

Microtubule organisation



Our overall goal is to reach a comprehensive understanding of how cells generate, maintain, and remodel the microtubule cytoskeleton during cell cycle and cell differentiation. Defects in the structure and function of the microtubule cytoskeleton are linked to cancer and certain developmental disorders. To ensure proper microtubule organisation, cells control where and when microtubules are made. Microtubule organising centres, such as the animal centrosome, participate in this regulation by providing sites that nucleate the polymerisation of new microtubules. These sites are formed by interaction with a large, multi-subunit protein complex, the γ -tubulin ring complex, which functions as microtubule nucleator. Microtubule nucleation sites provide a unique molecular environment, not only for the control of microtubule nucleation but also for regulating microtubule behaviour, and thus are central to our understanding of microtubule organisation. However, the exact molecular composition of microtubule nucleation sites and the spatio-temporal regulation of their assembly are poorly understood. To address this issue, we study centrosomal and non-centrosomal microtubule nucleation pathways in several cell types by identifying the molecular players and by characterising their function and regulation *in vitro* and *in vivo*.

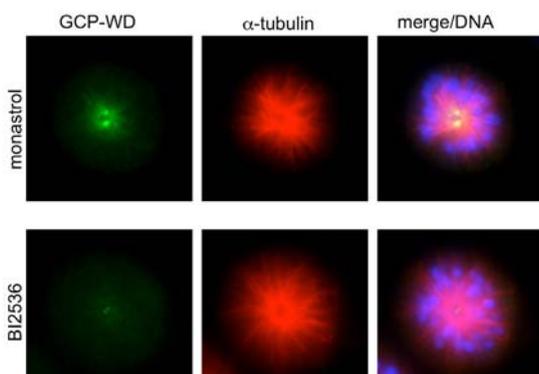


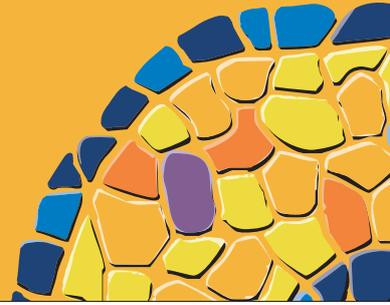
Figure 1. *Plk1* activity is required for localisation of the γ -tubulin targeting factor GCP-WD to centrosomes and spindle microtubules. HeLa cells treated with monastrol or the *Plk1* inhibitor BI2536 were fixed and immunostained for GCP-WD and α -tubulin to label microtubules. DAPI was used to label DNA. Both treatments produce monopolar spindles. Monastrol treatment, however, does not affect centrosome accumulation of GCP-WD, whereas BI2536 almost completely blocks recruitment of GCP-WD to the centrosomes.

In proliferating cells, the major microtubule organising centre (MTOC), the centrosome, organises the mitotic spindle. Numerical and functional centrosome abnormalities including aberrant size and shape, and microtubule (MT) nucleation activity are frequently found in cancer cells. Such centrosomal defects can impair proper spindle assembly and function and result in genomic instability. In addition to centrosomal MT nucleation, our previous work and studies by others show that proper mitotic spindle assembly requires MT nucleation from non-centrosomal sites. Non-centrosomal nucleation pathways are poorly characterised, but might be crucial for rapidly dividing cancer cells. These pathways may therefore provide great potential for future anti-cancer therapies.

Centrosomal microtubule nucleation in mitosis

The centrosome comprises a pair of barrel-shaped centrioles surrounded by a dense proteinaceous matrix, the pericentriolar material (PCM). The nucleation of MT polymerisation occurs within the PCM and requires the recruitment of γ -tubulin ring complexes (γ TuRCs) from the cytoplasm. These complexes contain γ -tubulin, a paralogue of α - and β -tubulin that is not incorporated into the MT polymer but functions as MT nucleator. We have previously shown that the interaction of γ TuRCs with centrosomes is mediated by a protein named GCP-WD (also

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known as NEDD1) (Lüders *et al*, 2006; Haren *et al*, 2006). When cells prepare for mitosis in late G2 phase of the cell cycle, size and microtubule nucleating activity of the duplicated centrosomes increase. This is accomplished by the recruitment of additional PCM to the centrosomes, including proteins involved in microtubule nucleation and organisation, such as γ -tubulin. This process, also termed centrosome maturation, is critical for the function of centrosomes as MTOCs in mitosis, and depends on the activity of mitotic kinases such as Polo-like kinase 1 (Plk1). Interference with Plk1 function by RNAi or specific inhibitors prevents the recruitment of γ -tubulin to mitotic centrosomes, thereby essentially inactivating their MT nucleation activity, and impairing bipolar spindle formation. Plk1 inhibitors are currently being studied in clinical trials as potential agents for cancer therapy. To date, a Plk1 substrate that controls γ -tubulin recruitment in a phosphorylation-dependent manner has not been identified.

We discovered that Plk1 associates with GCP-WD, the γ -tubulin targeting factor, and Plk1 activity contributes to mitotic phosphorylation of GCP-WD (Haren *et al*, 2009, in preparation). Plk1 depletion or inhibition revealed that accumulation of γ -tubulin at centrosomes is regulated by controlling the levels of centrosomal GCP-WD (Figure 1). Surprisingly, GCP-WD mutants that are defective in Plk1 binding and phosphorylation still accumulate at mitotic centrosomes and recruit γ -tubulin. At present, we are studying whether the Plk1-dependent phosphorylation of GCP-WD serves other functions and whether it affects spindle assembly and function.

Interestingly, our studies revealed that Plk1 also controls the recruitment of other PCM proteins implicated in centrosomal γ -tubulin attachment. Our results support a model in which Plk1-dependent recruitment of γ -tubulin to mitotic centrosomes is regulated upstream of GCP-WD, and involves multiple PCM proteins and potentially multiple Plk1 substrates (Haren *et al*, 2009, in preparation). Our next goal is to identify these substrates and, through phospho-mutant analysis, shed light on the Plk1-dependent pathway that triggers centrosome maturation and γ TuRC recruitment.

To study the role of Plk1 and other mitotic kinases in the regulation of microtubule nucleation during spindle formation, we have initiated a collaboration project with Carme Caelles and Joan Roig, researchers in the Molecular Medicine Programme at IRB Barcelona.

In addition to the general importance of this pathway for proliferating cells, two of the proteins involved in γ -tubulin recruitment to centrosomes have recently been implicated in micro-

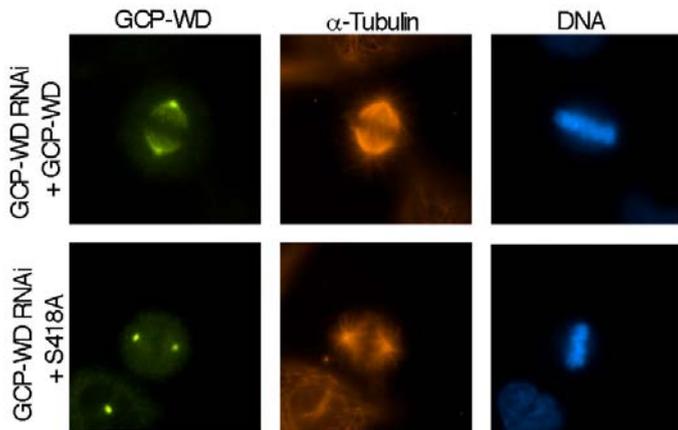


Figure 2. Mitotic phosphorylation of GCP-WD is required for spindle targeting but not for centrosome targeting. HeLa cells were cotransfected with a plasmid expressing shRNA targeting endogenous GCP-WD and plasmids expressing either RNAi-insensitive GCP-WD-MycHis or the phosphorylation mutant GCP-WD S418A-MycHis. Cells were stained with anti-Myc and anti- α -tubulin antibodies and DAPI to visualise DNA.

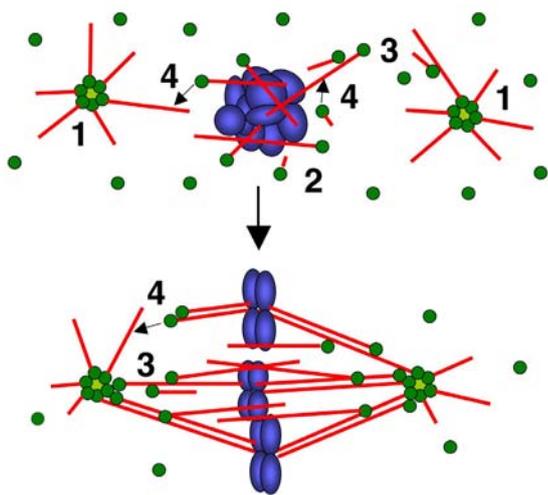


Figure 3. 'Amplification model' for the function of the γ TuRC in spindle assembly. Microtubules are nucleated by γ TuRC at the two centrosomes (1) and near the mitotic chromatin (2). A third pathway depends on γ TuRC bound to the sides of existing spindle microtubules (3). This pathway 'amplifies' the process of spindle assembly by nucleating additional microtubules where needed, within the forming spindle. Correct orientation is achieved by directional nucleation, or by motor-mediated orientation soon after nucleation. Minus end-bound γ TuRC facilitates the capture of free microtubules by directing their ends to the sides of other microtubules (4). γ TuRCs are depicted in green, microtubules in red, and chromosomes in blue.

cephaly, a condition associated with certain neurodevelopmental disorders. Mutations in the genes of Cep215/Cdk5Rap2 and pericentrin cause primary microcephaly and Seckel syndrome, respectively. In both cases, affected individuals have abnormally small brains and show mental retardation. It has been speculated that microcephaly is caused by defects in the proliferation of neuronal progenitor cells, but mechanistic insight is still missing. We hope that our studies will contribute to a better understanding of these diseases.

Non-centrosomal microtubule nucleation in mitosis

γ TuRCs typically interact with MT minus ends at centrosomes, where they nucleate and stabilise MTs. However, a large number of γ TuRCs are also found in the soluble fraction of the cytoplasm and associated with mitotic spindle microtubules. In mitosis, these γ TuRCs participate in non-centrosomal MT nucleation pathways, such as the chromatin-mediated nucleation pathway, which is controlled by the small GTPase Ran. Recently, we discovered that mitotic phosphorylation of GCP-WD at a Cdk1 consensus phosphorylation site targets the protein to a subset of spindle MTs (Figure 2). Mutation of this phosphorylation site interferes specifically with the recruitment of γ TuRC to spindle MTs.

The lack of spindle-associated γ TuRCs results in defective spindles that assemble less efficiently and have a lower density of MTs, but does not seem to affect centrosomal or chromatin-mediated nucleation (Lüders *et al*, 2006). On the basis of these findings, we proposed that spindle-bound γ TuRC is required to nucleate additional MTs within the spindle to allow proper spindle formation. Studies in *Drosophila* have identified components of a protein complex termed augmin, which function upstream of GCP-WD in mediating the association of γ TuRCs with mitotic spindle microtubules (Goshima *et al*, 2008). Together, these studies support a model in which γ TuRCs are recruited to the sides of pre-existing spindle MTs to nucleate additional MTs, thereby "amplifying" MT nucleation and promoting spindle formation (Lüders and Stearns, 2007; Figure 3).

We have started to characterise this new pathway in human cells. One of our goals is to achieve a molecular understanding of how these novel, non-centrosomal MT nucleation sites are assembled. Using a cell line that stably expresses γ -tubulin fused to photoactivatable GFP, we demonstrated that, compared to the interaction of γ -TuRCs with centrosomes, the interaction of γ TuRCs with spindle microtubules is much more dynamic (Archinti, Lacasa and Lüders, in preparation), thereby hindering direct analysis in living cells. We are using bioinformatics, yeast two-hybrid screening, biochemical and RNAi-based approaches to identify the components of this pathway. We have already identified several candidate genes, which we are currently analysing for a role in non-centrosomal MT nucleation within the spindle. In addition, we have generated tools, such as stable cell lines and antibodies, for the analysis of this pathway *in vitro* and *in vivo*.

Microtubule nucleation during differentiation

During the differentiation of muscle cells, myoblasts fuse to form multi-nucleated myotubes. This process involves a reor-

ganisation of the MT network from a radial, centrosome-based MT array to an elongated array composed of parallel MTs in myotubes. Interestingly, centrosomes degenerate during myotube formation and differentiation. Several PCM proteins, including γ -tubulin, redistribute to the surface of the nuclear envelope, which functions as MTOC. Whether the nuclear envelope-associated MTOC is critical for myotube formation or differentiation is not known.

Using lentivirus-mediated infection and expression of shRNA to deplete endogenous GCP-WD in mouse C2C12 cells, an *in vitro* model for muscle cell differentiation, we found that, in contrast

to its role in centrosome attachment, the γ -tubulin targeting factor GCP-WD is not required for nuclear envelope localisation of γ TuRC. Moreover, a few days after differentiation is initiated, GCP-WD expression is down-regulated, thereby suggesting that its function is not required in differentiated muscle cells. γ -tubulin expression remains relatively constant, suggesting that the γ TuRC participates in MT organisation throughout the differentiation process. To address this question, we have also established conditions for efficient RNAi-mediated depletion of γ -tubulin in muscle cells. These studies will provide the first insight into the role of non-centrosomal MT nucleation pathways in the reorganisation of the MT cytoskeleton during cellular differentiation.

SCIENTIFIC OUTPUT

Research networks and grants

Microtubule organizing centers and microtubule nucleation in mitosis (MTOC function)

European Commission, PEOPLE-2007-4-3-IRG (2008-2012)

Principal investigator: Jens Lüders

Regulation of microtubule nucleation through phosphorylation

Carme Caelles and Joan Roig, Molecular Medicine Programme, IRB Barcelona (Barcelona, Spain)

Collaborations

Recruitment of γ -tubulin complexes to mitotic centrosomes

Andreas Merdes and Laurence Haren, Institut de Sciences et Technologies du Médicament de Toulouse, Centre National de la Recherche Scientifique/Pierre Fabre (Toulouse, France)