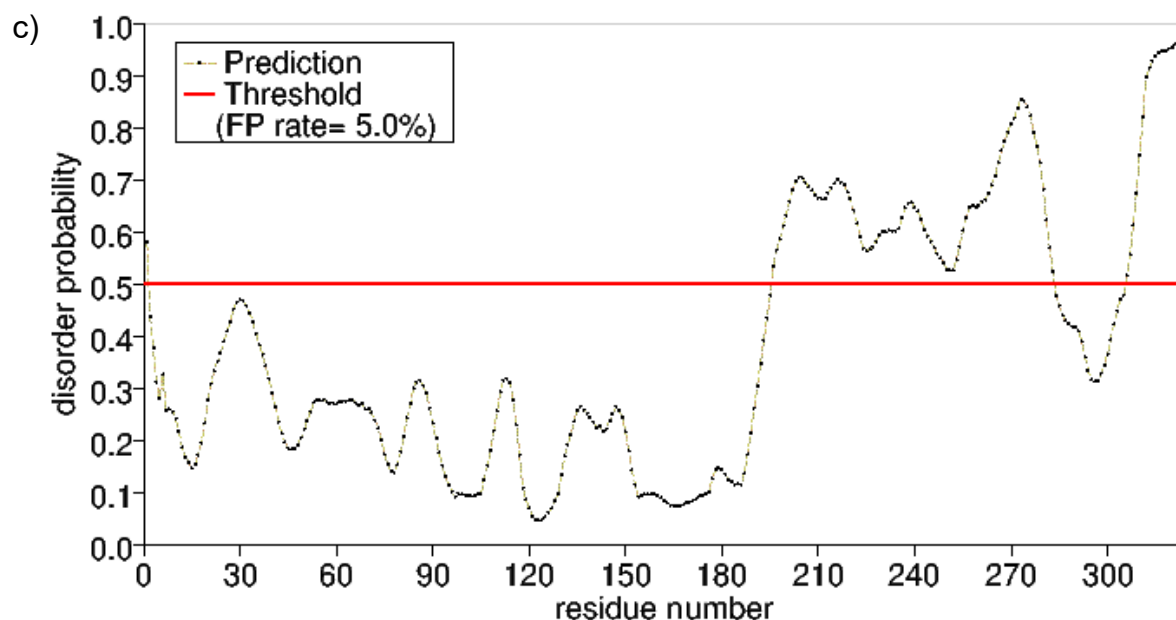
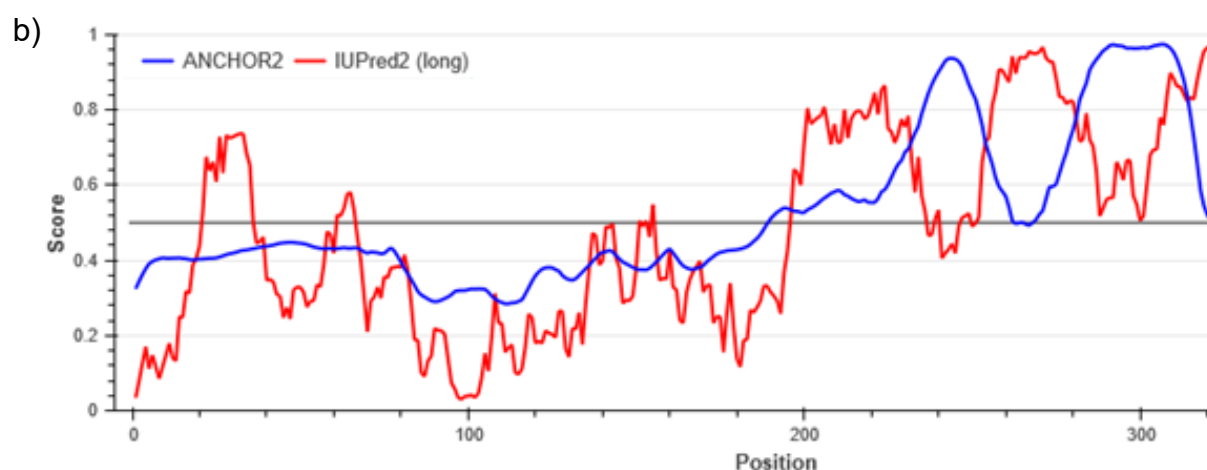
⁴Université Paris Descartes, Sorbonne Paris Cité, 12 Rue de l'École de Médecine, 75006 Paris, France.

* Correspondence:

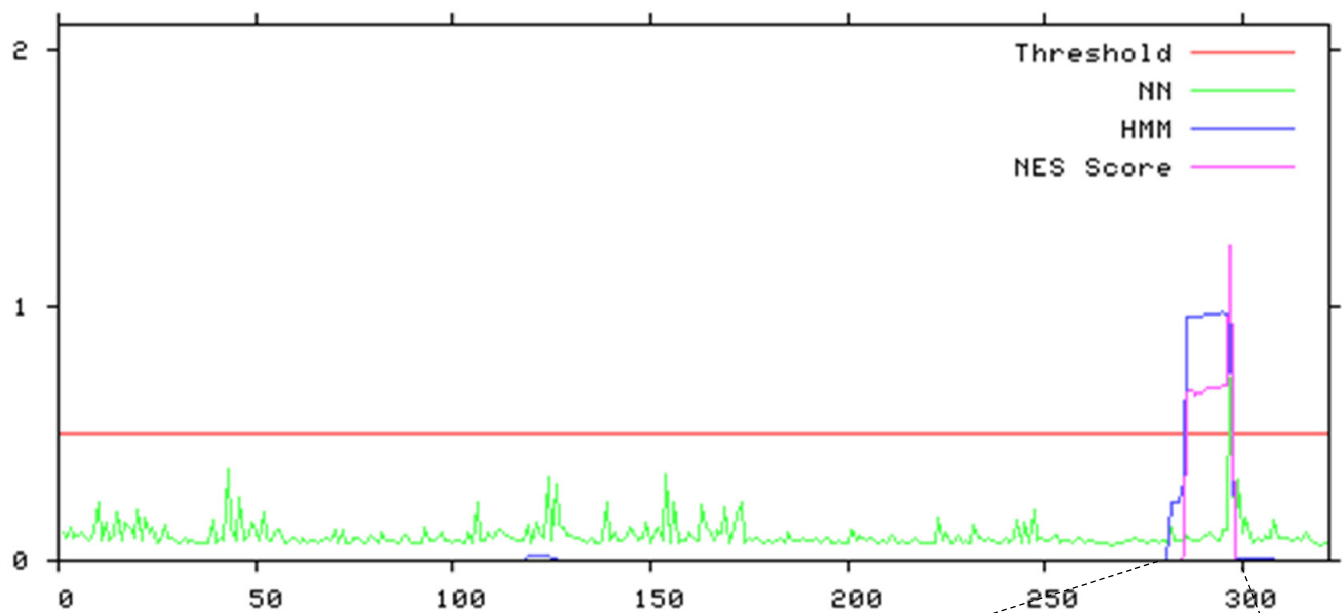
Abhijit Deb Roy abhijit.debrov@gmail.com

Takanari Inoue ictinoue@jhmi.edu

a) Query: α -TAT1 Isoform 7
 MEFPFDVDALFPERITVLDQHLRPPARRPGTTTTPARVDLQQQIMTIIDELGKASAKAQNLSAPITSA
 SRMQSNRHVVYILKDSSARPAGKGAIGFIKVGKFLVDDREAHNEVEPLCILDYIHESVQRH
 GHGRELFQYMLQKERVEPHQLAIDRPSQKLLKFLNKHYNLETTVPQVNNFVIFEGFFAHQHRPPA
 PSLRATRHSRAAAVDPTPAAPARKLPPKRAEGDIKPYSSSDREFLKVAVEPPWPLNRPARRATP
 PAHPPPRSSSLGNSPERG**PLRPFVPEQE****LLRSLRL**CPHPPTARLLLAADPGG**SPAQRRT**³²²R



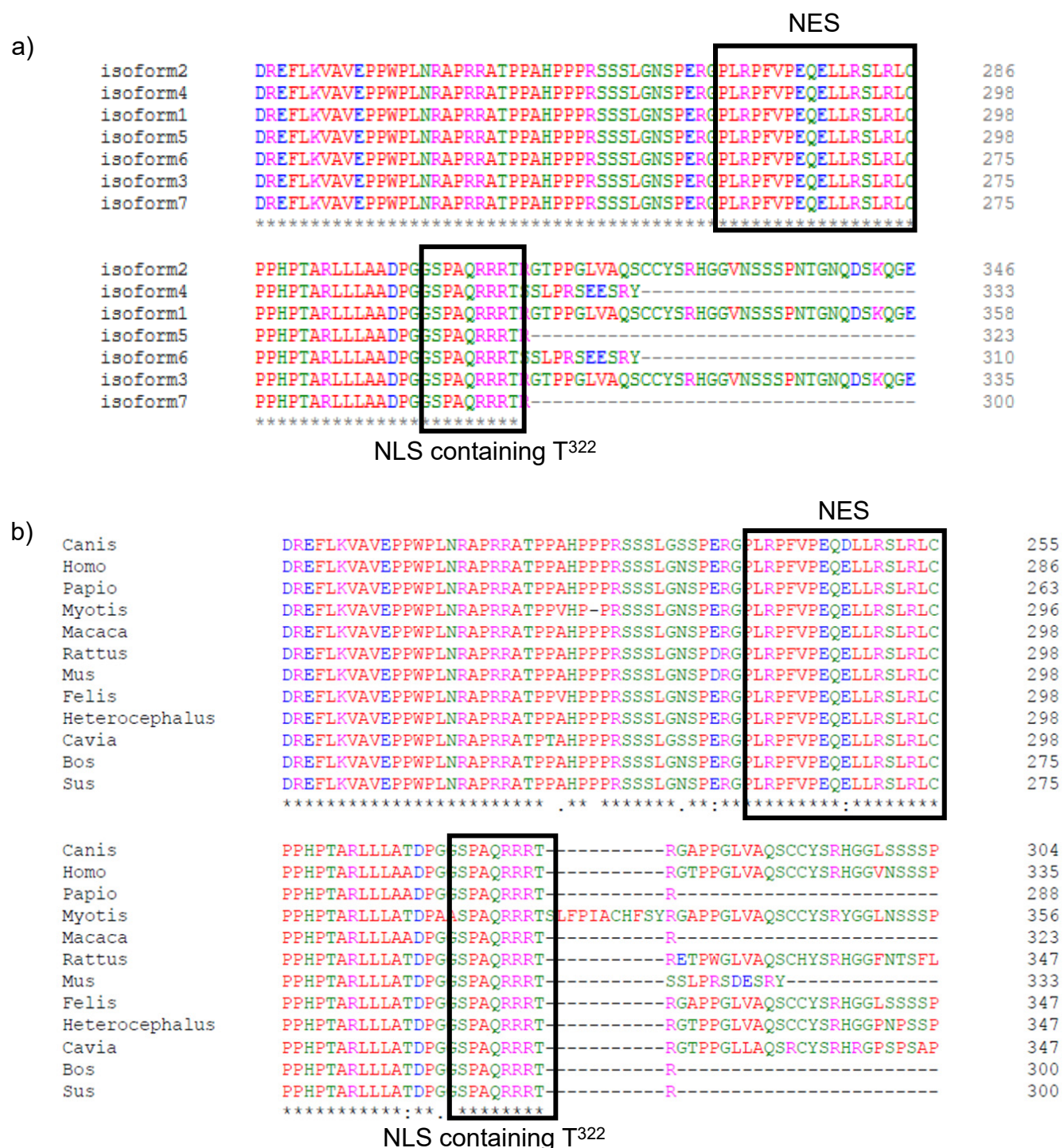
Supplementary Figure S1. α TAT1 C-terminus is disordered. a) amino acid sequence of α TAT1 isoform 7 used as the query, the putative NES and NLS are in bold, b) IUPred2 (and ANCHOR2) and c) PrDOS results suggesting that α TAT1 C-terminus is disordered. The threshold to be considered disordered is 0.5 for both.



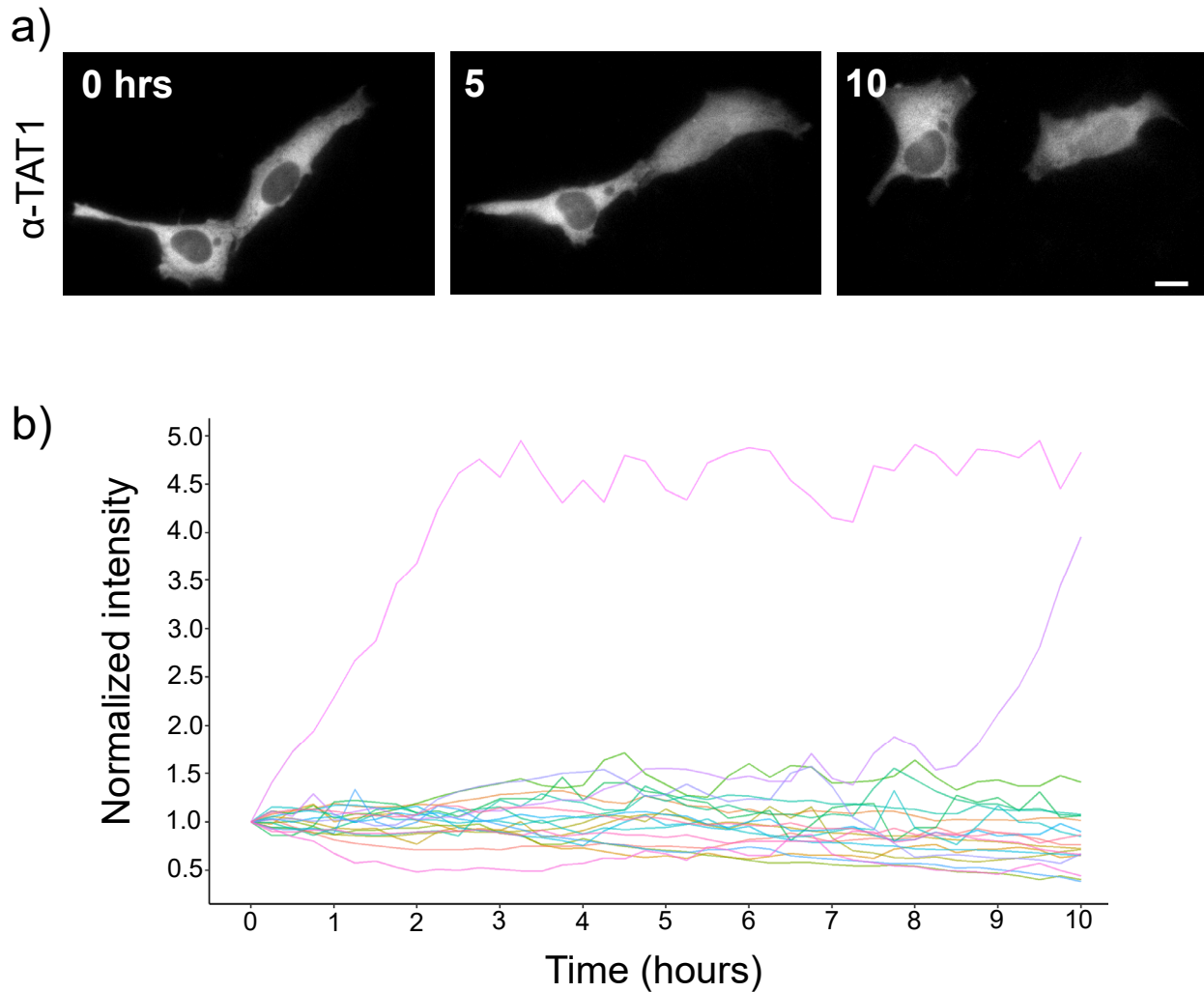
NetNES optimized prediction: LRPF**V**PEQELLRSRL

Hidden Markov Model (HMM) algorithm prediction: LRPF**V**PEQELLRSRL

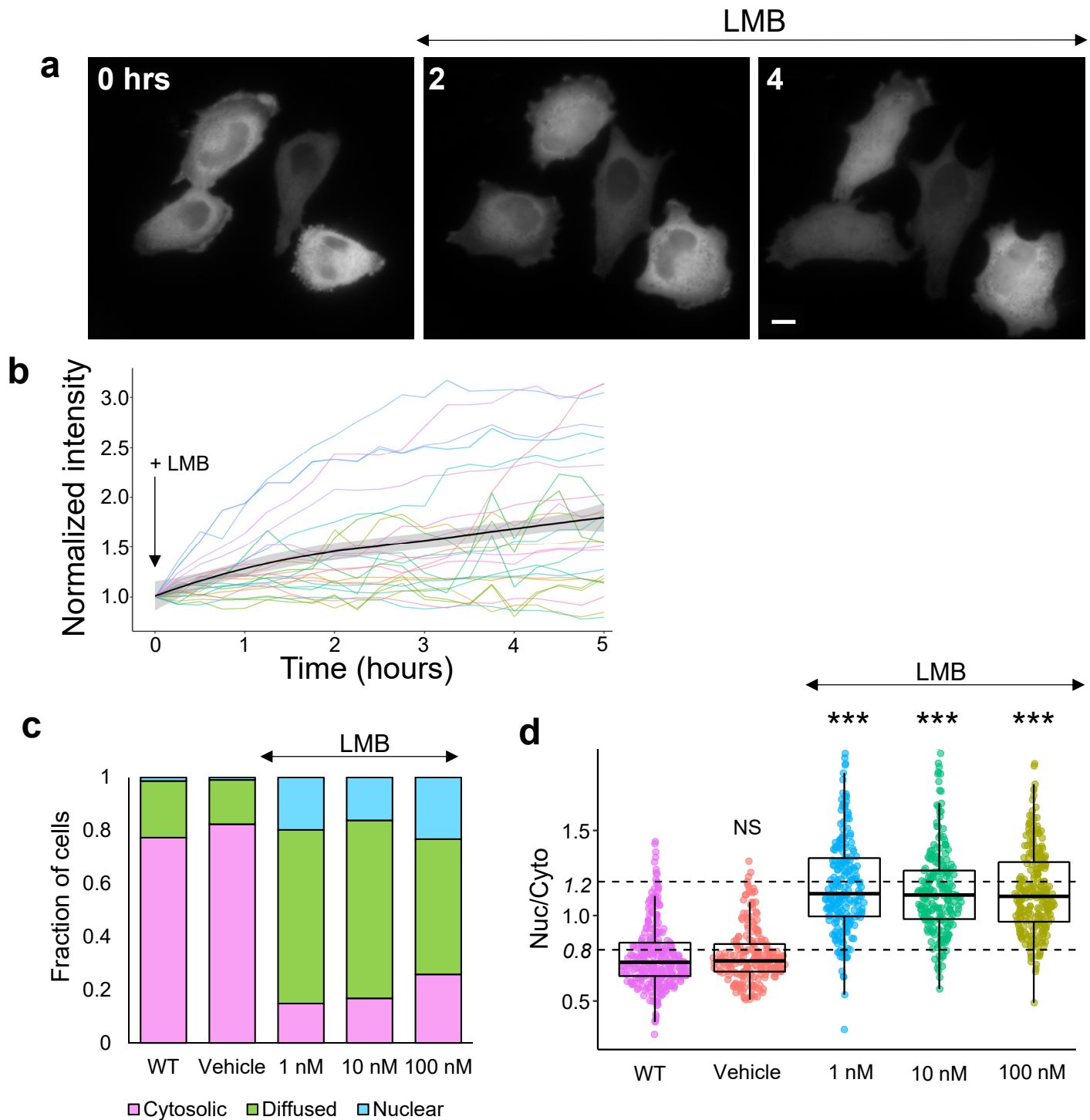
Supplementary Figure S2. NetNES prediction for NES in α TAT1 identifying the putative NES, the predicted NES from NetNES optimized algorithm (pink) and that from Hidden Markov Model (blue) are shown with the hydrophobic residues indicated in bold.



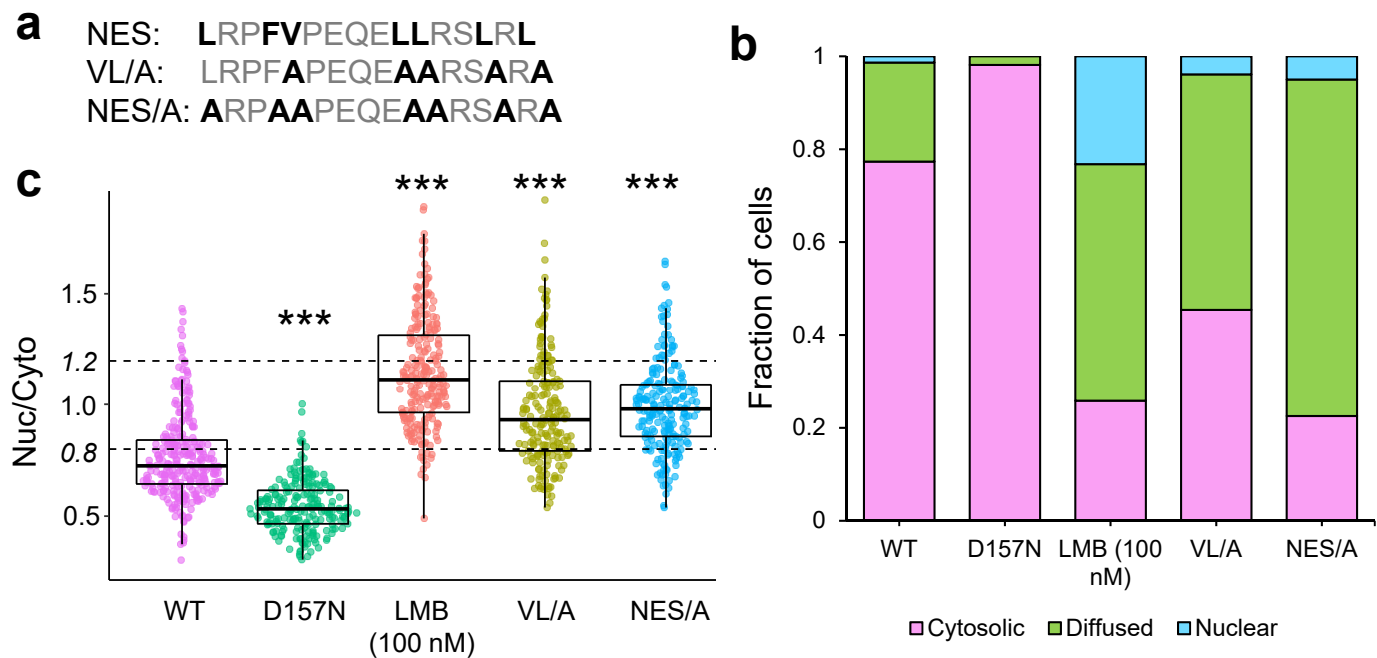
Supplementary Figure S3. The putative NES and NLS in α -TAT1 are evolutionarily conserved. a) Alignment of human α -TAT1 isoforms, b) alignment of different mammalian α -TAT1 showing the C-terminal region including the putative NES and NLS (enclosed in boxes), genus of the mammalian α -TAT1 are shown. Alignments were performed using ClustalW (<https://www.ebi.ac.uk/Tools/msa/clustalo/>)



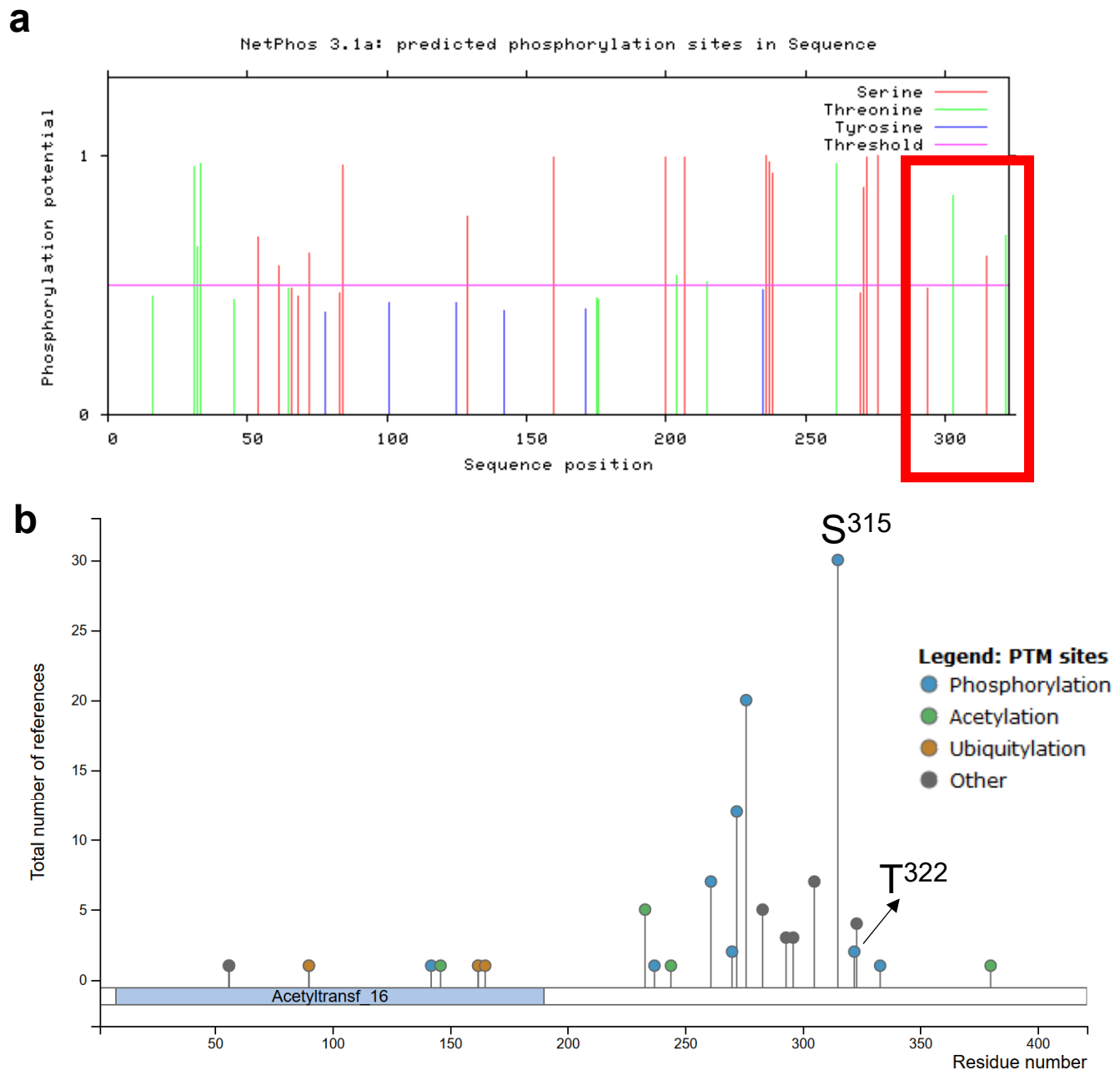
Supplementary Figure S4. α-TAT1 intracellular localization is dynamic in nature. a) Spontaneous temporal changes in spatial distribution of mVenus-α-TAT1, scale bar = 10 μm b) temporal changes in nuclear fluorescence intensity of mVenus-α-TAT1 in HeLa cells, each line indicates normalized nuclear intensity of mVenus- α-TAT1 in a single cell, n = 20 cells



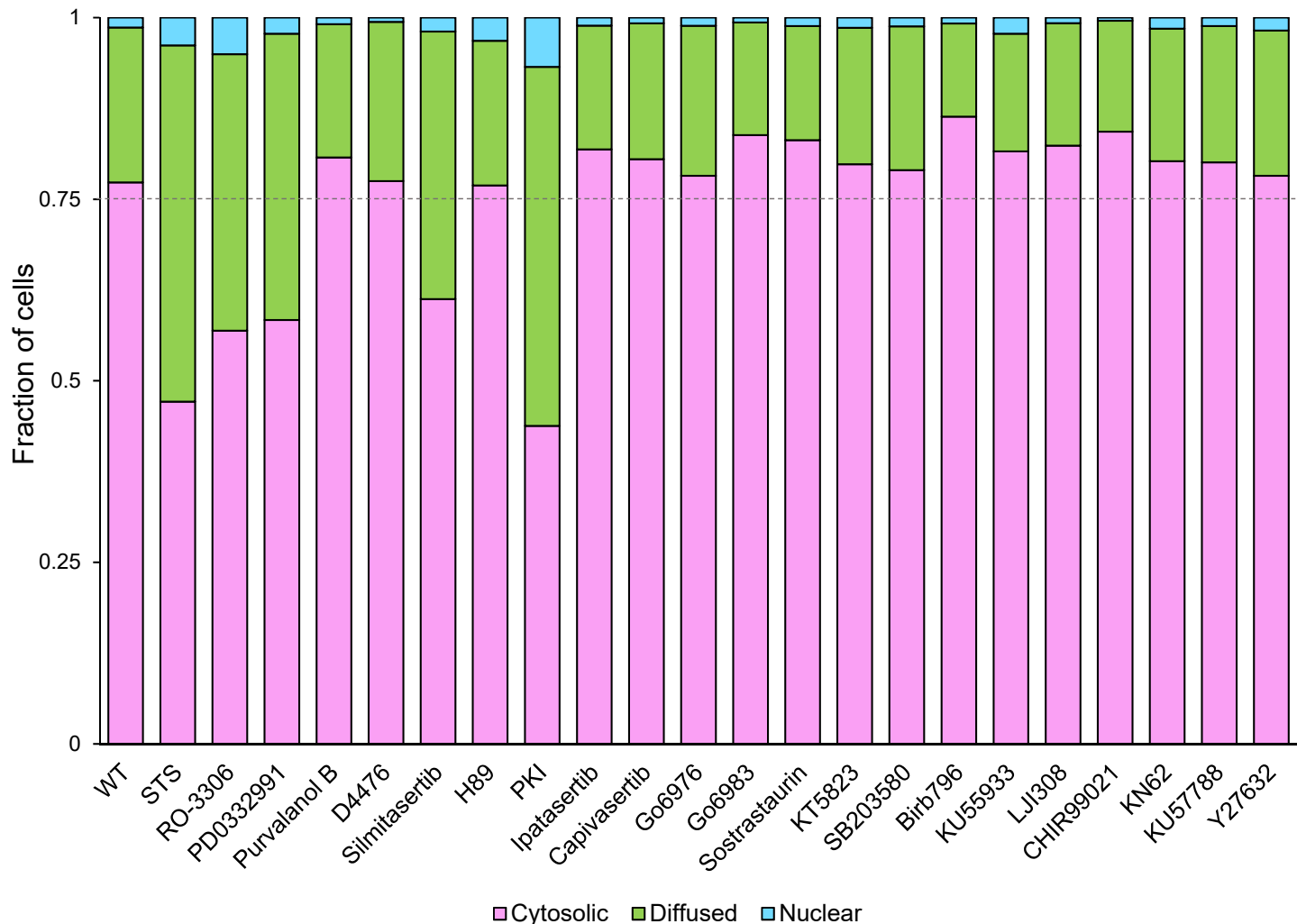
Supplementary Figure S5. α -TAT1 undergoes CRM1 dependent nuclear export. a), b) Temporal changes in mVenus- α -TAT1 localization on 100 nM LMB treatment after LMB addition at time 0, scale bar = 10 μ m, for b), each line indicates normalized nuclear intensity of mVenus- α -TAT1 in a single cell, black line shows the mean and gray line indicate the 95% C.I., n = 26 cells , c) categorical analysis (WT: 1032, vehicle: 450, 1 nM: 329, 10 nM: 291, 100 nM: 495 cells) and d) ratiometric analysis (WT: 304, vehicle: 210, 1 nM: 203, 10 nM: 200, 100 nM: 240 cells) of mVenus- α -TAT1 localization on vehicle (EtOH) or LMB treatment at 1, 10 and 100 nM. *** P <0.001 and NS, not significant, Student's t -test.



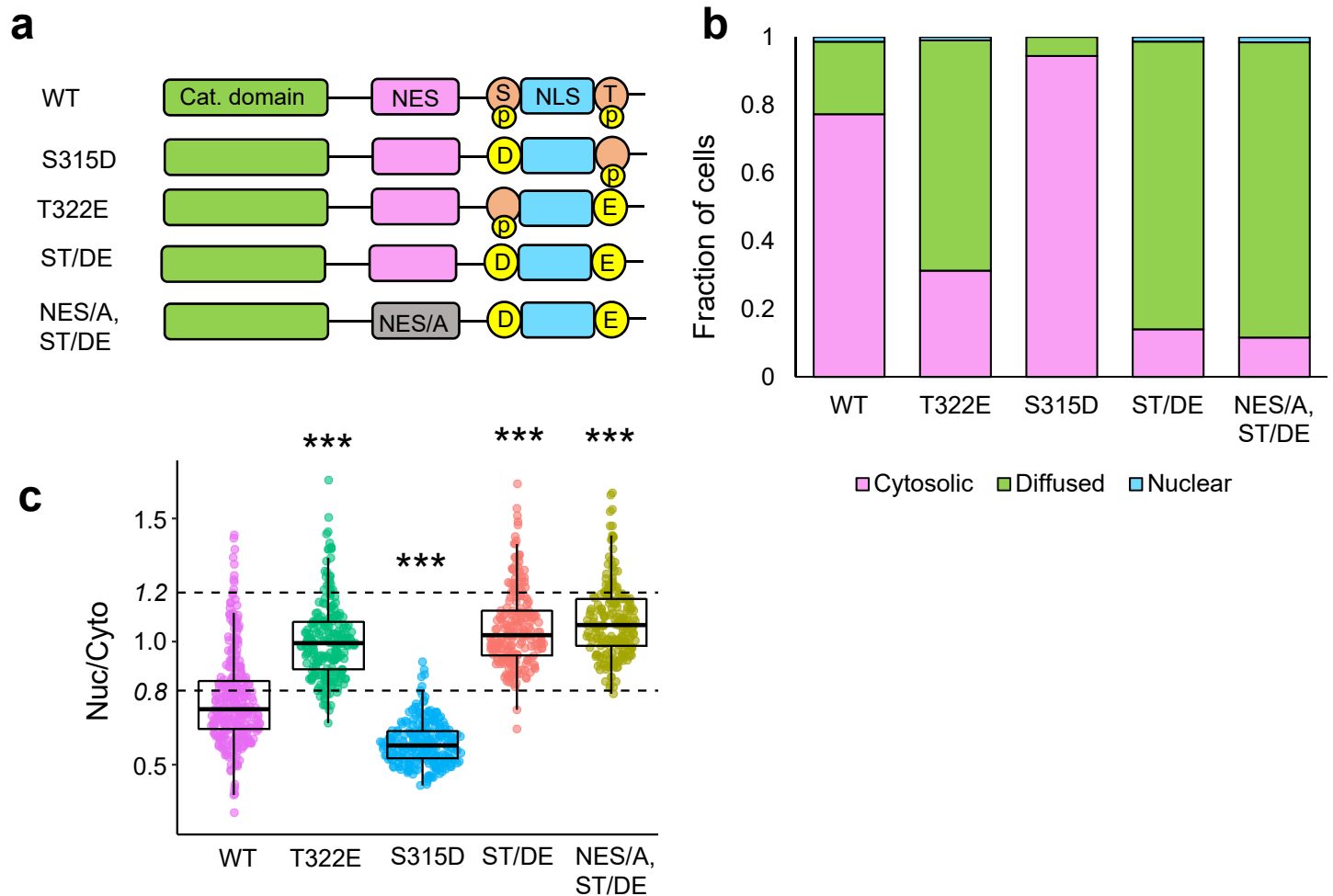
Supplementary Figure S6. α -TAT1 has a C-terminal NES. a) α -TAT1 NES predicted by NetNES and mutants using alanine substitution, b) categorical (WT: 1032, D157N: 429, LMB: 495, VL/A: 436, NES/A: 501 cells), and c) ratiometric (WT: 304, D157N: 217, LMB: 240, VL/A: 201, NES/A: 212 cells) analyses of intracellular localization of mVenus- α -TAT1 and mutants as indicated. *** P <0.001 and NS, not significant, Student's t -test.



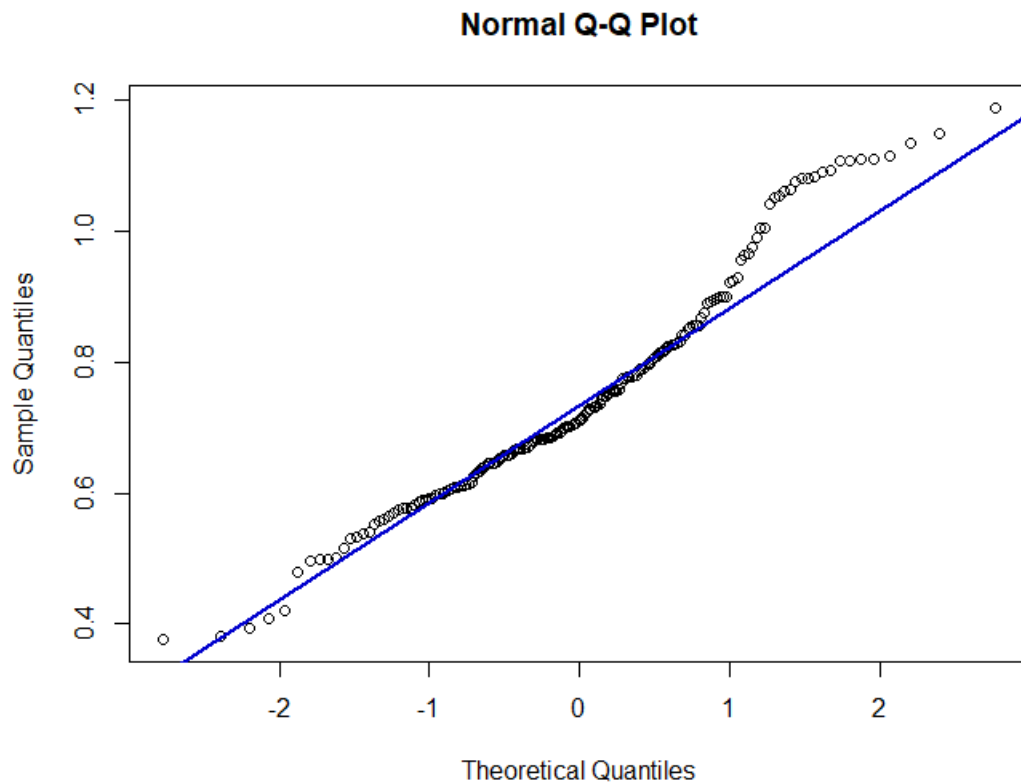
Supplementary Figure S7. a) Putative phosphosites in α -TAT1 predicted by NetPhos, those in F285-R323 are highlighted, b) currently reported post-translational modifications in α -TAT1 curated by PhosphoSitePlus®, www.phosphosite.org.



Supplementary Figure S8. Categorical analysis of α -TAT1 localization on treatment with kinase inhibitors. WT: 1032, STS: 157, RO-3306: 796, PD0332991: 543, Purvalanol B: 992, D4476: 689, 418, H89: 251, PKI: 354, Ipatasertib: 546, Capivasertib: 385, Go6976: 712, Go6983: 606, Sostrastaurin: 605, KT5823: 570, SB203580: 505, Birb796: 991, KU55933: 815, LJ1308: 522, CHIR99021: 484, KN62: 531, KU57788: 949, Y27632: 340 cells.



Supplementary Figure S9. α -TAT1 has a C-terminal phospho-inhibited NLS. a) Cartoon showing α -TAT1 NES and NLS flanked by potential phosphosites and mutants, b) categorical (WT:1032, S315A: 442, S315D: 412, T322E: 310, ST/DE: 258 cells) and c) ratiometric (WT: 304, S315A: 212, S315D: 223, T322E: 232, ST/DE: 195 cells) analyses of intracellular localization of mVenus- α -TAT1 mutants. *** P <0.001 and NS, not significant, Student's t -test.



Supplementary Figure S10. Normal probability plot of nuclear/cytosolic ratio of mVenus- α -TAT1 expressed in HeLa cells.

Drug	Conc. Used	target kinase	Incubation time	p value (t-test)
STS	100 nM	Pan kinase	4 hours	1.20187E-11
RO3306	10 μ M	Cdk1, Cdk2	4 hours	1.87845E-10
PD0332991	1 μ M	Cdk4, Cdk6	4 hours	1.29985E-10
Purvalanol B	1 μ M	Cdk1, Cdk2, Cdk5	4 hours	0.086698844
D4476	1 μ M	CK1	4 hours	0.244864719
Silmitasertib	10 μ M	CK2	4 hours	7.34743E-12
H89	10 μ M	PKA	4 hours	0.001868139
PKI	NA	PKA	Overnight	8.76993E-08
Ipatasertib	10 μ M	PKB (Akt)	4 hours	0.026614985
Capivasertib	1 μ M	PKB (Akt)	4 hours	0.928873535
Go6976	250 nM	PKC	4 hours	0.900843024
Go6983	250 nM	PKC	4 hours	0.068144954
Sostrastaurin	10 μ M	PKC	4 hours	0.008916116
KT5823	5 μ M	PKG	4 hours	0.581314971
SB203580	10 μ M	p38 MAPK	4 hours	0.007945499
Birb796	10 μ M	p38 MAPK	4 hours	0.768174955
KU55933	10 μ M	ATM	4 hours	0.26994365
LJI308	10 μ M	RSK	4 hours	9.62544E-07
CHIR99021	1 μ M	GSK3	4 hours	0.309440886
KN62	10 μ M	CaMK-II, P2RX7	4 hours	0.021582087
KU57788	1 μ M	DNA-PK	4 hours	0.385979985
Y27632	10 μ M	ROCK	4 hours	0.375473038

Supplementary Table T1. Details of kinase inhibitors used.