ABSTRACT
Defects in mitosis can lead to aneuploidy, which is a common feature of human cancers. Spindle Assembly Checkpoint (SAC) controls fidelity of chromosome segregation in mitosis to prevent aneuploidy. The ubiquitin receptor protein Ubiquitin Associated and SH3 Domain Containing B (UBASH3B) was recently found to control SAC silencing and faithful chromosome segregation by relocating Aurora B kinase to the mitotic microtubules. Accordingly, loss and gain of function of UBASH3B have strong effects on mitotic progression. Downregulation of UBASH3B prevents SAC satisfaction leading to inhibition of chromosome segregation, mitotic arrest, and cell death. In contrast, increased cellular levels of UBASH3B trigger premature and uncontrolled chromosome segregation. Interestingly, elevated levels of UBASH3B were found in aggressive tumors. Therefore, we raised the question whether the oncogenic potential of UBASH3B is linked to its role in chromosome segregation. Here we show that in cancer cells expressing high levels of UBASH3B and SAC proteins, downregulation of UBASH3B, can further potentiate SAC response inducing mitotic arrest and cell death. Moreover, data mining approaches identified a correlation between mRNA levels of UBASH3B and SAC components in a set of primary patient tumors including kidney and liver carcinomas. Thus, inhibition of UBASH3B may offer an attractive therapeutic perspective for cancers.

Introduction
Cell division requires duplication of genetic material during S-phase, which is incorporated into chromosomes and equally partitioned between 2 daughter cells during mitosis. The assembly of the mitotic spindle and its attachment to the sister kinetochores allows for proper chromosome segregation. The kinetochore attachment is a stochastic process and is strictly controlled by the Spindle Assembly Checkpoint (SAC) (also known as the mitotic checkpoint), which ensures that chromosome segregation in anaphase does not take place before all chromosomes are properly aligned at the metaphase plate. The kinetochore attachment defects prevent SAC satisfaction and inhibit chromosome segregation, often leading to prolonged mitotic arrest and ultimately cell death. The SAC activity implies assembly of the Mitotic Checkpoint Complex (MCC), which includes mitotic arrest deficient 2 (MAD2) and Bub1-related kinase (BubR1) proteins that localize to the kinetochores of misaligned chromosomes and inhibit onset of the anaphase. Defects in SAC response or MCC complex are associated with premature and unequal chromosome segregation. Aberrant segregation of chromosomes could result in chromosomal instability (CIN), a persistently high rate of gain and loss of whole chromosomes, a phenomenon, which may in turn lead to a state of aneuploidy, or abnormal number of chromosomes in a cell. CIN cells are very often characterized by increased misorientation of chromosomes, which could be a result of defects in the sister chromatid cohesion, spindle assembly, and inability to resolve errors of microtubule-kinetochore attachments. Indeed, kinetochore microtubule attachments are abnormally stabilized in CIN positive cell lines. Overall, impaired expression of MCC components and altered SAC signaling could be associated with an increased risk of aneuploidy and tumor formation. For example, mutations in SAC protein BubR1 lead to mosaic variegated aneuploidy, an extremely rare syndrome associated with microcephaly, growth deficiency, and childhood cancer. Possible connections between aneuploidy and tumorigenesis have been demonstrated already over 100 years ago by Theodor Boveri. In the 1960s the karyogram of cancer tissues was shown to differ from that of normal tissues. Through systematic genetic analysis of many cancer tissues over the last decades, the presence of severe chromosome abnormalities was demonstrated in thousands of cancer samples. Consistent with these observations, aneuploidy and CIN were suggested to drive tumorigenesis. However, increased rates of aneuploidy and CIN were...
rather implicated in tumor suppression, suggesting that the level of damage might determine the oncogenic or oncosuppressor role of aneuploidy and CIN. Consequently, deregulated expression of chromosome segregation factors observed in various cancers stimulated development of therapeutic anti-mitotic drugs. However, inhibitors of microtubule function, the first generation of antimitotics currently used in therapies, are very nonspecific, which apparently leads to targeting of noncancer cells. Furthermore, none of the specific small molecule inhibitors of mitotic kinases entered into clinic routine so far. These rather disappointing clinical outcomes and low therapeutic potential of antimitotics might be due to a general cell toxicity. Thus, the current challenge is to identify the factors that control mitosis of cancer cells but at the same time have no effect on the normal noncancer cells. Since aneuploidy is the feature that distinguishes cancer from normal cells, the future drugs may need to exploit this tumor-specific vulnerability. The idea is that such drugs can overwhelm cancer cells with intolerable levels of chromosome instability, which are not compatible with cell survival. Defects in cell cycle regulation, chromosome cohesion, dynamics of kinetochore–microtubule attachment can all lead to chromosome instability in cancer cells. Interestingly, in many cancer cells the SAC is weaker but not absent, which is related to chromosome instability-driving malignancy and at the same time secures the cancer cells from acquiring too many errors in chromosome segregation which impacts cell viability. It was therefore suggested that more severe inactivation of SAC components may cause a higher level of aneuploidy that exceeds the adaptation capacity of cancer cells.

In accordance with this, prolonged mitotic progression that often leads to mitotic catastrophe and cell death is a desired outcome for targeting cancer cells. Indeed, as compared with normal cells, cancer cells appear to be more sensitive to mitotic catastrophe. This suggests that upregulation of SAC response resulting in a prolonged mitosis and mitotic catastrophe should also be exploited as a potential anticancer therapeutic strategy. In particular, the regulators but not the core components of SAC might represent the attractive therapeutic targets, as such regulators are preferentially expressed in cancer cells and promote tolerable levels of aneuploidy and survival.

Our recent findings show that UBASH3B is the ubiquitin receptor protein for mitotic kinase Aurora B, which drives Aurora B recruitment to the microtubules in cancer cells. Spatiotemporal regulation of Aurora B localization plays a crucial role in the control of mitotic progression and SAC function. Thus, UBASH3B is a negative SAC regulator that might be specifically used by cancer cells to promote their propagation and survival. Levels of UBASH3B protein were shown to be elevated in highly aggressive breast and prostate cancers, promoting malignant growth, invasion, and metastasis. We hypothesize that downregulation of UBASH3B in cancer cells will prevent SAC satisfaction, induce mitotic arrest, and promote cancer cell death.

Results

During mitosis all chromosomes have to be attached to the mitotic spindle before they are segregated to the opposite poles during anaphase. Our recent findings in cervical cancer-derived HeLa cells show that ubiquitin receptor protein UBASH3B plays a critical role in this process. Upon microtubule attachment UBASH3B forms a complex with the microtubule motor protein Mitotic kinesin-like protein 2 (MKlp2) and drives relocalization of Aurora B kinase from chromosomes to spindle microtubules. UBASH3B acts in concert with MKlp2 motor protein allowing for dynamic Aurora B localization and microtubules targeting, which controls the timing and fidelity of chromosome segregation and Spindle Assembly Checkpoint (SAC). Downregulation of UBASH3B levels leads to the loss of the centromeric localization of Aurora B and its accumulation on the chromosome arms, impairing kinetochore functions of Aurora B. SAC response is high and cells arrest in mitosis without chromosome segregation, causing cell death. High levels of UBASH3B are sufficient to induce relocalization of centromeric Aurora B to microtubules, a signal that silences SAC and induces premature and uncontrolled chromosome segregation, which may lead to aneuploidy.
microtubules. This event satisfies SAC signaling and triggers chromosome segregation (Fig. 1A). Downregulation of UBASH3B leads to the accumulation of Aurora B on chromosomes, thus preventing SAC satisfaction and inducing mitotic arrest and apoptosis (Fig. 1B). In contrast, overexpression of UBASH3B drives premature recruitment of Aurora B to microtubules, SAC silencing, erroneous chromosome segregation, and aneuploidy (Fig. 1C).

**Figure 2.** (For figure legend, see page 4).
Since high levels of UBASH3B were detected in highly aggressive breast and prostate cancers,\textsuperscript{18} we therefore wondered whether different cancer cells can utilize UBASH3B to override SAC. If this is the case, depletion of UBASH3B is expected to cause a mitotic delay and accumulation of cells in preanaphase stages. HeLa cells are characterized by high levels of SAC due to increased expression of BubR1.\textsuperscript{20} Likewise, adenocarcinoma-derived MDA-MB-231 cells express high MAD2 and BubR1 levels,\textsuperscript{20} suggesting the presence of a strong SAC response (Fig. 2A). Interestingly, and similar to HeLa cells,\textsuperscript{17} UBASH3B knockdown in MDA-MB-231 cells markedly increased numbers of prometa- and metaphase-like cells indicating a delay in mitosis (Fig. 2B and C). In contrast, in UBASH3B-negative colorectal adenocarcinoma-derived Colo 320DM (human Dukes’ type C, colorectal adenocarcinoma with double minutes) cell line the numbers of preanaphase cells were not altered upon downregulation of UBASH3B. Moreover, epidermoid carcinoma-derived A431 cells, characterized by low levels of SAC components did not increase preanaphase cell numbers upon UBASH3B knockdown (Fig. 2C), in line with SAC-dependent mechanism of UBASH3B-mediated mitotic progression. Importantly, protein levels of SAC components were not affected by depletion of UBASH3B, which however efficiently reduced the protein levels of UBASH3B by at least 70% in all analyzed cell lines (Ref.\textsuperscript{17}, Fig. 2B and data not shown). These observations suggest that mitotic progression in UBASH3B-expressing cancers characterized by strong SAC is sensitive to alterations of UBASH3B levels. To test this hypothesis, we have analyzed the level of cell death in the four cancer cell lines (HeLa, MDA-MB-231, Colo 320DM, A431). Interestingly, downregulation of UBASH3B induced approximately twofold increase in the death rate in HeLa and MDA-MB-231 cell lines but not in Colo 320DM and A431 cells (Fig. 2D) as compared with the corresponding control siRNA treated cells. To further corroborate these findings, we have analyzed the strength of the SAC response in the four cancer cell lines and in the primary fibroblasts (F31, R5). Interestingly, strong SAC response and high numbers of mitotic figures following treatment with the microtubule stabilizing agent paclitaxel were identified in HeLa and MDA-MB-231 in contrast to Colo 320DM, A431, and F31, R5 cells (Fig. 2E). Accordingly, the levels of SAC protein BubR1 were found low in the 2 primary cell lines (Fig. 2E). Importantly, despite high levels of UBASH3B found in the primary fibroblasts, their treatment with control or UBASH3B siRNAs did not lead to any differences in the cell death rate (Fig. 2F). These preliminary correlative findings suggest that cancer cells with a strong SAC response and expressing high levels of SAC as well as UBASH3B are specifically vulnerable to reduction of UBASH3B levels. Thus, downregulation or inhibition of UBASH3B in these cancer cells could be a mechanism to interrupt mitosis in tumors and serve as a novel therapeutic avenue in cancer treatment.

To further explore therapeutic perspectives of UBASH3B-mediated SAC silencing and to identify targetable cancer types, we analyzed the relative mRNA expression levels of UBASH3B as well as SAC components MAD2 (MAD2L1) and BubR1 (BUB1B) in different cancers. To this end we performed expression correlation analyses using data from the The Cancer Genome Atlas (TCGA) database. The positive correlation between MAD2L1 and BUB1B expression was statistically significant in all the tumors analyzed (Fig. 3A). A positive correlation between UBASH3B and BUB1B expression was statistically significant in Luminal B breast cancer, melanoma, pancreatic adenocarcinoma, kidney renal cell carcinoma, and liver hepatocellular carcinoma, while a positive correlation between UBASH3B and MAD2L1 expression was statistically significant in melanoma, pancreatic adenocarcinoma, kidney renal cell carcinoma, and liver hepatocellular carcinoma. In particular, kidney renal cell carcinoma and liver hepatocellular carcinoma tissue samples showed the strongest positive correlation in the mRNA expression levels of the three genes (Fig. 3B and C). This analysis suggests that these two cancer types may possess the molecular profiles suitable for therapeutic targeting of UBASH3B.

**Discussion**

Aneuploidy is one of the hallmarks of tumorigenesis. Aneuploidy observed in human cancers may arise from the defects in the mitotic checkpoint, which normally protects the cells from chromosome missegregation.\textsuperscript{21} Cancer cells may override the SAC either by downregulation of expression levels of the SAC components or their mutations. Indeed, defects in the mitotic checkpoint signaling are frequently observed in different human cancers.\textsuperscript{22,24} However mutations in the SAC genes have been rarely reported as the majority of them is very likely to be nonviable.

Here we propose that UBASH3B can indirectly contribute to the mitotic checkpoint defect by triggering the relocalization event of the essential mitotic kinase and SAC regulator Aurora B.\textsuperscript{17} Interestingly, sustained kinase activity of Aurora B by overexpression of Bub1 kinase induced aneuploidy and formation of various tumors in mice.\textsuperscript{23} Likewise, high expression of Aurora B promoted aneuploidy and tumor development in a xenograft model.\textsuperscript{26}

We believe that UBASH3B-mediated mechanism can be adopted by cancer cells, which due to high rates of proliferation accumulate chromosomal abnormalities that under normal circumstances prevent SAC satisfaction and lead to apoptosis.
Figure 3. Correlation analysis of mRNA expression of UBASH3B and SAC factors. (A) Spearman’s rank correlation coefficient ($r$) was calculated to analyze the relationship between normalized mRNA expression levels of SAC components (MAD2L1 = MAD2, BUB1B = BubR1), and UBASH3B in several human tumors using expression data from the TCGA database. The results were considered statistically significant (indicated in bold) when $P < 0.05$. The number of available patients is indicated. Note that kidney renal cell carcinoma and liver hepatocellular carcinoma tissue samples (red) show the strongest positive correlation in the mRNA expression levels of the three genes. (B and C) Scatter plot of UBASH3B, MAD2L1, and BUB1B expression correlation analysis for kidney renal cell carcinoma (B) and liver hepatocellular carcinoma (C).
These cancers may induce expression of UBASH3B to promote SAC silencing and induce aneuploidy and CIN in a manner that is compatible with their survival. It is particularly intriguing that downregulation of UBASH3B in cancer cell lines with high levels of SAC components and strong SAC response leads to Aurora B mislocalization, further potentiation of SAC and accumulation of cells in preanaphase stages (Fig. 2B and C) and finally to cell death (Fig. 2D). On the other hand, overexpression of UBASH3B leads to SAC silencing in these cells (Ref.17 and data not shown). High levels of UBASH3B were observed in highly aggressive forms of breast and pancreatic cancers in humans and in mouse models, in which UBASH3B promoted metastasis.19 Our correlative studies with SAC components presented here suggest that high levels of UBASH3B can be observed in other cancer types, in particular hepatocellular carcinoma (HCC) and kidney renal cell carcinoma (RCC). Hepatocellular carcinoma (HCC) is associated with the chronic exposure to toxins, alcohol, or viral infection and is one of the most common causes of cancer-related death in the world. Moreover, aneuploidy is frequently observed in HCC and is implicated in carcinogenesis process.27–29 Some HCC cell lines also showed impaired SAC response and failed to arrest in mitosis following exposure to microtubule drugs.29 Likewise, the incidence of HCC recurrence after surgical resection was higher in patients with lost expression of SAC MAD1 protein.30 A high risk of kidney RCC is often associated with the von Hippel-Lindau (VHL) disease and alteration in the VHL gene is a frequent event in sporadic clear cell RCC. Interestingly, recent studies in the VHL-deficient renal carcinoma showed impaired mitotic checkpoint and aneuploidy due to reduced expression of MAD2 both in cells31 and in mice.32 Lowering levels of MAD2 can indeed weaken the SAC response in a manner that is compatible with the cellular survival but leads to tumor development.9 Future studies are needed to confirm if these two cancer types are also dependent on the high levels of UBASH3B and sensitive to its downregulation. However, we strongly believe that the oncogenic potential of UBASH3B can be, at least partially, correlated with its role in chromosome segregation in these cancer cells.

High therapeutic potential of UBASH3B inactivation in cancer is further supported by other recent findings, claiming that UBASH3B may promote cancer cell growth and invasion by inactivating E3 Ubiquitin Ligase Casitas B-lineage Lymphoma (CBL), which subsequently leads to upregulation of epidermal growth factor receptor.19,33 Therefore inactivation of UBASH3B might interfere with both cancer cells growth and proliferation.

Irrespective of the precise mechanisms, UBASH3B function might be restricted to aggressive, aneuploid cancer cells offering an interesting therapeutic perspective exploiting this tumor-specific vulnerability. This is confirmed by our results in the primary fibroblasts where weak SAC response and low levels of SAC protein BubR1 are found (Fig. 2E) and where downregulation of UBASH3B does not affect cellular survival (Fig. 2F). Interestingly, other nonpathogenic cells, in particular embryonic cells are characterized by lack of persistent SAC.34–37 Thus, regulation of faithful chromosome segregation in the primary fibroblasts and other normal cells might not be dependent on UBASH3B. This is also supported by the fact that UBASH3B knockout animals are viable and do not exhibit any developmental phenotypes.38 In future, it will be important to uncover the precise molecular mechanisms of control of UBASH3B function during normal mitosis as well as in tumorigenesis.

**Materials and methods**

Cell culture, siRNA transfections, and western blotting were performed as described previously.17 Primary fibroblasts (R5 and F31) were obtained from Institut de Genetique et de Biologie Moleculaire et Cellulaire (IGBMC) Cell Culture Facility. Analysis of the percentage of preanaphase cells was performed in cells synchronized in mitosis by double thymidine block and release and by immunofluorescence microscopy as described previously.17 The strength of the SAC response was analyzed by treatment of cells with Taxol (paclitaxel) (at 1 μM) for 18 hours and immunofluorescence microscopy using rabbit polyclonal antibodies to the mitotic marker phospho histone H3 Ser10 (Merck Millipore 06–570, 1:500).

Cellular death was analyzed by LIVE/DEAD® Fixable Dead Cell Stain kit (Thermo Fischer Scientific, catalog number L34969). Cell were grown on the coverslips, treated with indicated siRNAs for 48h as described previously and stained with the fluorescent reactive dye according to the manufacturer’s protocol. Following treatment, cells were fixed with 3% ParaFormAldehyde (PFA) in Phosphate-buffered saline (PBS) and analyzed by immunofluorescence microscopy as described previously.17

Gene expression data from The Cancer Genome Atlas database (http://cancergenome.nih.gov/) were used to analyze UBASH3B, BUB1B, and MAD2L1 expression in 17 types of cancer. Normalized mRNA expression data were downloaded from cBioportal and analyzed as described previously.39,40 Spearman correlation was performed using GraphPad Prism (GraphPad Software, San Diego, CA, www.graphpad.com). All statistical tests were two-sided, and results were considered statistically significant when P < 0.05.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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Author contributions

K.K., I.R-H., V.S-M. and I.S. designed the study. K.K., C.K., S.A. I.R-H. conducted the experiments. I.S. and K.K. wrote the manuscript.

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