

# A phospho-regulated ensemble signal motif of $\alpha$ -TAT1 drives dynamic microtubule acetylation

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

- 2 acetylation
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#### 20 Abstract:

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22 Spatiotemporal patterns of microtubule modifications such as acetylation underlie diverse cellular functions. While the molecular identity of the acetylating agent, α-tubulin N-23 24 acetyltransferase 1 ( $\alpha$ -TAT1), as well as the functional consequences of microtubule acetyla-25 tion have been revealed, the molecular mechanisms that regulate multi-tasking α-TAT1 action 26 for dynamic acetylation remain obscure. Here we identified a signal motif in the intrinsically 27 disordered C-terminus of  $\alpha$ -TAT1, which comprises three functional elements - nuclear export, 28 nuclear import and cytosolic retention. Their balance is tuned via phosphorylation by serine-29 threonine kinases to determine subcellular localization of α-TAT1. While the phosphorylated 30 form binds to 14-3-3 adapters and accumulates in the cytosol for maximal substrate access, 31 the non-phosphorylated form is sequestered inside the nucleus, thus keeping microtubule 32 acetylation minimal. As cancer mutations have been reported to this motif, the unique ensem-33 ble regulation of α-TAT1 localization may hint at a role of microtubule acetylation in aberrant 34 physiological conditions.

35

#### 37 Introduction:

#### 38

39 Acetylation of Lysine-40 of α-tubulin is an evolutionarily conserved post-translational modifica-40 tion observed across eukaryotic species<sup>1-3</sup>, which is involved in diverse physiological and pathological states<sup>4–6</sup>. Acetylation is mainly observed in polymerized microtubules<sup>7–9</sup> and may 41 42 provide structural flexibility to stabilize microtubules against bending forces<sup>10–13</sup>. In cultured 43 cells, microtubule acetylation mediates focal adhesion dynamics, adaptation to extracellular 44 matrix rigidity as well as regulation of tissue stiffness<sup>14–17</sup>. Additionally, acetylated microtu-45 bules regulate touch sensation in *M. musculus*, *C. elegans* and *D. melanogaster*, suggesting a role in mechano-response<sup>18-21</sup>. Microtubule acetylation has been implicated in axonal 46 47 transport in neurons<sup>22–24</sup>, migration in cancer cells<sup>5,25–27</sup>, autophagy<sup>6,28,29</sup>, podosome stabilization in osteoclasts<sup>30</sup> and viral infections<sup>31–34</sup>.  $\alpha$ -TAT1 is the only known acetyltransferase for  $\alpha$ -48 tubulin<sup>35,36</sup> in mammals. α-TAT1 predominantly catalyzes α-tubulin in stable polymerized mi-49 50 crotubules<sup>19,37</sup>, and may have additional effects on microtubules independent of its catalytic activity<sup>38</sup>. 51

52 Although microtubule acetylation is spatially and temporally regulated downstream of 53 many molecular signaling pathways, little is known about how these pathways converge on  $\alpha$ -54 TAT1 to achieve such dynamic patterns. In the present study, we used computational 55 sequence analyses and live cell microscopy to identify a conserved motif in the intrinsically 56 disordered C-terminus of  $\alpha$ -TAT1, consisting of an NES and an NLS, that mediates its spatial distribution. We show that cytosolic localization of  $\alpha$ -TAT1 is critical for microtubule 57 58 acetylation. We further demonstrate that nuclear localization of  $\alpha$ -TAT1 is inhibited by the 59 action of serine-threonine kinases, specifically cyclin dependent kinases (CDKs), Protein 60 Kinase A (PKA) and Casein kinase 2 (CK2) and identify 14-3-3 proteins as binding partners of 61  $\alpha$ -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.

#### 65 **Results**:

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67 **α-TAT1 localization mediates microtubule acetylation:** α-TAT1 has an N-terminal catalytic 68 domain that shows homology to other acetyltransferases, while its C-terminus was not resolved in crystal structures<sup>39</sup> (Fig. 1a). Based on its amino acid sequence, α-TAT1 C-69 70 terminus was predicted to be intrinsically disordered by both IUPred2A<sup>40</sup> and PrDOS<sup>41</sup> 71 prediction servers (Fig. 1a, Supplementary Fig. S1a, b, c). To explore if the intra-cellular 72 localization of α-TAT1 is regulated, we sought to identify any localization signals present in the 73 α-TAT1 amino acid sequence (Supplementary Fig. S1a). The prediction program NetNES<sup>42</sup> 74 identified a putative NES in α-TAT1 C-terminus (Fig. 1a, Supplementary Fig. S2). On the other 75 hand, PSORT-II<sup>43</sup> subcellular localization program predicted that α-TAT1 should be predominantly localized to the nucleus due to the presence of a putative class 4 NLS<sup>44</sup> in its 76 77 C-terminus (Fig. 1a). The region encompassing the putative NES and NLS is conserved 78 across the human α-TAT1 isoforms (Supplementary Fig. S3a), as well as across mammalian 79  $\alpha$ -TAT1 proteins (Supplementary Fig. S3b). To test whether  $\alpha$ -TAT1 indeed showed any 80 intracellular distribution pattern, we expressed mVenus-α-TAT1 in HeLa cells, and observed 81 distinct nuclear exclusion in most cells, although a subset of cells showed lack of exclusion 82 (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 83 84 diffused pattern (i.e., both cytosolic and nuclear distribution) and 1% of cells showed nuclear enrichment of mVenus- $\alpha$ -TAT1 (Fig. 1d). These observations were consistent with ratiometric 85 86 analysis (see Methods, Fig 1c) of mVenus- $\alpha$ -TAT1 distribution (Fig. 1e). Time lapse 87 microscopy showed temporal changes in fluorescence intensity of mVenus- $\alpha$ -TAT1 in cell 88 nuclei and cytosol (Supplementary Fig. S4a, b), suggesting that  $\alpha$ -TAT1 localization is 89 dynamic by nature.

90  $\alpha$ -TAT1 preferentially acetylates polymerized microtubules, which are typically 91 cytosolic. Based on this, we hypothesized that spatial regulation of  $\alpha$ -TAT1 may control its 92 function. Exogenous expression of mVenus-α-TAT1 or its catalytic domain (residues 1-93 236)<sup>19,39</sup> (Fig. 1f) was sufficient to significantly increase  $\alpha$ -tubulin acetylation in HeLa cells 94 compared to non-transfected cells (Fig. 1g, h). To test whether nuclear localization may 95 sufficiently sequester a-TAT1 from microtubules, we tethered the NLS from cMyc to a-TAT1 catalytic domain and thus localized it to the nucleus (Fig. 1f, g top panel). Exogenous 96 97 expression of NLS-mVenus- $\alpha$ -TAT1(1-236) did not increase  $\alpha$ -tubulin acetylation levels 98 compared to non-transfected cells (Fig. 1 g, h), suggesting that nuclear sequestration of  $\alpha$ -99 TAT1 inhibits its function.

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101 a-TAT1 undergoes Exportin 1 dependent nuclear export: To dissect the molecular 102 mechanisms of  $\alpha$ -TAT1 localization in the cytosol, we speculated that  $\alpha$ -TAT1 is actively 103 exported out of the nucleus, and/or that  $\alpha$ -TAT1 binds to a protein that keeps the complex out 104 of the nucleus. We began by testing the first possibility by assessing involvement of the 105 nuclear export machinery. Exportin 1 (Exp1), also called Chromosome region maintenance 1 106 protein homolog (CRM1), mediates nuclear export of many proteins<sup>45,46</sup>. GFP-α-TAT1, but not 107 GFP, co-immunoprecipitated with endogenous Exp1 (Fig. 2a), indicating an interaction 108 between these two proteins. Treatment with 100 nM Leptomycin-B (LMB), an inhibitor of Exp1 109 mediated nuclear export<sup>47</sup>, significantly decreased the number of cells displaying nuclear 110 exclusion of mVenus-α-TAT1 compared to vehicle (Fig 2b, c, d). Inhibition of nuclear export 111 was initiated within an hour of LMB treatment, although some cells were refractory to the 112 treatment (Supplementary Fig. S5a, b). Decreased LMB concentrations (1 nM and 10 nM) 113 had a comparable impact as 100 nM dosage (Supplementary Fig. S5c, d). Furthermore, LMB 114 treatment induced significant reduction in  $\alpha$ -tubulin acetylation levels in HeLa cells within 4

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

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121 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 122 123 of a-TAT1 is regulated by its catalytic activity, we expressed a catalytic dead mutant, mVenus-124 α-TAT1(D157N)<sup>39</sup>, in HeLa cells. mVenus-α-TAT1(D157N) did not display any loss of nuclear 125 exclusion (Supplementary Fig. S6a, b, c). The catalytic domain, mVenus- $\alpha$ -TAT1(1-236), 126 displayed a complete loss of nuclear exclusion; whereas the C-terminus, mVenus-a-127 TAT1(236-323), displayed a distribution pattern comparable to that of WT (Fig. 3a, b, c, d). In 128 addition, inhibition of Exp1 by 100 nM LMB significantly reduced the nuclear exclusion of mVenus- $\alpha$ -TAT1 C-terminus (Fig. 3e). Exclusion of  $\alpha$ -TAT1 C-terminus (size  $\approx$  38 kDa) further 129 130 indicates that nuclear exclusion of mVenus- $\alpha$ -TAT1 (size  $\approx$  63 kDa) is not simply due to size 131 exclusion of passive diffusion into nuclei, and demonstrates that nuclear exclusion of α-TAT1 132 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<sup>42,45</sup> 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  $\alpha$ -TAT1 C-terminus (Supplementary Fig S2). Interestingly, this region is also predicted to be a site of protein-protein interactions by ANCHOR2 prediction software<sup>40</sup> (Supplementary Fig. S1b). Truncation of this putative NES,  $\alpha$ -TAT1delNES, abrogated its nuclear exclusion (Fig. 3a, b, c, d). Although alanine substitution of the

140 hydrophobic residues in the V286-L297 region,  $\alpha$ -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, we observed that the NES predicted by the Hidden Markov Model (Supplementary Fig. S2) 143 144 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 (Fig. 3a, b, c, d, Supplementary Fig. S6a, b, c), suggesting that these residues contribute to 146 147 the nuclear export of a-TAT1. Taken together, our data suggest that a-TAT1 has a 148 hydrophobic NES in its C-terminus.

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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 NLS<sup>48–50</sup>. Part of the putative NLS sequence "PAQRRRTR" bears similarity to 14-3-3 binding 154 motif RXX(pS/pT)XP<sup>51</sup>, and 14-3-3-Pred<sup>52</sup>, a 14-3-3 interaction prediction server identified 155 156 T322 as a potential 14-3-3 binding site. To examine whether  $\alpha$ -TAT1 bound to 14-3-3 proteins. 157 we co-expressed GFP- $\alpha$ -TAT1 with HA-tagged 14-3-3- $\beta$  or 14-3-3- $\zeta$  in HEK-293T cells and performed a co-immunoprecipitation assay. GFP-α-TAT1, but not GFP alone, co-precipitated 158 159 with HA-14-3-3- $\beta$  and HA-14-3-3- $\zeta$  (Fig. 4a). These observations are consistent with our observations in mass spectrometry analysis of  $\alpha$ -TAT1 in HEK cells that identified 14-3-3- $\beta$ 160 161 and 14-3-3-ζ as potential interactors<sup>53</sup>.

162

163 Serine/threonine kinase activities mediate cytosolic localization of  $\alpha$ -TAT1. 14-3-3 164 binding to proteins is mediated by phosphorylated serine and threonine residues<sup>51</sup>. NetPhos

165 prediction server identified over 30 putative phosphosites in  $\alpha$ -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 nuclear localization of  $\alpha$ -TAT1 (Fig. 4b, c, Supplementary Fig. S8), suggesting that 168 169 phosphorylation negatively regulated nuclear localization of  $\alpha$ -TAT1. To identify the specific 170 kinases that regulate  $\alpha$ -TAT1 localization, we treated cells expressing mVenus- $\alpha$ -TAT1 with 171 several serine-threonine kinase inhibitors (summarized in Supplementary Table T1). Both RO-172 3306 (inhibitor for CDK1 and CDK2<sup>54,55</sup>) and PD0332991 (also called Pablociclib, inhibitor for 173 CDK4 and CDK6<sup>56,57</sup>), but not Purvalanol-B (inhibits CDK1, CDK2 and CDK5<sup>55</sup>), 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 CK2<sup>58</sup>) but not D-4476 (inhibitor for CK1<sup>59</sup>) 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 PKA<sup>60</sup>, considerably increased nuclear localization of mVenus-179 α-TAT1 (Fig 4c, Supplementary Fig. S8, Supplementary Table T1). Finally, treatment with 180 LJI308 (inhibitor for Ribosomal S9 kinase) appeared to further increase nuclear exclusion of 181 mVenus- $\alpha$ -TAT1 (Fig. 4c, Supplementary Table T1). These data demonstrate that nuclear 182 localization of α-TAT1 is negatively regulated by CDKs, CK2 and PKA.

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

190 they also flank the putative NLS (Fig. 5a). Importin- $\alpha$  binds with NLS enriched in basic residues through a charge-based interaction<sup>44</sup>. Phosphorylation of amino acids adjacent to 191 192 such an NLS may inhibit the association of Importin- $\alpha$  binding through a disruption of the 193 charge balance in the NLS region<sup>61</sup>. 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  $\alpha$ -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-TAT1. These observations suggest that the phosphate moiety in phosphorylated T322 and 205 206 S315 is critical for inhibition of  $\alpha$ -TAT1 nuclear import.

207 One possible explanation of increased nuclear localization of T322A mutant is that 208 phospho-T322 mediates nuclear export of  $\alpha$ -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  $\alpha$ -TAT1. Furthermore, alanine substitution of the 211 hydrophobic residues in  $\alpha$ -TAT1 NES as well as S315 and T322,  $\alpha$ -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.  $\alpha$ -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  $\alpha$ -TAT1 NLS and that the  $\alpha$ -TAT1 NES and NLS act independently of one another.
- 217

#### 218 **Discussion**:

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220 One of the bottlenecks in elucidating the role of microtubule acetylation in biological 221 phenomena is the knowledge gap of how upstream molecular signaling pathways control α-222 TAT1 function to modulate microtubule acetylation. Our study demonstrates that intracellular 223 α-TAT1 localization is a dynamically regulated process, orchestrated by a balance of nuclear 224 export and import, which modulates microtubule acetylation levels (Fig. 5e). To our 225 knowledge, this is the first study to identify the molecular mechanisms that spatially regulate 226  $\alpha$ -TAT1. We have demonstrated a hitherto unknown role of the inherently disordered  $\alpha$ -TAT1 227 C-terminus and identified novel interactions with 14-3-3 proteins and several kinases. TAK1 228 dependent phosphorylation of  $\alpha$ -TAT1 Serine-237 has been reported to stimulate its catalytic property<sup>62</sup>. In neurons, p27<sup>kip1</sup> directly binds to α-TAT1 and stabilizes it against proteasomal 229 230 degradation<sup>24</sup>, thus enhancing  $\alpha$ -tubulin acetylation. Our observation that spatial 231 sequestration of  $\alpha$ -TAT1 from microtubules modulates acetylation dynamics suggests a role of 232 the nucleus as a reservoir or sequestration chamber to control protein access of substrates. 233 Regulated spatial sequestration of biomolecules can control their action<sup>63–67</sup> and aberrant 234 localization of proteins have been reported in many diseases<sup>68–71</sup>. Our study further highlights 235 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 NLS<sup>44</sup>. 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

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

246 Our data demonstrate that nuclear localization of  $\alpha$ -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  $\alpha$ -TAT1. Such phospho-regulation of  $\alpha$ -TAT1 249 provides a possible mechanism for the changes in α-TAT1 localization and microtubule acetylation observed at different stages of the cell cycle<sup>72</sup>. We identified Threonine-322 to be 250 251 a putative binding site for 14-3-3 proteins and demonstrated that  $\alpha$ -TAT1 binds to 14-3-3- $\beta$ 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 NLS<sup>73</sup>. Furthermore, 14-3-3 proteins may significantly alter the structure of their 255 binding partners to align along their rigid  $\alpha$ -helical backbone, to expose or hide critical binding 256 sites<sup>74</sup>. Comparable kinase-mediated regulation of nuclear export and nuclear import has previously been reported in a few transcription regulators<sup>75–78</sup>. In particular, regulation of 257 258 Cdc25 localization by Checkpoint kinase1 (Chk1) mediated phosphorylation of and 259 subsequent recruitment of 14-3-3- $\beta$  to an NLS-proximal phosphosite is virtually the same as our proposed model (Fig. 5e) of  $\alpha$ -TAT1 localization<sup>78,79</sup>, suggesting that such kinase-260 261 mediated balancing of nuclear export and import is a general strategy for protein localization. 262  $\alpha$ -TAT1 is unique in this aspect in that unlike the other proteins that are spatially regulated in this manner, α-TAT1 has no known substrates in the nucleus and that its nuclear localization 263 264 appears to be primarily to sequester it from microtubules. Of course, it is possible that nuclear import of α-TAT1 facilitates interactions with presently unidentified substrates located in the 265 nucleus. In a similar vein, 14-3-3 proteins and Exp1 are also acetylated<sup>80,81</sup>, and it is intriguing 266 267 to speculate that these might be substrates of  $\alpha$ -TAT1.

268 It is worthwhile to consider that a significant number of post-translational modifications 269 of  $\alpha$ -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 pathways<sup>82</sup>. We have 272 demonstrated the presence of an NES, NLS, phosphorylation sites and putative 14-3-3 273 binding sites within the  $\alpha$ -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  $\alpha$ -TAT1 C-terminus 275 in its function. Indeed, numerous cancer-associated mutations curated in COSMIC<sup>83</sup> 276 (www.cancer.sanger.ac.uk) and TCGA Research Network (www.cancer.gov/tcga) databases, in the ATAT1 gene are located in the intrinsically disordered C-terminal region. More 277 278 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  $\alpha$ -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  $\alpha$ -TAT1 may 283 be present in other diseases as well.

In conclusion, we propose a new model for regulation of microtubule acetylation through spatial sequestration of  $\alpha$ -TAT1 (Fig. 5e), which include three key aspects: presence of an NES that facilitates Exp1 mediated nuclear export, presence of an NLS to mediate nuclear import and finally, modulation of this nuclear import by kinases. Further investigation into the role of specific kinases on  $\alpha$ -TAT1 localization may yield a better understanding of its 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% CO<sub>2</sub>). 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  $\alpha$ -TAT1 at the C terminus. H2B-mCherry and CFP-PKI 304 constructs were respectively subcloned into mCherry-C1 and mCer3-C1 vectors (Clontech). GFP-aTAT1 construct was a gift from from Dr. Philippe Chavrier and Dr. Guillaume 305 306 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, mVenus and mCherry were appended to facilitate detection. Unless specified otherwise, the 308 309 termini of tagging were positioned as in the orders they were written. Truncations of  $\alpha$ -TAT1 310 were generated by PCR. Point mutations of  $\alpha$ -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-W<sup>84</sup>
 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 # S2248), KU-55933 (Sigma Aldrich, catalog # SML1109) were generous gifts from Dr. Sergi 320 321 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), LJI308 (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- $\alpha$ -TAT1. 330 mVenus- $\alpha$ -TAT1 catalytic domain and NLS-mVenus- $\alpha$ -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  $\alpha$ -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. after which they were washed thrice with PBS and images were captured by microscopy. For 336 337 LMB treatment, HeLa cells were dosed with 100 nM LMB or equal volume of vehicle (EtOH),

incubated for 4 hours, after which methanol fixation and immunostaining was performed as

described above.

340 Immunoprecipitation assays: HEK293T cells were transiently transfected with pEGFP-c1 341 (GFP-Ctl) or GFP- $\alpha$ TAT1 with HA-14-3-3 $\beta$  or HA-14-3-3 $\zeta$  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 the supernatant was used as the soluble input. Soluble detergent extracts were incubated 346 347 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% milk and incubated 1 h with the primary antibody and 1 h with HRP-conjugated secondary 353 354 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 molecular weights. Antibodies used: GFP-HRP (NB600-313, Novus Biologicals), anti-HA (rat; 356 357 Merck; 11867423001), anti-exportin-1 (mouse; BD Transduction Laboratories<sup>™</sup>; 611832). 358 Secondary HRP antibodies were all purchased from Jackson ImmunoResearch.

Microscopy and image analyses: All imaging was performed with an Eclipse Ti microscope (Nikon) with a 100X objective (1.0X zoom and 4X4 binning) and Zyla 4.2 sCMOS camera (Andor), driven by NIS Elements software (Nikon). Time lapse imaging was performed at 15 min intervals for 10-15 hours. All live cell imaging was conducted at 37°C, 5% CO<sub>2</sub> and 90%

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

368 For categorical analysis of mVenus- $\alpha$ -TAT1 localization, images were visually inspected and classified as displaying either cytosolic, diffused, or nuclear localization of 369 370 mVenus fluorescence signal. For ratiometric analysis, the ratio of the fluorescence intensity 371 from region of interest ( $\approx$  10 µm 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 for cytosolic (<0.8) and nuclear (>1.2), we visually identified WT mVenus-α-TAT1 cells, with or 373 374 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.

For immunofluorescence assays with exogenous expression of mVenus- $\alpha$ -TAT1 plasmids, transfected cells were identified by the presence of mVenus fluorescence signal. The ratio of acetylated  $\alpha$ -Tubulin over  $\alpha$ -Tubulin (Ac.  $\alpha$ -Tub/ $\alpha$ -Tub) for transfected cells was normalized against that for non-transfected cells averaged over 20 untransfected cells from the same dish. For LMB and vehicle treatment, Ac.  $\alpha$ -Tub/ $\alpha$ -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-aTAT1. Extreme outliers (<Q1 - 3xIQR or 397 >Q3+3xIQR) 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**

ADR initiated the project and designed and performed most of the experiments and data analyses. GSP and EGG performed experiments and data analyses under the guidance of ADR and TI. SS performed the immunoprecipitation assays under the guidance of SEM. ADR 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.

## 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  $\alpha$ -TAT1, 424 adapted from PDB: 4GS4, b) intracellular distribution of mVenus- $\alpha$ -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  $\alpha$ -TAT1 distribution, d) categorical analysis (n = 1032 cells) and e) ratiometric analysis (n = 304 cells) of mVenus- $\alpha$ -TAT1 localization, f) cartoon showing 427 428  $\alpha$ -TAT1 mutants used in g) immunofluorescence assays showing levels of acetylated  $\alpha$ -tubulin 429 and total  $\alpha$ -tubulin, transfected cells are indicated with red arrowheads, h) ratio of acetylated 430  $\alpha$ -tubulin to total tubulin intensities with exogenous expression of  $\alpha$ -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 = 10µm. \*\*\*: P<0.001 and NS: not significant, Student's *t*-test.

433

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

a) Co-immunoprecipitation of endogenous Exp1 with GFP-α-TAT1, b) intracellular distribution 435 436 of mVenus- $\alpha$ -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 acetvlated to total  $\alpha$ -tubulin with 441 vehicle or LMB treatment (vehicle:120, LMB: 130 cells). Scale bar = 10µm. \*\*\*: P<0.001 and 442 NS: not significant, Student's *t*-test.

443

# 444 Figure 3. Intracellular distribution of $\alpha$ -TAT1 is mediated by its C-terminus.

a) Cartoon showing α-TAT1 mutant design, b) categorical analysis (WT: 1032, Cat. Dom: 371, C-term: 290, delNES: 343 and NES/A: 501 cells) and c) ratiometric analysis (WT: 304, Cat. Dom: 213, C-term: 220, delNES: 221 and NES/A: 212 cells) of mVenus-α-TAT1 mutant localization as indicated, d) representative images showing intracellular distribution of mVenus-α-TAT1 mutants as listed, red dotted lines outline nuclei, e) ratiometric analysis of intracellular distribution of mVenus-α-TAT1 C-term with 100 nM LMB (C-term: 220, LMB: 209 cells. Scale bar = 10µm. \*\*\*: *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-14-3-3β and HA-14-3-3ζ 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 mVenus-α-TAT1 localization with kinase inhibitors (WT:304, STS: 183, RO-3306: 221, 457 458 PD0332991: 234, Purvalanol-B: 243, D4476:253, Silmitasertib: 253, H89: 219, PKI: 208, Ipa-459 tasertib: 259, Capivsertib: 218, Go6976: 297, Go6983: 241, Sostrastaurin: 401, KT-5823: 269, SB203580: 259, Birb796: 262, KU55933: 317, LJI308: 229, CHIR99021: 227, KN-62:210, KU-460 461 57788: 249, Y-27632: 161 cells). Scale bar = 10µm. \*\*\*: 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.

a) Cartoon showing α-TAT1 putative NLS flanked by potential phosphosites and corresponding mutants, b) intracellular distribution of mVenus-α-TAT1 mutants as indicated, nuclei are
outlined in red dotted lines, c) ratiometric analysis (WT: 304, T322A: 228, ST/A: 194, delNLS:
208, NES/A, ST/A: 225 cells) and d) categorical analysis (WT: 1032, T322A: 346, ST/A: 290,
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 =  $10\mu m$ . \*\*\**P*<0.001 and NS, not significant, Student's *t*-test.

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1	Supplementary Information
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3	A phospho-regulated signal motif of $\alpha$ -TAT1 drives dynamic microtubule acetylation
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a) Query: α-TAT1 Isoform 7

MEFPFDVDALFPERITVLDQHLRPPARRPGTTTPARVDLQQQIMTIIDELGKASAKAQNLSAPITSA SRMQSNRHVVYILKDSSARPAGKGAIIGFIKVGYKKLFVLDDREAHNEVEPLCILDFYIHESVQRH GHGRELFQYMLQKERVEPHQLAIDRPSQKLLKFLNKHYNLETTVPQVNNFVIFEGFFAHQHRPPA PSLRATRHSRAAAVDPTPAAPARKLPPKRAEGDIKPYSSSDREFLKVAVEPPWPLNRAPRRATP PAHPPPRSSSLGNSPERGP**LRPFVPEQELLRSLRL**CPPHPTARLLLAADPGGS**PAQRRRT**<sup>322</sup>R



**Supplementary Figure S1.**  $\alpha$ TAT1 C-terminus is disordered. a) amino acid sequence of  $\alpha$ 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  $\alpha$ TAT1 C-terminus is disordered. The threshold to be considered disordered is 0.5 for both.



**Supplementary Figure S2.** NetNES prediction for NES in  $\alpha$ 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.

NES

#### isoform2 DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGNSPERG PLRPFVPEOELLRSL 286 DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGNSPERG PLRPFVPEOELLRSLRI 298 isoform4 DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGNSPERCPLRPFVPEQELLRSLRL 298 isoform1 isoform5 DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGNSPERCPLRPFVPEQELLRSLRL 298 DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGNSPERG PLRPFVPEQELLRSLRL 275 isoform6 isoform3 DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGNSPERG PLRPFVPEQELLRSLRL 275 DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGNSPERG PLRPFVP<mark>EQE</mark>LLRSLRI isoform7 275 \*\*\*\*\*\* isoform2 PPHPTARLLLAADPGGSPAQRRRTNGTPPGLVAQSCCYSRHGGVNSSSPNTGNQDSKQGE 346 PPHPTARLLLAADPGGSPAQRRRTSSLPRSEESRY-----isoform4 333 isoforml PPHPTARLLLAADPGGSPAQRRTIGTPPGLVAQSCCYSRHGGVNSSSPNTGNQDSKQGE 358 isoform5 PPHPTARLLLAADPGGSPAQRRRTI \_\_\_\_\_ 323 isoform6 PPHPTARLLLAADPGGSPAQRRTISLPRSEESRY------310 isoform3 PPHPTARLLLAADPGSSPAQRRRTRGTPPGLVAQSCCYSRHGGVNSSSPNTGNQDSKQGE 335 isoform7 PPHPTARLLLAADPG GSPAQRRRT 300 \*\*\*\*\* \*\*\*\*\*\*\*

NLS containing T<sup>322</sup>

a)

	NES	
Canis	DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGSSPERGPLRPFVPEQDLLRSLRL	С
Homo	DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGNSPERGPLRPFVPEQELLRSLRL	С
Papio	DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGNSPERGPLRPFVPEQELLRSLRL	С
Myotis	DREFLKVAVEPPWPLNRAPRRATPPVHP-PRSSSLGNSPERGPLRPFVPEQELLRSLRL	С
Macaca	DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGNSPERGPLRPFVPEQELLRSLRL	С
Rattus	DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGNSPDRGPLRPFVPEQELLRSLRL	С
Mus	DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGNSPDRGPLRPFVPEQELLRSLRL	C
Felis	DREFLKVAVEPPWPLNRAPRRATPPVHPPPRSSSLGNSPERGPLRPFVPEQELLRSLRL	С
Heterocephalus	DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGNSPERGPLRPFVPEQELLRSLRL	С
Cavia	DREFLKVAVEPPWPLNRAPRRATPTAHPPPRSSSLGSSPERGPLRPFVPEQELLRSLRL	С
Bos	DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGNSPERGPLRPFVPEQELLRSLRL	С
Sus	DREFLKVAVEPPWPLNRAPRRATPPAHPPPRSSSLGNSPERGPLRPFVPEQELLRSLRL	С
	***************************************	*
Comia		D
Canis	PPHPTARLLLATDPG-SPAQKRRTRGAPPGLVAQSCC1SRnGGLSSSS	2°
Pomio	PPHPTARLLLAADPG-SPAQKRRTRGTPPGLVAQSCCISRHGGVNSSS	P
Papio	PPHPTARLLLAADPG 5PAQKRRT	-
Myotis	PPHPTARLLLATDPAKSPAQRRRTSLFFIACHFSIRGAPFGLVAQSCCISRIGGLNSSS	P
Macaca	PPHFTARLLLAADPG-SPAQRRRTRRRRRR	-
Rattus	PPHFTARLLLATDPG-SPAQRRRTRETPWGLVAQSCHYSRHGGFNTSF	ь
Mus	PPHFTARLLLATDPG SPAQRRRTSSLPRSDESRY	-
Felis	PPHFTARLLLATDPG SPAQRRRTRGAPPGLVAQSCCYSRHGGLSSSS	P
Heterocephalus	PPHFTARLLLATDPGGSPAQRRRTRGTPPGLVAQSCCYSRHGGPNPSS	P
Cavia	PPHPTARLLLATDPGGSPAQRRRTRGTPPGLLAQSRCYSRHRGPSPSA	P
Bos	PPHPTARLLLATDPG SPAQRRRTRRRR	-
Sus	PPHPTARLLLATDPG SPAQRRRTRRRR	-
	*********	

NLS containing T<sup>322</sup>

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



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



**Supplementary Figure S5.**  $\alpha$ -TAT1 undergoes CRM1 dependent nuclear export. a), b) Temporal changes in mVenus- $\alpha$ -TAT1 localization on 100 nM LMB treatment after LMB addition at time 0, scale bar = 10 µm, for b), each line indicates normalized nuclear intensity of mVenus- $\alpha$ -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- $\alpha$ -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.**  $\alpha$ -TAT1 has a C-terminal NES. a)  $\alpha$ -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- $\alpha$ -TAT1 and mutants as indicated. \*\*\**P*<0.001 and NS, not significant, Student's *t*-test.



**Supplementary Figure S7.** a) Putative phosphosites in  $\alpha$ -TAT1 predicted by NetPhos, those in F285-R323 are highlighted, b) currently reported post-translational modifications in  $\alpha$ -TAT1 curated by PhosphoSitePlus<sup>®</sup>, www.phosphosite.org.



□Cytosolic □Diffused □Nuclear

**Supplementary Figure S8.** Categorical analysis of α-TAT1 localization on treatment with kinase inhibitors. WT: 1032, STS: 157, R0-3306: 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, LJI308: 522, CHIR99021: 484, KN62: 531, KU57788: 949, Y27632: 340 cells.



**Supplementary Figure S9.**  $\alpha$ -TAT1 has a C-terminal phospho-inhibited NLS. a) Cartoon showing  $\alpha$ -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- $\alpha$ -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.

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

Supplementary Table T1. Details of kinase inhibitors used.