TacT is a GCN5-related *N*-acetyltransferase (GNAT) that inhibits translation through covalent modification of amino acids coupled to tRNA. Although the three paralogs of TacT in *Salmonella* Typhimurium (TacT1, TacT2 and TacT3), as well as their corresponding antitoxins (TacA1, TacA2 and TacA3), are highly similar, previous work had demonstrated that in each case, the antitoxin can inhibit only its cognate toxin[5,6]. To understand the basis for this specificity, Grabe et al. used X-ray crystallography to determine structures of TacT1 and TacT3 in complex with the neutralization domains from their cognate antitoxins. On the surface, the toxins are inhibited in very similar ways; but as usual, the devil is in the details, and close inspection and comparison of the structures revealed subtle differences in a particular region of the toxin (the $\alpha4^T$ helix), which is recognized specifically by each antitoxin. More importantly, by swapping the interacting recognition helix between antitoxins and analyzing toxicity in vivo, the authors showed that specificity for the cognate toxins can also be exchanged, clearly demonstrating that they had identified molecular basis for this specificity.

Next, the authors took a closer look at a structure of an intact TacAT2 complex, which includes two DNA-binding domains on the antitoxin and forms a higher-order 2:4 toxin:antitoxin complex. Here, two dimers of antitoxin are associated with two toxin molecules in such a way that each toxin molecule makes contacts with both antitoxin dimers at two main interfaces and two minor points (Fig. 1a). The two C-terminal regions from one antitoxin dimer fold in different manners, thus forming two separate interfaces—primary (P) and secondary (S)—with a single toxin molecule (Fig. 1b). Two additional contact points exist between the antitoxin's DNA-binding domain and the toxin, the tertiary (T) and quaternary (Q) sites. Surprisingly, and in contrast to the results obtained with the isolated interaction regions, swapping parts of the P and S regions in the context of the full-length complex still conferred partial toxin inactivation, suggesting that the higher-order complex was intact despite a mismatch at the primary interface. This supports a hypothesis in which the multiplicity of interaction interfaces creates a safe space for evolution of the main interface, and thus evolution of insulation between TA paralogs, without risking accidental toxin activation (Fig. 1c). According to this model, the process of evolving insulation between paralogous TA systems involves several phases, including an initial mismatch at the main interface—for example, to allow the activated toxin to target a different molecule or insulation to evolve—followed by compensatory adaptations of the binding partner to restore a fully cognate interaction.

Although these new results clearly demonstrate how insulation is achieved in the TacAT system specifically, the implications of the work are much wider. Most TA systems (such as VapBC, Xre-RES and YefM-YoeB) form higher-order complexes, and in several cases, two antitoxins bind two sites on the same toxin, much like what is observed for TacAT[7–9]. It is thus possible that the principles for the coevolution of paralogous systems described by Grabe et al. are more general and relevant to a wide range of TA systems. In fact, creating space for the safe evolution of toxin activity may, along with the ability to bind DNA, constitute the main driver for the formation of higher-order complexes in these molecular systems. ❐

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Competing interests
The authors declare no competing interest.

CELL DIVISION

Crotonylation directs the spindle

Controlled positioning of the mitotic spindle is key to tissue development and homeostasis. A recent study uncovered an EB1 crotonylation event that orchestrates microtubule dynamics in late mitosis, thus ensuring correct division orientation in vertebrate cells.

Stefano Santaguida and Marina Mapelli

Proper segregation of sister chromatids during mitosis is crucial to maintaining cell homeostasis[1]. Central to this process is the ability of cells to dictate proper localization and functioning of key proteins. An elegant mechanism enabling this timely and rapid control hinges on post-translational modifications (PTMs)[2]. In this issue of *Nature Chemical Biology*, Song et al.[3] describe the crotonylation of end-binding protein 1 (EB1) as a novel and critical PTM required for correct spindle positioning during mitosis, adding an extra layer of PTM regulation to the known repertoire of modifications that cells can utilize to finely modulate protein activity and localization in mitosis (Fig. 1).

In multicellular organisms, proper spindle positioning is important to ensure accurate chromosome segregation and daughter cell positioning, and thereby sustain correct morphogenesis and homeostasis[4]. This requires appropriate organization and functioning of the microtubule (MT) network forming the mitotic spindle (and its associated proteins) (Fig. 1). MTs emanate from centrosomes and reach either the kinetochores or (for astral MTs) the plasma

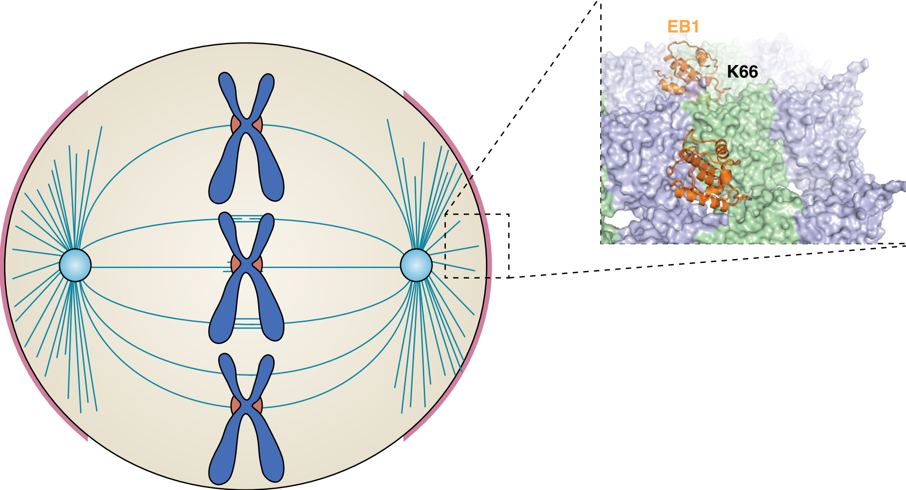

Fig. 1 | EB1 crotonylation on K66 controls mitotic spindle orientation. Dynamic crotonylation of EB1-K66 by TIP60 in metaphase regulates astral microtubule dynamic instability by disrupting the EB1–tubulin interface. The cartoon shows a mitotic cell with sister chromatids aligned at the metaphase plate (in blue) and microtubules of the mitotic spindle (in cyan) reaching either the kinetochore (brown) or the cell cortex. NuMA (in pink) recruits dynein to the cortex above the spindle poles to exert traction forces on astral microtubules positioning the spindle. EB1 at microtubule plus ends promotes the anchoring of astral MTs at the NuMA-enriched cortical region. Close-up shows the binding interface between the calponin-homology domain of EB1 (in orange cartoon representation) and the MT lattice (in surface representation: α-tubulin in light violet and β-tubulin in pale green) that is regulated by K66 crotonylation. EB1-K66 is shown in ball-and-stick representation facing β-tubulin subunits (PDB ID 3JAL was used for the close-up).

membrane. MT ends undergo continuous cycles of polymerization (growth) and depolymerization (shrinkage), interspersed with pauses, that are finely controlled by numerous regulatory MT-associated proteins (MAPs) interacting with tubulin or MTs. Among these, MT-plus-end-tracking proteins (+TIPs) constitute a family of structurally and functionally diverse MT regulators that accumulate at growing plus ends to control the fate of MT tips. EB1 is perhaps the best-characterized plus-end astral microtubule tracker[5,6]; it recruits other proteins to plus ends but also alters mechanical lattice properties[7] to regulate dynamic instability.

Lysine crotonylation is a histone PTM recently reported to influence gene expression[8], but its involvement in other cell biological processes remained unclear. By carrying out a systematic screen for EB1 PTMs, Song et al.[3] have now provided compelling evidence for EB1 crotonylation at lysine 66 (CrK66-EB1) in metaphase and anaphase through the mitotic enzymatic activity of TIP60 and its reversion through the action of HDAC3.

Given the similar chemistry of crotonyl and acetyl groups, a major caveat in addressing the role of EB1 crotonylation is to ensure that the phenotypic effects associated to this modification are specific, meaning that they cannot be ascribed to EB1-K66 acetylation due to the acetyltransferase activity of TIP60. Song et al.[3] used a genetic encoding method to generate an HeLa cell line expressing EB1 with an acetylated K66 and compare it to chemically engineered CrK66-EB1 in an EB1 knockout background. Time-lapse imaging revealed that cells expressing CrK66EB1 exhibited chromosome segregation errors and misoriented spindles, leading to prolonged mitosis, and that these effects were indeed specific to CrK66-EB1, as they did not occur in the AcK66-EB1-expressing HeLa cells. The mitotic defects observed in the CrK66-EB1 HeLa cells were consistent with improper control of late mitosis events, when crotonylation of EB1 by TIP60 peaks. Kymographs of quantitative total internal reflection fluorescence (TIRF) microscopy experiments showed that in vitro, CrK66-EB1 exhibits a decreased affinity for microtubules and reduced dwell time at plus ends, accompanied by altered dynamic instability. The reduced affinity results in fewer, shorter and more unstable astral microtubules that barely reached the cortex, causing the observed misorientation defects (Fig. 1). Again, these effects were not observed in analogous experiments conducted in vitro and in cells with AcK66-EB1. The authors conducted a proteomic screen to identify CrK66-specific EB1 interactors and identified the dynein adaptor NuMA, a master regulator of spindle orientation[9]. Although the EB1–NuMA interaction does not depend on EB1 crotonylation, this finding further corroborates the idea that crotonylation of EB1 localizes EB1 to the plus end, providing a link between astral microtubules and the cortex.

Collectively, the findings from Song and colleagues identify a mitotic crotonylation event that guides correct mitotic progression and spindle orientation. This discovery enriches the scenario of mitotic PTMs orchestrating correct chromosome segregation and daughter-cell positioning. The importance of EB1-crotonylation and de-crotonylation rates, and its interplay with other tubulin PTMs that influence microtubule dynamics[10], remain interesting quests to be pursued. Further studies will be required to understand whether other crotonylation events affect cell division and how they undergo cross-talk with one another and with other PTMs, especially those affecting tubulin polymerization and microtubule dynamic instability. Studying and understanding the implications of crotonylation during mitosis holds the promise of unveiling unexplored paths crucial for proper chromosome segregation and adding an important layer of regulation to our picture of the fascinating process of cell division. ❐

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Competing interests

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