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Report

A Role for NuSAP in Linking Microtubules to Mitotic Chromosomes

Katharina Ribbeck,[1,*] Tim Raemaekers,[2] Geert Carmeliet,[3] and Iain W. Mattaj[1]
[1] European Molecular Biology Laboratory
Meyerhofstrasse 1
D-69117 Heidelberg
Germany
[2] Membrane Trafficking Laboratory
Center for Human Genetics
Flanders Interuniversity Institute for Biotechnology/Katholieke Universiteit Leuven
B-3000 Leuven
Belgium
[3] Laboratory for Experimental Medicine and Endocrinology
Katholieke Universiteit Leuven
Gasthuisberg
B-3000 Leuven
Belgium

Summary

The spindle apparatus is a microtubule (MT)-based machinery that attaches to and segregates the chromosomes during mitosis and meiosis. Self-organization of the spindle around chromatin involves the assembly of MTs, their attachment to the chromosomes, and their organization into a bipolar array. One regulator of spindle self-organization is RanGTP. RanGTP is generated at chromatin and activates a set of soluble, Ran-regulated spindle factors such as TPX2, NuMA, and NuSAP [1]. How the spindle factors direct and attach MTs to the chromosomes are key open questions. Nucleolar and Spindle-Associated Protein (NuSAP) was recently identified as an essential MT-stabilizing and bundling protein that is enriched at the central part of the spindle [2, 3]. Here, we show by biochemical reconstitution that NuSAP efficiently adsorbs to isolated chromatin and DNA and that it can directly produce and retain high concentrations of MTs in the immediate vicinity of chromatin or DNA. Moreover, our data reveal that NuSAP-chromatin interaction is subject to Ran regulation and can be suppressed by Importin α (Impα) and Imp7. We propose that the presence of MT binding agents such as NuSAP, which can be directly immobilized on chromatin, are critical for targeting MT production to vertebrate chromosomes during spindle self-organization.

Results

We previously reported that NuSAP accumulates at chromatin-proximal MTs during mitosis in HeLa cells, mouse fibroblasts (MC3T3E1 cells), and *Xenopus laevis* oocytes [2, 3]. Importantly, NuSAP remains localized to the chromosomes after removal of MTs by nocodazole treatment [2], suggesting that its ability to interact with chromatin may be independent of MTs.

To better understand the interplay of NuSAP and chromatin, we compared NuSAP localization on chromosomes with established markers such as CREST antigens for kinetochores and Aurora B for inner centromeres at different stages of mitosis in fixed MC3T3E1-cultured cells. Throughout mitosis, NuSAP localized to numerous restricted regions on chromatin (Figures 1A and 1B). Its colocalization with the kinetochores and Aurora B was only partial, suggesting that the NuSAP-interacting domains on chromosomes did not correspond to the kinetochores or the inner centromeres (Figures 1A and 1B). To determine whether NuSAP localizes to one or multiple positions on individual chromosomes, we immunostained spread HeLa chromosomes. As shown in Figure 1C, NuSAP was not uniformly distributed but was present at multiple sites along each chromosome, whereas the kinetochores were detectable at one distinct location. Thus, NuSAP appears to have multiple binding sites on chromosomes that are distinct from kinetochores or centromeres.

We wished to determine whether NuSAP by itself is able to interact with chromatin. To test this, we added recombinant NuSAP to sperm head chromatin [4] in BRB80 buffer (Figure 2A). For direct detection, NuSAP was covalently labeled with Alexa488. As specificity controls, Alexa488-labeled maltose binding protein (MBP) and H_{10}-tagged GFP (recombinant NuSAP itself carries a H_{10} tag) were used. Although the two control proteins accumulated only weakly on chromatin, NuSAP was enriched approximately 50-fold at the chromatin surface, as determined by fluorescence intensity (Figure 2A and data not shown). Thus, NuSAP itself has the capacity to interact with and accumulate at chromatin.

We previously showed that NuSAP can directly interact with MTs and efficiently stabilize and crosslink them in vitro [2, 3]. In these experiments, NuSAP was present in solution. Given that NuSAP is highly enriched on the chromosomes in vivo, we now asked how NuSAP would function when concentrated on chromatin. Isolated sperm chromatin was incubated with 0.2 μM recombinant NuSAP in a solution of pure tubulin (15 μM), and the reaction was incubated at 37°C for 15 min (Figure 2B, right panels). Under these conditions, thick MT fibers emerged at the surface of the chromatin samples. In contrast, when the MBP control protein was present, very few MTs were detectable in the reaction, and none were chromatin-associated (Figure 2B, left panels). As a second control, the effect of the MT-associated protein TPX2 (0.2 μM), which can induce MT assembly in solution [5], was also studied (Figure 2B). TPX2 formed tubulin aggregates both around chromatin and in solution, but no bundled fibers were detectable.

NuSAP-mediated fiber formation at chromatin was highly efficient and was detectable on >90% of the chromatin samples. The length of the fibers depended on the

*Correspondence: katharina_ribbeck@hms.harvard.edu

reaction time and the tubulin concentration in the reaction medium. Together, these results show that NuSAP can efficiently adsorb to chromatin and from here generate a local high concentration of MTs. Importantly, the NuSAP-generated MTs appear to remain associated with chromatin.

The chromatin samples in the previous experiment were in the mitotic state [4, 6] and contained histones and other sperm-specific DNA-binding proteins that may influence NuSAP's ability to generate and crosslink MTs. We wished to determine whether NuSAP might be able to interact with DNA directly. To test this, we coated magnetic beads with plasmid DNA and incubated the DNA-beads with Alexa488-labeled NuSAP, Alexa488-MBP, or H_{10}-GFP. Figure S1 shows that whereas the control proteins H_{10}-GFP and Alexa488-MBP only weakly accumulated at the beads, Alexa488-NuSAP efficiently adsorbed to them. NuSAP did not significantly bind to control beads without DNA (data not shown, but see below). This result suggests that NuSAP can directly interact with DNA.

To test whether NuSAP can produce MT fibers while it is bound to DNA, we combined 0.2 μM recombinant NuSAP, DNA beads, and 15 μM of tubulin and incubated the reaction at 37°C for 15 min. Figure 3A shows that in the absence of NuSAP, the DNA beads remained empty. In the presence of TPX2, tubulin aggregates were detectable both around the beads and in solution, but no fibers formed. In contrast, in the presence of NuSAP, MT fibers efficiently assembled at the surface of the DNA beads. Reproducibly, more than 90% of the DNA beads acquired prominent MT fibers.

If the DNA at the beads indeed provides binding sites for NuSAP, and if NuSAP interacts with MTs while it is bound to DNA, it should be possible to compete for fiber formation at the beads with soluble plasmid DNA. Figure 3B shows that indeed, in the presence of both DNA beads and soluble plasmid DNA, NuSAP no longer induced MT fiber formation exclusively at the surface of the DNA-beads. Instead, fibers still assembled, but many were no longer associated with the DNA-beads. Importantly, the fibers that assembled at a distance from the beads appeared associated with compacted plasmid DNA (Figure 3B). This result suggests that NuSAP can indeed interact with both pure DNA and MTs and thereby directly interconnect both. It remains unclear whether one NuSAP molecule can interact with MTs and DNA simultaneously or whether oligomerization of NuSAP is required to link both structures.

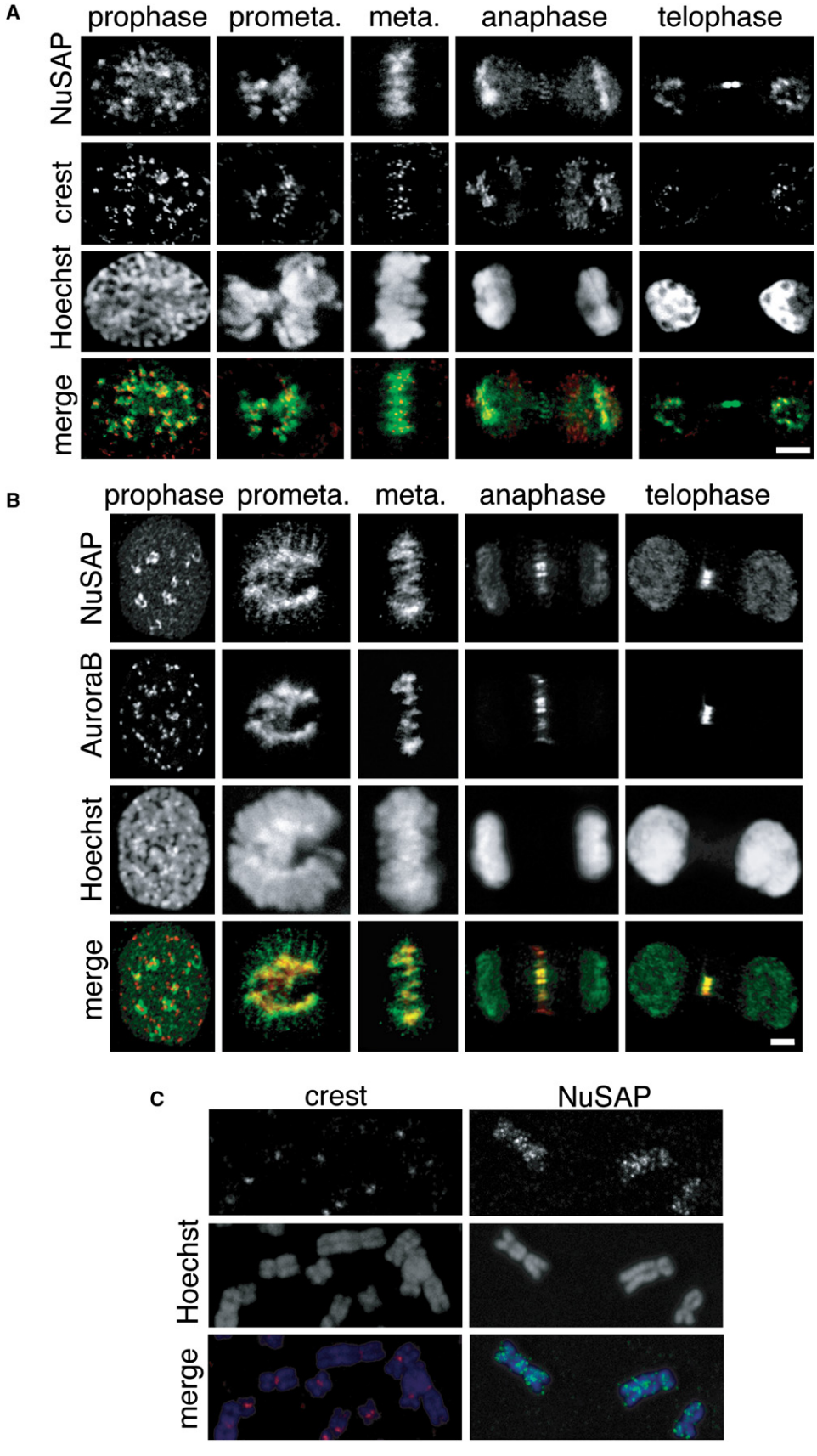
Importantly, NuSAP could also induce MT crowding in the absence of DNA, for example when it was attached via a histidine tag to Nickel beads (Figure S2). This suggests that it is not the direct interaction of NuSAP with DNA, but rather its attachment to and concentration on a localized surface, that is required to locally accumulate MTs.

A key recent finding in understanding Ran function was that Ran-regulated transport receptors not only change the intracellular localization of a cargo but also directly regulate cargo activity [1, 7, 8]. It has been shown that certain spindle-assembly factors are inactivated by importin binding and are activated by RanGTP-dependent importin release [1, 7]. The production of RanGTP is stimulated by RCC1 localized on chromatin and thus activates mitotic regulators and triggers spindle assembly around chromatin [3].

We recently showed that the activity of NuSAP on MTs is directly regulated by three importins: Impα, Impβ, and Imp7 [3]. Whereas Impα and Imp7 block the MT-stabilizing activity of NuSAP, Impβ appears to specifically suppress the crosslinking activity of NuSAP. Here we asked whether the three importins would affect the ability of NuSAP to interact with chromatin and to link it to MTs.

First, we tested whether the importins affect the ability of NuSAP to interact with isolated chromatin in the absence of tubulin. Alexa488-labeled NuSAP was incubated with isolated sperm chromatin in the absence of any other component or in the presence of the individual importins (Figure S3). As already shown, Alexa488-NuSAP strongly accumulated at chromatin. In contrast, in the presence of either Impα or Imp7, the binding of NuSAP to chromatin was significantly reduced. Quantitation of three representative chromatin samples revealed that the signal of NuSAP was reduced approximately 5-fold in the presence of Impα and by a factor of 10 in the presence of Imp7. Importantly, this inhibitory effect was also observed when DNA beads were used instead of chromatin (data not shown). In contrast, Impβ did not detectably alter the ability of NuSAP to accumulate at chromatin.

We next investigated whether the importins would affect the ability of NuSAP to link MT fibers to chromatin by performing the described reaction in the presence of tubulin (Figure 4). As before, in the absence of any importin, NuSAP efficiently produced thick MT fibers at the surface of chromatin. In contrast, in the presence of Impα, the majority of chromatin samples remained naked with respect to associated MTs (Figure 4, third row). This confirms the earlier observation that Impα impairs NuSAP's ability to bind chromatin and that, as a consequence, it inhibits the production of MT structures in contact with chromatin. In the presence of Impβ, long individual MT fibers were detected in the background, and occasionally also at chromatin. However, the formation of MTs at chromatin was highly inefficient as compared to control samples. Thus, although NuSAP can still interact with chromatin in the presence of Impβ (Figure 4, fourth row), some other functional aspect that is necessary for MT fiber formation appears to be suppressed. When Imp7 was present, many small aster-like structures emerged in solution. However, only a small fraction (<20%) of the chromatin samples was associated with thick MT fibers (Figure 4, fifth row). This indicates that Imp7 also blocks the ability of NuSAP to efficiently link MTs to chromatin. The strongest inhibitory effect of NuSAP activity at chromatin was observed when all three receptors were present (Figure 4, bottom row). In this case, less than 5% of the chromatin samples were associated with MT fibers. Importantly, when importins were present in the absence of NuSAP, no MT structures were detected (Figure S4). Two conclusions can be drawn from this experiment. First, Impα and Imp7 appear to reduce the ability of NuSAP to interact with chromatin and DNA. Second, not only the interaction with chromatin but also a different aspect of NuSAP function, which is specifically blocked by Impβ, appears to be required for NuSAP to efficiently produce MT fibers at chromatin.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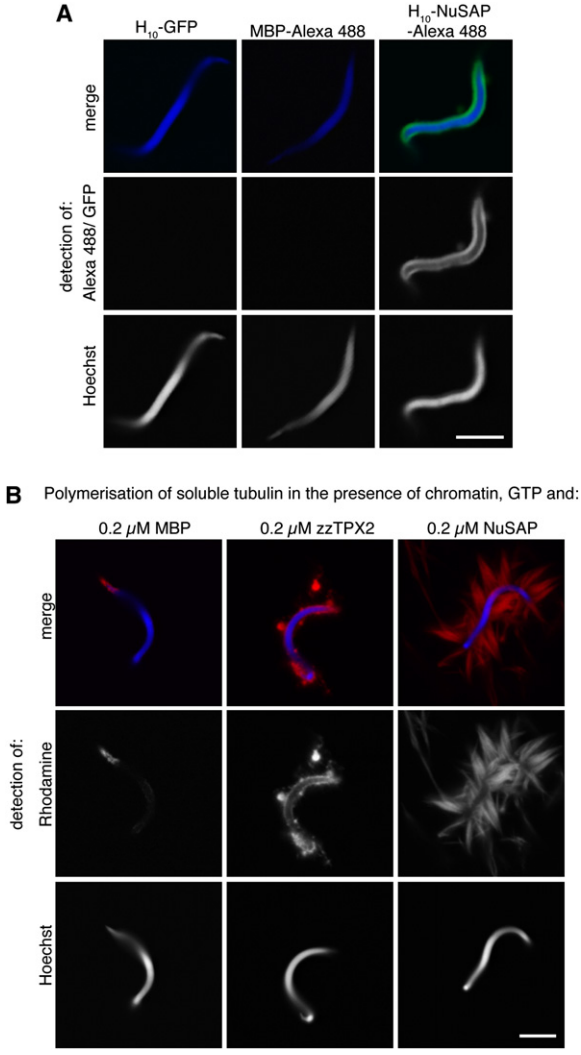

Figure 2. NuSAP Can Adsorb to Chromatin and from Here Generate a Local High Concentration of MTs

(A) Isolated sperm chromatin was placed in BRB80 buffer and incubated with 0.2 μM of recombinant Alexa488-labeled NuSAP, Alexa488-labeled MBP, or H_{10}-tagged GFP, as indicated.
(B) Isolated sperm chromatin was incubated with 0.2 μM recombinant NuSAP, zzTPX2, or MBP in a solution of pure Rhodamine-labeled tubulin (15 μM) at 37°C for 15 min.
Scale bars represent 10 μm.

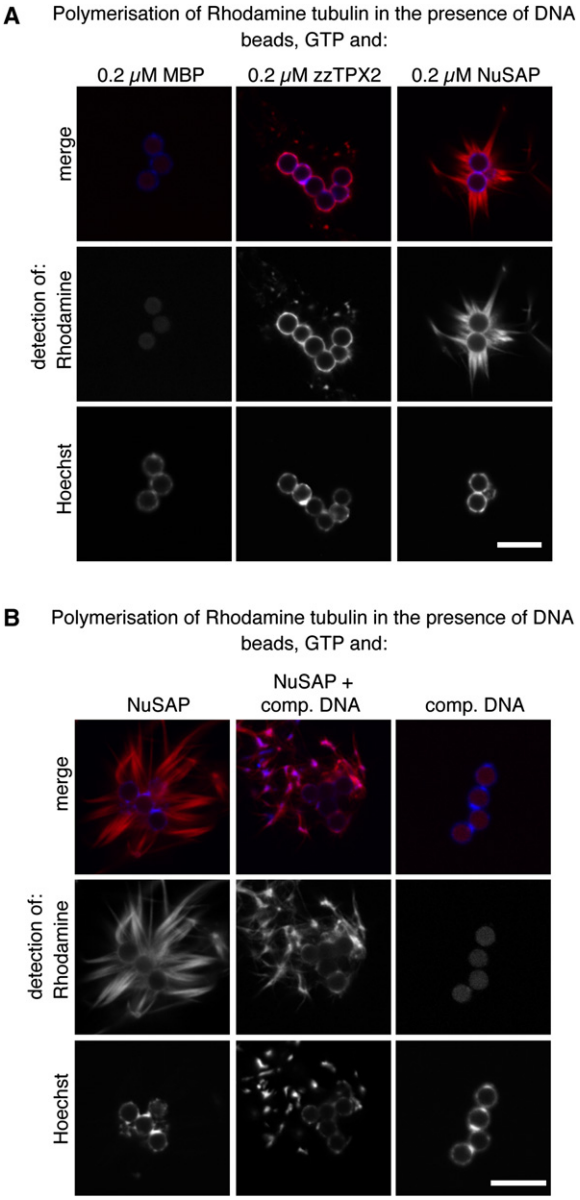

Figure 3. NuSAP Can Interact with Both Pure DNA and MTs and Thereby Interconnect Both

(A) DNA beads were incubated with 15 μM pure Rhodamine-labeled tubulin and 0.2 μM recombinant NuSAP, zzTPX2, or MBP at 37°C for 15 min.
(B) As in (A). In addition, where indicated, competitor plasmid DNA (30 ng/μl) was present.
Scale bars represent 5 μm.

Discussion

In mitotic HeLa cells, mouse fibroblasts, and *Xenopus* oocytes, NuSAP is enriched at the central part of the spindle, where MTs are in close proximity to the chromosomes [2, 3]. Here we show by reconstitution that NuSAP can efficiently produce high concentrations of MTs in the immediate vicinity of chromatin or DNA. Furthermore, we extend the function of Importinα, Importinß, and Importin 7 to include regulation of the interaction of NuSAP with chromatin and/or MTs.

What is the function of NuSAP at the spindle? It has been proposed that a soluble RanGTP gradient produced at chromatin triggers localized spindle

Figure 1. NuSAP Localizes along Chromosome Arms to Multiple Sites that Are Distinct from Kinetochores and Centromeres

(A and B) Mouse fibroblasts (MC3T3E1 cells) were fixed at different stages of mitosis and immunostained for NuSAP and kinetochores (CREST serum) or inner centromeres via Aurora B.
(C) Spread metaphase chromosomes from HeLa cells were immunostained for kinetochores and NuSAP, respectively.
Scale bars in (A) and (B) represent 5 μm.

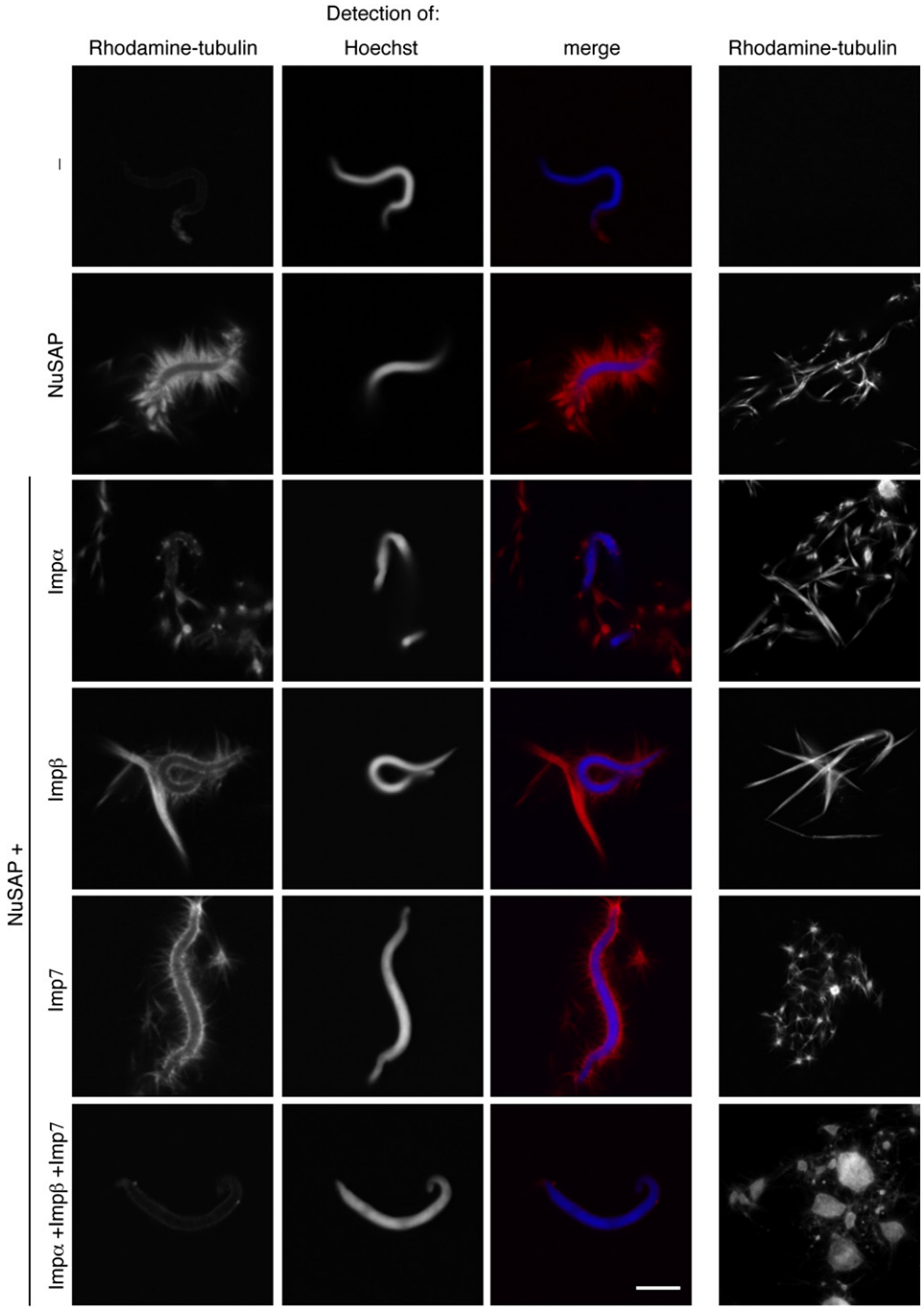

Figure 4. Impα, Impβ, and Imp7 Suppress the Ability of NuSAP to Generate a Local Crowding of MTs at Chromatin

Isolated sperm chromatin was incubated in BRB80 buffer with 0.2 μM recombinant NuSAP, 15 μM pure Rhodamine-labeled tubulin, and 3 μM of the indicated importins. The right column shows the configuration of tubulin in the same sample as in the left panels but at distance from chromatin. The reaction was incubated for 15 min at 37°C before it was fixed and analyzed by confocal microscopy. The scale bar represents 10 μm.

assembly and guides MT attachment to the chromosomes [1, 7, 9–16]. A RanGTP gradient may indeed explain the local activation of Ran-regulated spindle factors in the vicinity of chromatin. However, diffusible RanGTP probably surrounds the chromosomes in a volume that is large relative to the size of both kinetochores (around 100 nanometers) and even entire chromosomes [17–21]. Therefore, precise MT targeting to chromosomes and kinetochores probably necessitates nondiffusible activities that directly produce MTs at the chromosomes or rapidly attach them there. We propose that MT-binding agents, such as NuSAP, that are directly immobilized on chromatin provide a critical missing link to allow precise targeting of the MTs to the vertebrate chromosomes during spindle self-organization.

Chromokinesins share the ability to distribute along the chromosome arms and to interact with both DNA and MTs [22–28]. What is the difference between the function of chromokinesins and NuSAP at the spindle? Spindles that form upon NuSAP depletion show a reduced density of chromatin-proximal MTs and are frequently accompanied by misaligned chromosomes [2, 3]. This suggests that NuSAP functions to stabilize or assemble chromosome-associated MTs, or both, as well as to stabilize the interaction between chromosomes and spindle MTs. In contrast, inhibition of chromokinesins in HeLa cells or *Xenopus* egg extract results in the spindle's failure to organize MTs according to their polarity [29] or to establish and maintain chromosome positioning at the metaphase plate [26, 28]. Importantly, the initial concentration of MTs around chromatin at early steps of spindle assembly remains largely unaffected when chromokinesins are absent [26, 28, 29]. Thus, NuSAP and chromokinesins appear to perform nonredundant and qualitatively distinct functions in spindle assembly. We propose that NuSAP acts to establish and retain high numbers of MTs at the chromosomes, whereas chromokinesins sort these chromosome-proximal MTs according to their polarity [29] and use them as tracks to correctly position the chromosomes [22, 26, 28].

Clearly, chromokinesins and other motor proteins will only be able to organize MTs efficiently with respect to chromosomes if NuSAP-MT interactions are transient; irreversible association of MTs with chromosome arms would be deleterious to spindle assembly and chromosome movement. The exact nature of NuSAP-MT interactions is currently unknown, but they are probably dynamic and are possibly subject to disruption by other MAPs, motors, and regulators. One possible mode of interaction is that NuSAP could crosslink chromosomes to the spindle in a mobile fashion by "skating" along the microtubule lattice, comparable to a behavior that has recently been revealed for a microtubule-binding domain in dynactin [30]. This specialized type of microtubule interaction could tether microtubules to chromatin without obstructing chromokinesin activity while providing a sustained link between chromosomes and the spindle.

Supplemental Data

Supplemental Data include additional Discussion, Experimental Procedures, and four figures and are available online at http://www.current-biology.com/cgi/content/full/17/3/230/DC1/.

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