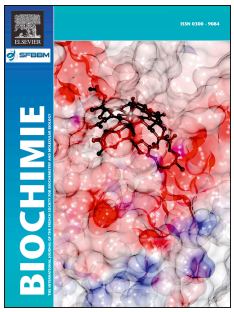

AKAP350 enables p150glued /EB1 interaction at the spindle poles

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PII: S0300-9084(20)30199-1

DOI: https://doi.org/10.1016/j.biochi.2020.08.014

Reference: BIOCHI 5955

To appear in: *Biochimie*

Received Date: 1 July 2020

Revised Date: 14 August 2020

Accepted Date: 19 August 2020

Please cite this article as: E. Almada, A. Pariani, Tomá.Rivabella. Maknis, F. Hidalgo, R. Vena, Cristiá. Favre, M.C. Larocca, AKAP350 enables p150glued /EB1 interaction at the spindle poles, *Biochimie* (2020), doi: https://doi.org/10.1016/j.biochi.2020.08.014.

Manuscript Type: Short Communication

Title: AKAP350 enables p150glued /EB1 interaction at the spindle poles

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Running Title: AKAP350 and p150glued /EB1 at the spindle poles

ABSTRACT

A-kinase anchoring protein 350 (AKAP350) is a centrosomal/Golgi scaffold protein, critical for the regulation of microtubule dynamics. AKAP350 recruits end-binding protein 1 (EB1) to the centrosome in mitotic cells, ensuring proper spindle orientation in epithelial cells. AKAP350 also interacts with p150^{glued}, the main component of the dynactin complex. In the present work, we found that AKAP350 localized p150^{glued} to the spindle poles, facilitating p150^{glued}/ EB1 interaction at these structures. Our results further showed that the decrease in AKAP350 expression reduced p150^{glued} localization at astral microtubules and impaired the elongation of astral microtubules during anaphase. Overall, this study provides mechanistic data on how microtubule regulatory proteins gather to define microtubule dynamics in mitotic cells.

Key-words: AKAP450, AKAP350, microtubule, p150^{glued}, EB1, centrosome.

1. Introduction

P150^{glued} is the main subunit of dynactin, which is a protein complex essential for dynein-mediated retrograde transport of vesicles and organelles along microtubules [1]. By localizing at microtubule plus-ends, p150^{glued} also has a prominent role in the regulation of microtubule dynamic instability [2]. Although p150^{glued} can directly interact with microtubules, its association with microtubule plus-ends is predominantly dependent on its interaction with EB1 [3], a key member of the family of microtubule plus-end tracking proteins [4]. p150^{glued} also interacts with EB1 at the centrosome. This interaction is required for microtubule minus-end anchoring during the assembly and maintenance of the radial microtubule array of interphase cells [5]. EB1 and p150^{glued} also participate in mitotic spindle formation and orientation [6, 7] and in the initiation of

cytokinesis [8]. Nevertheless, to our knowledge, there is no mechanistic data on how p150^{glued} is recruited to the spindle poles. AKAP350 (AKAP450, AKAP9 or CG-NAP) is an A-kinase-anchoring protein that scaffolds protein complexes at the Golgi apparatus and at the centrosome [9], playing a central role in the regulation of microtubule dynamics [10-12]. AKAP350 interacts with p150^{glued}, which is relevant to AKAP350 localization at the Golgi apparatus [13]. Considering that AKAP350 interacts with EB1 at the spindle poles [14], in the present study, we analysed AKAP350 participation in p150glued localization at the centrosome and in p150^{glued}/EB1 interaction at the spindle poles, and evaluated the relevance of this protein complex in the regulation of astral microtubule dynamics during mitosis.

2. Materials and methods

2.1. Cell culture

MDCK cells, obtained from Keith Mostov lab (UCSF, CA), were cultured and tested for contamination [14]. Cells were treated and cell lines were prepared as described in Supplementary Methods.

2.2. *Immunofluorescence.*

Cells were grown on glass coverslips, fixed with 4% paraformaldehyde or 100% methanol, permeabilized and blocked [14]. Cell staining was performed as described in Supplementary Methods.

2.3. Image acquisition and analysis.

Images of p150^{glued} or EB1 and γ-tubulin or α-tubulin co-staining were obtained by confocal laser microscopy (Nikon C1SiR with inverted microscope NikonTE200). Images were acquired and analyzed as described in Supplementary methods. The colocalization between EB1 and p150glued was estimated by measuring the Pearson

coefficient at the spindle poles of mitotic cells. For the analysis of p150^{glued}/EB1 *in situ* interaction, the acceptor photo-bleaching FRET method was used [16], using a LSM880 confocal with an ObserverZ1 inverted microscope and proper controls [14].

2.4. Preparation of centrosome fractions.

Centrosome-enriched fractions were prepared by centrifugation on a Fycoll cushion, using a method based on Blomberg-Wirschell and Doxsey [14, 17].

2.5. Statistical analysis.

Data are expressed as mean ± s.e.m. and they are representative of at least three experiments. Paired Student's t-test or non-parametric Mann–Whitney test were used for comparison among experiments or within each experiment, respectively. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. AKAP350 facilitates p150^{glued} localization at the spindle poles.

MDCK cells with decreased AKAP350 expression (AKAP350KD) were generated using a lentiviral-based short hairpin RNA expression system. Western blot analysis of AKAP350 expression in AKAP350KD cells demonstrated a reduction to 5%-20% of the control levels (Fig. 1A). Subsequently, p150^{glued} levels in centrosome-enriched fractions of control and AKAP350KD cells were analysed by western blot. Our results showed that centrosomal p150^{glued} was dramatically reduced in AKAP350KD cells (Fig. 1B). The immunofluorescence analysis of p150^{glued} localization at the centrosomes in interphase cells indicated similar p150^{glued} levels in control and AKAP350KD cells (data not shown). Considering that the preparation of centrosome-enriched fractions requires previous cell treatment with nocodazole, we analysed p150^{glued} localization at the centrosome in nocodazole-treated cells by immunofluorescence. Our results showed

that the decrease in AKAP350 expression inhibited p150^{glued} localization at the centrosome in nocodazole-treated cells (Fig. 1C). Considering that interphase microtubules subjected to nocodazole treatment and washout suffer rapid remodelling, we speculated that AKAP350 was relevant for p150^{glued} recruitment to the centrosome in conditions of extensive microtubule remodelling. During mitosis, the microtubule network undergoes intense reorganization. The duplicated centrosomes segregate, forming two opposing microtubule organizing centres at the spindle poles, with a markedly increased nucleation capacity. P150^{glued} becomes robustly enriched around the spindle poles at the beginning of mitosis [18]. Therefore, we explored if AKAP350 was involved in p150^{glued} recruitment to the spindle poles. The analysis of mitotic cells showed that p150^{glued} levels at spindle poles were increased in metaphase control cells, and that they were markedly reduced in AKAP350KD cells at the same phase (Fig 1D).

Besides localizing at the centrosome, AKAP350 localizes at the Golgi apparatus [9] and at microtubule plus-ends [19]. In order to evaluate if the decrease in p150^{glued} centrosomal localization in AKAP350KD cells was directly related to the reduction of AKAP350 expression at the centrosome, we prepared cells with stable expression of the pericentrin-AKAP450 centrosomal targeting (PACT) domain, which delocalizes AKAP350 from the centrosome [15, 20]. Metaphase PACT cells showed a remarkable reduction in p150^{glued} levels at their spindle poles (Fig. 1E), suggesting that centrosomal AKAP350 participates in p150^{glued} recruitment to these structures. It is noteworthy that the decrease in p150^{glued} localization at the spindle poles appears to be more significant than the decrease generated by the reduction of AKAP350 expression (Fig. 1F). Expression of the PACT domain can also delocalize pericentrin from the centrosome [11, 20]. Even though pericentrin regulates the centrosomal localization of several proteins involved in mitotic spindle organization, p150^{glued} subcellular distribution is not

affected by the decrease in pericentrin expression [21, 22]. It will be interesting to study if the greater effect on p150^{glued} delocalization observed in PACT cells is intrinsically related to the expression of the PACT domain, which may interact with regulatory proteins such as calmodulin [20].

3.2. AKAP350 enables p150^{glued}/EB1 interaction at the spindle poles.

EB1/p150^{glued} interaction is required for microtubule minus-end anchoring at the centrosomes [5] and regulates EB1 function in the regulation of microtubule dynamics [23]. The analysis of *in situ* EB1/p150^{glued} interaction by immuno FRET showed that, in metaphase cells, EB1 interacted with p150^{glued} at the spindle poles and that this interaction was reduced in AKAP350KD cells (Fig. 2A).

Considering that p150^{glued} is the main dynactin subunit and that the dynein/dynactin complex is involved in minus-end-directed protein trafficking [1], we evaluated if the defective recruitment of EB1 to the spindle poles in AKAP350 KD cells [14] could be rescued by p150^{glued} overexpression. We generated control and AKAP350KD cells with stable overexpression of p150^{glued} (p150^{glued}OE). Although p150^{glued} levels at the spindle poles were significantly increased in AKAP350KD-p150^{glued}OE cells, this overexpression did not rescue EB1 localization at the spindle poles (Fig. 2B). Furthermore, in accordance with an AKAP350-dependent p150glued/EB1 interaction at this location (Fig. 2A), p150^{glued} overexpression led to increased p150^{glued} colocalization with EB1 at the spindle poles in control but not in AKAP350KD cells.

AKAP350KD cells express lower levels of EB1 at astral microtubules [14]. Similarly, we found that the presence of p150^{glued} in astral microtubules was significantly decreased in AKAP350KD metaphase cells (Fig. 2 C). This evidence suggests that AKAP350 recruitment of p150^{glued} to the spindle poles and/or its interaction with EB1

has a direct impact on p150^{glued} localization at astral microtubules. Inhibition of p150^{glued}/EB1 interaction precludes anaphase microtubule-elongation [8]. Considering that EB1/p150^{glued} interaction at the spindle poles, as well as their localization at astral microtubules, was impaired in AKAP350KD metaphase cells, we analysed astral microtubules in AKAP350KD mitotic cells. Our results showed that astral microtubule length was reduced in AKAP350KD anaphase cells (Fig. 2 D), suggesting that AKAP350 could regulate astral microtubule dynamics by enabling EB1/p150 interaction at the spindle poles.

4. Conclusion

The role of p150^{glued} in the regulation of microtubule remodelling and spindle orientation has been widely characterized [2, 5-8]. In this regard, p150^{glued} interaction with EB1 is highly relevant for the regulation of p150^{glued} function [3, 5-8]. Even though it has been shown that p150^{glued} localizes at the spindle poles [18], the factors governing p150^{glued} localization and interaction with EB1 at these structures remained unknown. In the present study, we describe that AKAP350 facilitates p150^{glued} localization at the spindle poles during metaphase, and we provide strong evidence that supports that AKAP350 plays a central role in enabling p150^{glued} interaction with EB1 at that location. Our results further demonstrate that this protein complex participates in the regulation of astral microtubule dynamics during mitosis. Future studies will be aimed at characterizing the events determined by the AKAP350/EB1/p150^{glued} complex that regulate EB1 and p150^{glued} localization at astral microtubules.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Conceived and designed the experiments: EA, MCL. Performed the experiments: EA, AP, TRM, FH, RV. Supervised the experiments: CF, MCL. Analysed the data: EA, TRM, MCL. Wrote the paper: EA, MCL. All authors read and approved the final manuscript.

Acknowledgments

We thank A. B. Almada for the language correction of the manuscript.

This work was supported by Grants PUE 0089 from CONICET and PICT PICT2015-2755 from ANPCyT to MCL. The funders had no role in study design, data collection and analysis, decision to publish, or paper preparation.

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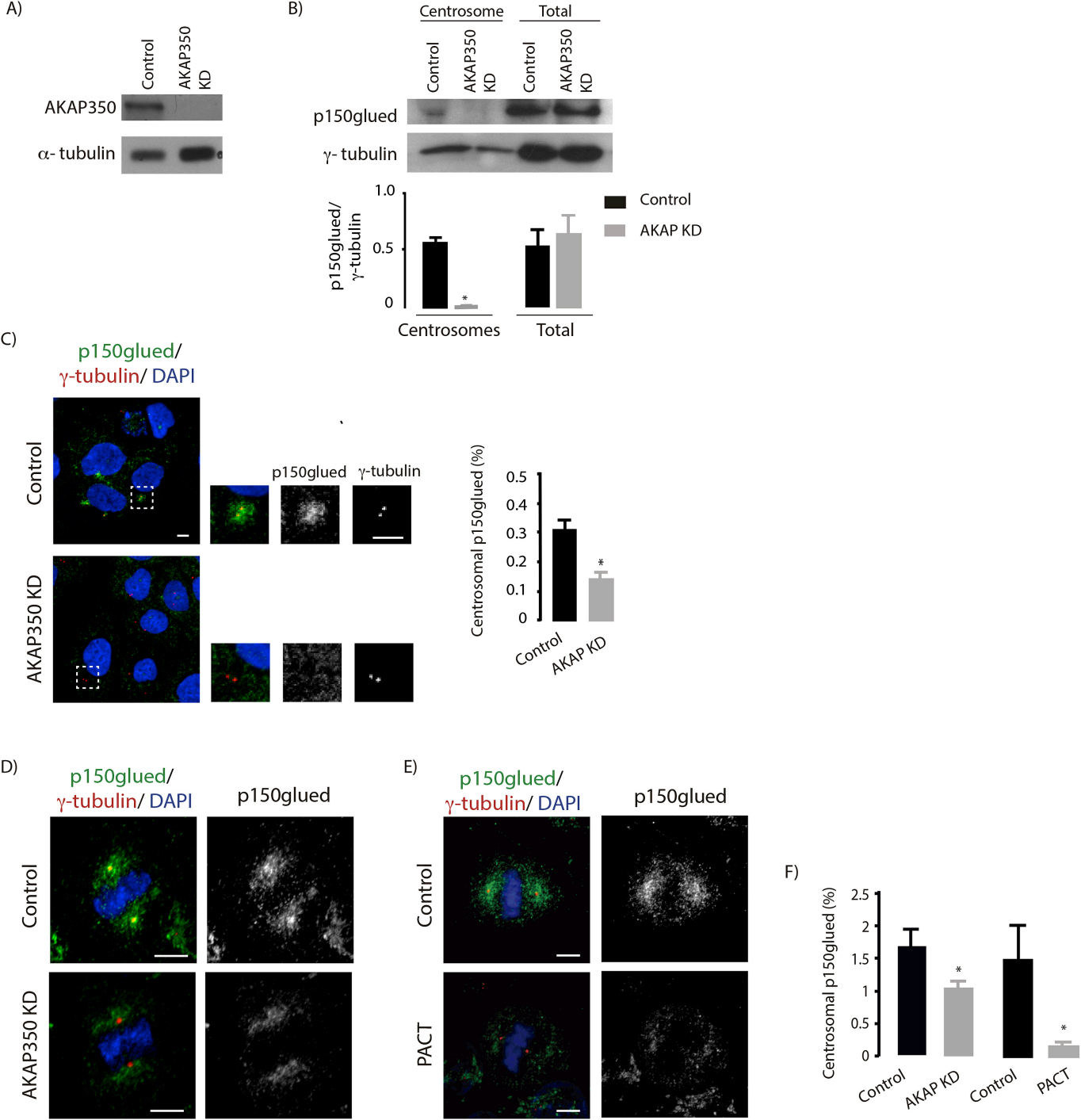
Figure legends

Figure 1. Role of AKAP350 in p150^{glued} localization at the centrosome. A) Western blot analysis of AKAP350 expression in control and AKAP350KD cells. α-tubulin was used as loading control. **B)** Western blot analysis of p150^{glued} expression in cell-lysates and centrosomal fractions in control and AKAP350KD cells. Bars represent p150^{glued}-bands relativized to γ-tubulin-bands density. **C)** Merged images show p150^{glued} (green),

γ-tubulin (red) and DAPI (blue) staining in control and AKAP350KD cells subjected to Nocodazole treatment. The inset images show magnified views of centrosome areas (boxed areas). Bars represent centrosomal $p150^{glued}$ fluorescence, expressed as percentage of total cell fluorescence. **D,E)** Merged images show $p150^{glued}$ (green) and γ-tubulin (red) staining in AKAP350KD metaphase cells (D) or in metaphase cells with stable expression of the PACT-domain (E) and their respective controls. **F**) Bars represent the percentage of $p150^{glued}$ fluorescence associated to the spindle poles. Scale bars, 5 μm. *$p < 0.05$.

Figure 2. P150glued/EB1 at the spindle poles. A) Analysis of $p150^{glued}$/EB1 *in situ* interaction. Merged images show EB1 (green) and $p150^{glued}$ (red) staining in metaphase control and AKAP350KD cells, pre- and post- photobleaching in the acceptor ($p150^{glued}$) channel. FRET images illustrate in fire pseudocolor the increase in intensity in the bleached area in the donor (EB1) channel. Bars represent FRET efficiency [14]. **B)** Merged images show $p150^{glued}$ (green), EB1 (red) and DAPI (blue) staining in control, AKAP350KD, p150gluedOE and AKAP350KD-p150gluedOE mitotic cells. Inset merge and pseudocolor images show magnified views of EB1 and $p150^{glued}$ fluorescence at the centrosome (boxed areas). Bars represent the percentage of EB1 fluorescence present at the centrosomes in metaphase cells (first graph) and Pearson coefficients estimating EB1/$p150^{glued}$ colocalization at the centrosome region in metaphase cells. **C)** Merged images show $p150^{glued}$ (green), EB1 (red) and DAPI (blue) staining in control and AKAP350KD cells. Inset merged and pseudocolor images show magnified views of astral microtubules (boxed areas). **D)** Merged images show α-tubulin (green), and DAPI (blue) staining in metaphase control and AKAP350KD cells. Bars represent average microtubule length. Inset images show magnified views of astral microtubules (boxed areas) as represented in the outputs of aster microtubule analysis

obtained with the ImageJ plugin “Microtubule tools”. Scale bars, 5 μm (**C, E**) or 2.5 μm (**A, B, D**). *$p < 0.05$.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AKAP350 KD
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1.0
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Control
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Centrosomes
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p150glued/
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AKAP350 KD
p150glued
γ-tubulin
Centrosomal p150glued (%)
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AKAP350 KD
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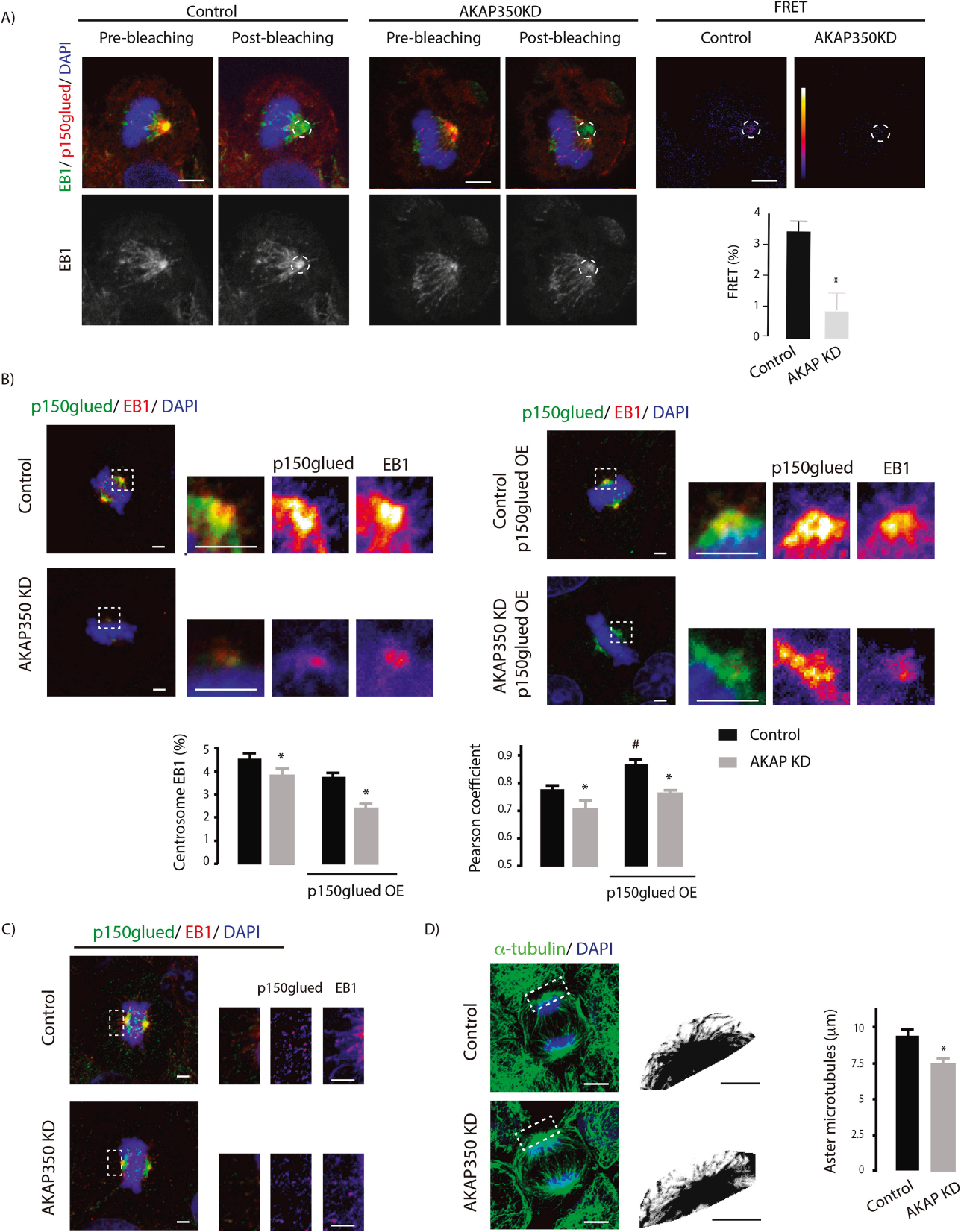
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EB1
FRET (%)
Control
AKAP KD
B)
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Control
AKAP350 KD
p150glued
EB1
p150glued/ EB1/ DAPI
Control
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AKAP350 KD
p150glued OE
p150glued
EB1
Centrosome EB1 (%)
p150glued OE
Pearson coefficient
p150glued OE
Control
AKAP KD
C)
p150glued/ EB1/ DAPI
Control
AKAP350 KD
p150glued
EB1
D)
α-tubulin/ DAPI
Control
AKAP350 KD
Aster microtubules (μm)
Control
AKAP KD

Highlights

- AKAP350 knock down (AKAP350KD) reduces $p150^{glued}$ localization at the spindle poles
- $P150^{glued}$/EB1 interaction at the spindle poles is diminished in AKAP350KD cells
- $P150^{glued}$ overexpression does not rescue EB1 spindle pole-levels in AKAP350KD cells
- AKAP350 knock down delays astral microtubule-elongation during anaphase

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