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Cdk1 and BRCA1 target γ-tubulin to microtubule domains

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ABSTRACT

DNA damage is a critical event that requires an appropriate cellular response. This is mediated by checkpoint proteins such as Cdk1 that controls S/G2 and G2/M transition. Cdk1 is required for BRCA1 transport to DNA damage sites inside the nucleus where BRCA1 functions as a scaffold to initiate a signaling cascade. BRCA1 is a multifunctional protein that also ubiquitinates γ-tubulin and, consequently, inhibits microtubule nucleation at the centrosome. Here, we report that γ-tubulin also localizes at confined areas in the microtubule network. Nocodazole-mediated microtubule depolymeration results in disappearance of this γ-tubulin fraction, while microtubule stabilization by taxol preserves this structure. Surprisingly, overexpression of Cdk1 or BRCA1 greatly expands the γ-tubulin coating of microtubules, suggesting that the microtubule-bound γ-tubulin is involved in DNA damage response. This is in accordance with numerous reports of microtubule-associated DNA damage proteins, such as p53, that are transported to the nucleus when DNA damage occurs. γ-Tubulin itself has been reported to form complexes with DNA repair proteins in the nucleus.

1. Introduction

Microtubules are a cytoskeleton type mostly needed for transport events of, for example, organelles, neurotransmitter vesicles, sister chromosomes and sensory stimuli at primary cilia [1–4]. It is composed of 13 protofilaments assembled into a hollow cylindrical polymer [5]. The basic units of microtubules are dimers of α- and β-tubulin [6]. In addition, microtubules serve as cytoplasmic retention devices for DNA damage proteins such as p53, mOGG1, hGTSE-1, Bim, B99, MAP4 and Cyclin G2 [7–14]. Once DNA damage has been detected, the microtubules serve, in combination with motor proteins such as dynein, as highways to reach the nucleus [9,15].

Microtubules are nucleated by the γ-Tubulin Ring Complex (γ-TuRC) at the centrosome, the mitotic spindle, the kinetochores, the Golgi and the midbody [2,16–21]. The γ-TuRC is composed of several units of γ-Tubulin Small Complexes (γ-TuSCs) and additional regulation and targeting factors: Gamma-TuRC Component Protein 4 (GCP4), GCP5, GCP6, GCP-WD and GCP8 [18,22–24]. The γ-TuSC is a heterotetramer consisting of two copies of γ-tubulin, one GCP2 and one GCP3 [24]. Multiple γ-TuSCs assemble into γ-TuSC ring structures with 13 γ-tubulins per turn, matching microtubule architecture [17]. In this structure, γ-tubulins interact laterally to form a ring that makes longitudinal contacts with α- and β-tubulin dimers [17]. Thus, γ-tubulin functions as a template for α- and β-tubulin dimer recruitment in the process of microtubule nucleation.

γ-Tubulin is a multifunctional protein which is also involved in microtubule minus-end capping, microtubule plus-end regulation and mitotic spindle checkpoint function [25–28]. In addition, γ-tubulin forms a complex with the DNA repair protein Rad51 in the nucleus and with the DNA damage checkpoint protein C53 in the nucleolus [29,30]. γ-Tubulin also interacts with the DNA damage proteins BRCA1 and ATR [31].

In the course of cell division, the amount of microtubule nucleation activity dramatically increases at the onset of mitosis when the mitotic spindle is assembled, and is subsequently reduced when cells exit mitosis. The level of microtubule nucleation activity is regulated by multiple mechanisms, including BRCA1-mediated ubiquitination of γ-tubulin [32–35]. Ubiquitination of γ-tubulin inhibits its ability to nucleate microtubules and also disrupts the anchoring of γ-tubulin to the centrosome [32–34,36]. The centrosomal presence of other DNA damage proteins such as ATM, ATR, Chk1 and Chk2 inversely correlates with γ-tubulin levels at the centrosome [31].

The hereditary breast and ovarian cancer type 1 susceptibility gene product BRCA1 is a tumor suppressor and a master regulator of genome stability [37]. BRCA1 forms a stabilizing heterodimer with BRCA1-associated RING domain protein BARD1 and this complex acts as an E3 ubiquitin ligase [38]. BRCA1 exerts its function through regulation of cell cycle checkpoints, DNA damage

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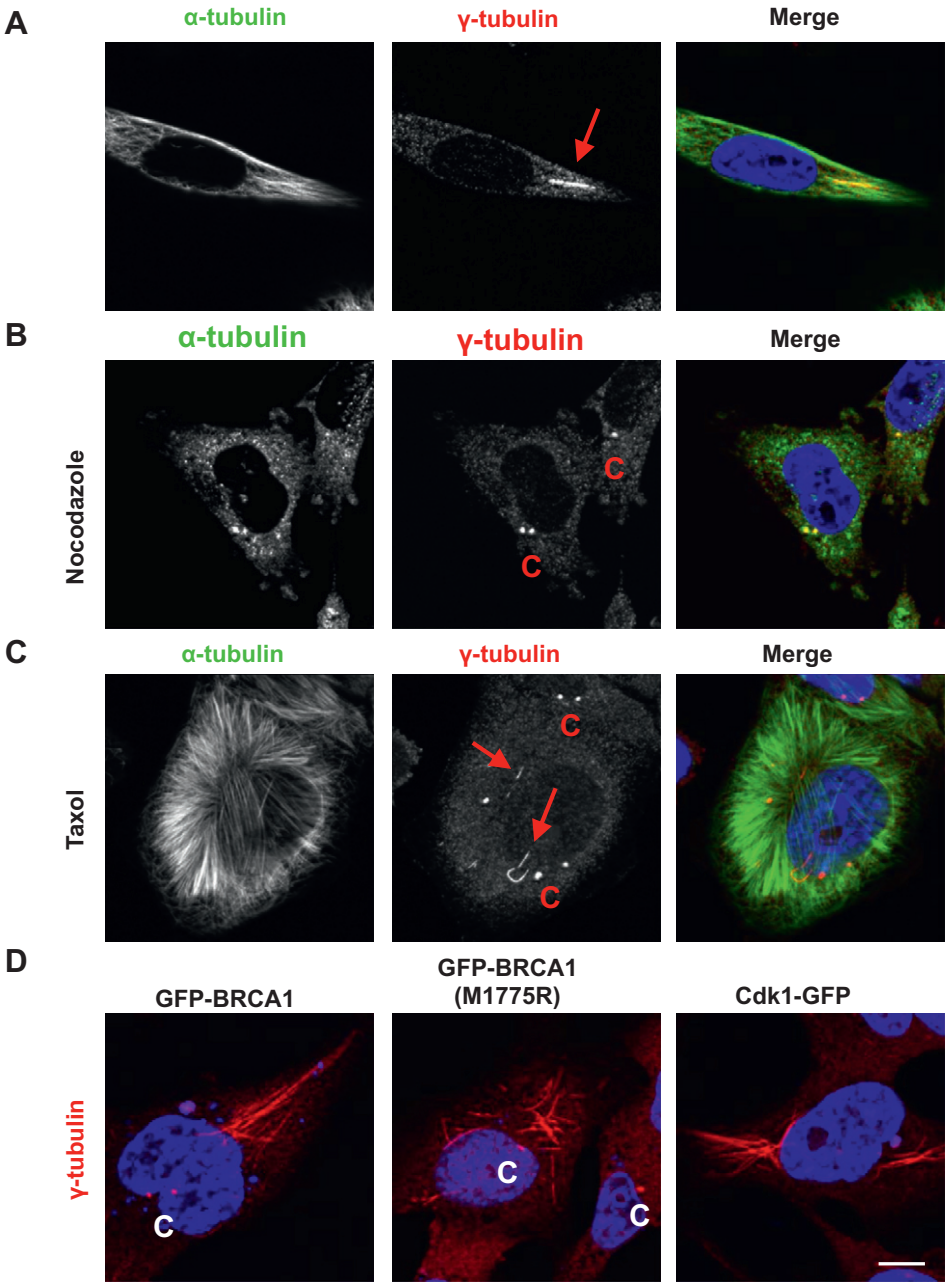

Fig. 1. Cdk1 and BRCA1 target γ-tubulin to microtubule domains in MDA-MB-231 cells. (A) A γ-tubulin filament colocalizes with microtubules (α-tubulin). (B) Nocodazole-mediated depolymerization of microtubules disrupts γ-tubulin filaments. (C) Taxol-treatment of cells to stabilize microtubules preserves γ-tubulin filaments. (D) Expression of GFP-tagged BRCA1, BRCA1(M1775R) and Cdk1 give rise to a burst of γ-tubulin filaments in the cytoplasm. Red arrows indicate γ-tubulin filaments. "C" indicates centrosomes. Scale bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

signaling, DNA damage repair, DNA decatenation, chromatin remodeling, transcription, RNA processing and mitotic spindle assembly [39–49]. Besides its well characterized role in mitosis, cyclin-dependent kinase 1 (Cdk1) functions in DNA damage response [50–52].

Importantly, Cdk1 is required for the targeting of BRCA1 to sites of DNA damage in the nucleus where BRCA1 acts as a scaffold to recruit ATM and ATR that phosphorylate proteins such as Chk1 and Chk2 [53].

In this manuscript, we will provide for the first time evidence that the microtubule nucleation factor γ-tubulin is an integral component of the DNA damage response.

2. Materials and methods

2.1. Reagents

Nocodazole and taxol were purchased at Sigma–Aldrich (#M1404; #T1912).

2.2. Plasmids

EGFP-BRCA1 was a kind gift from Dr. O. Sibon (Department of Radiation and Stress Cell Biology, University of Groningen, The

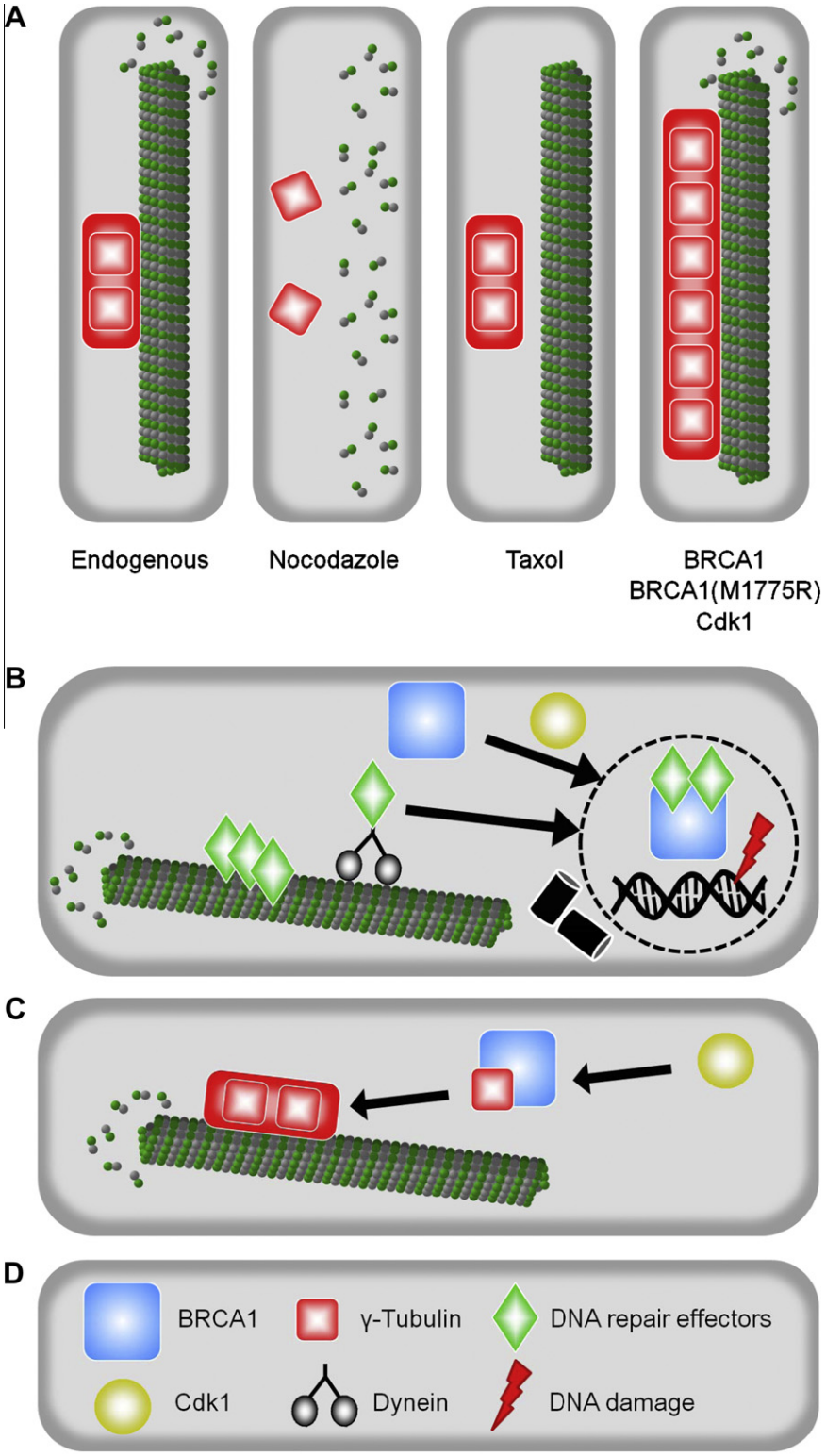

Fig. 2. Hypothetical model. (A) The results from Fig. 1 are summarized. Sketches correspond, from left to right, with Fig. 1 A, B, C and D. Green and grey globes represent α-tubulin–β-tubulin heterodimers that form microtubules. Red blocks represent γ-tubulin. (B) Literature summary. Symbols are illustrated in (D). DNA damage induces microtubule-associated DNA damage proteins, such as p53, to be transported along microtubules to the nucleus. Cdk1 mediates targeting of BRCA1 to DNA damage foci in the nucleus. BRCA1 forms complexes at DNA damage foci. (C) Hypothetical model explaining the obtained results. BRCA1 and its regulator Cdk1 target γ-tubulin to microtubule domains. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Netherlands). EGFP-BRCA1 was subjected to point mutagenesis using the Quikchange Site-Directed Mutagenesis kit (Agilent Technologies). Cdk1-GFP was a kind gift from Dr. R. Muschel (Children's Hospital of Philadelphia, USA).

2.3. Antibodies

Mouse monoclonal anti α-tubulin antibody was from Sigma–Aldrich (#T6557). Rabbit polyclonal anti γ-tubulin antibody was pur-

chased at Abcam (#ab16504). Alexa Fluor 594-conjugated goat anti-rabbit antibody and Alexa Fluor 488-conjugated goat anti-mouse antibody were from Molecular Probes (#A11037, #A11001).

2.4. Cell culture and cell tranfection

MDA-MB-231 cells were maintained at 37 °C in a humidified 10% CO_2 incubator and grown in DMEM (Gibco) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 IU/ml penicillin. Cells were transfected with jetPRIME following the manufacturer's instructions (Polyplus Transfection, #114–15).

2.5. Immunostaining and immunofluorescence microscopy

Cells were washed with PBS, fixed with formalin (3% paraformaldehyde solution) for 25 min at room temperature and permeabilized with 100% methanol for 5 min at −20 °C. Formalin was neutralized with 0.75% glycine for 20 min. Cells were then blocked in 1% BSA in PBS for 30 min and incubated with primary antibody for 1 h at 37 °C. Cells were washed in PBS, then incubated with secondary antibody and 4,6-diamidino-2-phenylindole (DAPI) for 30 min at room temperature. Following immunostaining, samples were analysed using a Carl Zeiss Axiovert 200 M Apotome epifluorescence microscope ⁎⁎(63× 1.4NA oil objective) equipped with an Axiocam cooled CCD camera and processed using Axiovision software (Zeiss).

3. Results and discussion

We previously reported the localization of γ-tubulin at membrane protrusions and observed γ-tubulin-induced suppression of stress-fibers [54]. Interestingly, BRCA1 also localizes at membrane protrusions and functions in cell spreading and cell motility [55]. γ-Tubulin was also found in the nucleus and the nucleolus, where it functions in DNA damage response [29,30]. Independently, we also observed endogenous γ-tubulin in the nucleoplasm and the nucleolus of HEK293T cells (Fig. S1A left panels). We also report the original finding that the nucleolar localization of γ-tubulin is dependent on active RNA Polymerase I-mediated transcription of ribosomal genes, as the nucleolar γ-tubulin staining disappears after actinomycin D treatment (Fig. S1A right panels). These results were confirmed by expression of V5-tagged γ-tubulin (Fig. S1B). In contrast, V5-tagged β-tubulin, which has a comparable size, was poorly enriched in the nucleoplasm and was excluded from the nucleoli (Fig. S1C). This suggests that γ-tubulin is actively transported through the nuclear pore complex (NPC), while β-tubulin is weakly imported in the nucleus through passive diffusion. To confirm the nuclear localization of γ-tubulin, we searched for a nuclear localization signal (NLS) in the amino acid sequence of γ-tubulin [56]. Although no potential NLS was uncovered, an intriguing tandem motif was observed between amino acids 399 and 418 of γ-tubulin. The consensus motif is RKx(D/E)xFx(D/E)xF (Fig. S1D). Intriguingly, the degree of conservation of this motif duplication correlates with organism complexity (Fig. S2). The duplicated motif localizes in a loop between two α-helices and is exposed on the surface, except for the phenylalamines that constitute the hydrophobic core of the protein (Fig. S3). We created a mutant γ-tubulin in which all acidic and basic amino acids in the motifs were substituted by alanines and called the resulting mutant protein γ-tubulin BAF (Basic-Acidic-Phenylalanine). This γ-tubulin BAF mutant was uniformly and almost exclusively localized in the cytoplasm, implying that γ-tubulin is not passively transported to the nucleus (Fig. S1E). We further examined the strength of γ-tubulin localization in the nucleolus by coexpression of γ-tubulin and the nucleolar proteins nucleostemin and borealin. In this experiment, nucleostemin and borealin failed to reduce nucleolar γ-tubulin levels, suggesting nucleolar retention of γ-tubulin (Fig. S1F). These findings indicate that γ-tubulin is an authentic nuclear and nucleolar protein. The sole known role of γ-tubulin in the nucleus is to function in DNA damage response [29,30].

We also report the presence of γ-tubulin filaments in the cytoplasm of MDA-MB-231 and HeLa cells (Fig. 1A and Fig. S4). These γ-tubulin filaments colocalized with microtubules (Fig. 1A). Incubation of MDA-MB-231 cells with the microtubule depolymerizating drug nocodazole resulted in loss of the γ-tubulin filaments, indicating that γ-tubulin is associated with microtubules (Fig. 1B). Stabilization of microtubules with taxol did not suppress the γ-tubulin filaments, indicating that microtubule dynamics is not necessary for γ-tubulin filaments (Fig. 1C). Altogether, we hypothesize that microtubules are coated with γ-tubulin at specific locations.

The reported role of γ-tubulin in the nucleus and nucleolus prompted us to study the effect of the master regulator of DNA damage response BRCA1 on γ-tubulin localization on microtubules. Expression of GFP-tagged BRCA1 resulted in a dramatic expansion of γ-tubulin coating on microtubules (Fig. 1D, left panel). We repeated the experiment with a well characterized, cancer-associated M1775R substitution in BRCA1, which led to the same phenotype (Fig. 1D, middle panel). Since Cdk1 acts as a transport factor for BRCA1, we also expressed GFP-tagged Cdk1 (Fig. 1D, right panel) [53]. This resulted in the same increase of γ-tubulin coating on microtubules (Fig. 1D, right panel). Several other GFP-fusion constructs had no effect on γ-tubulin localization on microtubules, ensuring that the observed phenotype is not a result of GFP expression (unpublished results). Our experimental results, literature data and a hypothetical model corresponding with our observations are presented in Fig. 2.

Expression of Cdk1 or BRCA1 greatly expands the γ-tubulin coating of microtubules, suggesting that microtubule-bound γ-tubulin is involved in DNA damage response. This is in accordance with numerous reports of microtubule-associated DNA damage proteins, such as p53, that are transported along microtubules to the nucleus once DNA damage has occurred [9]. Our new findings induce several questions. How does γ-tubulin associate with microtubule domains? Of special interest is the interaction between γ-tubulin and MARK4, a microtubule-associated kinase that regulates binding of proteins to microtubules [57]. Microtubule domains arise from post-translational modification such as detyrosination and acetylation, which modulate microtubule turn over and motor protein function [58–61]. What is the role of γ-tubulin in DNA damage response? BRCA1 interacts directly with γ-tubulin and inhibits its microtubule nucleation activity at the centrosome [33,36]. However, it is possible that BRCA1-mediated ubiquitination of γ-tubulin does not result in an inert protein but, on the contrary, switches new functions on that contribute to the DNA damage response.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.09.064.

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