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Reversible arginine methylation of PI3KC2 α controls mitotic spindle dynamics

Yena Cho^{1,2}, Jee Won Hwang², Mark T. Bedford³, Dong Hee Na⁴, Dae-Geun Song^{5,6}, Su-Nam Kim^{5,6} and Yong Kee Kim^{1,2*}

Abstract

Microtubules, composed of $\alpha\beta$ -tubulin dimers, undergo dynamic polymerization and are fundamental to cell structure and function. In the current study, we discovered that phosphatidylinositol 3-kinase class 2 α (PI3KC2 α) acts as a novel regulatory factor in microtubule dynamics. Specifically, asymmetric dimethylation of PI3KC2 α at the R175 residue (R175me2a) by coactivator-associated arginine methyltransferase 1 (CARM1) enhances its interaction with α -tubulin, stabilizing microtubule assembly. Furthermore, lysine Demethylase 4 A (KDM4A) serves as an arginine demethylase for PI3KC2 α R175me2a. During mitosis, protein kinase C (PKC)-mediated phosphorylation of KDM4A results in its dissociation from PI3KC2 α , preventing demethylation and increasing R175me2a levels. This facilitates spindle formation and highlights the critical role of reversible arginine methylation in regulating mitotic spindle dynamics. Cumulatively, these findings reveal the coordinated interplay between CARM1 and KDM4A in modulating microtubule behavior through PI3KC2 α R175 methylation, offering new insights into the regulatory mechanisms of mitotic progression.

Keywords CARM1, PI3KC2 α , Tubulin, Microtubule, Autoregulation, Mitosis

Introduction

Microtubules, the primary components of the cytoskeleton, are long and thin filaments composed of repeating $\alpha\beta$ -tubulin dimers [1]. They are crucial in determining cell shape and facilitating various cell movements, including certain forms of locomotion, intracellular organelle transport, and chromosome separation during mitosis [2, 3]. Their dynamics are tightly regulated by intracellular levels of soluble tubulins, microtubule-associated proteins, and post-translational modifications (PTMs) of tubulin [4, 5]. For example, acetylation of α -tubulin subunits enhances microtubule stability, while deacetylation causes destabilization [5]. Additionally, end-binding protein-1 (EB1) and stathmin 1 (STMN1) are essential in regulating microtubule dynamics. EB1 stabilizes microtubules by localizing at their growing plus ends [6, 7], whereas STMN1 interacts directly with soluble tubulin, preventing microtubule formation and increasing the

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pool of unpolymerized free tubulin [8]. When cells detect an excess of free tubulin, tubulin mRNA is degraded through an mRNA decay pathway known as tubulin autoregulation [9, 10], helping maintain the intracellular tubulin pool.

Recent studies have identified the pivotal role of tetratricopeptide repeat domain 5 (TTC5) [11] and phosphatidylinositol 3-kinase class 2 α (PI3KC2 α) in tubulin autoregulation [12]. PI3KC2 α acts as a sequestering factor for TTC5, which is controlled by coactivator-associated arginine methyltransferase 1 (CARM1)-mediated methylation at the R175 residue [12]. Thus, arginine methylation is an important PTM associated with various biological processes, including transcription, splicing, and cell signaling [13–16]. Moreover, the TTC5-mediated tubulin autoregulation pathway is triggered by unmethylated PI3KC2 α binding to and sequestering TTC5; however, this form is unstable and quickly degrades, leading to TTC5 release. Free TTC5 recognizes and binds to nascent tubulin polypeptides emerging from translating ribosomes, subsequently recruiting factors such as the adaptor protein (SCAPER), the substrate recruitment subunit (CNOT11), and the deadenylase complex (CCR4–NOT). This process results in the degradation of associated mRNAs [11, 12, 17]. Meanwhile, methylated PI3KC2 α also increases free TTC5 due to its limited binding [12]. This observation raises two fundamental questions. First, why was tubulin autoregulation not activated in this context? Since newly translated soluble $\alpha\beta$ -tubulin dimers sequester TTC5, inhibiting tubulin autoregulation [18], methylated PI3KC2 α may promote tubulin polymerization. Second, is the arginine methylation of PI3KC2 α dynamically regulated? To address these questions, we investigated the role of the CARM1–PI3KC2 α axis in tubulin polymerization and microtubule stabilization. Moreover, we sought to identify the arginine demethylase (RDM) that serves as CARM1's counterpart.

Results

PI3KC2 α R175-dimethylation by CARM1 promotes tubulin polymerization and stabilizes microtubules

To determine whether PI3KC2 α R175me2a is essential for tubulin polymerization (Fig. 1A), we first examined if the physical interaction between PI3KC2 α and tubulin depends on R175 methylation by CARM1. PI3KC2 α interacted with α -tubulin (Figs. 1B, S1A) in an R175me2a-dependent manner (Fig. 1B and D). Specifically, when CARM1 was depleted or inhibited, PI3KC2 α R175me2a levels decreased, leading to a loss of its interaction with α -tubulin (Figs. 1C, S1B). Moreover, unlike in CARM1 wild-type (WT) cells, overexpressed PI3KC2 α in CARM1 knockout (KO) cells did not interact with α -tubulin due to the lack of R175 methylation (Fig. 1D).

This was further confirmed by the loss of interaction between the R175K/A mutant and α -tubulin, with an R175me2a-dependent interaction observed only in the WT cells (Figs. 1E, S1C).

Next, to investigate the effect of PI3KC2 α R175me2a on microtubule dynamics, we overexpressed the R175 WT or R175K/A mutant and assessed the levels of microtubules (insoluble tubulin polymers) and soluble tubulins. In CARM1 WT cells, overexpression of the R175K/A mutant reduced the total, soluble, and insoluble levels of tubulins, whereas the R175 WT increased the levels of microtubules and soluble tubulins (Figs. 1F, 1G, S1D). Interestingly, in CARM1 KO cells, overexpression of R175 WT only increased soluble tubulin levels (Figs. 1H, S1E). This suggested that intact R175 is essential for maintaining intracellular tubulin levels by suppressing tubulin autoregulation (Fig. 1I and K, S1F–S1H). These results also indicate that even with sufficient soluble tubulins, tubulin polymerization does not occur when PI3KC2 α is not methylated. Indeed, the PI3KC2 α R175me2a specifically appeared to increase microtubule levels (Figs. 1F, 1G), supporting the hypothesis that R175 methylation of PI3KC2 α promotes tubulin polymerization.

To further validate this hypothesis, we performed an *in vitro* tubulin polymerization assay. As expected, the presence of methylated form of PI3KC2 α (WT) significantly enhanced tubulin polymerization, while unmethylated forms of PI3KC2 α (R175K and R175A mutants) failed to promote tubulin polymerization (Figs. 2A, S2A). Moreover, the methylated PI3KC2 α exclusively cosedimented with polymerized tubulin (microtubules), whereas the non-methylated form did not associate with microtubules (Fig. 2B). We next examined markers of growing microtubule. At the growing ends of microtubules, α -tubulin undergoes C-terminal tyrosination, and the protein EB1 is recruited, leading to microtubule stabilization [6, 7, 19]. CARM1 overexpression promoted microtubule formation as evidenced by increased insoluble tubulin levels and EB1 recruitment without affecting the total amount, coinciding with an increase in PI3KC2 α R175me2a recruitment (Figs. 2C, S2B). Similar results were observed when PI3KC2 α was overexpressed (Figs. 2D, S2C). On the contrary, depletion of CARM1 or PI3KC2 α reduced EB1 recruitment at the microtubule growth ends and decreased tyrosinated α -tubulin levels (Fig. 2D and F, S2D). Furthermore, significantly fewer EB1 comets were observed in cells with depleted CARM1 or PI3KC2 α compared to control cells (siCON = 62.5, siCARM1 = 28.4, siPI3KC2 α = 21.6; Figs. 2G, S2E). Acetylated α -tubulin levels—another marker of microtubule stabilization [5]—were markedly decreased and increased in cells with depleted or overexpressed PI3KC2 α , respectively (Fig. S2F).

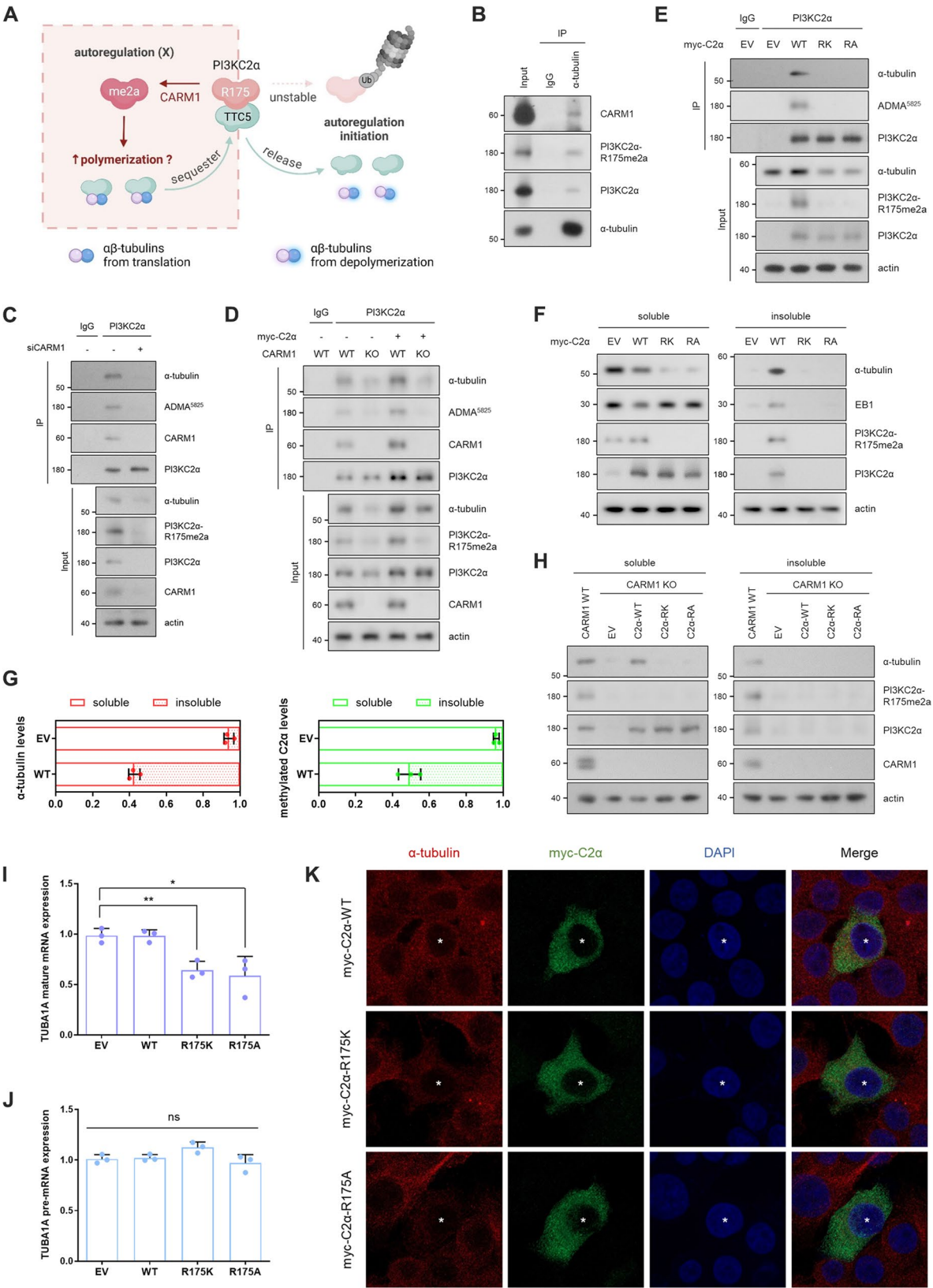


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Fig. 1 PI3KC2 α R175-dimethylation by CARM1 promotes tubulin polymerization and stabilizes microtubules. **A** Schematic image suggesting the role of the CARM1–PI3KC2 α axis in microtubule dynamics. **B** Immunoblotting with anti- α -tubulin antibody to detect the interaction between PI3KC2 α and tubulin depends on R175 methylation by CARM1. **C** After transfection of CARM1 siRNAs for 72 h, lysates were immunoprecipitated using anti-PI3KC2 α antibody. **D** CARM1 WT or KO MEF cells were transfected with myc-PI3KC2 α for 48 h. The cell lysates were immunoprecipitated with an anti-PI3KC2 α antibody. **E** Cells were transfected with myc-PI3KC2 α -WT, -R175K, or -R175A, and lysates were immunoprecipitated with anti-PI3KC2 α antibody. **F** and **G** Cells were transfected with myc-PI3KC2 α -WT, -R175K, or -R175A, and soluble and insoluble fractions were analyzed via immunoblotting (**F**). Band intensities were quantified from three independent experiments and plotted as a fraction of total (soluble + insoluble) protein levels (**G**). **H** Soluble and insoluble fractions were prepared and immunoblotted to determine α -tubulin localization after transfection of CARM1 KO MEF cells with myc-PI3KC2 α -WT, -R175K, or -R175A. **I** and **J** After transfecting cells with myc-PI3KC2 α -WT, -R175K, or -R175A, mature *TUBA1A* mRNA (**I**) and pre-mRNA (**J**) levels were determined using quantitative real-time PCR. Data are presented as mean $\pm$ standard deviation ($n=3$). (**K**) MCF7 cells were transfected with myc-PI3KC2 α -WT, -R175K, or -R175A, and co-stained with antibodies against α -tubulin (red) and myc-PI3KC2 α (green) as well as DAPI (blue). Representative images are shown

To visualize the regulation of tubulin polymerization through the CARM1–PI3KC2 α axis, we designed a nocodazole release experiment. Nocodazole treatment depleted microtubules by inhibiting their formation. Upon release, the depleted microtubules rapidly reformed (Fig. 2H and I). During this process, microtubule reformation was markedly delayed in cells with depleted CARM1 or PI3KC2 α (Fig. 2H and I). Our findings suggest that PI3KC2 α methylation by CARM1 promotes microtubule formation and stabilization.

CARM1-mediated R175 dimethylation of PI3KC2 α regulates spindle dynamics during mitosis

As tubulin polymerization is most active at the growing ends of the spindle during mitosis, we investigated the role of CARM1-mediated R175 dimethylation of PI3KC2 α in spindle formation. The interaction between CARM1 and PI3KC2 α was substantially increased during mitosis compared to interphase (Figs. 3A, 3B). Co-IP analysis revealed that treatment with nocodazole—which inhibits spindle formation and causes its collapse—reduced the binding of the PI3KC2 α –CARM1 complex to tubulin, whereas paclitaxel—which excessively stabilizes the spindle—increased this binding (Figs. 3B, S3A). Furthermore, nocodazole treatment decreased the levels of PI3KC2 α and CARM1 in the insoluble fraction, while paclitaxel treatment increased these levels (Fig. 3C and D). This suggested that the CARM1–PI3KC2 α complex supports spindle formation during mitosis. Indeed, the formation of this complex and its interaction with tubulin increased during the late G2 phase (BI2536) and metaphase (nocodazole release with MG132), coinciding with a marked increase in PI3KC2 α R175me2a (Fig. 3E and F, S3B–D). The increase in PI3KC2 α methylation during mitosis was further corroborated by the proximity ligation assay (PLA) results (Fig. 3F). This increase in methylation correlated with enhanced interaction between PI3KC2 α and spindle during mitosis (Fig. 3G and H), implying that CARM1-mediated methylation of PI3KC2 α facilitates spindle formation.

PI3KC2 α methylation is reversely controlled by CARM1 and KDM4A cooperation

The PI3KC2 α R175me2a levels increase as cells enter mitosis and decrease as they exit, indicating that PI3KC2 α methylation is reversible during mitosis. Although the mechanisms of reversible arginine methylation are not fully understood, some KDMs (KDM3A, 4A, 4E, 5C, and 6B) reportedly exhibit RDM activity [20]. Among them, only KDM4A overexpression reduced PI3KC2 α methylation levels (Figs. 4A, S4A). Moreover, an in vitro demethylation assay using a synthetic PI3KC2 α peptide containing R175me2a showed its clear demethylation by KDM4A (Fig. 4B). This was evidenced by the peptide spectra displaying –28 Da mass shifts, corresponding to the loss of two methyl groups from the methylated residue (Fig. 4B). Additionally, we observed that KDM4A overexpression decreased PI3KC2 α (Fig. 4A) and mature *TUBA1A* mRNA levels without affecting pre-mRNA levels (Fig. 4C and D), accompanied by reduced α -tubulin protein levels (Fig. 4E). These observations result from demethylation of PI3KC2 α R175me2a, supporting that KDM4A functions as an RDM in vivo (Figs. 4E, S4B).

During mitosis, the binding of PI3KC2 α to the spindle increased until metaphase and then decreased (Fig. 4F). This was further supported by a gradual increase in the PI3KC2 α – α -tubulin PLA foci per cell until metaphase and subsequent decrease during ana-/telophase (Fig. 4G and H). The recruitment of PI3KC2 α to the spindle during mitosis closely correlated with changes in PI3KC2 α methylation (Fig. 4F). These changes in PI3KC2 α methylation can be explained by the interaction between PI3KC2 α and CARM1, which increases and is maintained until ana-/telophase, while the interaction between PI3KC2 α and KDM4A is abruptly lost during pro-/prometaphase and then recovered after metaphase (Fig. 4E, I and K, S4C, S4D). As cells exit mitosis, the interaction between CARM1 and PI3KC2 α gradually decreases, whereas the interaction between KDM4A and PI3KC2 α remains stable (Fig. 4I). Thus, the interaction between PI3KC2 α and CARM1 during early mitosis leads

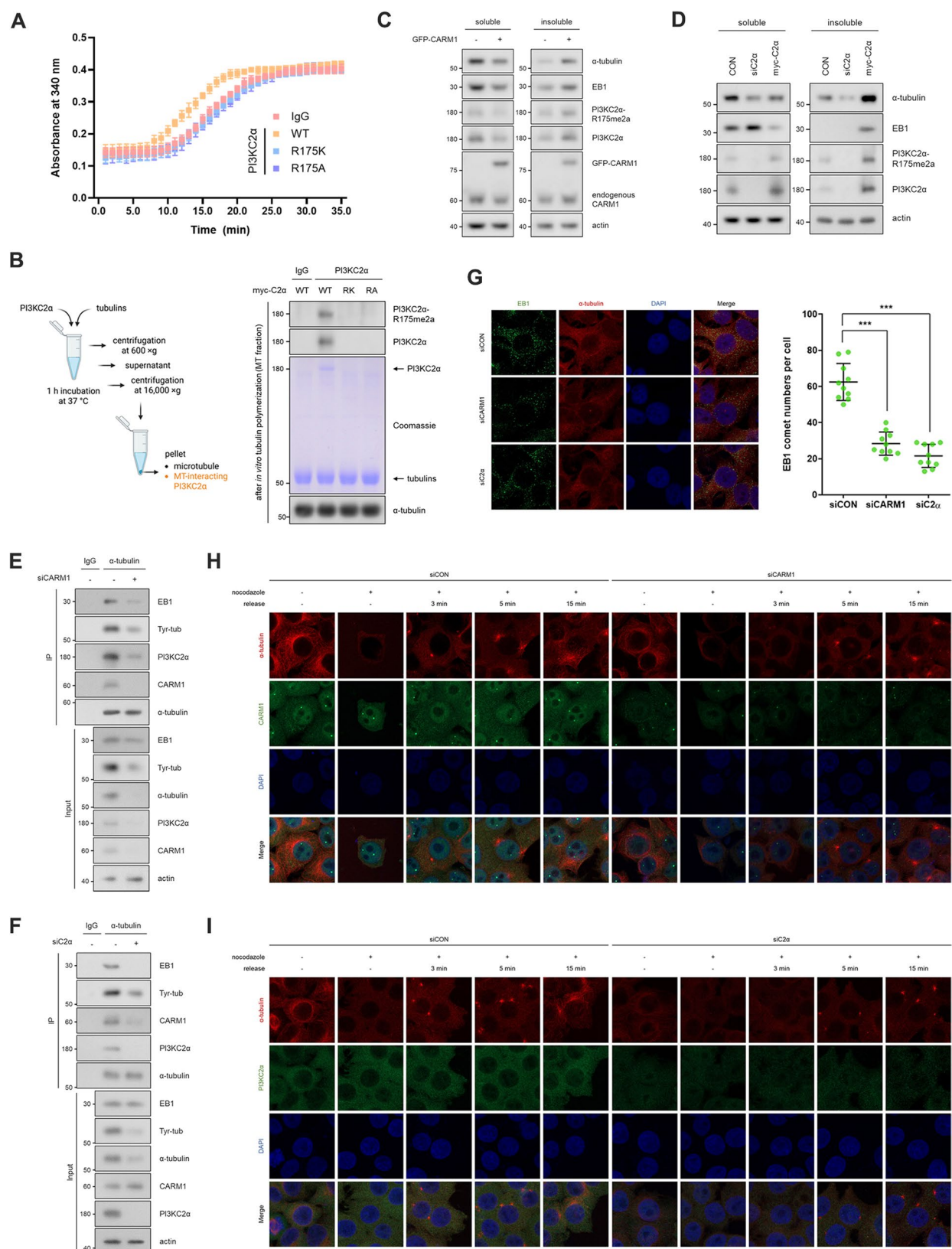


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Fig. 2 PI3KC2 α R175-dimethylation by CARM1 stabilizes microtubules. **A** and **B** In vitro tubulin polymerization assay in the presence of myc-PI3KC2 α -WT, -R175K, or -R175A. Data are presented as mean $\pm$ standard deviation ($n=3$) (**A**). Following polymerization, microtubule fractions were separated by centrifugation and analyzed by immunoblotting (**B**). **C** Immunoblotting of proteins in soluble and insoluble fractions after transfection of GFP-CARM1 for 72 h. **D** Protein expression in the soluble and insoluble fractions after PI3KC2 α knockdown or overexpression. **E** and **F** Immunoprecipitation of lysates using an anti- α -tubulin antibody after transfection of CARM1 (**E**) or PI3KC2 α (**F**) siRNAs for 72 h. EB1 and tyrosinated α -tubulin were used as markers of growing microtubules. **G** Cells were co-stained with antibodies against EB1 (green), α -tubulin (red), and DAPI (blue) after transfection of CARM1 or PI3KC2 α siRNAs. Representative images are shown. Data are presented as mean $\pm$ standard deviation. (**H** and **I**) CARM1- (**H**) or PI3KC2 α - (**I**) Depleted cells were treated with 1 μ g/mL nocodazole for 1 h and then released for indicated time points (3, 5, and 15 min). The cells were immediately fixed and immunostained. Representative confocal images of α -tubulin (red), CARM1 (**H**), PI3KC2 α (**I**) (green), and nuclei (blue)

to R175 dimethylation, promoting tubulin polymerization and spindle formation. After metaphase, PI3KC2 α binds to KDM4A and returns to a demethylated state, inducing microtubule depolymerization and spindle disassembly. These findings support the hypothesis that PI3KC2 α methylation is dynamically regulated through the cooperation between CARM1 and KDM4A to control tubulin polymerization.

PKC α -mediated phosphorylation of KDM4A disrupts its interaction with PI3KC2 α during mitosis

Given that a massive wave of phosphorylation occurs during mitosis [21], we examined whether KDM4A undergoes phosphorylation during this phase. Interestingly, KDM4A phosphorylation peaked at prometaphase and gradually decreased (Fig. 5A), showing an inverse correlation with its interaction with PI3KC2 α (Figs. 4K and 5A). This suggests that the phosphorylation of KDM4A may disrupt its interaction with PI3KC2 α , preventing PI3KC2 α demethylation. Moreover, we identified PKC α as the kinase responsible for KDM4A phosphorylation, supported by two key observations: first, *PRKCA* mRNA levels broadly correlated with those of various tubulin isoforms (*TUBA1A*, *TUBA1B*, *TUBA1C*, *TUBB*, *TUBB2A*, *TUBB3*, *TUBB6*, *TUBG1*, and *TUBG2*) in 1,517 cell lines from the DepMap database (Figs. 5B, S5A); second, the selective inhibition of PKC with calphostin C, not other mitotic kinases, reduced nocodazole-induced phosphorylation of KDM4A (Fig. 5C). We thus propose that during mitosis, PKC α -mediated phosphorylation of KDM4A reduces its interaction with PI3KC2 α , leading to an accumulation of PI3KC2 α R175me2a and promoting spindle formation (Fig. 5D). This hypothesis aligns with recent findings that PKC α promotes microtubule formation by phosphorylating the S165 of α -tubulin [22–24]. Supporting this hypothesis, only PKC-selective inhibition decreased PI3KC2 α R175me2a, inhibiting tubulin polymerization and triggering tubulin autoregulation (Fig. 5E and K, S5B–S5D). Conversely, activation of PKC with phorbol 12-myristate 13-acetate (PMA) increased PI3KC2 α methylation, producing the opposite effect (Fig. 5E and K). To validate the role of PKC α in regulating KDM4A phosphorylation and its demethylase activity, we employed constitutively active (CA) and dominant negative (DN) forms of PKC α . Overexpression of PKC α -CA

enhanced KDM4A phosphorylation, increased PI3KC2 α methylation, and enhanced its tubulin binding. In contrast, overexpression of PKC α -DN resulted in reduced KDM4A phosphorylation, diminished PI3KC2 α methylation, and weakened tubulin association (Figs. S5E and S5F). Collectively, these data provide compelling evidence that phosphorylation of KDM4A by PKC α plays a critical role in regulating the reversible methylation of PI3KC2 α and in modulating microtubule dynamics.

Discussion

Intracellular tubulin levels are strictly controlled by an autoregulatory mechanism mediated by TTC5 [11, 17]. When free TTC5 recognizes and binds to nascent tubulin polypeptides translated at ribosomes, several factors, including deadenylase, are recruited, leading to its mRNA decay [17]. Recent studies have identified PI3KC2 α as a sequestering factor for TTC5 [12]. Our findings show that microtubule dynamics are regulated by PI3KC2 α R175 methylation (Fig. 6), which aligns with the established tubulin autoregulation pathway. During microtubule depolymerization, the unmethylated PI3KC2 α binds to and sequesters TTC5, impeding tubulin autoregulation. However, unmethylated PI3KC2 α is unstable and rapidly degrades *via* the proteasome pathway, at which point TTC5 is released, allowing autoregulation to commence. This mechanism may function as a buffering system that stabilizes tubulin levels during rapid changes caused by microtubule depolymerization. Notably, methylated PI3KC2 α promotes microtubule formation and stabilization, emphasizing its role during mitosis. Previous studies have detected PI3KC2 α within the spindle-associated clathrin-transforming acidic coiled-coil containing protein 3 (TACC3) complex [25], with spindle enrichment mediated by the TACC3 binding site (aa 512–670) [26]. However, this interaction was observed only in mitotic-arrested cells, not interphase-arrested cells [26], paralleling the R175 methylation pattern by CARM1. This implies that methylation at R175 may enhance the interaction between PI3KC2 α and TACC3, leading to microtubule stabilization through subsequent recruitment of clathrin heavy chain [26–28].

To finely regulate microtubule dynamics, PI3KC2 α methylation at R175 must be reversible. Unlike lysine methylation, which is well-regulated by methylating

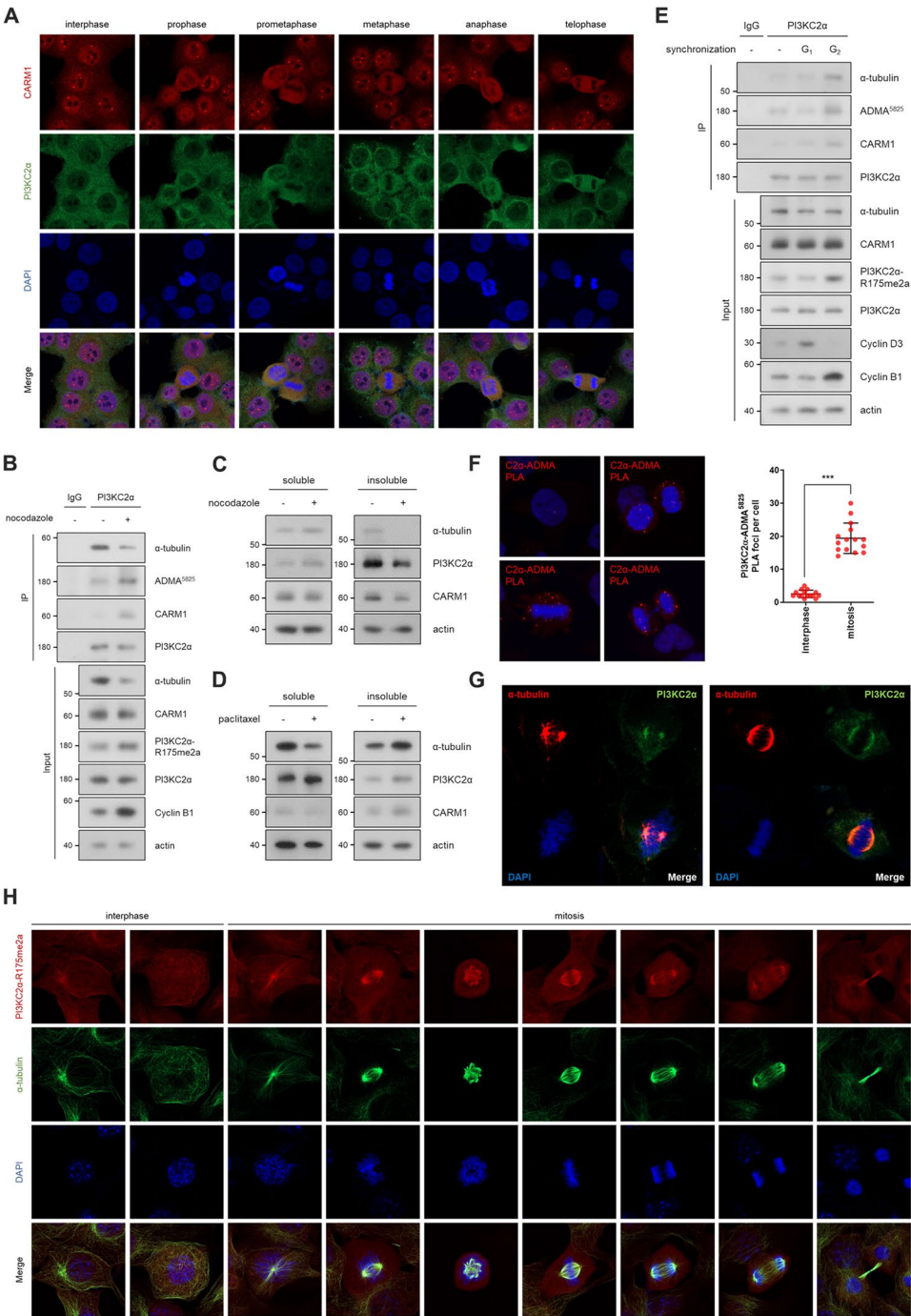


Fig. 3 CARM1-mediated R175 dimethylation of PI3KC2α regulates spindle dynamics during mitosis. **A** Confocal images of MCF7 cells co-stained with antibodies against CARM1 (red), PI3KC2α (green), and DAPI (blue) in each cell cycle phase. **B** After treatment of 100 ng/mL nocodazole for 24 h, lysates were immunoprecipitated using an anti-PI3KC2α antibody. **C** and **D** Soluble and insoluble fractions were separated and analyzed by immunoblotting after 100 ng/mL nocodazole (**C**) or 100 nM paclitaxel (**D**) treatment for 24 h. **E** Thymidine (G1 phase) or BI2536 (G2 phase, PLK1 inhibitor) was used to arrest the cell cycle. Each cell lysate was immunoprecipitated using an anti-PI3KC2α antibody. **F** After fixation and permeabilization of cells, PLA was performed with antibodies against PI3KC2α and ADMA⁵⁸²⁵. Red dots indicate PI3KC2α methylation and represent mean ± standard deviation. **G** Confocal images of α-tubulin (red), PI3KC2α (green), and DAPI (blue) in mitotic HeLa cells. PI3KC2α is co-localized to the mitotic spindle. **H** Confocal images of PI3KC2α R175me2a (red), α-tubulin (green), and DAPI (blue) in MEF cells at interphase and mitosis

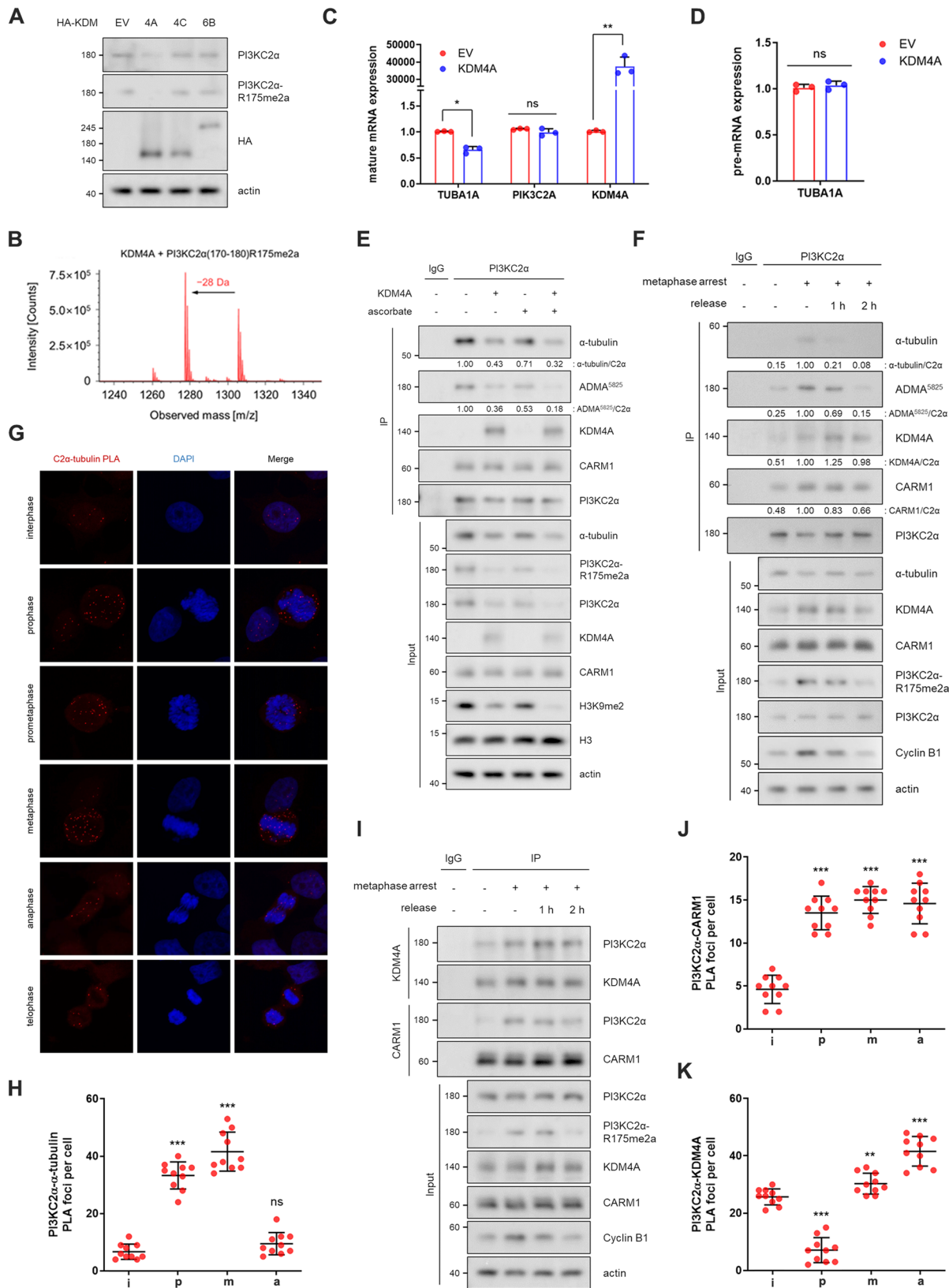


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Fig. 4 PI3KC2 α methylation is reversely controlled by CARM1 and KDM4A cooperation. **A** Immunoblotting of PI3KC2 α and its R175me2 α levels following transfection of 293T cells with HA-KDM4A, -KDM4C, or -KDM6B. **B** In vitro demethylation assay using KDM4A recombinant protein and PI3KC2 α (170–180) R175me2 α peptide (NGFNPR(me2 α)MPTFP) in the presence of the 2-oxoglutarate, ascorbate, and Fe²⁺. **C** and **D** Mature *TUBA1A* mRNA (**C**) and pre-mRNA (**D**) levels measured in KDM4A-overexpressing cells. Error bars indicate standard deviation ($n=3$). **E** Lysates were immunoprecipitated using an anti-PI3KC2 α antibody after transfection of cells with KDM4A. H3K9me2 is a representative KDM4A substrate. **F** Cells arrested at metaphase were released via incubation with fresh media for 1 h (anaphase) or 2 h (telophase or interphase). The cell lysates were immunoprecipitated using an anti-PI3KC2 α antibody, followed by immunoblot analysis of the indicated proteins. **G** and **H** Cells were incubated with antibodies against PI3KC2 α and α -tubulin. After recognition with PLA probe, ligation, and amplification, PLA signals (red dots) were counted. The nuclei were stained with DAPI (blue). Data are shown as mean $\pm$ standard deviation. **I** Cells arrested at metaphase were released via incubation with fresh media for 1 h (anaphase) or 2 h (telophase or interphase). The cell lysates were immunoprecipitated using an anti-CARM1 or -KDM4A antibody to assess its interaction with PI3KC2 α . (**J** and **K**) Cells were incubated with antibodies against PI3KC2 α and CARM1 (**J**) or KDM4A (**K**). After recognition with PLA probe, ligation, and amplification, PLA signals (red dots) were counted. Data are shown as mean $\pm$ standard deviation

and demethylating enzymes [29], arginine methylation is catalyzed by nine enzymes (PRMT1–9), while a bona fide demethylase has not yet been identified [30]. Although JMJD6 was initially reported to have RDM activity [31], it also functions as a lysyl hydroxylase [32]. Additionally, various KDMs have been suggested to possess RDM activity; however, this was primarily demonstrated using artificial substrates [20]. In this study, we report the novel role of KDM4A as an RDM that mediates arginine demethylation in vivo. While KDM4A, like JMJD6, may not be a pure RDM, our findings demonstrate that arginine methylation is dynamically regulated within cells. Specifically, KDM4A overexpression led to PI3KC2 α demethylation, reducing its stability and initiating tubulin autoregulation. This effect was further supported by observations during mitosis, during which the interaction between PI3KC2 α and KDM4A decreased as the mitotic spindle formed, while its interaction with CARM1 increased, leading to higher levels of methylation that stabilized the mitotic spindle. How are the interactions between PI3KC2 α and KDM4A/CARM1 regulated during mitosis? During interphase, CARM1 is primarily located in the nucleus, but as cells enter mitosis and the nuclear envelope breaks down, the likelihood of its interaction with PI3KC2 α in the cytoplasm increases. Moreover, several kinases are activated during mitosis, with Ca²⁺-dependent PKC playing a particularly significant role by directly phosphorylating α -tubulin proteins, promoting their polymerization [22–24]. KDM4A can also be phosphorylated by PKC, disrupting the interaction between KDM4A and PI3KC2 α . This allows PI3KC2 α to remain methylated, stabilizing the mitotic spindle. As mitosis progresses, KDM4A is dephosphorylated, increasing its interaction with PI3KC2 α , leading to PI3KC2 α demethylation, spindle depolymerization, and the initiation of tubulin autoregulation. Despite these findings, a limitation of our study is that the specific phosphorylation site(s) on KDM4A responsible for regulating its interaction with PI3KC2 α have not yet been identified. Elucidating these site(s) will be critical to fully understand the dynamic regulation of arginine

methylation by KDM4A, and thus represents an important avenue for future investigation.

In conclusion, our study clarifies the detailed mechanisms by which PI3KC2 α regulates microtubule dynamics, demonstrating that this process is precisely controlled through reversible arginine methylation mediated by the cooperative actions of CARM1 and KDM4A. Understanding these dynamics could offer valuable insights into how cells ensure the proper regulation of critical processes such as spindle assembly and tubulin homeostasis.

Methods

Chemicals, plasmids, and antibodies

Calphostin C, MG132, nocodazole, paclitaxel, PMA, RO-3306, and thymidine were purchased from Sigma-Aldrich (St. Louis, MO, USA). BI2536, hesperadin, and TP-064 were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Flag-KDM (KDM3A, KDM4E, KDM5C, KDM7B, and JMJD6) plasmids were generated by GenScript Biotech Corporation (Piscataway, NJ, USA). HA-KDM4A (#24180), HA-KDM4C (#24214), HA-KDM6B (#24167), HA-PKC α (#21232), HA-PKC α -CA (#21234), and HA-PKC α -DN (#21235) plasmids were purchased from Addgene (Watertown, MA, USA). The GFP-CARM1 plasmid was provided by Dr. Mark T. Bedford (University of Texas MD Anderson Cancer Center), and myc-PI3KC2 α was provided by Dr. Emily Kim Malmberg (Biotech Research and Innovation Centre, University of Copenhagen). As described previously [12], PI3KC2 α R175K and R175A mutants were generated using the Muta-Direct Site-Directed Mutagenesis Kit (iNtRON Biotechnology, Seongnam, Korea).

The following antibodies were used for immunoblotting, immunoprecipitation, or immunostaining: actin (sc-47778; Santa Cruz Biotechnology, Dallas, TX, USA), CARM1 (A300-421 A; Bethyl Laboratories, Montgomery, TX, USA), Cyclin B1 (#12231; Cell Signaling Technology, Danvers, MA, USA), Cyclin D3 (#2936; Cell Signaling Technology), EB1 (#2164; Cell Signaling Technology), GFP (sc-9996; Santa Cruz Biotechnology), HA

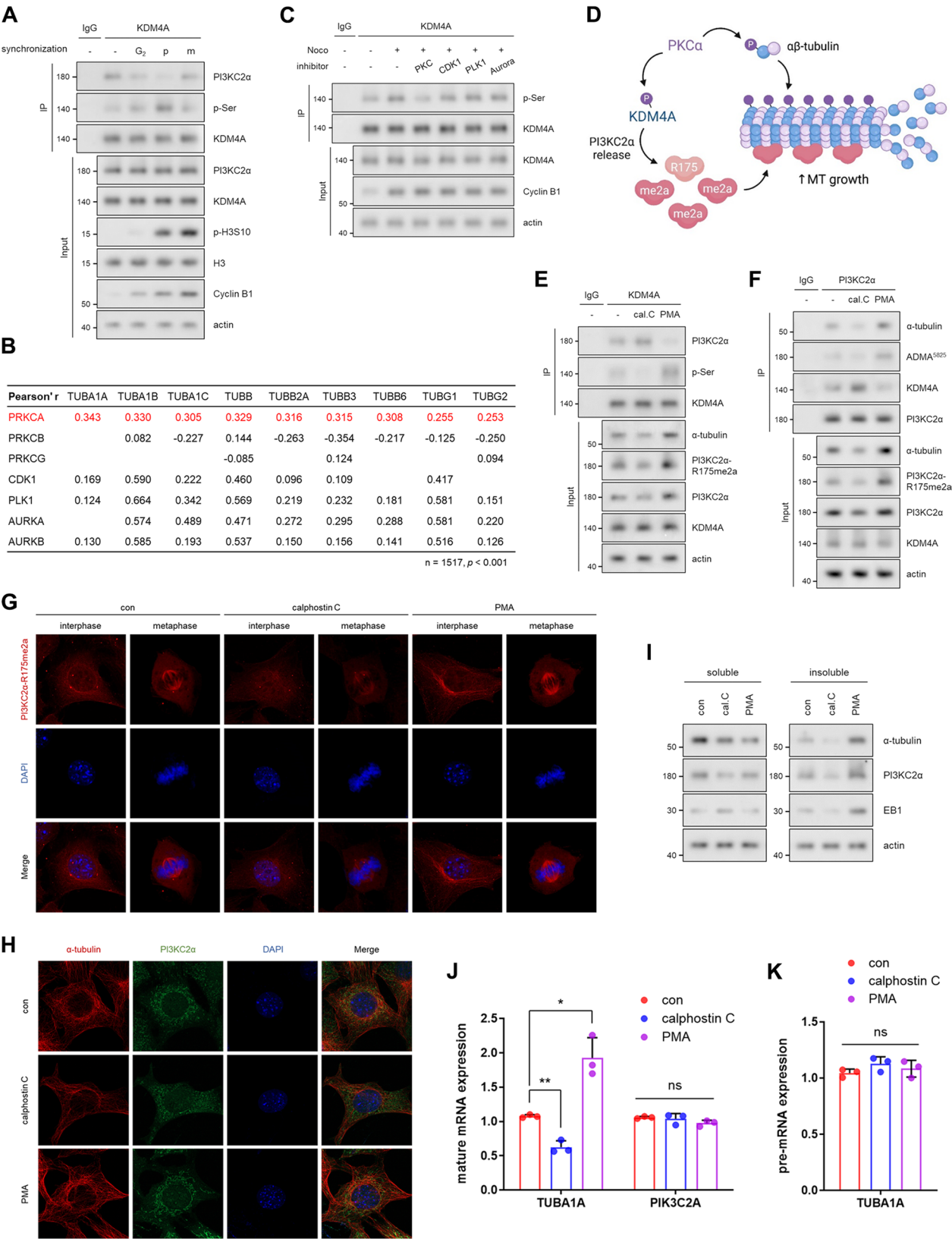


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Fig. 5 PKC α -mediated phosphorylation of KDM4A disrupts its interaction with PI3KC2 α during mitosis. **A** After cell synchronization at G2 (BI2536), pro-metaphase (nocodazole), or metaphase (nocodazole release with MG132), lysates were immunoprecipitated using an anti-KDM4A antibody to assess its phosphorylation levels. **B** Pearson correlation coefficient between indicated proteins was calculated using 1,517 cell lines derived from DepMap Expression Public 24Q2 data. **C** After nocodazole pretreatment (100 ng/mL, 12 h), calphostin C (0.5 μ M), RO-3306 (10 μ M), BI2536 (100 nM), or Hesperadin (100 nM) was added. The cell lysates were immunoprecipitated using an anti-KDM4A antibody to assess its phosphorylation levels. **D** Schematic image showing that PKC α promotes microtubule growth by phosphorylating KDM4A and reducing its interaction with PI3KC2 α . **E** and **F** After treatment with calphostin C (0.5 μ M) or PMA (100 nM) for 12 h, lysates were immunoprecipitated using KDM4A (**E**) or PI3KC2 α (**F**) antibodies. **G** Confocal images of PI3KC2 α R175me2a (red) and DAPI (blue) in MEF cells under the indicated condition. **H** Cells were treated with calphostin C or PMA and co-stained with antibodies against α -tubulin (red) and PI3KC2 α (green) as well as DAPI (blue). Representative images are shown. **I** Soluble and insoluble fractions were separated and analyzed by immunoblotting after calphostin C or PMA treatment. **J** and **K** Mature *TUBA1A* mRNA (**J**) and pre-mRNA (**K**) levels were measured in calphostin C- or PMA-treated cells. Error bars indicate standard deviation ($n=3$)

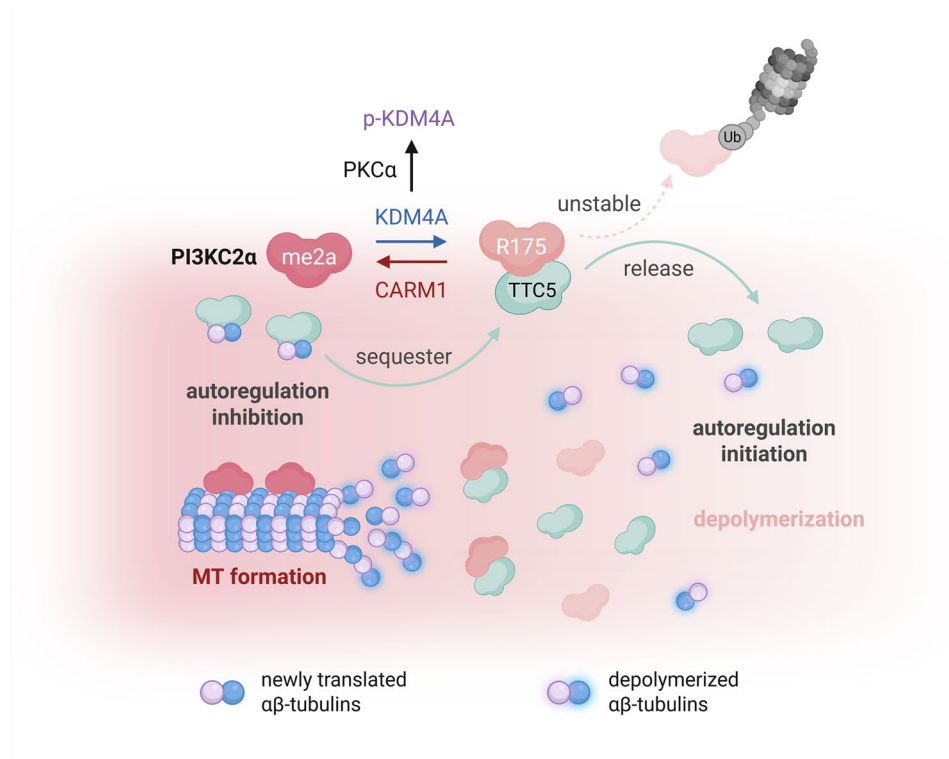


Fig. 6 Reversible arginine methylation of PI3KC2 α controls mitotic spindle dynamics. CARM1 asymmetrically dimethylates PI3KC2 α at R175 (R175me2a), enhancing its interaction with α -tubulin and promoting microtubule stabilization. This methylation is reversibly regulated by KDM4A-mediated demethylation. However, during mitosis, KDM4A is phosphorylated by PKC α , leading to its dissociation from PI3KC2 α . As a result, R175me2a levels increase, further stabilizing microtubules

(#3724; Cell Signaling Technology), Histone H3 (#9715; Cell Signaling Technology), H3K9me2 (A-4035; Epigentek, Farmingdale, NY, USA), H3K9me3 (07-442; Sigma-Aldrich), p-H3S10 (#9701; Cell Signaling Technology), KDM4A (29977-1-AP; Proteintech, Rosemont, IL, USA), c-Myc (sc-40; Santa Cruz Biotechnology), PI3KC2 α (#611046; BD Biosciences, Franklin Lakes, NJ, USA; 22028-1-AP: Proteintech; sc-365290: Santa Cruz Biotechnology), p-Ser (sc-81514; Santa Cruz Biotechnology), α -tubulin (sc-5286; Santa Cruz Biotechnology; #2144; Cell Signaling Technology), acetyl- α -tubulin (T7451; Sigma-Aldrich), tyrosinated- α -tubulin (MAB-1864-I; Sigma-Aldrich), and β -tubulin (T8660; Sigma-Aldrich). The ADMA⁵⁸²⁵ antibody, which recognizes

CARM1 substrates, was provided by Dr. Mark T. Bedford. The PI3KC2 α -R175me2a antibody (AbClon, Seoul, Republic of Korea, AC230110-161) was generated in rabbits using the asymmetrically dimethylated peptide (NH₂-NGFNPR(me2a)MPTEP-COOH). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Cell culture and transfection

HeLa, MCF7, and 293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and MEF (CARM1 WT and KO) cells were provided by Dr. Mark T. Bedford. All cells were grown in Dulbecco's

modified eagle medium (DMEM; HyClone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories) and 100 units/mL of penicillin/streptomycin (HyClone Laboratories). The cells were maintained at 37 °C in a humidified cell culture incubator containing 5% CO₂. Further, siRNAs were transfected using TransIT-X2 (Mirus Bio, Madison, Wisconsin, USA), and plasmids were transfected using TransIT-2020 (Invitrogen, Waltham, MA, USA) per the manufacturer's instructions.

Immunoblotting and immunoprecipitation

As described previously [33], cells were lysed using RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 0.5% sodium Deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100) supplemented with 1× protease and phosphatase inhibitor cocktails (Roche, Basel, Switzerland). Sonication was used to lyse cells, and the obtained lysate was centrifuged at 16,000 ×g for 10 min at 4 °C. For immunoprecipitation, an appropriate antibody was added to the lysate-adjusted concentration (1 mg/mL); the mixture was incubated overnight at 4 °C on a rotator. Thereafter, the antibody–protein complexes were obtained using protein A/G Sepharose beads (Santa Cruz Biotechnology) at 4 °C by placing the mixture on a rotator for 2 h. After washing twice with lysis buffer, the complex was eluted and analyzed using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins subjected to SDS-PAGE were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and blocked with 0.1% Tween 20/TBS (TBS-T) containing 5% skim milk for at least 1 h at room temperature (RT). The membrane was incubated with primary antibodies overnight at 4 °C. After washing thrice with TBS-T, the membrane was incubated with HRP-conjugated secondary antibody for 1 h at RT. The signal was detected using enhanced chemiluminescence (ECL) western blotting substrate (Advansta, Menlo Park, CA, USA).

Microtubule co-sedimentation assay

Cells were incubated with 1 μM paclitaxel at 37 °C for 30 min and dissolved in a microtubule polymerization buffer (80 mM K-PIPES pH 6.8, 1 mM EGTA, 1 mM MgCl₂, 0.5% NP-40, 20 mM NaF, 0.5% sodium Deoxycholate, 0.1 mM PMSE, and 10 mM paclitaxel) supplemented with 1× protease inhibitor cocktail at 37 °C for 5 min in the dark. The microtubule fraction was separated by centrifugation at 16,000 ×g for 15 min at 30 °C. The pellet was washed with microtubule polymerization buffer without detergent and resuspended with the same buffer in an equal volume of supernatant. Both fractions were loaded for SDS-PAGE, followed by immunoblotting.

In vitro tubulin polymerization assay

As described previously [34], Tubulin Polymerization Assay kit (Cytoskeleton, Inc., Denver, CO, USA) was used according to the manufacturer's instructions. Briefly, beads conjugated with PI3KC2α (WT, R175K, or R175A) were incubated with porcine tubulin (3 mg/mL) in a reaction buffer (80 mM PIPES pH 6.9, 0.5 mM EGTA, 2 mM MgCl₂, 10% glycerol, and 1 mM GTP) in a final volume of 110 μL per well in a 96 well plate. Tubulin polymerization was monitored by measuring absorbance at 340 nm every minute for 1 h at 37 °C using a SpectraMax i3x microplate reader (Molecular Devices, San Jose, CA, USA).

Quantitative real-time PCR

Total cellular RNA was extracted using TRIsure (Bio-line, London, UK), and cDNA was synthesized using the SensiFAST cDNA synthesis kit (Bioline). Levels of mRNA were analyzed *via* quantitative real-time PCR using SensiFAST SYBR No-ROX kit (Bioline) and Eco Real-Time PCR system (Illumina, San Diego, CA, USA). Reaction parameters were as follows: cDNA synthesis at 40 °C for 60 min, transcriptase inactivation at 95 °C for 5 min, and PCR cycling at 95 °C for 10 s, 58 °C for 20 s, and 72 °C for 20 s (40 cycles). Data were normalized to that of *ACTB*; the primer set sequences were as follows; *ACTB* (human): 5'-ACCTTCTACAATGAGCTGCG-3' and 5'-CCTGGATAGCAACGTACATGG-3', (mouse): 5'-ACCTTCTACAATGAGCTGCG-3' and 5'-CTGGA TGGCTACGTACATGG-3'; *PIK3C2A* (human/mouse): 5'-GGAAAGCAAGGACTGATTTGG-3' and 5'-AGGG ATTTTCCATTACCTTTTC-3'; *TUBA1A* (human): 5'-T TGTAGACTTGGAACCCACAG-3' and 5'-ATCTCCTT GCCAATGGTGTAG-3', (mouse): 5'-CAGTGTTCGTA GACCTGGAAC-3' and 5'-ATCTCCTTGCCAATGGT GTAG-3'; *TUBB* (human): 5'-GAAGCCACAGGTGGC AAATA-3' and 5'-CGTACCACATCCAGGACAGA-3'; *TUBA1A* pre-mRNA: 5'-GCAGCATTTGTAGCAGGT GA-3' and 5'-GCATTGCCAATCTGGACAC-3'; *TUBB* pre-mRNA: 5'-CTGGACCGCATCTCTGTGTGA-3' and 5'-GGTTCACGAAAGGGACAAAA-3'; *GAPDH* pre-mRNA: 5'-GGGAGGTAGAGGGGTGATGT-3' and 5'-GAGGCAGGGATGATGTTCTG-3'. To measure pre-mRNA levels, random hexamer primers were used instead of oligo(dT)₁₈ primers when synthesizing cDNA.

Immunofluorescence and confocal microscopy

Cells were plated on coverslips and then incubated with CARM1 or PI3KC2α siRNA for 72 h. The cells were fixed with 4% paraformaldehyde for 15 min, washed thrice with phosphate-buffered saline (PBS), and permeabilized using 0.5% Triton X-100 for 15 min at RT. Thereafter, the cells were washed thrice with PBS and incubated with primary antibody overnight at 4 °C, followed by

secondary antibody conjugated with fluorescent probes (Bethyl Laboratories, A90-116D4, A90-138D2, A120-101D4, and A120-101 F). Staining was visualized using Zeiss LSM 710 Confocal Microscopy (Carl Zeiss, Oberkochen, Germany), and image analysis was performed using ZEN software.

Microtubule regrowth assay

To disrupt the microtubule network, cells were treated with a high concentration of nocodazole (1 $\mu\text{g/mL}$) for 1 h. Thereafter, the drugs were washed out, and the cells were incubated with fresh media to induce microtubule regrowth for 3, 5, and 15 min. The cells were immediately fixed and subjected to immunofluorescence staining with an anti- α -tubulin antibody.

In situ proximity ligation assay

We detected PTM and protein–protein interactions using the Duolink In Situ Red Starter kit (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, cells were incubated with anti-PI3KC2 α and either anti-ADMA⁵⁸²⁵ or anti- α -tubulin antibodies overnight at 4 °C, followed by rabbit- and mouse-specific secondary antibodies conjugated with oligonucleotides. After ligation and amplification reactions, the samples were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min at 200 nM. The cells were washed thrice with PBS and mounted onto glass slides. The images were visualized and analyzed using Zeiss LSM 710 Confocal Microscopy and ZEN software.

Cell synchronization

As described previously [35], cells were seeded at 30% confluence and were maintained in growth media supplemented with 2 mM thymidine for 18 h. The cells were washed with PBS and placed in the fresh growth media for 9 h. Thereafter, 2 mM thymidine was re-added to the media, and cells were incubated for 15 h (G1/S arrest). To induce G2/M arrest, cells were treated with 100 nM BI2536 for 24 h or 100 ng/mL nocodazole for 12 h. To induce metaphase arrest, cells synchronized in the G2/M phase were released in fresh media containing 20 μM MG132 for 2 h.

In vitro demethylation assay

The PI3KC2 α (170–180)R175me2a peptide (NGFNPR (me2a)MPTFP; 20 μM) was incubated with KDM4A recombinant protein (Active Motif, Carlsbad, CA, USA; 2 μM), 2-oxoglutarate (Sigma-Aldrich; 100 μM), sodium ascorbate (Sigma-Aldrich; 100 μM), and ammonium ferrous sulfate hexahydrate (Sigma-Aldrich; 50 μM) for 1 h at 37 °C. Reactions were quenched with 1:1 (v/v) methanol and analyzed with liquid chromatography–mass

spectrometry (LC-MS) using the Waters BioAccord LC-MS system (Milford, MA, USA), which includes the ACQUITY UPLC I-Class plus system with an ACQUITY RDa detector (a compact time-of-flight mass detector) controlled by the UNIFI Scientific Information System Software Platform (Waters Corporation). Chromatography was conducted on an ACQUITY UPLC Peptide BEH C18 column (2.1 $\times$ 100 mm, 1.7 μm ; Waters Corporation) at 40 °C. Gradient elution was performed using water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) as the mobile phase. The gradient was initiated with (B) 10% and maintained until 0.23 min, raised to 90% until 4.63 min, maintained until 5.09 min, Decreased to 10% until 5.33 min, and maintained until 7.00 min. The flow rate was 0.3 mL/min, and the sample injection volume was 2 μL . MS was performed by positive electrospray ionization in the mass range of m/z 50–2,000. The acquisition mode was set as full scan with fragmentation mode at a scan rate of 1 Hz. The capillary voltage was set to 1.5 kV, and the cone voltage was set to 30 V.

Quantification and statistical analysis

All statistical analyses were performed in GraphPad Prism software. The data from independent experiments were included and presented as mean $\pm$ standard deviation (SD; $n \geq 3$). Data from two groups were compared using an unpaired t-test for independent samples, and a p -value < 0.05 was considered statistically significant.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12964-025-02419-1>.

Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions

Y.C. planned and conducted experiments and analyzed data. J.W.H. performed and analyzed in vitro methylation assays. M.T.B. performed and analyzed CARM1 substrate screening. D.K.S. and D.H.N. performed and analyzed mass spectrometry experiments. Y.C., M.T.B., S.N.K. and Y.K.K. designed the research and wrote the manuscript with input and contributions from all authors. All authors reviewed the manuscript.

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Data availability

All the other data supporting the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests

The authors declare no competing interests.

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