1 **Effects of α-tubulin acetylation on microtubule structure and stability**

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ABSTRACT

29 Acetylation of K40 in α-tubulin is the sole post-translational modification to mark the

30 luminal surface of microtubules. It is still controversial whether its relationship with

31 microtubule stabilization is correlative or causative. We have obtained high-resolution

32 cryo-electron microscopy reconstructions of pure samples of αTAT1-acetylated and

33 SIRT2-deacetylated microtubules to visualize the structural consequences of this

34 modification and reveal its potential for influencing the larger assembly properties of

35 microtubules. We modeled the conformational ensembles of the unmodified and

36 acetylated states by using the experimental cryo-EM density as the structural restraint in

37 molecular dynamics simulations. We found that acetylation alters the conformational

38 landscape of the flexible loop that contains αK40. Modification of αK40 reduces the

39 disorder of the loop and restricts the states that it samples. We propose that the change

40 in conformational sampling that we describe, at a location very close to the lateral contacts

41 site, is likely to affect microtubule stability and function.

42

ABBREVIATIONS

44 MT, microtubule; PF, protofilament; PTM, post-translational modification; MAP,

45 microtubule-associated protein; αTAT1, acetyltransferase TAT1 for α-tubulin; SIRT2

46 deacetylase SIRT2.

47

48   **INTRODUCTION**

49   Microtubules (MTs) are essential cytoskeletal polymers important for cell shape

50   and motility and critical for cell division. They are built of αβ-tubulin heterodimers that

51   assemble head-to-tail into ~13 polar protofilaments (PFs), which associate laterally to

52   form a hollow tube[1]. Lateral contacts involve key residues in the so-called M-loop

53   (between S7 and H9) in one tubulin monomer and the H2-H3 loop and β-hairpin structure

54   in the H1'-S2 loop of the other tubulin subunit across the lateral interface. These lateral

55   contacts are homotypic (α-α and β-β contacts), except at the MT "seam", where the

56   contacts are heterotypic (α-β and β-α contacts)[2]. MTs undergo dynamic instability, the

57   stochastic switching between growing and shrinking states[3,4]. These dynamics are highly

58   regulated *in vivo* by multiple mechanisms that affect tubulin and its interaction with a large

59   number of regulatory factors.

60   One mechanism that cells can use to manipulate MT structure and function

61   involves the post-translational modification of tubulin subunits. Through the spatial-

62   temporal regulation of proteins by the covalent attachment of additional chemical groups,

63   proteolytic cleavage or intein splicing, post-translational modifications (PTMs) can play

64   important roles in controlling the stability and function of MTs[5]. Most of tubulin PTMs alter

65   residues within the highly flexible C-terminal tail of tubulin that extends from the surface

66   of the MT and contributes to the binding of microtubule-associated proteins (MAPs)[6,7].

67   These PTMs include detyrosination, Δ2-tubulin generation, polyglutamylation, and

68   polyglycylation[8]. However, acetylation of α-tubulin on the K40 stands out as the only

69   tubulin PTM that localizes to the inside of the MT, within a loop of residues P37 to D47,

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70　　often referred to as the αK40 loop. This modification is carried out by α-tubulin

71　　acetyltransferase αTAT1 and removed by the NAD^{+}-dependent deacetylase SIRT2 and

72　　by HDAC6[6,9]. How the enzymes interact with the αK40 luminal loop, and whether this

73　　"hidden PTM" has a causative or correlative effect on MT properties remain elusive.

74　　　　　Shortly after its discovery over 30 years ago[10], acetylation of αK40 was found to

75　　mark stable, long-lived ($t_{1/2}$ >2 hours) MT subpopulations, including the axonemes of cilia

76　　and flagella or the marginal bands of platelets[6,9], and to protect MTs from mild treatments

77　　with depolymerizing drugs, such as colchicine[11] and nocodazole[12]. Multiple studies have

78　　shown that reduced levels of αK40 acetylation cause axonal transport defects associated

79　　with Huntington's disease, Charcot-Marie-Tooth disease, amyotrophic lateral sclerosis,

80　　and Parkinson's disease[13–16]. These defects can be reversed by restoring αK40

81　　acetylation levels[17]. On the other hand, elevated levels of αK40 acetylation promote cell-

82　　cell aggregation, migration and tumor reattachment in multiple aggressive, metastatic

83　　breast cancer cell lines[18,19].

84　　　　　Whether acetylated MTs are stable because they are acetylated or whether stable

85　　structures are better at acquiring this modification remains a point of contention. For

86　　example, a previous study showed that acetylation did not affect tubulin polymerization

87　　kinetics *in vitro*[20]. However, this study was confounded by two factors: (i) microtubules

88　　acetylated by flagellar extract were compared to native brain tubulin, which is

89　　approximately 30% acetylated and (ii) only a single round of

90　　polymerization/depolymerization was performed after *in vitro* acetylation, which is

91　　insufficient to remove αTAT1 or other MAPs. Thus, the results of this study may be limited

4

92   by the purity and preparation of the sample. Our previous structural work also found no

93   significant differences between 30% acetylated and 90% deacetylated MTs at a resolution

94   of ~9 Å, particularly at the modification site, the αK40 residue within the αK40 loop, which

95   was invisible in both cases due to the intrinsic disorder and/or to the remaining

96   heterogeneity of the loop (i.e. that study may have been limited by the low purity of the

97   samples)[9]. More recent *in vitro* studies, using pure samples of 96% acetylated and 99%

98   deacetylated MTs, argue that αK40 acetylation induces a structural change that improves

99   the flexibility and resilience of MTs[21,22]. These studies find that acetylated MTs maintain

100 their flexural rigidity, or persistence length, after repeated rounds of mechanical stress,

101 while deacetylated MTs show a 50% decrease in rigidity and are 26% more likely to suffer

102 from complete breakage events[21,22].

103   Since the αK40 residue is less than 15 Å away from the lateral interface between

104 protofilaments, a possible model for the molecular mechanism of acetylation is that it

105 alters inter-protofilament interactions by promoting a conformation of the αK40 loop that

106 confers flexural rigidity, thus increases its resistance to mechanical stress—a

107 phenomenon called protofilament sliding[21,22]. Molecular dynamics simulations have

108 suggested a model where αK40 forms a stabilizing salt bridge with αE55 within the core

109 of the α-tubulin monomer that in turn stabilizes αH283 within the M-loop of its neighboring

110 α-tubulin monomer[23]. Another study proposed that αK40 acetylation may specify 15-PF

111 MTs, which are known to be 35% stiffer than 13PF MTs and more effective at forming

112 microtubule bundles[24].

113   Given the uncertainties remaining concerning the effect of αK40 acetylation on

114   MTs, we decided to characterize the conformational properties of the αK40 loop in the

115   acetylated and deacetylated MTs that could have an effect on MT structure and

116   properties. To that end, we produced near atomic-resolution cryo-EM maps of 96%

117   acetylated (Ac[96]) and 99% deacetylated (Ac[0]) MTs. By improving sample purity, we were

118   able to visualize more density for the αK40 loop in the acetylated state. Using new

119   molecular dynamics methods, we found that acetylation shifts the conformational

120   landscape of the αK40 loop by restricting the range of motion of the loop. In contrast, in

121   the Ac[0] state, the αK40 loop extends deeper into the lumen of the MT, and samples a

122   greater number of conformations. These motions are likely to increase the accessibility

123   of the loop to αTAT1, in agreement with the hypothesis that αTAT1 acts by accessing the

124   MT lumen[25], and likely influence lateral contacts, in agreement with the causative effect

125   of acetylation on the mechanical properties of microtubules[21].

126

127   **RESULTS AND DISCUSSION**

128   ***High-resolution cryoEM reconstructions of pure acetylated (Ac[96]) and deacetylated***

129   ***(Ac[0]) MTs***

130   Using recent biochemical schemes designed to enrich for specific acetylation

131   states[21], we generated Ac[96] and Ac[0] MTs for use in our cryo-EM studies. We prepared

132   cryo-EM samples as previously described[2,26] of Ac[96] and Ac[0] MTs in the presence of end-

133   binding protein 3 (EB3). EB3 served as a fiducial marker of the dimer that facilitated

134   alignment of MT segments during image processing[27]. Table S1 summarizes the data

135   collection, refinement, and validation statistics for each high-resolution map we visualized

136   (see also Supplemental Figures 1 and 2). Using the symmetrized MT reconstruction,

137   which takes advantage of the pseudo-helical symmetry present in the MT, we extracted

138   a 4x3 array of dimers for further B-factor sharpening, refinement[28], and model-building

139   (Figure 1a). This array includes all possible lateral and longitudinal non-seam contacts

140   for the central dimer, which was later extracted for model building and map analysis

141   (Figure 1b,1c).

142   The αK40 loop has been poorly resolved in previous EM reconstructions, and

143   existing models contain a gap between residues Pro37 and Asp48 (Supplemental Figure

144   3a)[25]. While the loop has been resolved in a number of X-ray crystallographic structures,

145   the conformations stabilized in the crystal lattice are likely artifacts due to the presence

146   of calcium and/or crystal contacts (Supplemental Figure 3b). For our symmetrized maps,

147   we were able to build residues S38-D39 and G44-D47 into the Ac^{96} state and S38 and

148   D46-D47 into the Ac^0 state (Figure 1d, 1e). Qualitatively, the maps suggest that the αK40

149   loop is slightly more ordered in the Ac^{96} state, with the protrusion of density following

150   Pro37 extending away from or toward Asp48 in the Ac^{96} or Ac^0 states, respectively.

151   However, it is likely that multiple conformations of the loop, perhaps as a function of each

152   loop's individual position around a helical turn, are averaged together and result in the

153   low signal-to-noise levels we observe in the map.

154   ***Conformational differences across MT states are confirmed by non-symmetrized***

155   ***reconstructions***

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156   We considered the possibility that the symmetrizing procedure used to improve

157   signal and resolution in our image analysis was averaging different αK40 loop

158   conformation within different PFs and thus interfering with our interpretation of the loop

159   structure in the two states. To test the hypothesis, we analyzed the non-symmetrized

160   maps calculated with C1 symmetry for the Ac^{96} and Ac^{0} states. We extracted a full turn of

161   13 adjacent dimers. This full-turn map revealed additional density extending out further

162   along the loop in the Ac^{96} state when compared to the symmetrized maps filtered to the

163   same resolution (4 Å) (Figure 2). Furthermore, the density for the loop observed at the

164   seam was distinct from that at the non-seam contacts. To maximize the interpretability of

165   the subunits making non-seam contacts, we used non-crystallographic symmetry (NCS)

166   averaging as an alternative method to increase the signal-to-noise levels in the maps.

167   This procedure improved the density for non-Glycine backbone atoms in the αK40 loop

168   in the Ac^{96} state, allowing us to trace an initial Cα backbone for this region, while in the

169   Ac^{0} state the loop remained unmodelable (Figure 2c, 2d). This interpretation agrees with

170   the qualitative difference in the density, which indicate less disorder for the Ac^{96} state than

171   Ac^{0} state, of the traditionally symmetrized and C1 maps.

172   This NCS averaging method had multiple advantages over the traditional

173   averaging technique for pseudo-helical processing implemented in FREALIGN[26]. First,

174   the model coordinates used for the averaging are based on the matrix of α-tubulin

175   monomers along a full turn rather than the single α-tubulin monomer. Second, in the

176   FREALIGN averaging approach the signal from the dimers at the seam are down-

177   weighted, whereas NCS averaging allows us to separate the signal from the seam, and

178   thus to deconvolute the signal from the non-seam locations. Third, this procedure also

179   acts to low-pass filter the map to 4 Å (the high-resolution limit of the C1 map,

180   Supplemental Figure 5), which should suppress noise from the more disordered parts of

181   the map, including alternative conformations of the αK40 loop. Using this NCS-based

182   approach, we were able to resolve density and build a model for three additional residues,

183   the acetylated K40, T41, and I42. These residues pack towards the globular domain of α

184   tubulin, consistent with the favorability of burying these relatively hydrophobic residues in

185   the Ac^{96} state. Despite observing only very weak density, we have modeled the glycine-

186   rich region that extends into the lumen as a tight turn, which we note is only possible due

187   to the expanded Ramachandran space accessible to glycine residues (Figure 2c). In

188   contrast, and despite better global resolution, we did not observe any density consistent

189   with a stable conformation of the loop in the Ac^{0} map. Based on this result, which is

190   consistent across the NCS-averaged and traditionally symmetrized maps, we did not build

191   any additional residues into the Ac^{0} density (Figure 2d).

Ensemble modeling of the loop in each state using density-restrained molecular

dynamics

194   For regions that exhibit a high degree of disorder, like the αK40 loop, a single,

195   static structure is a poor description of the native state. Ensemble models can help to

196   elucidate how populations of conformations change upon perturbations, such as post-

197   translational modifications[29,30]. To derive an ensemble of conformations representing the

198   Ac^{96} and Ac^{0} states, we used the atomic structure built into the Ac^{96} map as the starting

199   model to initiate metainference-based molecular dynamics (MD) simulations, which

200 augment a standard forcefield with a term representing the density derived from the EM

201 map[31]. In contrast to Molecular Dynamics and Flexible Fitting (MDFF) and other

202 refinement methods that seek to converge on a single structure[32], this method models a

203 structural ensemble by maximizing the collective agreement between simulated and

204 experimental maps, and accounts for noise using a Bayesian approach[33]. Initiating

205 simulations for both the Ac^{96} and Ac^0 states from starting models that differ only in the

206 acetyl group and distinct input experimental density maps allowed us to test whether

207 acetylation restricts the motion of the loop, trapping it in a tighter ensemble of

208 conformations.

209 To analyze the conformational dynamics of the loop, we analyzed the root mean

210 square fluctuations of residues 36-48 within replicas for each simulation. This analysis

211 shows that the αK40 loop fluctuations are more restricted in the Ac^{96} state than in the Ac^0

212 state (Figure 3a). Next, we analyzed the distribution of conformations adopted by the loop

213 by analyzing the distance between K40 and the globular domain of α-tubulin (represented

214 by L26) and by clustering together the snapshots from all replicas of both simulations

215 based on the root mean square deviations of residues 36-48. Similar to the starting

216 reference model, where the distance is 10.6 Å, Ac^{96} is enriched in conformations that

217 pack close to the globular domain of the α-tubulin core (Figure 3b). These conformations,

218 exemplified by clusters 1, 4, and 6, position the acetylated lysine to interact with residues

219 along H1. In contrast, the Ac^0 state favors conformations that extend towards the MT

220 lumen, as exemplified by clusters 0, 2, 5, 7, and 8 (Figure 3b). Clusters 3, 9, 10, 11,

221 labeled in grey, had equal numbers of frames enriched in Ac^{96} and Ac^0 and sampled rare

10

222   (<5%) extreme states on both the exposed and packed ends of the conformational

223   spectrum (Figure 3b).

224   These computational results are consistent with the visual analysis of the density

225   for both the NCS and traditionally symmetrized maps, which indicated that the loop is

226   more ordered after acetylation. The residual disorder identified by the simulations using

227   the Ac[96] map may be important for de-acetylation by SIRT2. On the other hand, the

228   increased flexibility we observe for the Ac[0] state suggests a potential mechanism by which

229   αTAT1 could acetylate K40. Previous proposals argue that acetylation can occur from the

230   outside or inside of the lumen[25]. However, to catalyze the modification, a flexible region

231   within αTAT1 would have to extend approximately 25 Å through a MT wall fenestration

232   between four tubulin dimers to reach αK40, or the MT would have to undergo a major

233   structural rearrangement in the lattice to allow αTAT1 to enter the lumen. Previous work

234   demonstrated that the αTAT1 active site and its MT recognition surface is concave and

235   could not stretch through the lumen[25]. Our findings support the idea that αTAT1 modifies

236   the loop from within the lumen of the MT because the deacetylated loop samples

237   extended structures that would be accessible to αTAT1 and because the structural

238   rearrangement caused by acetylation is small and local to the αK40 loop.

239   ***Acetylation induces a local structural rearrangement of the αK40 loop that***

240   ***promotes stability by weakening lateral contacts***

241   Collectively our structural and MD results show that acetylation restricts the motion

242   of the αK40 loop. These results led us to hypothesize that the change in the structural

243   ensemble of the αK40 loop upon acetylation, while subtle and local, may affect lateral

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244   contacts. These local changes may disrupt the small lateral interface between α-tubulin

245   subunits. The origin of this effect may be highly distributed, as we do not visualize any

246   stable interactions between the Ac^0 state of the loop and the globular domain. However,

247   upon acetylation, the structural ensemble becomes more restricted and the potential for

248   the loop to strengthen any of these interactions between monomers is lost. For example,

249   in many of the extended conformations favored by the Ac^0 state, K40 in a α1-monomer is

250   close to the M-loop of the neighboring α2-monomer and may buttress the H1'-S2 loop,

251   providing support for the vital $_{α1}K60:_{α2}H283$ lateral interaction (Figure 4). In contrast, when

252   K40 is acetylated it packs ~10 Å closer to the globular domain of the α1-monomer,

253   reducing the potential for inter-monomer interactions (Figure 4).

254   We tested whether the loss of the positive charge of the lysine upon acetylation

255   alters the electrostatic interaction energy and the hydrogen-bonding network at the lateral

256   interface using MD simulations based on the Debye-Hückel (DH) formula[34,35]. We found

257   that acetylation does indeed weaken lateral interactions (Supplemental Figure 4).

258   Additionally, the Ac^0 ensemble contains conformations with strong DH interaction

259   energies that do not exist in the Ac^{96} ensemble (Supplemental Figure 4). While the effects

260   of acetylation are subtle, the local effects at the lateral contacts site may have an additive

261   effect that stabilizes the MT lattice. This idea is consistent with previous work that argues

262   that the weakening of lateral interactions is a protective mechanism to prevent pre-

263   existing lattice defects from spreading into large areas of damage under repeated

264   stress—a mechanism that could be exploited by cancer cells[21,22].

265   In conclusion, this comprehensive approach combines the structural insight of

266   cryoEM with the sampling efficiency and global scope of MD to investigate how PTMs

267   can transform a conformational ensemble[36,37]. Our high-resolution maps serve as a

268   blueprint for the scale of conformational change and relevant degrees-of-freedom that the

269   αK40 loop can sample with all-atomistic metainference MD[36]. We show that αTAT1

270   induces a site-specific electrostatic perturbation that restricts the motion of the loop. αK40

271   acetylation may function as an evolutionarily conserved 'electrostatic switch' to regulate

272   MT stability[36,37].

273

274   **MATERIALS AND METHODS**

275   Sample Preparation for Cryo-Electron Microscopy

276   Porcine brain tubulin was purified as previously described[38] and reconstituted to 10 mg/ml

277   in BRB80 buffer (80 mM 1,4-piperazinediethanesulfonic acid [PIPES], pH 6.9, 1 mM

278   ethylene glycol tetraacetic acid [EGTA], 1 mM $MgCl_2$) with 10% (vol/vol) glycerol, 1 mM

279   GTP, and 1 mM DTT, and flash frozen in 10 μl aliquots until needed. The acetylated and

280   deacetylated MTs (15 μM) were co-polymerized with end-binding protein 3 (EB3, 25 μM),

281   at 37°C for ~15 min in the presence of 10% NP-40, 1mM dithiothreitol (DTT), and BRB80

282   buffer. The EB3 decorated MTs were added to glow-discharged C-flat holey carbon grids

283   (CF-1.2/1.3-4C, 400 mesh, Copper; Protochips, Morrisville, NC) inside a Vitrobot (FEI,

284   Hillsboro, OR) set at 37°C and 85% humidity before plunge-freezing in ethane slush and

285   liquid nitrogen, respectively, as previously described[2].

286   Cryo-Electron Microscopy

287 Micrographs were collected using a Titan Krios microscope (Thermo Fisher Scientific,

288 Inc., Waltham, MA) operated at an accelerating voltage of 300 kV. All cryo-EM images

289 were recorded on a K2 Summit direct electron detector (Gatan, Pleasanton, CA), at a

290 nominal magnification of x22,500, corresponding to a calibrated pixel size of 1.07 Å. The

291 camera was operated in super-resolution mode, with a dose rate of ~2 e$^-$ per pixel per s

292 on the detector. We used a total exposure time of 4 s, corresponding to a total dose of 25

293 electrons/Å^2 on the specimen. The data were collected semi-automatically using the

294 SerialEM software suite[39].

295 <u>Image Processing</u>

296 Stacks of dose-fractionated image frames were aligned using the UCSF MotionCor2

297 software[40]. MT segments were manually selected from the drift-corrected images

298 (acetylated dataset: 205 images, deacetylated MT dataset: 476 images) using the

299 APPION image processing suite[41]. We estimated the CTF using CTFFIND4[42] and

300 converted the segments to 90% overlapping boxes (512 × 512 pixels) for particle

301 extraction. The remaining non-overlapping region is set to 80 Å and corresponds to the

302 tubulin dimer repeat (asymmetric unit). Consequently, there are ~13 unique tubulin

303 dimers per MT particle. To determine the initial global alignment parameters and PF

304 number for each MT particle, raw particles were compared to 2D projections of low-

305 passed filtered MT models (~20 Å, 4° coarse angular step size) with 12, 13, 14 and 15

306 PFs[43] using the multi-reference alignment (MSA) feature of EMAN1[44]. Finally, 13-PF MT

307 particles (acetylated dataset: 20,256 particles, deacetylated MT dataset: 29,396) were

308 refined in FREALIGN v. 9.11[45,46] using pseudo-helical symmetry to account for the

14

309   presence of the seam. To verify the location of the seam, we used the 40 Å shift approach

310   to categorize MTs based on their azimuthal angle, as previously described[27].

311   <u>Atomic Model Building and Coordinate Refinement</u>

312   COOT[47] was used to build the missing polypeptides of the αK40 loop in α-tubulin, using

313   the available PDB 3JAR as a starting model. Successively, all novel atomic models were

314   iteratively refined with phenix.real_space_refine into EM maps sharpened with

315   phenix.autosharpen[28,48]. For visual comparisons between states, potential density

316   thresholds were interactively adjusted in Coot to maximize iso-contour similarity around

317   backbone atoms distant from the αK40 loop. For Figures 1 & 2, all densities are

318   represented in Chimera at a threshold of 1.1.

319   <u>Molecular Dynamics Simulations</u>

320   Code for map preparation, simulation execution, and analysis is available at:

321   https://github.com/fraser-lab/plumed_em_md

322   To prepare the cryoEM maps, we fitted the maps with a Gaussian Mixture Model (GMM)

323   by applying a divide-and-conquer approach[33], using generate_gmm.py and

324   convert_GMM2PLUMED.sh. Cross-correlations to the experimental maps were greater

325   than 0.99. All simulations were performed with GROMACS 2016.5[34] and the PLUMED-

326   ISDB module[49] of the PLUMED library[50] using the Charmm36-jul2017 forcefield[51] with

327   patches for acetylated lysine (aly)[52] and the TIP3P water model. For the deacetylated

328   simulations, the same starting model was used with a manual edit of the PDB to eliminate

329   the acetylation (with all hydrogens replaced by GROMACS during model preparation).

330   The initial model was minimized then equilibrated for 2ns, using prep_plumed.py. MD

331   simulations were performed on a metainference ensemble of 8 replicas for an aggregate

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332 simulation time of 96ns for each acetylation state, using prep_plumed2.py and

333 prep_plumed3.py. Contributions of negative scatterers (atoms OD1 and OD2 of Asp

334 residues; OE1 and OE2 of Glu) were excluded from contributing to the predicted maps

335 during the simulation. This modification effectively eliminates the contribution of these

336 side chains to the agreement between density maps, in keeping with the non-existent

337 density of negatively charged side chains in EM maps, while allowing them to contribute

338 to the simulation through the energy function. Clustering and convergence analyses[31]

339 were performed and analyzed using MDAnalysis[53].

340   Changes in the electrostatic interaction energies at the lateral contacts were

341 determined using the using the Debye-Hückel (DH) formula:

346
$$\frac{1}{4\pi\varepsilon_r\varepsilon_0}\sum_{i\epsilon A}\sum_{j\epsilon B}q_iq_j\frac{e^{-\kappa|r_{ij}|}}{|r_{ij}|}$$

342 where ϵ_0 is the vacuum's dielectric constant, ϵ_r the dielectric constant of the solvent, q_i

343 and q_j the charges of the *i*-th and *j*-th atoms, respectively, $|r_{ij}|$ the distance between

344 these two atoms, and κ is the DH parameter[35] defined in terms of the temperature T

345 and the ionic strength of the solution I_S.

347   The DH energy is calculated between the following two groups of atoms, denoted

348 as A and B in the formula above: (i) all atoms in residue range 30-60 of chain A (α1

349 subunit) and (ii) all atoms in residue range 200-380 of chain E (α2 subunit) in PDBs

350 **XXYA** and **XXYB**. Residues not included in this range do not significantly contribute to

351 the DH interaction energy between adjacent α-subunits. Parameters used in the

352 calculation of the DH energy are: temperature (T=300K), dielectric constant of solvent

353 (ϵ_r=80; water at room temperature), and ionic strength (I_S=1M).

354

ACCESSION NUMBERS

All electron density maps have been deposited in the EMDB accession numbers **EMD-X1**, **EMD-X2**, **EMD-X3**, and **EMD-X4**. Atomic models are deposited in the PDB accession numbers **XXYA**, **XXYB, XXYC**, and **XXYD, XXYE.**

AUTHOR CONTRIBUTIONS

D.P. performed the tubulin purification and modification to generate the Ac^0 and Ac^{96} samples. L.E., R.Z., and D.T. performed the cryo-EM sample preparation, electron microscopy and data processing. L.E. and J.S.F. engineered the NCS-averaging technique, performed iterative model-building/refinement. L.E., J.S.F., T. L., M. V. and M.B. executed the metainference MD simulations. All authors contributed to structure interpretation, model development and manuscript writing.

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527   **FIGURE LEGENDS**

528

529   **Figure 1. High-resolution maps of 96% acetylated (Ac^{96}) and <1% acetylated (Ac^{0})**

530   **microtubules. (a)** Schematic of the model-building and refinement process in PHENIX.

531   We sharpened a representative 4x3 lattice, refined the corresponding atomic structure

532   (3JAR) into our map, and extracted out the central dimer to build additional residues into

533   the αK40 loop. We performed this process iteratively for both the Ac^{96} and Ac^{0}. The

534   structure of the Ac^{96} **(b)** and Ac^{0} **(c)** αβ-tubulin heterodimers, respectively, are shown from

535   the outer and luminal views with close-ups of αK40 loop in each state **(d)** and **(e)** low-

536   pass filtered to 3.7 Å.

537

538   **Figure 2. Symmetrized and NCS-averaged C1 maps of Ac^{96} and Ac^{0} microtubules**

539   **reveal the αK40 loop is more ordered in the Ac^{96} state.** Close-up views of the αK40

540   loop (P37-D47) in the **(a)** Ac^{96} and **(b)** Ac^{0} states in the symmetrized maps low-pass

541   filtered to 4 Å and the **(c)** Ac^{96} and **(d)** Ac^{0} states in the NCS averaged C1 maps low-pass

542   filtered to 4 Å. Dotted lines indicate missing residues.

543

544   **Figure 3. Acetylation restricts the motion and alters the conformational ensemble**

545   **of the αK40 loop.** (a) Per-residue root mean square fluctuations (RMSF) analyses were

546   determined over the course of 12 ns for residues 34-50 the C1 maps using GROMACs in

547   PLUMED and graphed using the MDAnalysis. (b) Ensemble modeling of the loop across

548   Ac^{96} and Ac^{0} states using density restrained MD. Frames were classified into one of 11

549   clusters by conformation. Clusters either had a greater number of Ac^{96} frames (red), Ac^{0}

550   frames (blue), or an equal number of frames from both states (grey). The reference is

551   shown in green. The unique conformations of each of the 11 clusters are shown to the

552   right.

553

554   **Figure 4. Acetylation may weaken lateral interactions.** Close-up view of the lateral

555   contacts between two α-tubulin monomers at a non-seam location (α1, light green; α2,

556   dark green). K40 in α1 of the Ac^0 state is 8 Å closer to the M-loop of α2 and appears to

557   buttress the H1'-S2 loop, providing support for the vital α1K60-α2H283 lateral interaction.

558   By contrast, that support is lost in the Ac^{96} state because the acetylated K40 now packs

559   much closer to the hydrophobic, inner core.

560

561   **Supplemental Figure 1. Schematic of the experimental workflow for sample**

562   **preparation and pseudo-helical image processing.** EB3 decorated MTs were added

563   to glow-discharged C-flat holey carbon grids (CF-1.2/1.3-4C, 400 mesh, Copper;

564   Protochips, Morrisville, NC) inside a Vitrobot (FEI, Hillsboro, OR) set at 37°C and 85%

565   humidity before plunge-freezing in ethane slush and liquid nitrogen. Images were

566   collected with the Titan Krios electron microscope (Thermo Fisher Scientific, Inc.,

567   Waltham, MA) operated at 300kV and equipped with a K2 direct detector (Gatan,

568   Pleasanton, CA). The micrographs were collected at a nominal magnification of

569   x22,500. Stacks of dose-fractionated image frames were aligned using the UCSF

570   MotionCor2 software and CTF-corrected with CTFFIND4. MT segments were manually

571   selected and converted to 90% overlapping boxes (512 × 512 pixels) for particle

572   extraction. The remaining non-overlapping region is set to 80 Å and corresponds to the

573   tubulin dimer repeat (asymmetric unit). These raw particles were compared to 2D

23

574   projections of low-passed filtered MT models (~20 Å, 4° coarse angular step size) with

575   13 and 14 PFs using the multi-reference alignment (MRA) feature of EMAN1. Next, 13-

576   PF MT particles were refined in FREALIGN v. 9.11 using pseudo-helical symmetry to

577   account for the presence of the seam. To verify the location of the seam, MTs were

578   categorized based on their azimuthal angle and refined again.

579

580   **Supplemental Figure 2. Sample preparation, data collection and image**

581   **processing of acetylated and deacetylated MT samples. (a)** Ac^{96} and Ac^{0} tubulin

582   preparations were produced by treating purified mammalian brain tubulin (Ac^{30}) with

583   acetyltransferase αTAT1 and deacetylatase SIRT2. Samples were resolved on SDS-

584   page and Coomassie-stained (top panel) or immunoblotted for αK40 acetylation (bottom

585   panel). Axomenal preparations from Tetrahymena cilia provide a 100% acetylation

586   calibrator. Adapted from Portran[21]. **(b)** Representative cryo-EM images of acetylated, in

587   the left panel, and deacetylated MTs, in the right panel. Scale bar = 200 nm. Images

588   were collected with the Titan Krios electron microscope (FEI, Hillsboro, OR) operated at

589   300kV and equipped with a K2 direct detector (Gatan, Pleasanton, CA). The

590   micrographs were collected at a nominal magnification of 22,500x, resulting in a final

591   pixel size of 1.07 Å per pixel and dose rate of 8 e-/pixel/s. **(c)** Schematic of data

592   collection. Using EB3, we generated >80% homogeneous samples to push the

593   resolution to ~3.5 Å.

594

595   **Supplemental Figure 3. Previous proposed αK40 loop models. (a)** Published PDBs

596   with incomplete models of the loop: 5NQU (Chain A), 5EYP (Chain A), 3RYC (Chain A),

24

597   3RYC (Chain C), 5NQT (Chain A), 3RYI (Chain A), 3RYI (Chain A), 3RYF (Chain A),

598   3RYF (Chain C). **(b)** Example of the a published PDB with the complete loop stabilized

599   by calcium: 5YL4 (Chain C).

600

601   **Supplemental Figure 4. Acetylation weakens lateral interactions.** By analyzing the

602   distribution of Debye-Hückel (DH) electrostatic energy between adjacent α-subunits

603   across the AC0 (blue) and AC96 ensembles (red), we find that acetylation weakens

604   lateral interactions. The DH energy is calculated between the following two groups of

605   atoms: (i) all atoms in residue range 30-60 of chain A (α1 subunit) and (ii) all atoms in

606   residue range 200-380 of chain E (α2 subunit) in PDBs **XXYA** and **XXYB.** The plot

607   shows the probability density function, or Pdf, as a function of the DH interaction

608   energy.

609

610   **Supplemental Figure 5. Fourier Shell Correlation Plots.**

611   The $FSC_{half-map}$ resolution, using **0.143** as the gold standard criterion, represents how

612   well the two half-maps from each dataset correlate as a function of spatial frequency.

613   The two half-maps were generated by dividing the final dataset into two independent

614   3D-reconstructions. The $FSC_{map\ vs.\ model}$ resolution, using **0.5** as the gold standard

615   criterion, represents how well the final map correlated with the refined atomic model. All

616   plots were generated in PHENIX.

617

618

619 SUPPLEMENTAL TABLE 1. CryoEM data collection, refinement parameters, and

620 validation statistics.

Parameters	Ac^{96} Symmetrized (EMDB-#, PDB-#)	Ac^0 Symmetrized (EMDB-#, PDB-#)	Ac^{96} C1 (EMDB-#, PDB-#)	Ac^0 C1 (EMDB-#, PDB-#)
Magnification	22500x	22500x	22500x	22500x
Voltage	300	300	300	300
Electron exposure (e-/A^2)	25	25	25	25
Defocus range (μm)	-1.5 to -2.5	-1.5 to -2.5	-1.5 to -2.5	-1.5 to -2.5
Pixel size (Å)	1.07 Å	1.07 Å	1.07 Å	1.07 Å
Symmetry imposed	HP	HP	C1	C1
Initial particle images (no.)	20256	29396	20256	29396
Final particle images (no.)	18432	24692	18432	24692
Helical Rise			9.3	9.3
Helical Twist			27.7	27.7
Dimer Rise (Å)			80.5	81
Dimer Twist			-0.12º	-0.12º
Map resolution (Å)	**3.3**	**3.7**	**4.1**	**4.0**
FSC threshold	0.143	0.143	0.143	0.143
Map resolution range (Å)	3.5-4.1 Å	3.0-3.6 Å	3.8-5.4 Å	3.5-4.5 Å
Refinement				
Initial model used (PDB ID)	3JAR	3JAR	3JAR	3JAR

Model resolution (Å)	4 Å	3.6 Å	6 Å	5.8 Å
FSC threshold	0.5	0.5	0.5	0.5
Map sharpening method	*Phenix_auto_sharpen*	*Phenix_auto_sharpen*	*Phenix_auto_sharpen*	*Phenix_auto_sharpen*
Model composition				
Nonhydrogen atoms	40866	40866	320775	320775
Protein residues	5184	5184	40702	40702
Ligands (GTP, GDP)	12	12	94	94
B factors (Å^2)				
Protein	126.11	96.80	193.47	161.40
Ligand	122.25	89.44	192.42	156.32
Bond lengths: RMS (deviation)	0.007	0.007	0.007	0.006
Bond angles: RMS (deviation)	1.110	1.107	1.110	1.112
Validation				
MolProbity score	1.57	1.63	1.80	1.78
Clashscore	6.64	6.64	9.15	8.92
Rotamer outliers (%)	0.14	0.41	0.14	0.14
Ramachandran plot				
Favored (%)	96.74	96.18	95.48	95.6
Outliers (%)	0	0	0	0

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GRAPHICAL ABSTRACT
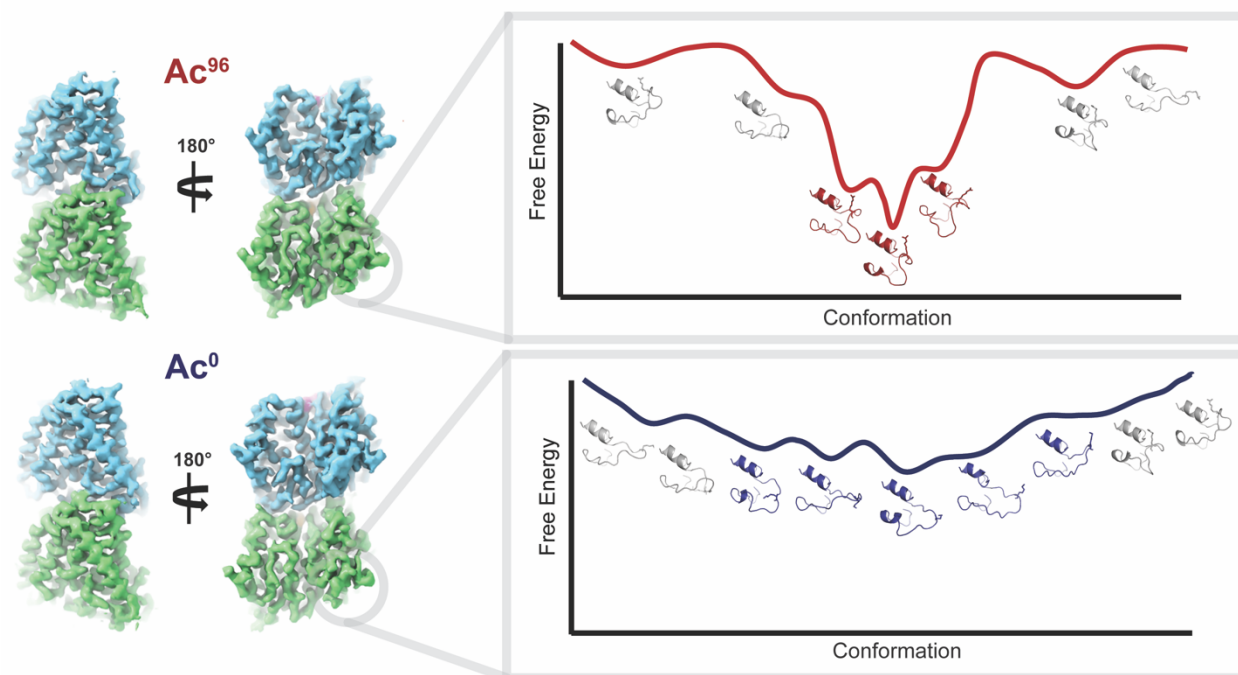
623   Acetylation alters the structural ensemble of the αK40 loop of α-tubulin.

Figure 1. High-resolution maps of 96% acetylated (Ac^{96}) and <1% acetylated (Ac^0) microtubules.
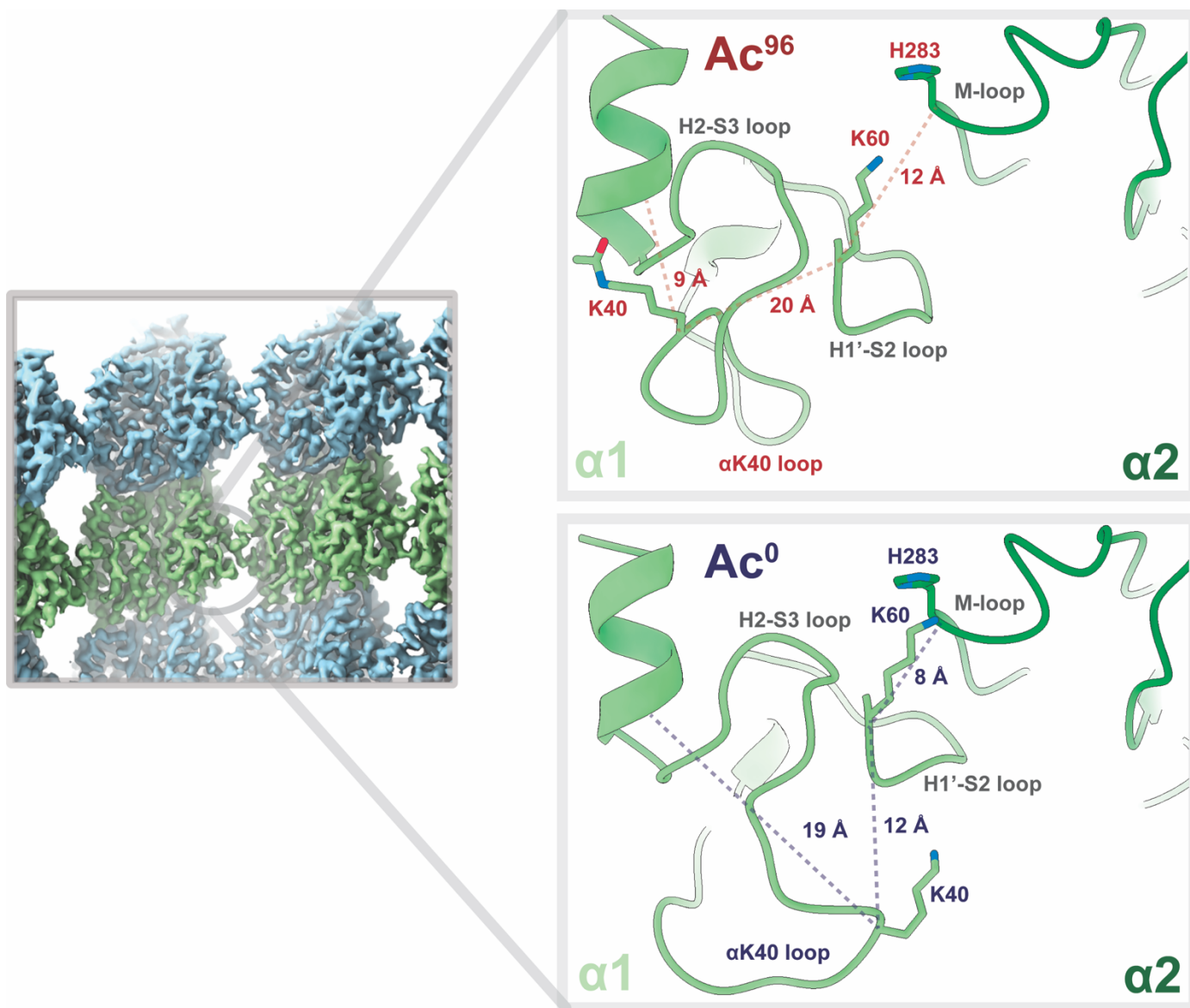
Figure 2. Symmetrized and NCS-averaged C1 maps of Ac[96] and Ac[0] microtubules reveal the αK40 loop is more ordered in the Ac[96] state.

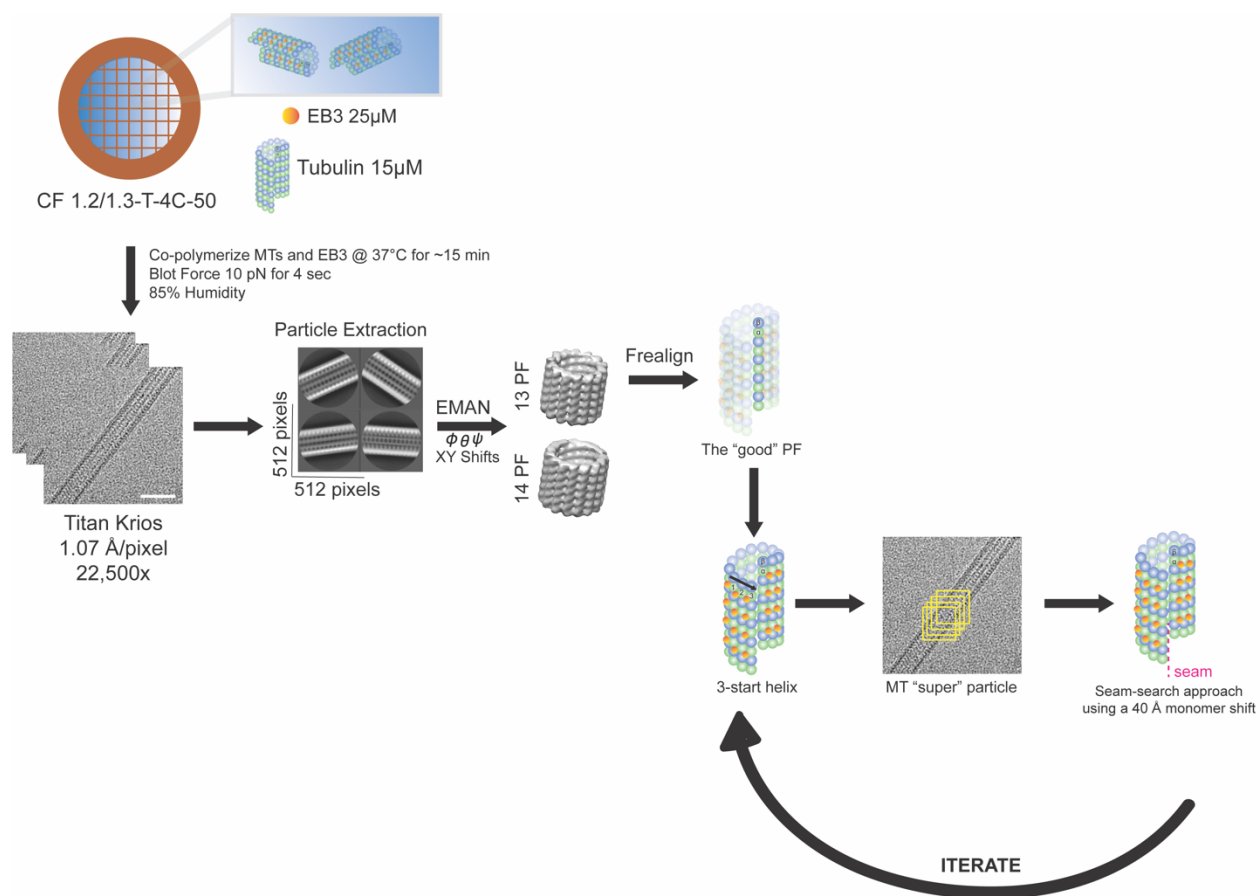**Figure 3. Acetylation restricts the motion and alters the conformational ensemble of the αK40 loop.**

Figure 4. Acetylation may weaken lateral interactions.

638

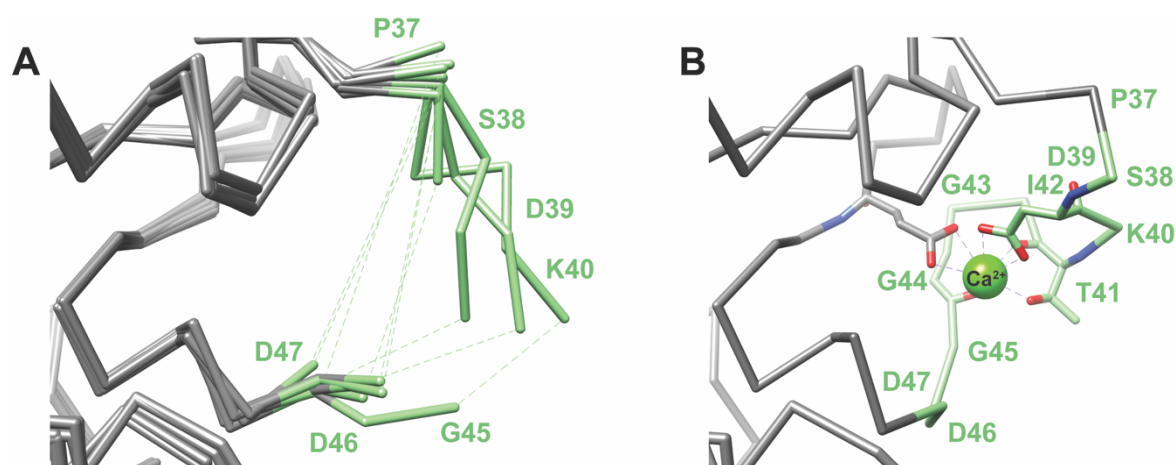**Supplemental Figure 1. Schematic of the experimental workflow for sample**
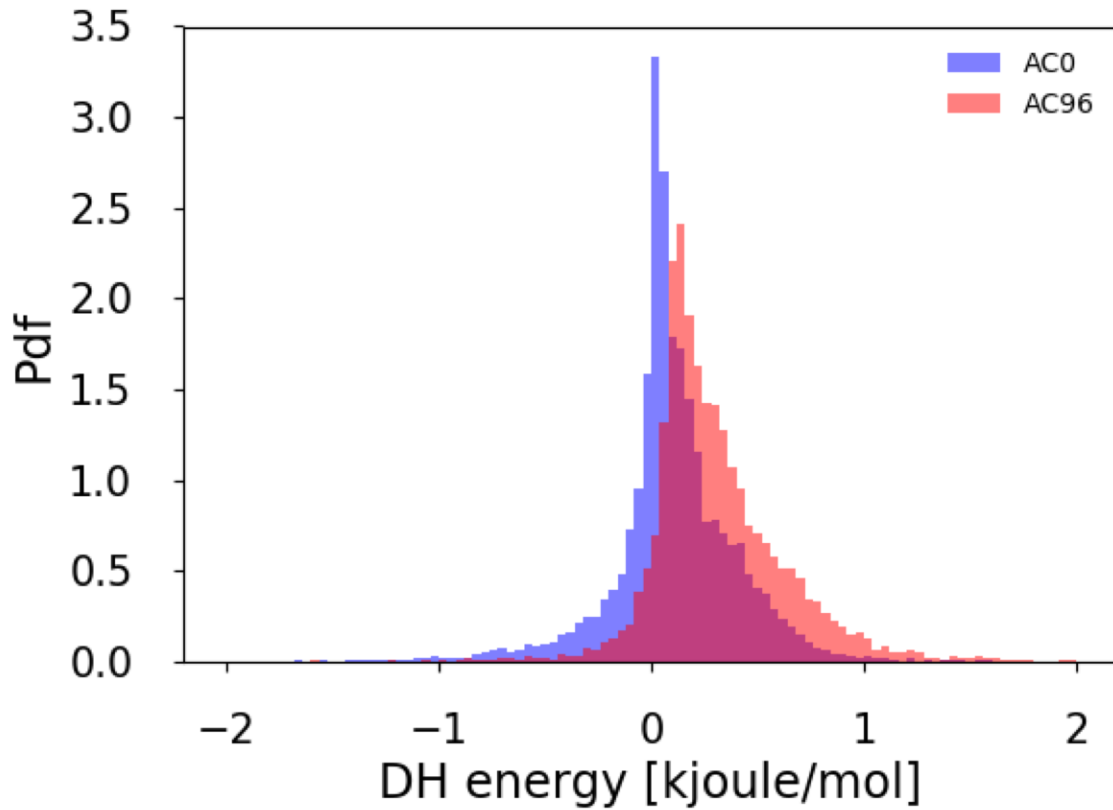
preparation and pseudo-helical image processing.

Supplemental Figure 2. Sample preparation, data collection and image processing of acetylated and deacetylated MT samples.
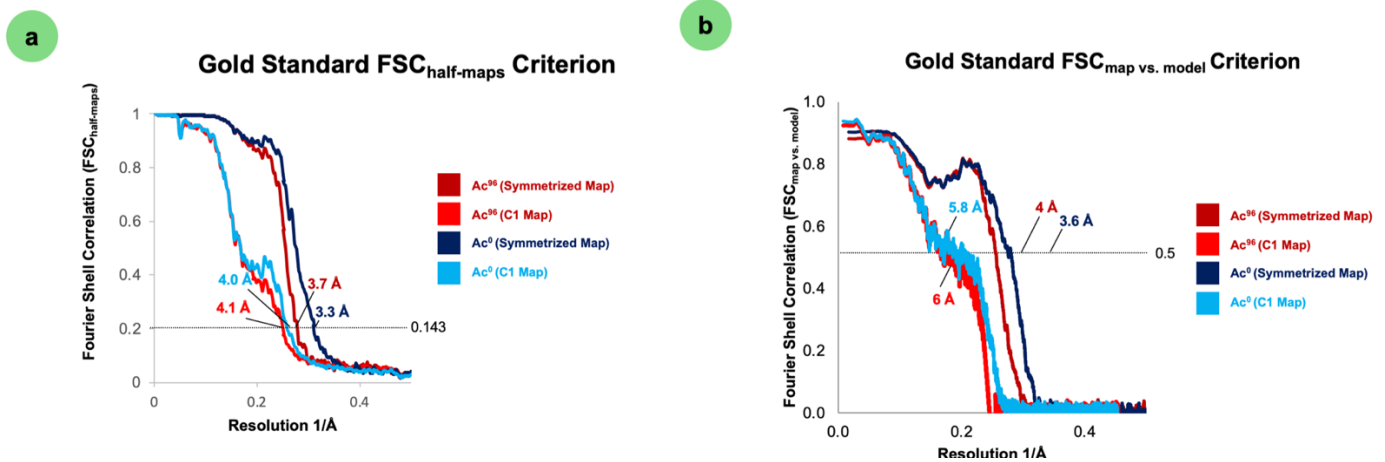

Supplemental Figure 3. Previous proposed αK40 loop models.

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Supplemental Figure 4. Acetylation weakens lateral interactions.

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Supplemental Figure 5. Fourier Shell Correlation Plots.

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