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Wnt-mediated protein stabilization ensures proper mitotic microtubule assembly and chromosome segregation

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Abstract

Wnt signaling stimulates cell proliferation by promoting the G1/S transition of the cell cycle through β-catenin/TCF4-mediated gene transcription. However, Wnt signaling peaks in mitosis and contributes to the stabilization of proteins other than β-catenin, a pathway recently introduced as Wnt-dependent stabilization of proteins (Wnt/STOP). Here, we show that Wnt/STOP regulated by basal Wnt signaling during a normal cell cycle is required for proper spindle microtubule assembly and for faithful chromosome segregation during mitosis. Consequently, inhibition of basal Wnt signaling results in increased microtubule assembly rates, abnormal mitotic spindle formation and the induction of aneuploidy in human somatic cells.

Introduction

Canonical Wnt signaling plays a crucial role in embryonic, somatic and stem cells and is required for normal development, organogenesis and tissue regeneration. Moreover, its deregulation has been implicated in human diseases, most notably in cancer, but also in neurodegenerative diseases [1–3]. The key effector of Wnt signaling is β-catenin, which acts together with its co-factor TCF4 as a Wnt-activated transcription factor [1]. In the absence of Wnt signaling, β-catenin is subject to ubiquitin–proteasome-mediated protein degradation, which requires its prior phosphorylation mediated by a cytoplasmic destruction complex comprising glycogen synthase kinase 3 (GSK3), casein kinase 1 (CK1), adenomatous polyposis coli (APC) and Axin-1 [1]. Upon Wnt ligand binding to frizzled receptors (Fzd) and their co-receptors LRP5 and LRP6, the intracellular domain of LRP6 is phosphorylated by GSK3 and CK1. This is followed by receptor internalization and the formation of so-called signalosomes containing Fzd, phospho-LRP6, disheveled (Dvl), GSK3 and Axin-1 [4–6]. Under this condition, GSK3 is inhibited directly by phospho-LRP6 and by sequestration into multivesicular endosomes [7–10]. This prevents β-catenin phosphorylation and, thus, its degradation by the cytoplasmic destruction complex. Accumulated β-catenin can then translocate into the nucleus where it binds TCF4 triggering gene expression [1]. Among the various *bona fide* target genes of β-catenin is *AXIN2*, a component of the cytoplasmic destruction complex, and *C-MYC* [11], which mediates the expression of *CYCLIN-D,* a key driver of the G1/S transition of the cell cycle [12]. Thus, Wnt signaling can stimulate cell proliferation at G1/S via triggering gene expression in a β-catenin-dependent manner. Interestingly, when cells enter mitosis, LRP6 is phosphorylated by a mitosis-specific cyclin-dependent kinase (CDK14-cyclin Y), indicating that endogenous Wnt signaling is under cell cycle control peaking at G2/M [13,14]. In line with this, protein levels of β-catenin and Axin-2 also reach their maximum levels at G2/M [15,16]. However, a physiological role for this basal and cell cycle-regulated Wnt signaling has not been revealed so far.

Intriguingly, most recently it was found that Wnt signaling can contribute to the stabilization of proteins other than β-catenin [9,17]. In particular, this occurs at G2/M and is now referred to as Wnt-dependent stabilization of proteins (Wnt/STOP) [18]. However, this novel role of Wnt signaling is yet poorly understood and a specific role for the entry into or for the progression of mitosis has not been identified so far.

In addition to that, several Wnt signaling proteins such as APC, Axin-2, Dvl and β-catenin have been implicated as direct regulators of mitosis [13,19]. For instance, APC together with Dvl localizes at the microtubule–kinetochore interface where they might contribute to proper microtubule binding to kinetochores [20–22]. This function seems to be independent of Wnt signaling. However, APC and Dvl2 also associate with the mitotic cell cortex where they might help to anchor astral microtubules to the cortex in order to ensure proper orientation of the mitotic spindle. This function also involves the Wnt receptor Fzd and its co-receptor LRP6 [21]. Furthermore, β-catenin and Axin-2 are present at mitotic centrosomes where they might be involved in centrosome function, microtubule nucleation and mitotic spindle assembly [23–25]. Thus, Wnt signaling as well as
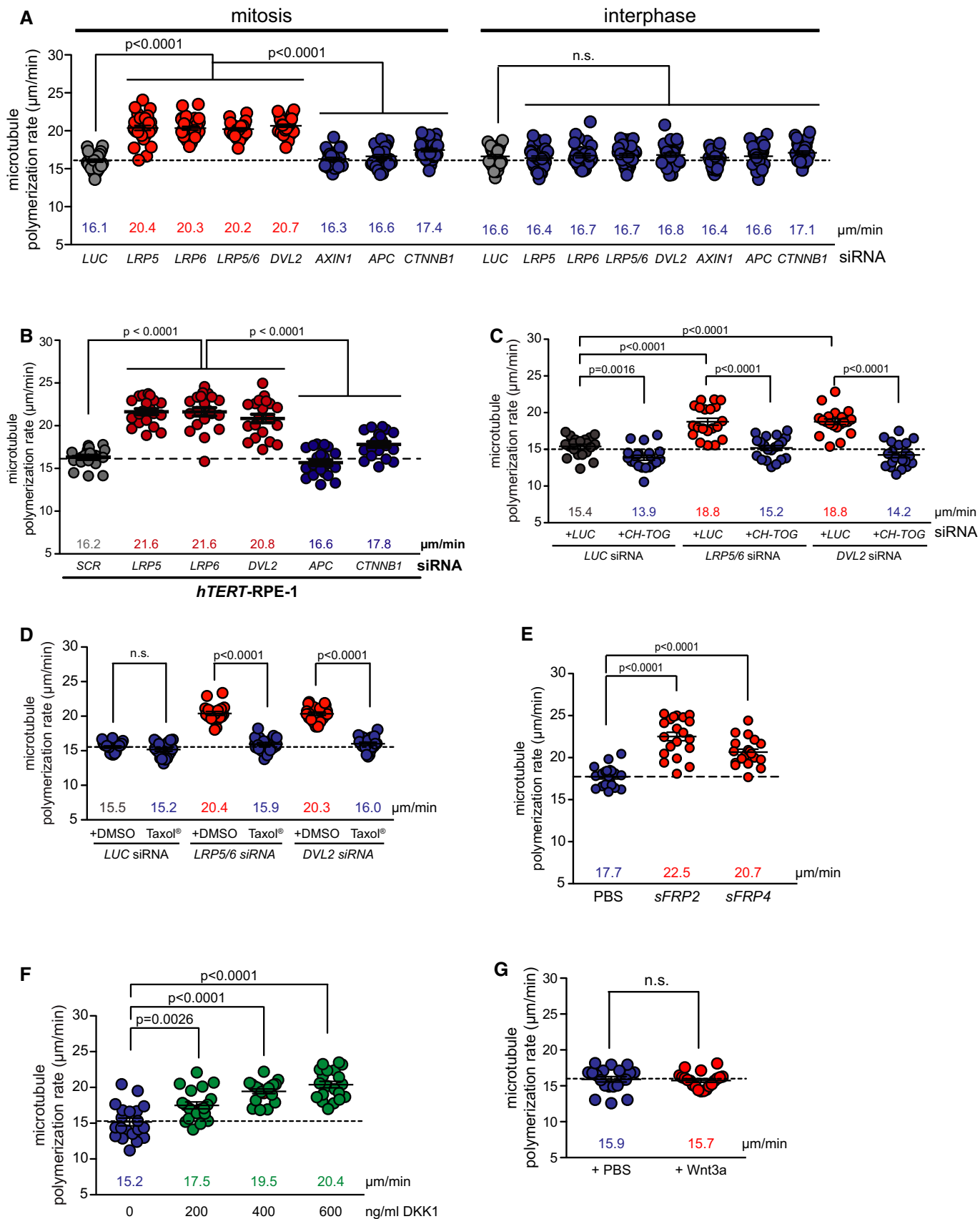
Figure 1.

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Figure 1. Loss of Wnt signaling causes an increase in microtubule plus end assembly rates during mitosis.

A   Measurements of microtubule plus end assembly rates in living HCT116 cells during mitosis or interphase after siRNA-mediated knockdown of different Wnt pathway components as indicated.

B   Microtubule plus end assembly rates in *hTERT*-RPE-1 cells during mitosis after the indicated siRNA transfections.

C   Mitotic microtubule plus end assembly rates in HCT116 cells with low expression of *LRP5/6* or *DVL2* and after knockdown of *CH-TOG/CKAP5*.

D   Mitotic microtubule plus end assembly rates in HCT116 cells after knockdown of *LRP5/6* or *DVL2* and in the absence or presence of 0.2 nM Taxol®.

E   Mitotic microtubule plus end assembly rates in HCT116 cells in response to treatment with 400 ng/ml sFRP2 or sFRP4.

F   Mitotic microtubule plus end assembly rates in HCT116 cells in response to treatment with DKK1.

G   Mitotic microtubule plus end assembly rates in HCT116 cells treated with 400 ng/ml of recombinant human Wnt3a for 24 h.

Data information: Scatter dot plots show average assembly rates (20 microtubules/cell; mean ± SEM; *t*-test; *n* = 30 cells in A, *n* = 20 cells in B–G).

particular Wnt signaling components appear to be involved in the regulation of mitosis, but the nature of their action remains largely elusive.

It is conceivable that the proper progression of mitosis is essential for faithful chromosome segregation and the generation of euploid progenitors in normal somatic cells. On the other hand, aneuploidy as a consequence of mitotic chromosome missegregation is often associated with human diseases including cancer and neurodegenerative diseases [26]. In particular, much effort has been undertaken to understand how chromosomes are missegregated in cancer cells, but the underlying mechanisms are still poorly understood [27]. Recently, we identified a key mechanism leading to perpetual chromosome missegregation and aneuploidy in human cancer cells [28]. In fact, we found that increased microtubule plus end assembly rates in mitosis are directly responsible for the generation of so-called lagging chromosomes during anaphase, which represent a common pre-stage of chromosome missegregation in somatic cells [28,29]. Thus, cells must ensure proper microtubule assembly rates during mitosis in order to maintain a stable karyotype. However, the molecular pathways that ensure proper microtubule plus end assembly during a normal mitosis are ill defined.

In our work presented here, we reveal a requirement for Wnt signaling during mitosis that is independent of canonical Wnt signaling for proper mitotic microtubule plus end assembly and for faithful chromosome segregation in human somatic cells.

Results and Discussion

Inhibition of basal Wnt signaling causes increased mitotic microtubule plus end assembly rates during mitosis

Our previous work established proper microtubule plus end assembly rates during mitosis as an essential determinant for proper mitotic progression and faithful chromosome segregation [28]. Therefore, we investigated a potential involvement of non-induced (= basal or baseline) Wnt signaling in this process. We transfected HCT116 and non-transformed human retinal pigment epithelial (hTert-RPE1) cells with siRNAs targeting different Wnt signaling components (Supplementary Fig S1A and B), which did not affect cell proliferation or cell cycle progression (Supplementary Fig S1C). Subsequently, we determined microtubule plus end assembly rates by tracking EB3-GFP fusion proteins [30] in living cells (Supplementary Fig S1D). Interestingly, we found that partial repression of *LRP5*, *LRP6* or *DVL2*, which led to inhibition of baseline Wnt signaling in the absence of additional Wnt treatment (Supplementary Fig S2A), triggered an

increase in microtubule plus end assembly rates in mitotic, but not in interphase cells (Fig 1A and B, Supplementary Fig S2B). In contrast, repression of *AXIN1* or *APC*, which activated Wnt signaling (Supplementary Figs S1A and B and S2A), did not alter microtubule assembly rates (Fig 1A and B). Interestingly, loss of β-catenin or TCF4 (Supplementary Fig S1A and E), which also inhibits Wnt activity (Supplementary Fig S2A), hardly affected mitotic microtubule assembly rates (Fig 1A and B, Supplementary Fig S1F) indicating a β-catenin/TCF4-independent function of Wnt signaling for proper microtubule dynamics in mitosis. It is of note that our former work showed that increased microtubule plus end assembly rates are not associated with alterations in other microtubule dynamics parameters per se [28].

Increased microtubule assembly rates can be efficiently suppressed by partial repression of *CH-TOG*, a microtubule polymerase involved in catalyzing the incorporation of α/β-tubulin subunits into the growing microtubule plus tip [31]. The same effect can be achieved by treatment of cells with sub-nanomolar concentrations of Taxol®, which stabilizes microtubule plus tips [28]. Both experimental approaches fully restored proper microtubule plus end assembly rates in cells with reduced expression of *LRP5/6* or *DVL2* (Fig 1C and D, Supplementary Fig S1G).

As an alternative approach to inhibit basal Wnt signaling, we treated cells with purified sFRP and DKK1 proteins [32] (Supplementary Fig S2C and D) and measured microtubule plus end assembly rates. In line with our first results, we found a significant increase in microtubule assembly rates upon sFRP- and DKK-mediated Wnt inhibition (Fig 1E and F). However, similar to repression of *APC* or *AXIN1*, hyper-activation of Wnt signaling by treatment with additional Wnt3a (Supplementary Fig S2E) did not affect microtubule assembly rates (Fig 1G). We conclude that basal Wnt signaling is required to ensure proper microtubule plus end assembly rates in mitotic cells. Thus, the regulation of microtubule dynamics during mitosis might represent an important function of basal and cell cycle-regulated Wnt signaling. Whether this novel mitotic function also involves the cell cycle-regulated and CDK14-cyclin Y-mediated mitotic phosphorylation of LRP6 [14] remains to be shown. Similarly, the role of multivesicular endosomes during mitosis that contribute to the inactivation of GSK3 in response to Wnt signaling merits future investigations [9,10].

Inhibition of Wnt signaling causes mitotic spindle defects and the generation of lagging chromosomes

Next, we investigated the consequences of increased microtubule assembly rates in response to the loss of Wnt signaling during an
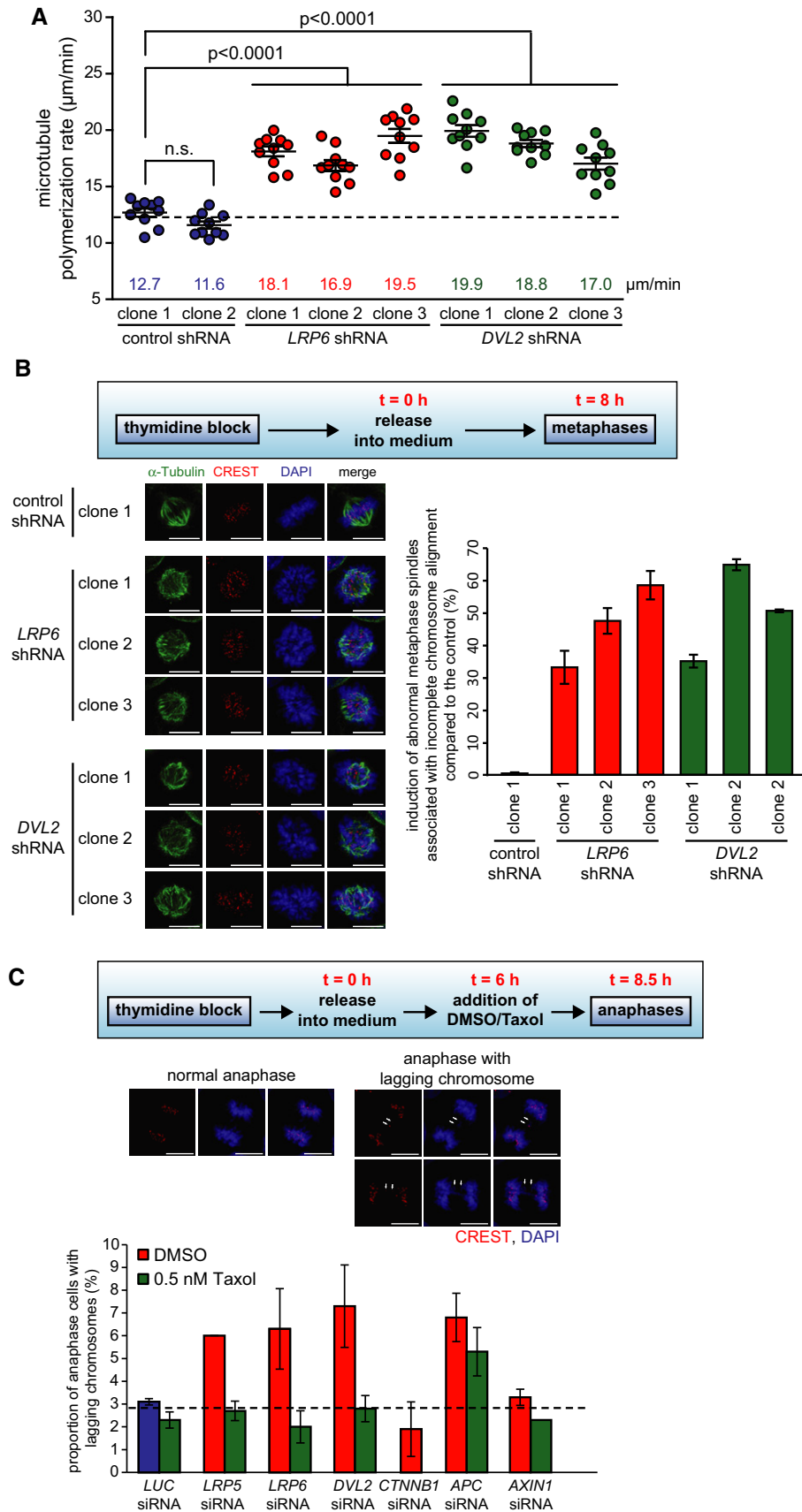
Figure 2.

◀ **Figure 2. Increased microtubule assembly rates in response to loss of Wnt signaling cause abnormal mitotic spindles and lagging chromosomes during anaphase.**

A Mitotic microtubule plus end assembly rates in different single cell clones derived from HCT116 cells stably expressing shRNAs targeting LRP6 or DVL2. Scatter dot plots show average assembly rates (20 microtubules/cell, mean ± SEM, t-test, n = 20 cells).

B Quantification of cells showing abnormal bipolar metaphase spindles. Cells were synchronized in metaphase as indicated. The graph shows the induction of abnormal spindles associated with incomplete chromosome alignment normalized to control cells (mean ± SD, n = 800–1,000 mitotic cells). Representative examples are shown (scale bar, 10 μm).

C Detection of lagging chromosomes in HCT116 cells transfected with siRNAs and synchronized in anaphase. Cells were treated with or without 0.5 nM Taxol®, and the proportion of cells exhibiting lagging chromosomes were quantified (mean ± SD, n = 371–711 anaphase cells). Representative examples are shown (scale bar, 10 μm).

unperturbed cell cycle. To this end, we generated cell lines stably expressing shRNAs targeting LRP6 or DVL2. Single cell clones were isolated (Supplementary Fig S3A and B), and live cell microscopy experiments verified an increase in mitotic microtubule plus end assembly rates in different cell clones (Fig 2A). Analyses of cells synchronized in metaphase (Fig 2B, outline) demonstrated an induction of abnormal metaphase spindles associated with incomplete chromosome alignment upon reduced expression of LRP6 or DVL2 (Fig 2B). Moreover, repression of LRP5, LRP6 or DVL2, but not of CTNNB1 or AXIN1, caused the generation of lagging chromosomes during anaphase (Fig 2C), which typically arise due to erroneous microtubule–kinetochore attachments [33] and represent a common pre-stage of chromosome missegregation in somatic cells [34]. Importantly, lagging chromosomes were greatly suppressed upon treatment with low doses of Taxol® (Fig 2C), which restores proper microtubule assembly rates (Fig 1D). Thus, these findings establish a causal relationship between the inhibition of basal Wnt signaling, an increase in mitotic microtubule assembly and the induction of chromosome missegregation. In contrast, we found that loss of APC caused lagging chromosomes, which were not suppressed by Taxol® treatment (Fig 2C). This clearly supports the notion that APC fulfills a role in chromosome segregation independent of microtubule plus end dynamics, most likely in mediating microtubule–kinetochore attachments as suggested earlier [22,35]. It is also of note that both APC and Dvl2 interact and have been implicated not only in mediating microtubule–kinetochore attachments, but also in regulating spindle orientation. Interestingly, in contrast to the microtubule–kinetochore attachments, their functions in spindle orientation appear to be dependent on Wnt signaling [21]. Since increased microtubule assembly rates can also cause spindle mis-orientation [28], it is possible that the role of Dvl2 in spindle orientation involves the regulation of microtubule plus end dynamics. On the other hand, our results showing that chromosome missegregation upon loss of DVL2 can be suppressed by low dose Taxol® treatment indicate that an additional role of Dvl2 in microtubule–kinetochore attachments is not responsible for the induction of aneuploidy. From these points, it is clear that further detailed investigations are needed in order to fully understand the various different functions of Wnt signaling components during mitosis.

Inhibition of Wnt signaling promotes aneuploidy in human somatic cells that is caused by increased microtubule plus end assembly rates

Next, we asked whether the mitotic abnormalities triggered by inhibition of basal Wnt signaling affect karyotype stability. Chromosome counting from metaphase spreads and interphase FISH

analyses were used to determine karyotype variability and the induction of aneuploidy in cell clones derived from HCT116 cells that maintain a near diploid karyotype [36,37]. Clearly, single cell clones stably expressing shRNAs targeting LRP6 or DVL2 and grown for 30 generations exhibited a more than twofold higher karyotype variability and an induction of aneuploidy than clones expressing control shRNAs while maintaining the same modal number of chromosomes (Fig 3A, Supplementary Fig S4A and B). To investigate whether the development of aneuploidy is indeed mediated by increased microtubule plus end assembly rates, we generated single cell clones in the absence or presence of low doses of Taxol®. In line with our experiments described above (Fig 1D), those single cell clones showed a full restoration of proper microtubule assembly rates upon treatment with Taxol® (Fig 3B). Importantly, the high karyotype variability and the generation of aneuploidy induced by repression of LRP6 or DVL2 were significantly suppressed by Taxol® treatment (Fig 3C and Supplementary Fig S4C). These results demonstrate that aneuploidy induction is indeed due to increased microtubule assembly triggered by inhibition of Wnt signaling.

Wnt-mediated protein stabilization is required for proper microtubule assembly during mitosis

Since our data indicate a role for β-catenin/TCF4-independent Wnt signaling at G2/M in the regulation of mitotic microtubule dynamics, we reasoned whether the recently identified Wnt/STOP pathway [18] might be involved in this particular regulation in mitosis. If so, one would expect that increased microtubule assembly rates induced by repression of LRP6 or DVL2 are restored to normal levels by concomitant inhibition of proteasome-mediated protein degradation or upon inhibition of the cytoplasmic Wnt destruction complex. Indeed, proteasome inhibition mediated by an 1-h MG132 treatment on mitotically synchronized cells fully restored normal microtubule assembly rates in HCT116 or RPE-1 cells with reduced levels of LRP6 or DVL2 (Fig 4A and B). Moreover, the concomitant repression of LRP6 or DVL2 together with APC or AXIN1 was sufficient to restore proper microtubule assembly rates in mitosis, both, in HCT116 and in RPE-1 cells (Fig 4C and D, Supplementary Fig S5A and B). To exclude the possibility that activation of β-catenin/TCF4 activity is involved in the suppression effect, we simultaneously repressed CTNNB1 in APC siRNA-treated cells, but found no reversal effect on the phenotype (Fig 4C, Supplementary Fig S5A). Finally, to provide a link between Wnt-mediated protein stabilization and chromosome missegregation, we also analyzed the generation of lagging chromosomes during anaphase. In fact, lagging chromosomes in LRP6- or DVL2-depleted cells were greatly suppressed when AXIN1 was also repressed (Fig 4E, Supplementary Fig S5C). Together, these results might point into
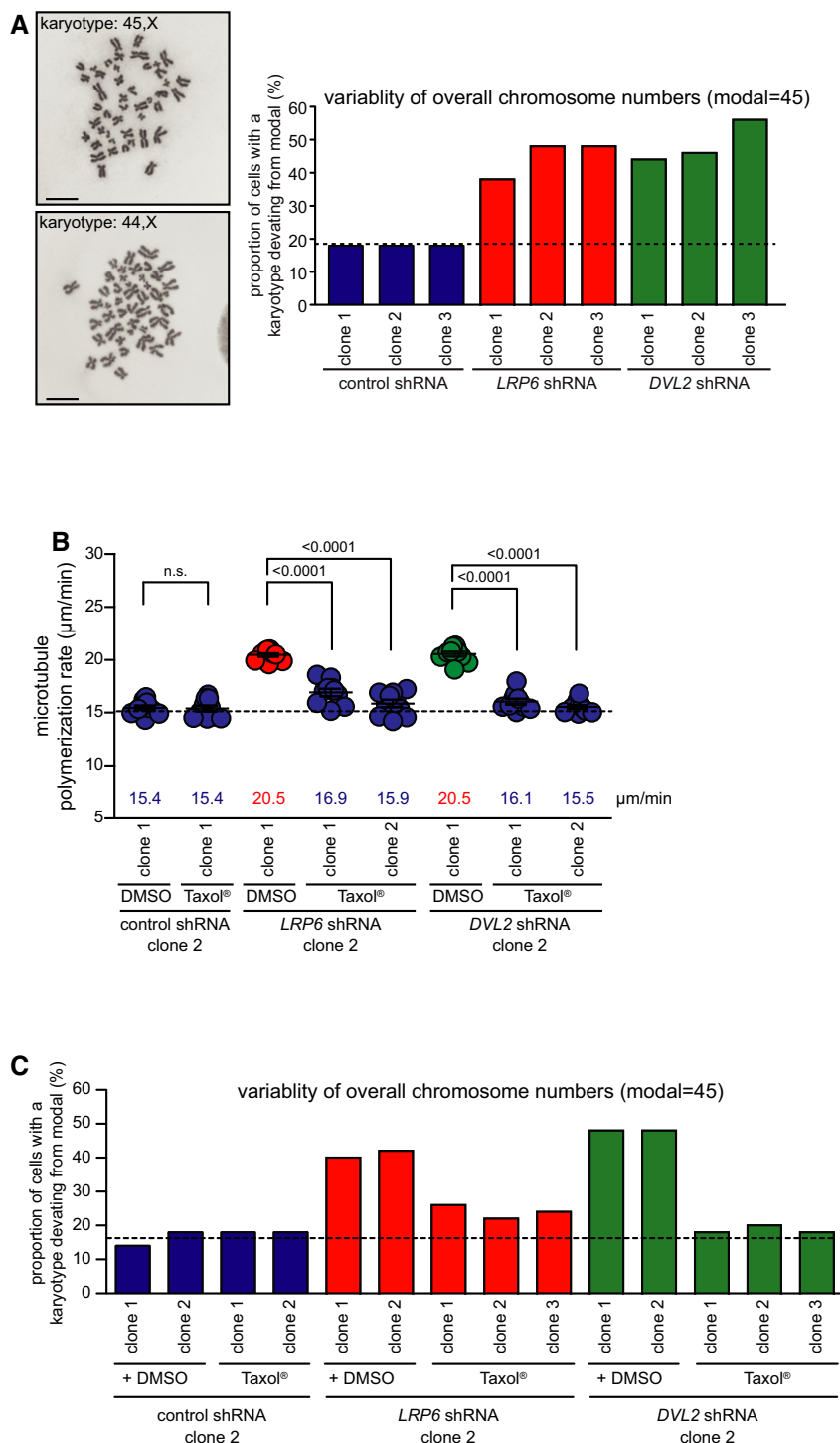
Figure 3. Loss of Wnt signaling induces aneuploidy mediated by increased mitotic microtubule assembly rates.

A   Chromosome number variability/aneuploidy of different single cell clones derived from HCT116 cells stably expressing control or shRNAs targeting *LRP6* and *DVL2* and grown for 30 generations. The graph shows the proportion of cells harboring a karyotype with chromosome numbers deviating from the modal (*n* = 50 cells; modal number = 45). Representative examples of metaphase chromosome spreads showing a normal (45,X) and an aneuploid (44,X) karyotype are shown.

B   Mitotic microtubule plus end assembly rates in different single cell clones derived from HCT116 cells stably expressing shRNAs targeting *LRP6* or *DVL2* and grown for 30 generations in the absence (DMSO) or presence of low doses of Taxol®. Scatter dot plots show average assembly rates (20 microtubules/cell, mean ± SEM, *t*-test, *n* = 10 cells).

C   Determination of the chromosome number variability/aneuploidy in different single cell clones grown in the absence or presence of Taxol® for 30 generations. The graph shows the proportion of cells harboring a karyotype with chromosome numbers deviating from the modal (*n* = 50 cells; modal number = 45).
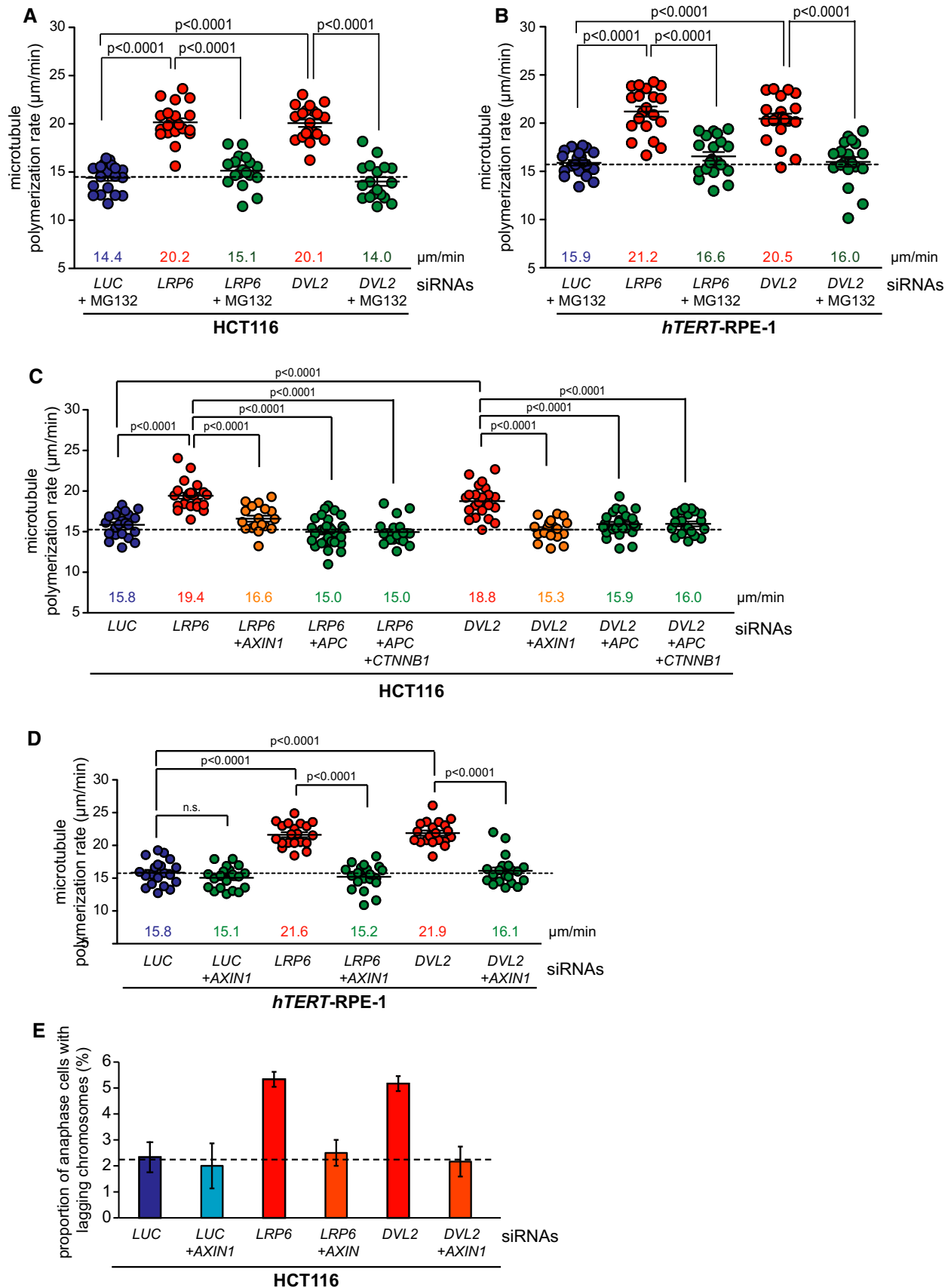
Figure 4.

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Figure 4. Wnt/STOP is required for proper microtubule plus end assembly and chromosome segregation during mitosis.

A   Mitotic microtubule plus end assembly rates in HCT116 cells with low expression levels of *LRP6* or *DVL2* in the presence or absence of the proteasome inhibitor MG132 (n = 18–20).
B   Mitotic microtubule plus end assembly rates in *hTERT*-RPE-1 cells with repressed *LRP6* or *DVL2* in the presence or absence of the proteasome inhibitor MG132 (n = 20 cells).
C   Mitotic microtubule plus end assembly rates in HCT116 cells with repressed *LRP6* or *DVL2* and concomitant repression of *AXIN1*, *APC* and/or *CTNNB1* (n = 18–29 cells).
D   Mitotic microtubule plus end assembly rates in *hTERT*-RPE-1 cells with low expression of *LRP6* or *DVL2* and concomitant repression of *AXIN1* (n = 20 cells).
E   Detection of lagging chromosomes in HCT116 cells with low expression of *LRP6* or *DVL2* and concomitant repression of *AXIN1*. The proportion of cells exhibiting lagging chromosomes during anaphase were quantified (mean ± SD, n = 600 anaphase cells).

Data information: In (A–D), 20 microtubules/cell; mean ± SEM; *t*-test .

the direction that Wnt-mediated stabilization of proteins other than β-catenin is required for the maintenance of proper microtubule assembly and for faithful chromosome segregation during a normal mitosis (see model in Supplementary Fig S6). Thus, our work might provide the first physiological role of Wnt/STOP during an unperturbed cell cycle. However, given the fact that apparently many different proteins are subject to Wnt/STOP [9,17,18], one might hypothesize that additional key regulatory steps during mitosis, other than microtubule plus end dynamics, might be controlled by the Wnt/STOP pathway. Therefore, it will be of paramount interest to systematically identify the Wnt/STOP target proteins. This will provide important new insights for a possible role of Wnt/STOP as a regulatory pathway for a normal cell cycle and, in particular, for the regulation of a normal mitosis and chromosome segregation.

Importantly, abnormal mitosis and the generation of aneuploidy are typical features of various human diseases, most notably cancer and neurodegenerative diseases [26]. Interestingly, those diseases have also been associated with de-regulated Wnt signaling. In human cancer, Wnt signaling is frequently over-activated and loss of APC is among the most frequent alterations in colorectal cancer [1]. In fact, hyper-active Wnt signaling and loss of APC might contribute to aneuploidy by interfering with spindle checkpoint function and by impairing proper microtubule attachments to kinetochores [14,20–22]. In our work, we also observed the generation of lagging chromosomes indicating chromosome missegregation upon loss of APC, but this was not dependent on an impairment of mitotic microtubule dynamics.

On the other hand, downregulation of LRP6 or induction of DKK1, both of which are associated with inhibition of Wnt signaling, has been implicated in pathogenesis of Alzheimer's disease (AD) where aneuploidy is highly prevalent [2,26,38]. It is intriguing that under these conditions, GSK3, which is under negative control of Wnt/STOP signaling, plays a central role for the hyper-phosphorylation of the microtubule-associated protein tau and for amyloid plaque formation [39]. Thus, it is tempting to speculate that hyper-phosphorylation of tau and production of Aβ peptide might be a consequence of loss of Wnt/STOP and could contribute to the induction of aneuploidy in AD brains.

Materials and Methods

Tissue culture

HCT116 cells were cultured at 37°C with 5% CO_2 in RPMI1640 containing 10% fetal calf serum, 1% glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin (Invitrogen, the Netherlands).

hTert-RPE-1 cells were cultured in DMEM/Nutrient F-10 Ham (1:1 mixture, PAA, Germany).

Transfections

DNA and siRNA transfections were carried out essentially as described [28]. Selection of single cell clones was carried out in medium containing 1 μg/ml puromycin. Details are provided in the Supplementary Methods.

Cell treatments

Where indicated, cells were treated with 400 ng/ml recombinant human Wnt3a (R&D Systems, USA) or with up to 600 ng/ml of recombinant human DKK1 or 400 ng/ml secreted Frizzled-related proteins 2 and 4 (sFRP2/sFRP4) (R&D Systems) for 24 h. To stabilize proteins during mitosis, cells were first synchronized in mitosis and then treated with 15 μM of the proteasome inhibitor MG132 (Enzo Life Sciences, Germany) for 1 h.

Western blotting

Western blotting was performed as described [28]. Details are provided in the Supplementary Methods.

Microscopy

Microscopy on fixed samples and determination of microtubule plus end assembly rates using tracking EB3-GFP protein in live cell microscopy experiments were carried out as described [28]. Details are provided in the Supplementary Methods.

Karyotype analyses

Karyotype analyses on single cell clones using chromosome counting and FISH analyses were performed as described [28]. Details are provided in the Supplementary Methods.

Statistical analyses

All data are shown as mean ± standard error of the mean (SEM or SD). Where indicated, unpaired Student's *t*-tests using the Prism software package, version 4, were applied. Assessment of data was performed blinded by different investigators in independent experiments.

Supplementary information for this article is available online: http://embor.embopress.org

Author contributions

AS, KN and NE planned and performed the experiments and analyzed the data. HB coordinated the project, assisted in planning the experiments and analyzed the data. AS, NE and HB wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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