Published in final edited form as:

Histochem Cell Biol. 2016 March ; 145(3): 275–286. doi:10.1007/s00418-015-1388-1.

Novel Subcellular Localization of the DNA Helicase Twinkle at the Kinetochore Complex during Mitosis in Neuronal-Like Progenitor Cells

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Abstract

During mitosis, the kinetochore, a multi-protein structure located on the centromeric DNA, is responsible for proper segregation of the replicated genome. More specifically, the outer kinetochore complex component Ndc80/Hec1 plays a critical role in regulating microtubule attachment to the spindle for accurate sister chromatid segregation. In addition, DNA helicases play a key contribution for precise and complete disjunction of sister chromatids held together through double-stranded DNA catenations until anaphase. In this study, we focused our attention on the nuclear-encoded DNA helicase Twinkle, which functions as an essential helicase for replication of mitochondrial DNA. It regulates the copy number of the mitochondrial genome, while maintaining its integrity, two processes essential for mitochondrial biogenesis and bioenergetic functions. Although the majority of the Twinkle protein is imported into mitochondria, a small fraction remains cytosolic with an unknown function. In this study, we report a novel expression pattern of Twinkle during chromosomal segregation at distinct mitotic phases. By immunofluorescence microscopy, we found that Twinkle protein colocalizes with the outer kinetochore protein HEC1 as early as prophase until late anaphase in neuronal-like progenitor cells. Thus, our collective results have revealed an unexpected cell cycle-regulated expression pattern of the DNA helicase Twinkle, known for its role in mtDNA replication. Therefore, its recruitment to the kinetochore suggests an evolutionary conserved function for both mitochondrial and nuclear genomic inheritance.

Keywords

Chromosomal Segregation; Hexameric DNA Helicases; Kinetochore Complex; Mitochondria; Mitosis; Twinkle

Introduction

During cell division, accurate genomic inheritance is essential for cellular viability and proper development. Missegregation of sister chromatids in daughter cells results in aneuploidy, a hallmark feature for most cancers and specific neurogenic disorders, such as

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Down syndrome and Turner syndrome (O'Doherty et al. 2005; Wiseman et al. 2009; Dierssen et al. 2009; Gordon et al. 2012; Siegel and Amon, 2012). Interestingly, mitotic neural progenitor cells exhibit mosaic aneuploidy during normal embryonic neurogenesis, giving rise to aneuploid postmitotic neurons well integrated into circuitry of normal mature brains (Rehen et al. 2001; Yang et al. 2003; Kingsbury et al. 2005; Rehen et al. 2005; Peterson et al. 2012). However, exacerbation of neural aneuploidy mediates abnormal brain development and brain dysfunction, leading to neurodegenerative diseases, such as Ataxia-telangiectasia and Alzheimer's disease (Kingsbury et al. 2006; Iourov et al. 2009).

The centromere-kinetochore complex is essential for chromosomal segregation on the mitotic spindle (Bergmann et al. 2011; Stimpson and Sullivan, 2011, Aldrup-MacDonald and Sullivan, 2014). Each sister chromatid has its own centromere embedded in long stretches of repetitive DNA, which recruits the kinetochore, a specialized proteinaceous structure, to allow precise positioning of the sister chromatids on the spindle microtubules in preparation for their physical separation at the onset of anaphase (Foley and Kapoor, 2013). By taking advantage of its trilaminar morphology, the inner plate of the kinetochore interfaces with the centromere whereas its outer plate makes contact with the spindle microtubules and its central plate connects the inner to the outer plate (Cleveland et al. 2003; Maiato et al. 2004). While the constitutive centromere-associated network (CCAN) is the core complex located in the inner plate throughout all the stages of the cell cycle, the KMN network (named according to the acronym for KNL1, Mis12 and Ndc80) is transiently assembled at the outer plate during the very early stages of mitosis (Liu et al. 2006; Cheeseman and Desai, 2008). DNA helicases are intimately involved in precise and complete disjunction of sister centromeres that are held together through double-stranded DNA (ds-DNA) catenations until anaphase.

Although more than 100 proteins have been reported to associate with the centromere-kinetochore complex, not all players involved in its molecular architecture are known (Foltz et al. 2006; Izuta et al. 2006; Okada et al. 2006). In this study, we examined the expression pattern of the hexameric DNA helicase Twinkle in proliferative neuronal-like progenitor cells. Twinkle shares structural homology with the bacteriophage T7 gene product 4 primase-helicase (T7 gp4) and unwinds ds-DNA in the 5' to 3' orientation using energy from nucleotide triphosphate (NTP) hydrolysis (Spelbrink et al. 2001; Korhonen et al. 2003). It contains two structural domains connected by a linker, with the C-terminus housing five conserved motifs for the helicase activity and the N-terminus containing some of the conserved motifs associated with the primase activity (Ziebarth et al. 2007; Holmlund et al. 2009). Although Twinkle is nuclear-encoded, it is largely imported into mitochondria via its mitochondrial signaling peptide to co-localize with mitochondrial nucleoids (Korhonen et al. 2004; Tyynismaa et al. 2004; Ylikallio et al. 2010). Its essential role in mtDNA replication is demonstrated by substantial mitochondrial DNA (mtDNA) depletion in Twinkle null mice resulting in early embryonic lethality (Milenkovic et al. 2013). Mutations in the human gene *Twinkle* (*C10orf2*) also lead to mtDNA depletion/deletion and defective mtDNA maintenance, resulting in severe neuromuscular diseases, such as autosomal dominant progressive external ophtalmoplegia (adPEO), hepatocerebral mtDNA depletion syndrome (MDS) and infantile-onset spinocerebellar ataxia (Zeviani et al. 1989; Suomalainen et al. 1997; Nikali et al. 2005; Hakonen et al. 2008; Van Hove et al. 2009; Fratter et al. 2010).

In this study, we report a novel temporal expression pattern of Twinkle during chromosomal segregation at distinct mitotic phases in neuronal-like progenitor cells. While discrete amount of Twinkle is present in the cytosolic compartment of non-mitotic cells, Twinkle accumulates around the nuclear membrane undergoing lamina disassembly at the early prophase. During late prophase, Twinkle protein translocates to the nuclear compartment. The transition into prometaphase is marked colocalization with the outer kinetochore protein HEC1. This dynamic expression pattern suggests converging properties of Twinkle in both mitochondrial and nuclear genomic inheritance in mitotic neuronal-like progenitor cells.

Materials and methods

Cell culture

PC12-NeuroD6 cells (PC12-ND6; previously called PC12-Nex1) were generated as described by Uittenbogaard and Chiaramello (2002) and grown on collagen I-coated plates (Becton Dickinson Labware). They were grown in the presence of F12K medium (Life Technology) containing 15% horse serum (Life Technology), 2.5% fetal bovine serum (Life Technology). Even though the three generated PC12-ND6 clones (PC12-Nex1-M A, B and C) displayed similar response upon NGF exposure and withdrawal of trophic factors (Uittenbogaard and Chiaramello, 2002; 2004; 2005), we used the PC12-ND6 clone A to remain consistent with our previous studies pertaining to regulation of mitochondrial biogenesis and bioenergetics during neuronal differentiation (Baxter et al. 2009; Uittenbogaard et al. 2010a; 2010b).

Immunocytochemistry

PC12-ND6 cells were grown on poly-D-lysine coated coverslips and fixed in 4.0% (w/v) paraformaldehyde for 5 min before permeabilization in 0.2 % Triton X-100 for 5 min. Cells were blocked in 10% goat serum (Life Technology) for 1 h at room temperature. Incubations with primary and secondary antibodies were performed at room temperature as described by Baxter et al. (2009). We used the following primary antibodies: anti-Twinkle immunogen affinity-purified rabbit polyclonal antibody (ab83329; Abcam; 1:180); anti-SOD2 mouse monoclonal (Abcam; 1:250); anti-Hec1 mouse monoclonal (Abcam; 1:300); anti- α tubulin mouse monoclonal (Santa Cruz; 1:500); anti-CoxVa mouse monoclonal (Abcam; 1:500). We used the following Alexa Fluor®-conjugated secondary antibodies (Molecular Probes) diluted 1:1000 in PBS: 488-labeled goat anti-rabbit IgG and 568-labeled anti-mouse IgG. Samples were mounted using Prolong Gold antifade reagent with the nuclear counterstain DAPI (Molecular Probes).

Immunoblot analysis

Total lysate proteins from the PC12-ND6 cells were isolated as described by Uittenbogaard et al. (2010). Proteins (40 μ g) were resolved on a 10% NuPAGE Bis-Tris gels (Invitrogen) and transferred to a nitrocellulose membrane as described by Baxter et al. (2012). The Twinkle protein was detected with the rabbit polyclonal antibody ab83329 (Abcam; 1:500), while the loading and transfer control protein GADPH was detected using the mouse monoclonal antibody AM4300 (Ambion; 1:10,000). The antigen-antibody complexes were detected using infrared dye-conjugated secondary antibodies with a 1:20,000 dilution

(IRDye® 680LT conjugated goat anti-mouse (827-11080; IRDye® 800 CW conjugated goat anti-rabbit (926-32211) from LI-COR. Bands were detected using the Odyssey Infra-Red Imaging System (LI-COR).

Image acquisition and analysis

Images were captured with a Zeiss LSM 710 confocal system equipped with an oil immersion 100 X Plan-Apochromat objective (numerical aperture 1.46) and Zen software (Zeiss). Stacked images were acquired in the linear range of the fluorescence intensity. During imaging, the duration and light exposure was kept constant for all samples and emissions at 520 nm and 590 nm were detected simultaneously. The laser power, gain, offset and iris were kept constant to allow direct comparison between samples and cells at different mitotic phases. Colocalization analysis was performed with the ImageJ software on at least ten fields of cells, each field containing about 50 cells, from three distinct experiments to ensure a good representation of PC12-ND6 cells in each mitotic phase. Colocalization of Twinkle with SOD2 or Hec1 was investigated using the “colocalization” plugin in ImageJ software. When indicated, we performed specific line scanning graph (Zen software) to visualize the relative spatial fluorescence intensities of co-expressed proteins in defined areas of mitotic chromosomes as illustrated on corresponding figures. The intensity of the pixels along the line was recorded to generate a histogram as described by Wu et al. (2009).

Results

Twinkle Transiently Accumulates at Discrete Regions of Mitotic Chromosomes at Specific Mitotic Phases

The study of Twinkle interfacing with the spindle microtubule during chromosomal segregation was performed in our established neuronal cell line, PC12-ND6, which constitutively expresses the neuronal differentiation basic Helix-Loop-Helix (bHLH) transcription factor NeuroD6 (Uittenbogaard and Chiaramello, 2002). PC12-ND6 cells behave as neuronal progenitor cells poised to withdraw from cell cycle and undergo terminal differentiation upon neurotrophic cues (Uittenbogaard and Chiaramello, 2004; 2005; Uittenbogaard et al. 2010), thereby mimicking the proliferation potential of NeuroD6⁺ committed neural progenitors during neurogenesis (Wu et al. 2005). Moreover, NeuroD6 coordinates neuronal differentiation with mitochondrial biogenesis to provide sufficient mitochondrial mass for proper neurite outgrowth (Baxter et al. 2009; Uittenbogaard et al. 2012; Uittenbogaard and Chiaramello, 2014).

To visualize Twinkle protein, we used a commercial polyclonal antibody raised against an epitope mapping in the H3 and H4 motifs of the DNA helicase domain, which shares high homology among the human, mouse and rat species (Fig. 1). The C-terminal helicase domain of Twinkle shares structural similarities with the bacteriophage T7 primase-helicase (T7 gp4) and contains five conserved motifs typical to the superfamily 4 (SF4) helicases, among them the H3 and H4 motifs (Spelbrink et al. 2001; Ziebarth et al. 2007). To rule out the existence of eukaryotic helicase proteins carrying a similar helicase domain with the H3 and H4 motifs, we carried out a homology search using an enhanced algorithm for Domain Enhanced Lookup Time Accelerated Blast (Delta Blast) using the reference proteins

database of the human, mouse and rat species. We found no BLAST hits other than with the Twinkle protein and its known isoforms from the three species (Fig. 1). In addition, we verified specificity of the immunogen-purified Twinkle antibody by immunoblot analysis using total lysate of PC12-ND6 cells, which revealed a single detected band with the predicted molecular weight of the Twinkle protein (Supplementary Fig. 1). This validates our choice of the polyclonal antibody raised against the peptide spanning the H3 and H4 motifs to investigate the subcellular distribution of Twinkle in PC12-ND6 neuronal-like cells.

In non-mitotic PC12-ND6 cells, co-localization analysis using the Zeiss Zen software not only revealed the expected co-expression of Twinkle and SOD2 in mitochondria, but also noticeable cytosolic Twinkle signal, which did not overlap with the mitochondrial marker SOD2 (Fig. 2). However, in mitotic PC12-ND6 cells, we observed an unexpected redistribution of Twinkle in the nuclear region. Following the nuclear membrane dissolution, right at the outset of prophase, we observed a robust and punctate pattern of Twinkle expression, suggestive of an oligomeric complex characteristic of active helicases (Fig. 3). More specifically, Twinkle colocalized with chromosomal DNA undergoing condensation during prophase. At prometaphase and metaphase, we found that Twinkle accumulated at discrete regions of mitotic chromosomes. During anaphase, Twinkle expression segregated at the mitotic poles and colocalized with microtubules of the mitotic spindles, as revealed by co-staining with an anti-alpha-tubulin antibody (Fig. 3). At telophase, twinkle was no longer associated with chromosomal DNA when chromosomal decondensation and kinetochore disassembly occurred (Fig. 3). Thus, our collective immunofluorescence results indicate that Twinkle decorates mitotic chromosomes at discrete regions with a staining pattern usually associated with kinetochore labeling.

Twinkle Colocalizes with the Kinetochore Marker Hec 1 during Specific Mitotic Phases

To test the hypothesis that Twinkle may participate in the molecular architecture of the kinetochore, we performed immunocytochemistry using an antibody against Hec1, a marker of the outer kinetochore essential for establishing stable kinetochore-microtubule spindle attachment and tension (Ciferri et al., 2005; DeLuca et al., 2005; Emanuele et al., 2005; Cheeseman et al., 2006). Figure 4 illustrates the spatio-temporal expression pattern of Twinkle and Hec1 throughout the mitotic phases of PC12-ND6 cells. Co-distribution of Twinkle and Hec1 could already be detected at prophase. As PC12-ND6 cells progressed to prometaphase and metaphase, colocalization between Twinkle and Hec1 became particularly striking. During separation of sister chromatids at anaphase, Twinkle and Hec1 remained colocalized at the spindle poles. As expected, Twinkle and Hec1 proteins were no longer expressed at telophase (Fig. 4). We corroborated our colocalization results by examining the expression patterns of Twinkle and Hec1 throughout the individual slices from a stacked confocal image of mitotic PC12-ND6 cells shown in figure 4. We focused on PC12-ND6 cells in prometaphase because Hec1 proteins have assembled at the core microtubule-attachment site in preparation for alignment of all the chromosomes in the middle of the mitotic spindle at metaphase (Cheeseman and Desai, 2008). Figure 5, which displays the nine slices making the stacked confocal image of prometaphase PC12-ND6 cells, shows the matching colocalization between Twinkle and Hec1 throughout all the slices. Figure 5 also

includes a colocalization analysis performed on slice #4 since it contains the most comprehensive number of kinetochores (Fig. 5). The colocalization histogram from the scanning line shown in the high magnification of slice #4 unambiguously indicates a perfect pattern of co-expression between Twinkle and Hec1 signals throughout all the kinetochores (Fig. 5). Thus, our collective observations indicate that nuclear expression of Twinkle is transiently restricted to discrete punctate structures mapping to the kinetochore of segregating chromosomes in mitotic neuronal-like PC12-ND6 cells.

Distribution of Twinkle at the Kinetochore Complex does not Involve Mitochondria

Given that Twinkle is localized in mitochondria, more specifically in the mitochondrial nucleoids, based on its well known function as a mitochondrial DNA helicase, we wanted to rule out that Twinkle expression was associated with mitochondria bound to the kinetochore-microtubule complex. To this end, we co-labeled PC12-ND6 cells with primary antibodies against Twinkle and the mitochondrial marker CoxVa and analyzed their co-localization by immunofluorescence confocal microscopy. We did not detect any mitochondrial CoxVa signal that colocalized with Twinkle signal associated with kinetochores in prometaphase PC12-ND6 cells (Fig. 6). Similarly, the mitochondrial marker CoxVa is absent from the mitotic poles of anaphase PC12-ND6 cells where kinetochores are located (Fig. 6). Therefore, Twinkle expression observed in the kinetochore complexes is independent of the mitochondrial population. Worth noting is the accumulation of Twinkle-positive mitochondria along the spindle microtubules in the equatorial plane of anaphase PC12-ND6 cells, which is congruent with a microtubule-mediated mitochondrial inheritance for accurate partitioning during the late mitotic anaphase/telophase between daughter cells (Lawrence and Mandato, 2013a; 2013b). Previous studies have demonstrated that this type of inheritance was also observed with other organelles, such as the Golgi, endosomes and lysosomes, which are inherited via an ordered mechanism requiring the mitotic spindle microtubules (Bergeland et al., 2001; Dunster et al., 2002; Wei et al., 2009).

Discussion

Our study on the expression pattern of the mitochondrial DNA helicase Twinkle in mitotic neuronal-like progenitor cells reports the novel observation of Twinkle recruitment to the kinetochore complex during chromosomal segregation, which has major implications. First, it is suggestive of a novel function of Twinkle as participant of genomic inheritance in addition to its well-established role as a regulator of mitochondrial nucleoid dynamics. Second, it highlights a new layer of regulation and function to the complex dynamic process and mechanistic aspect of mitosis. And third, the localization of Twinkle at the kinetochore structure in combination with its well-documented conserved sequence across species is highly reminiscent of the evolutionary conserved structure of the centromere-kinetochore complex, the most ancient and conserved cellular structure.

Twinkle, which shares striking similarity with the bacteriophage T7 primase/helicase gene 4 protein, belongs to the SF4 replicative helicase family, known to hydrolyze nucleotides to provide energy for DNA unwinding (Spelbrink et al, 2001). It is a key component of the mitochondrial nucleoid complex, which constitutes the unit of genetic inheritance in

mitochondria (Gilkerson, 2009). Its molecular architecture consists of: 1) the core domain with 1–2 mtDNA molecules and its associated proteins such as the transcription and histone-like protein Tfam, mtSSB, Twinkle, DNA polymerase γ , mtRNA polymerase, and mtTFB; and 2) the peripheral layer (Bogenhagen et al, 2008; Gilkerson et al, 2013) that is more heterogeneous and dynamic in nature and includes various proteins participating in the heat shock and metabolic pathways (Wang and Bogenhagen, 2006). Even though the nuclear and mitochondrial genomes are widely distinct in terms of size, architecture and replication, our results are suggestive of a parallel function of Twinkle as a member of the mtDNA nucleoid complex and the kinetochore complex of condensed chromosomes. In support of this premise is the recent evidence that some nuclear DNA helicases are transported into mitochondria to participate in the mtDNA replication, repair, maintenance and/or stability (Lin and Yilun, 2015). These include DNA helicases RECQ4 (Chi et al; Croteau et al, 2012) DNA2 (Zheng et al, 2008; Duxin et al, 2009; Ronchi et al, 2013), PIF1 (Futami et al, 2007) and SUV3 (Minczuk et al, 2002; Khidr et al, 2008; Wang et al, 2009). Thus, it is conceivable that Twinkle may be working in concert with other nuclear DNA helicases to regulate inheritance of the nuclear genome.

Together, our provocative observations raise several questions for future studies with respect to Twinkle functions in the nuclear compartment during chromosomal segregation. First, Twinkle may be located at the kinetochores for immediate availability for the mtDNA transcription/replication machinery after cell division, thereby bypassing the need for transcription and translation. Second, the localization of Twinkle upon the kinetochore formation may be a checkpoint for the timing of mitotic division to proceed, such as completion of mitochondrial biogenesis accompanied by mtDNA transcription and/or a critical threshold of mitochondrial mass, which would function as a retrograde signaling to trigger mitosis. And third, Twinkle helicase function may be involved in some unwinding activity associated with the separation of sister chromatids, which requires centromeric decatanation and nucleosome remodeling via a two-step process involving the coordinated actions among PICH (PLK1-interacting checkpoint DNA helicase, also known as ERCC6L), BLM (Bloom Syndrome hexameric DNA helicase) and the DNA topoisomerase II α (Topo II α) enzyme (Baxter et al., 2011; Ke et al., 2011). PICH localizes at the centromere-kinetochore complex in prometaphase, a step required for recruiting BLM before the onset of anaphase (Baumann et al., 2007; Rouzeau et al., 2012). Given the fact that Twinkle forms distinct punctate signals suggests that Twinkle protein may be assembled into the typical heptameric or hexameric ring structures, as previously described whether bound to ds or ss DNA, or in the absence of DNA (Sen et al, 2012). Such observations are not incompatible with our findings, suggesting that Twinkle may interact with the kinetochore complex rather than with DNA region of the centromere. In sum, Twinkle helicase activities may play a crucial role in synchronizing the timing of mitosis after completion of mitochondrial biogenesis, which would make Twinkle available for the next round of mitochondrial biogenesis once mitosis is completed.

Together, our findings highlight the fact that Twinkle mutations-associated diseases may have a more profound cellular impact than previously suspected, as certain mutations may progressively degrade the nuclear-mitochondrial cross-talk due to the combination of mtDNA deletion, timing of chromosomal segregation and/or missegregation. It is

conceivable that in the case of mitotic cells, such as stem cells and progenitors cells, the degradation of the nuclear-mitochondrial cross-talk may be more pronounced, which would in part explain the early embryonic lethality of Twinkle null mice (Milenkovic et al, 2013). This may contribute to the progressive neurodegenerative symptoms of Perrault syndrome and infantile-onset spinocerebellar ataxia, both due to recently identified causative compound heterozygous Twinkle mutations (Morino et al., 2014; Park et al., 2014). In these childhood-onset diseases, Twinkle autosomal recessive mutations alter the helicase activity of Twinkle, leading to increased severity of specific neurological symptoms with age.

In summary, our studies have revealed an unexpected expression pattern of Twinkle with striking accumulation in the kinetochores of segregating chromosomes in mitotic neuronal-like progenitor cells, which is independent of its known mitochondrial functions. Our observations have broad neurodevelopmental implications pertaining to progenitor cell propagation. Thus, our future studies will address whether Twinkle is present at the kinetochores in other cellular paradigms in the context of proliferation and differentiation. Elucidating how Twinkle regulates chromosomal segregation will be an important direction in the future, which will ultimately provide novel insights on the molecular mechanisms responsible for neurodevelopmental disorders associated with causative Twinkle mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work was supported in part by the National Institutes of Health, National Institute of Neurological Disorders and Stroke (grant number R21NS085282 to A.C.), by the National Center for Research Resources (S10RR025565), and by the National Institute of Child Health and Human Development (P30HD40677).

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Fig. 1.

Protein alignment of Twinkle homologues and isoforms of Human, Mouse and Rat. NCBI COBALT Alignment was used with “identity settings for strict homology”. Conserved sequence motifs are highlighted in yellow, while the species and isoforms are specified next to the corresponding protein accession NCBI numbers. At the N-terminus, the mitochondria target sequence is represented by a boxed-dotted line. The linker region, which is located between the primase domain at the N-terminus and helicase domain at the C-terminus is indicated by a black-boxed line, whereas the GP4d helicase sequence is highlighted in yellow. The GP4d helicase conserved domains are illustrated by a red-boxed line (H1-

Walker A; H1A; H2-Walker B; H3; and H40. The Human Twinkle peptide sequence (AA 540-589) used to raise rabbit polyclonal antibodies is highlighted in orange (Abcam # 83329). A sequence homology search in the NCBI Mammalian protein database with this specific peptide did not identify similar domains, which could have cross-reacted with this antibody.

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Fig. 2.

Co-localization analysis between Twinkle and the mitochondrial marker SOD2. Cells were fixed with paraformaldehyde and simultaneously stained with rabbit-anti-Twinkle and mouse anti-SOD2 antibodies, followed by 488-Alexa Fluor®-conjugated anti-rabbit antibodies and 588-Alexa Fluor®-conjugated anti-mouse antibodies. Chromosomal DNA was stained with DAPI. Stained cells were visualized by laser confocal microscopy. Colocalization was analyzed with the ImageJ software, as shown in the lower left panel. Although significant overlapping signals between Twinkle and SOD2 was observed, noticeable cytosolic Twinkle signal did not overlap with the mitochondrial marker SOD2. The SOD2 signal, which appears to be within the nucleus, is most likely due to projection of mitochondria located below and above the nucleus, as a result of stacking. The colocalization histogram derived from the white scanning line shown in the merge panel is shown with red and green lines indicating SOD2 or Twinkle labeling intensity, respectively. The blue line illustrates the DAPI signal intensity. Black arrows indicate non-overlapping signals between Twinkle and SOD2. Bar = 10 μm .

Fig. 3. Twinkle signal labels the kinetochore region in mitotic PC12-ND6 cells from prometaphase to anaphase. PC12-ND6 cells were simultaneously stained with rabbit-anti-Twinkle and mouse anti- α -Tubulin antibodies, followed by 488-Alexa Fluor®-conjugated anti-rabbit antibodies and 588-Alexa Fluor®-conjugated anti-mouse antibodies. Chromosomal DNA was stained with DAPI. Stained cells were visualized by laser confocal microscopy. Immunofluorescent signals of Twinkle and α -tubulin are shown at different mitotic phases. Bar = 5 μ m.

Fig. 4.

Twinkle colocalizes with the outer kinetochore marker Hec 1 in PC12-ND6 cells at specific mitotic phases. Cells were simultaneously stained with rabbit-anti-Twinkle and mouse anti-Hec1 antibodies, followed by 488-Alexa Fluor®-conjugated anti-rabbit antibodies and 588-Alexa Fluor®-conjugated anti-mouse antibodies. Chromosomal DNA was stained with DAPI. Stained cells were visualized by laser confocal microscopy. Insets show higher magnification views of colocalization between Twinkle (green) and Hec 1 (red).

Colocalization was analyzed with the ImageJ software and shown in the right panels.

Scanning lines are indicated in white in the merge panels. The corresponding histograms are shown with green and red lines indicating Twinkle or Hec1 labeling intensity, respectively.

The blue line illustrates the DAPI signal intensity. Bar = 5 μm .

Fig. 5. Cytological evidence of Hec1 and Twinkle interactions in prometaphase PC12-ND6 cells. The stacked confocal image of prometaphase PC12-ND6 cells shown in figure 4 contained a total of nine slices labeled with Hec1 (red) and Twinkle (green). Only merged pictures are shown. A high magnification of slice #4 with its corresponding scanning line is shown, as it contains the most comprehensive number of kinetochores during prometaphase. Colocalization was analyzed with the ImageJ software. The corresponding histograms are shown with green and red lines indicating Twinkle or Hec1 labeling intensity, respectively. Bar = 5 μm .

Fig. 6.

Twinkle expression at the kinetochore complex is not associated with mitochondria during the mitotic phases. Cells were simultaneously stained with rabbit-anti-Twinkle and mouse anti-CoxVa antibodies, followed by 488-Alexa Fluor®-conjugated anti-rabbit antibodies and 588-Alexa Fluor®-conjugated anti-mouse antibodies. Chromosomal DNA was stained with DAPI. Stained cells were visualized by laser confocal microscopy. The mitochondrial marker CoxVa was not detected at the kinetochore complex in PC12-ND6 cells at the prometaphase, as illustrated in the merge panel. Similarly, the CoxVa signal was not detected at the mitotic poles of cells in late anaphase, as indicated by white arrows in the lower panels. While Twinkle-positive mitochondria are evenly distributed within the cytoplasm of prometaphase PC12-ND6 cells, as shown in the top panels, Twinkle-positive mitochondria become enriched at the cell equator of anaphase PC12-ND6 cells indicated by red arrows while depleted at the mitotic poles indicated by white arrows in the low panels. Bar = 5 μ m.