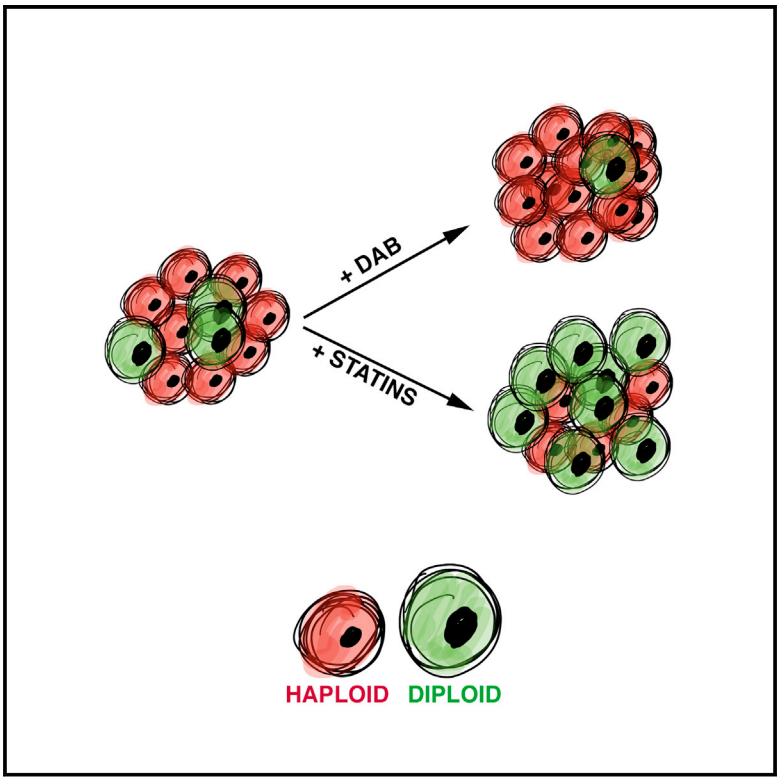
Cell Reports

A Chemical Screen Identifies Compounds Capable of Selecting for Haploidy in Mammalian Cells

Graphical Abstract



Highlights

- Mammalian haploid cell cultures become progressively enriched in diploid cells
- DAB, a precursor of Taxol, facilitates the maintenance of haploidy
- DAB selects for cells with lower ploidy in mixed cultures of mammalian cells
- Statins accelerate the gradual loss of haploid cells in culture

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In Brief

Cultures of haploid animal cell lines become progressively enriched in diploid cells. By conducting a chemical screen in HAP1 cells, Olbrich et al. identify compounds that facilitate the maintenance of haploid cells and a general strategy to select for cells with lower ploidy in mixed cultures of mammalian cells.

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Report

A Chemical Screen Identifies Compounds Capable of Selecting for Haploidy in Mammalian Cells

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SUMMARY

The recent availability of somatic haploid cell lines has provided a unique tool for genetic studies in mammals. However, the percentage of haploid cells rapidly decreases in these cell lines, which we recently showed is due to their overgrowth by diploid cells present in the cultures. Based on this property, we have now performed a phenotypic chemical screen in human haploid HAP1 cells aiming to identify compounds that facilitate the maintenance of haploid cells. Our top hit was 10-Deacetyl-baccatin-III (DAB), a chemical precursor in the synthesis of Taxol, which selects for haploid cells in HAP1 and mouse haploid embryonic stem cultures. Interestingly, DAB also enriches for diploid cells in mixed cultures of diploid and tetraploid cells, including in the colon cancer cell line DLD-1, revealing a general strategy for selecting cells with lower ploidy in mixed populations of mammalian cells.

INTRODUCTION

With a few exceptions like wasps or mites, haploidy in animals is confined to the germline (Wutz, 2014). A diploid genome increases genetic diversity and buffers the impact of mutations due to the presence of two copies of each gene. However, this feature has also limited genetic screenings in mammalian cells until recently, which were more efficiently conducted in model organisms that can grow as haploids such as yeast. Accordingly, the isolation of animal haploid somatic cell lines has been something attempted since the 1960s with initial efforts focusing on frogs and insects (Debec, 1978; Freed, 1962; Freed and Mezger-Freed, 1970). Through parthenogenesis (triggering development of unfertilized oocytes) or androgenesis (development of zygotes only containing a paternal genome), haploid mammalian embryonic stem cells (haESCs) have now been successfully derived from a wide range of species including zebrafish, mouse, rat, pig, monkey, and humans (Elling et al., 2011; Leeb and Wutz, 2011; Sagi et al., 2016; Yang et al., 2013; Yi et al., 2009). In addition to haESCs, a cell line (KBM7) harboring a near-haploid genome except for two copies of chromosome 8 was isolated from a leukemia patient (Andersson et al., 1995). KBM7 cells were subsequently used for the generation of an adherent derivative (HAP1) that has lost one copy of chromosome 8 and is thus virtually haploid (Carette et al., 2011). As expected, haploid mammalian cell lines rapidly became a powerful tool for forward genetic screenings and have been successfully used in many such approaches (Blomen et al., 2015; Carette et al., 2011; Jae et al., 2013, 2014; Papatheodorou et al., 2011; Rong et al., 2015; Wang et al., 2015).

Despite the usefulness of haploid cell lines, haploidy is an unstable state in mammalian somatic cells as these cultures rapidly become enriched in diploids, which demands frequent sorting in order to maintain a good percentage of haploid cells (reviewed in Yilmaz et al., 2016). While this phenomenon was originally thought to be a consequence of diploidization, we recently showed that it is caused by the activation of a p53-dependent response, which limits the viability of the haploid cells that are progressively overgrown by diploid cells present in the cultures (Olbrich et al., 2017). Accordingly, p53 deficiency facilitates the maintenance of haploidy in mouse haESCs (mhaESCs) or HAP1 cells. Besides the identification of genetic conditions that help to stabilize the haploid state, chemical strategies could also facilitate the maintenance of haploid cultures. While some efforts in this area have been made, they focused on a few selected compounds and came to opposing conclusions, as either activating (Takahashi et al., 2014) or inhibiting (He et al., 2017) CDK1 was reported to stabilize haploidy in mhaESCs. We here report on a chemical screen to identify compounds capable of facilitating the maintenance of haploid cells, both in mhaESCs and in HAP1 cells, and that has revealed a general

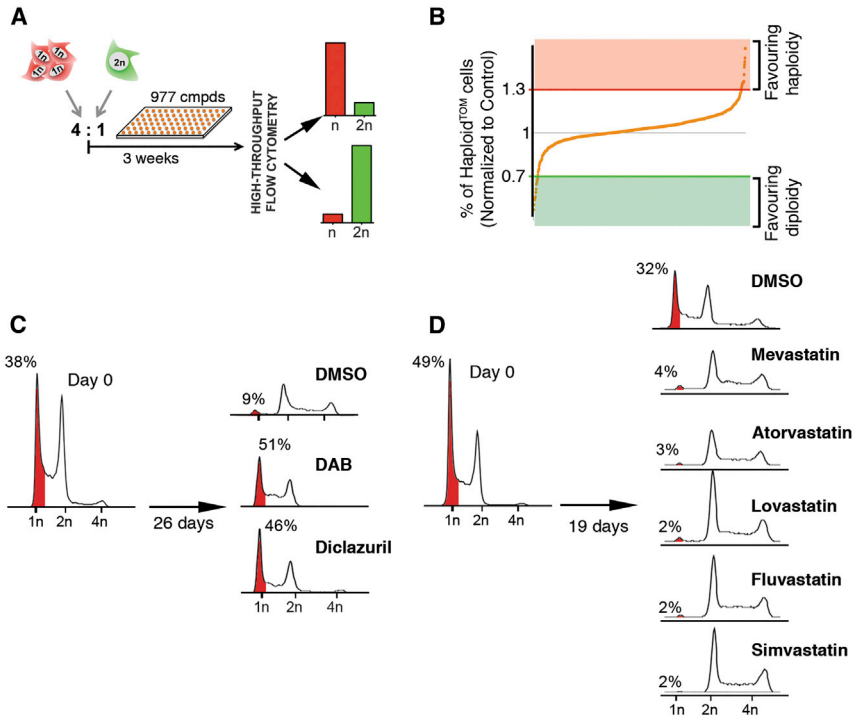

Figure 1. A Chemical Screen to Identify Compounds Stabilizing Haploidy in HAP1 Cells

(A) Schematic representation of the screening procedure. tdTomato-expressing haploid (Haploid^{TOM}) and EGFP-expressing diploid (Diploid^{EGFP}) HAP1 cells were mixed at a 4:1 ratio and cultured in media supplemented individually with 977 different compounds during the course of 21 days. After this period, the percentage of Haploid^{TOM} and Diploid^{EGFP} cells was evaluated by flow cytometry and compounds either increasing or decreasing the percentage of Haploid^{TOM} cells were selected for secondary validation.

(B) Graph representing the results of the screen defined in (A), indicating the thresholds at which compounds either increased or decreased the percentage of haploidy in more than 30% from that observed in control (DMSO treated) wells. The panel represents the data from 930 compounds, as 47 chemicals killed all cells during the experiment (see Table S1).

(C) Flow cytometry analysis of DNA content in HAP1 cells that were sorted for haploidy by FACS on day 0 and after 26 days of growth in the presence of DMSO (control), DAB (10 μM), or Diclazuril (10 μM). The fraction of haploid G1 cells is highlighted in red. One representative example out of three independent experiments is shown.

(D) Flow cytometry analysis of DNA content in HAP1 cells that were sorted for haploidy by FACS on day 0 and after 19 days of growth in the presence of DMSO (control), Mevastatin (10 μM), Atorvastatin (10 μM), Lovastatin (10 μM), Fluvastatin (10 μM), or Simvastatin (10 μM). The fraction of haploid G1 cells is highlighted in red. One representative example out of three independent experiments is shown.

strategy to select for cells with lower ploidy in mixed cultures of mammalian cells.

RESULTS AND DISCUSSION

A Chemical Screen for Drugs That Stabilize Haploidy in HAP1 Cells

As mentioned above, we recently identified that the progressive reduction in the percentage of haploid cells observed in haploid mammalian cell lines is due to their outgrowth by diploid cells existing in these cultures (Olbrich et al., 2017). Based on this property, and in order to identify chemicals that facilitate the maintenance of haploidy, we performed a chemical screen in HAP1 cells using a library of 977 bioactive compounds, including many US Food and Drug Administration (FDA)-approved agents. To do so, haploid and diploid HAP1 cells were isolated by fluorescence-activated cell sorting (FACS) and subsequently infected with lentiviruses encoding for tdTomato fluorescence protein (TOM) or EGFP, respectively. Haploid^{TOM} and diploid^{EGFP} populations of HAP1 cells were then mixed at a 4:1 ratio, seeded in 96-well plates, and treated individually with the chemicals from the library for 3 weeks (Figure 1A). During the screen, compounds were added twice per week and cells were split once per week. At the endpoint of the experiment, the percentage of haploid and diploid HAP1 cells was quantified by high-throughput flow cytometry on the basis of EGFP- and TOM-positive cell fractions.

The results of the screen were plotted as the percentage of haploid cells observed in the wells treated with compounds normalized to that observed in control wells (dimethylsulfoxide [DMSO]). This representation followed a normal distribution, with several compounds showing a clear impact on the percentage of TOM-positive HAP1 cells remaining after the 3-week period (Figure 1B). From the initial screen, we selected 37 compounds that either increased (n = 19) or decreased (n = 18) the percentage of haploid cells more than 30% from that observed in control wells for secondary validation. To validate our hits in an orthogonal assay, HAP1 cell cultures freshly sorted for haploidy were grown for up to 1 month in the continuous presence of each of the 37 compounds. Two independent rounds of validations led to the identification of seven compounds that consistently had an effect on the percentage of haploid HAP1 cells: two that stabilized haploidy and five that led to an almost complete loss of all haploid cells in the cultures (Figures 1C and 1D). Remarkably, all five compounds that led to the selective disappearance of haploid cells were statins, a widely used class of inhibitors of the hydroxymethyl glutaryl coenzyme A reductase (HMGCR) that are medically used for controlling cholesterol levels (Ramkumar et al., 2016). Nevertheless, since our objective was to identify chemicals capable of stabilizing haploidy, we focused on this class for subsequent analyses.

10-Deacetylbaccatin-III Selects for Haploid Cells in HAP1 and mhaESCs

The two compounds that consistently stabilized haploidy in HAP1 cells in validation experiments were 10-Deacetylbaccatin-III (DAB), a natural compound isolated from the yew tree

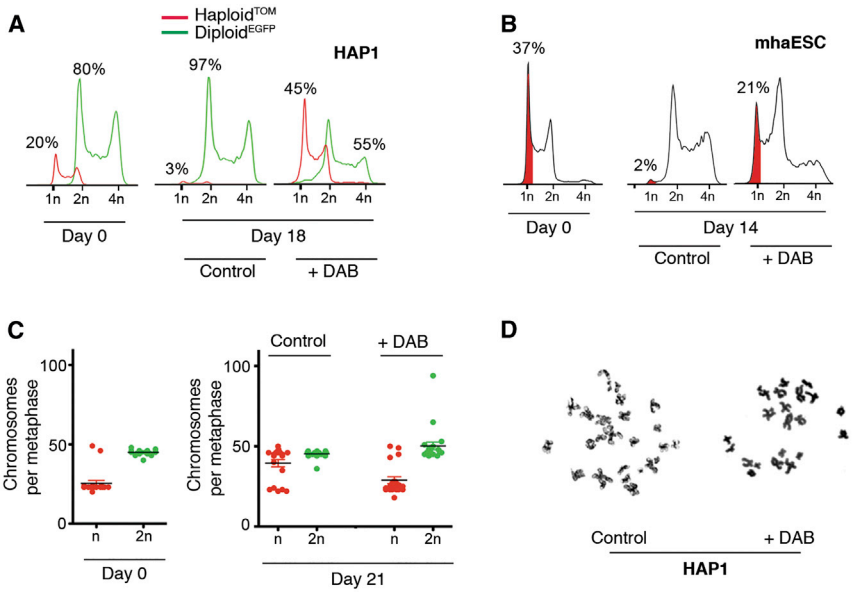

Figure 2. DAB Facilitates the Maintenance of Haploidy in HAP1 Cells and mhaESCs

(A) tdTomato-expressing haploid (Haploid^{TOM}) and EGFP-expressing diploid (Diploid^{EGFP}) HAP1 cells were mixed at a 1:4 ratio and cultured in media containing either DMSO (control) or DAB (10 μM) for 18 days. After this period, DNA content, EGFP, and TOM expression were quantified by flow cytometry. Numbers indicate the percentages of each population. One representative example out of three independent experiments is shown.

(B) Flow cytometry analysis of DNA content in mhaESCs that was sorted for haploidy by FACS on day 0 and after 14 days of growth in the presence of DMSO (control) or DAB (5 μM). The fraction of haploid G1 cells is highlighted in red. One representative example out of two different experiments made in independent mhaESC clones is shown.

(C) Quantification of the number of chromosomes found on metaphases of HAP1 cells that were sorted for haploidy by FACS on day 0 and grown in the presence of DMSO (control) or DAB (10 μM) for 3 weeks. The effect of the compound in a diploid clone of HAP1 cells is show for comparison of chromosome numbers. Black lines represent mean values.

(D) Representative examples of the metaphases that can be found on HAP1 cells after 3 weeks of continuous growth in the presence of DMSO (control) or DAB (10 μM).

and that is used as a precursor in the synthesis of paclitaxel (Guéritte-Voegelein et al., 1986), and Diclazuril, an antiparasitic used in the prevention of bovine coccidiosis (Zechner et al., 2015). However, while DAB was able to stabilize haploidy both in HAP1 as well as in mhaESCs (see below), the effects of Diclazuril were restricted to HAP1 cells. Thus, we selected DAB for mechanistic studies. Of note, we also observed mild effects in stabilizing haploidy in HAP1 cells with other compounds such as the anticancer kinase inhibitors Regorafenib or Nilotinib (Figure S1). However, these compounds overall decrease growth rates, which would simply slow down the overgrowth of haploid cells by diploids.

To further define the potency of DAB in stabilizing haploidy in mammalian cultures, we analyzed its effects in HAP1 cultures where haploid cells were a minority within the mixed population. To do so, haploid^{TOM} and diploid^{EGFP} HAP1 cells were mixed in a 1:4 ratio and grown in the presence or absence of DAB (Figure 2A). By 18 days, the population of haploid^{TOM} cells in control conditions had been reduced to 3% from the culture. Strikingly, when the mixed culture was grown in the presence of DAB, the population of haploid HAP1 cells not only did not decrease but actually increased to 45% by day 18. We next evaluated the effect of DAB in the growth of mhaESCs, where the progressive loss of haploid cells occurs significantly faster than in HAP1 cells (Olbrich et al., 2017). Accordingly, in 2 weeks of culture the haploid cells had almost disappeared from mhaESC that had been sorted for haploid content (Figure 2B). In contrast, mhaESCs grown in the presence of DAB for the same time kept a significant population of haploid cells. Of note, karyotype analyses of HAP1 cells grown in the presence of DAB for 3 weeks failed to detect any obvious evidences of chromosomal gains, breaks, or rearrangements induced by the treatment in haploid cells (Figures 2C and 2D). Moreover, exposure to DAB did not lead to any obvious activation of the DNA damage response in haploid HAP1 cells as measured by evaluating the phosphorylation of histone H2AX or the incorporation rates of 5-Ethynyl-2′-deoxyuridine (EdU) (Figure S2). In summary, these experiments confirmed that DAB facilitates the maintenance of the haploid state in mammalian somatic cell cultures.

DAB Selects for Lower Ploidy in Mixed Cultures of Mammalian Cells

Given that DAB selects for cells with a 1n DNA content in mixed cultures of haploid and diploid cells, we hypothesized that this could be a general property of the chemical to select for cells with lower ploidy in mixed cultures of mammalian cells. To do so, we first isolated a tetraploid population from HAP1 cultures by FACS and evaluated the effects of DAB in these cells. Consistent with our hypothesis, tetraploid HAP1 cells had nearly disappeared after 20 days of culture in the presence of DAB and were replaced by diploids, which were likely contaminants carried over during the isolation of the tetraploid fraction (Figure 3A). To further test the effects of DAB in selecting for lower ploidy, we infected tetraploid HAP1 cells with lentiviruses expressing blue fluorescent protein (BFP; tetraploid^{BFP}) and mixed them at 1:1:1 ratio with haploid^{TOM} and diploid^{EGFP} HAP1 cells. In agreement with the reduced fitness of mammalian haploid and tetraploid cells (Olbrich et al., 2017; Pfau et al., 2016), both populations were progressively overgrown by diploid^{EGFP} HAP1 cells (Figure 3B). In contrast, when the mixed cultures were grown in the presence of DAB, these became increasingly enriched in haploid^{TOM} cells, followed by diploid^{EGFP} and finally by tetraploid^{BFP} cells, confirming a general effect of DAB in selecting for cells with lower ploidy.

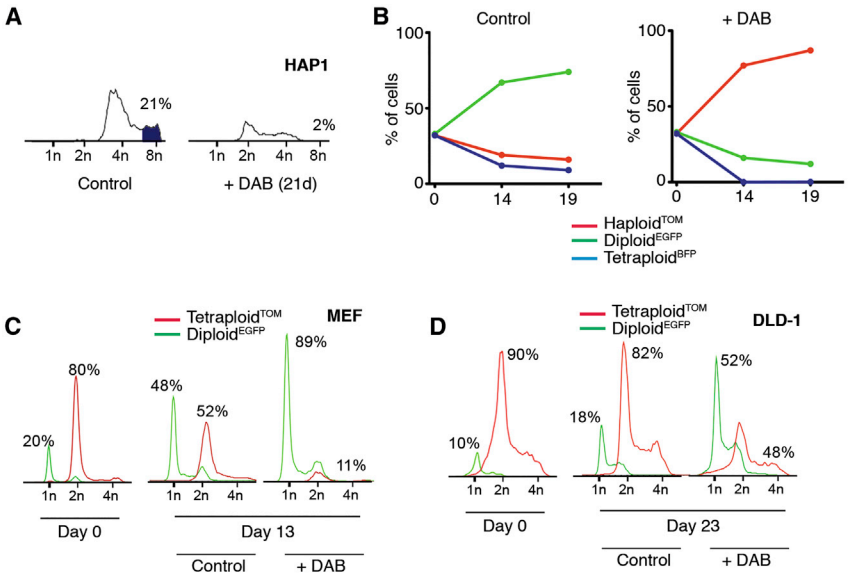

Figure 3. DAB Selects for Cells with Lower Ploidy in Mixed Cultures

(A) Flow cytometry analysis of DNA content in HAP1 cells that were sorted for tetraploidy by FACS on day 0 and after 21 days of growth in the presence of DMSO (control) or DAB (20 μM). The fraction of tetraploid G2 cells is highlighted in blue. One representative example out of two independent experiments is shown.

(B) tdTomato-expressing haploid ($Haploid^{TOM}$), EGFP-expressing diploid ($Diploid^{EGFP}$), and BFP-expressing tetraploid ($Tetraploid^{BFP}$) HAP1 cells were mixed at a 1:1:1 ratio and cultured in media containing either DMSO (control) or DAB (10 μM) for 19 days. The graph represents the percentage of $Haploid^{TOM}$, $Diploid^{EGFP}$, and $Tetraploid^{BFP}$ cells at days 0, 14, and 19.

(C) tdTomato-expressing tetraploid ($Tetraploid^{TOM}$) and EGFP-expressing diploid ($Diploid^{EGFP}$) MEF cells were mixed at a 1:4 ratio and cultured in media containing either DMSO (control) or DAB (10 μM) for 13 days. After this period, DNA content, EGFP, and TOM expression were quantified by flow cytometry. Numbers indicate the percentages of each population. One representative example out of three independent experiments is shown.

(D) tdTomato-expressing tetraploid ($Tetraploid^{TOM}$) and EGFP-expressing diploid ($Diploid^{EGFP}$) DLD-1 cells were mixed at a 1:9 ratio and cultured in media containing either DMSO (control) or DAB (30 μM) for 23 days. After this period, DNA content, EGFP, and TOM expression were quantified by flow cytometry. Numbers indicate the percentages of each population.

To determine if the effects of DAB were not restricted to HAP1 cells, we evaluated its effects in mouse embryonic fibroblasts (MEFs), which have a tendency to spontaneously tetraploidize. Cultures of diploid MEF expressing EFGP and tetraploid cells expressing tdTomato at a 1:4 ratio were grown in the presence or absence of DAB. While, in agreement with the reduced fitness of tetraploid mammalian cells (Pfau et al., 2016), the $tetraploid^{EGFP}$ MEF population had decreased from 80% to 52% by day 13, this nearly disappeared if the cultures were grown in the presence of DAB (Figure 3C). Finally, we evaluated if these findings were also conserved in cancer cells, as tetraploidy or whole genome duplication has been found to be a frequent event in cancer (Zack et al., 2013). To do so, we evaluated the effects of DAB in the colon cancer cell line DLD-1, for which an isogenic tetraploid derivative was recently isolated through the inhibition of cytokinesis (Drosopoulos et al., 2014). As before, diploid DLD-1 cells expressing EFGP were mixed in a 1:9 ratio with tetraploid DLD-1 cells expressing tdTomato and let to grow for 23 days. While, consistent with the reduced fitness of tetraploid cells, in control conditions the population of diploid cells increased from the original 10% to 18%, this increased to half of the culture when the cultures were grown in the presence of DAB (Figure 3D). Altogether, these findings indicate that DAB selects for cells with lower ploidy in mixed cultures of mammalian cells, including cancer cells.

DAB Promotes Mitotic Arrest in a Ploidy-Dependent Manner

Last, we sought to determine the mechanism by which DAB works. First, given that p53 deficiency stabilizes haploidy in mammalian cells, we evaluated the effects of DAB in HAP1 cells in which *P53* had been deleted by CRISPR-Cas9. However, DAB was able to increase the fraction of haploid cells both in wild-type and *P53*-deficient HAP1 cells upon 25 days in culture (Figure S3A). Next, given that DAB is a precursor in the synthesis of paclitaxel, which stabilizes microtubules by preventing their disassembly, we explored if DAB could have similar effects. In fact, evaluation of the intracellular distribution of α-tubulin after microtubule depolymerization induced by a cold shock revealed a clear effect of DAB in the microtubule dynamics of interphase cells (Figure S3B). Since microtubule reorganization is particularly relevant for the assembly of the mitotic spindle, we then evaluated the effects of DAB on the time that cells spend in mitosis. To do so, we infected haploid, diploid, and tetraploid HAP1 cells with a histone H2B-red fluorescent protein (RFP) fusion expressing lentivirus and monitored the effect of DAB in these cell lines by live-cell video-microscopy (Figure 4A). These analyses revealed that DAB extended the duration of mitosis in all three cell lines, with the severity of the arrest correlating with their ploidy (Figures 4A and 4B). Importantly, while most haploid cells could overcome the mitotic arrest induced by DAB and continue cell division, diploids and particularly tetraploid HAP1 cells presented very prolonged arrests that were often followed by cell death. Flow cytometry analyses of DNA content confirmed the ploidy-dependent toxic effects of DAB in HAP1 cells (Figure S4A). Accordingly, while DAB did not significantly affect the growth of haploid HAP1 cells, it had a higher impact on diploid and particularly tetraploid HAP1 cultures (Figure S4B). The ploidy-dependent toxicity of DAB provides a mechanism to explain its effects on selecting for cells with lower ploidy in mixed cultures of mammalian cells.

Further analyses of the images from the video microscopy experiment revealed that the extended duration of mitosis induced

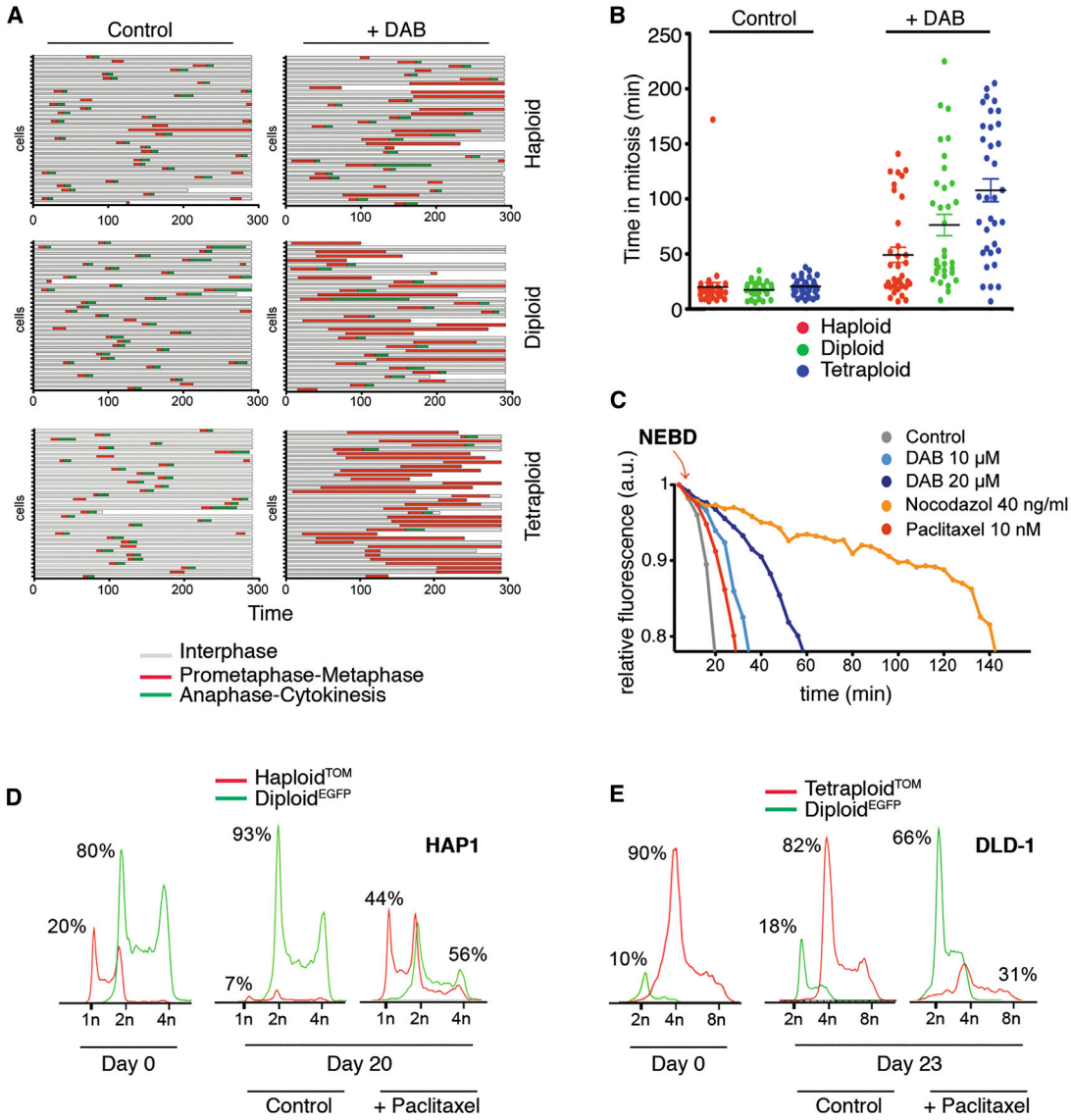

Figure 4. DAB Impairs Mitosis in a Ploidy-Dependent Manner

(A) Schematic representation of the time spent in mitosis (red and green) or interphase (gray) in individual RFP-H2B-expressing haploid, diploid, and tetraploid HAP1 cells grown in the presence of DMSO (control) or DAB (10 μM) for 16 h. Time spent in mitosis was defined as the time between chromosome condensation and cytokinesis. The time between chromosome condensation and the formation of the metaphase plate is indicated in red, and from anaphase onset to cytokinesis in green. At least 35 individual cells were analyzed per condition.

(B) Quantification of the time spent in mitosis from the experiment shown in (A). Black lines represent mean values.

(C) Degradation of cyclin B in U2OS expressing a cyclin B-mCherry fusion construct as cells quantified by live-cell imaging. The graph shows the relative fluorescence levels of cyclin B-mCherry from nuclear envelope breakdown (NEBD) until the onset of anaphase, in cells treated with the indicated compounds. Nocodazole was used as a positive control.

(D) tdTomato-expressing haploid ($Haploid^{TOM}$) and EGFP-expressing diploid ($Diploid^{EGFP}$) HAP1 cells were mixed at a 1:4 ratio and cultured in media containing either DMSO (control) or Paclitaxel (15 nM) for 20 days. After this period, DNA content, EGFP, and TOM expression were quantified by flow cytometry. Numbers indicate the percentages of each population.

(E) tdTomato-expressing tetraploid ($Tetraploid^{TOM}$) and EGFP-expressing diploid ($Diploid^{EGFP}$) DLD-1 cells were mixed at a 1:9 ratio and cultured in media containing either DMSO (control) or Paclitaxel (30 nM) for 23 days. After this period, DNA content, EGFP, and TOM expression were quantified by flow cytometry. Numbers indicate the percentages of each population.

by DAB was mainly due to an effect on the compound in delaying the formation of a metaphase plate (Figure 4A). Accordingly, while immunofluorescence analyses revealed normal metaphase and anaphase figures in haploid HAP1 cells treated with DAB, mitoses from diploids and even more so from tetraploids revealed that these were arrested at pro-metaphase with a high proportion of

lagging chromosomes (Figure S4C). To further analyze the effects of DAB in impairing mitotic progression, we used a U2OS cell line stably expressing a cyclin B-mCherry fusion (Gavet and Pines, 2010). Since cyclin B levels are highest at mitotic entry and lowest at the onset of anaphase (Clute and Pines, 1999), this system can be used to quantify the time needed to assemble a complete metaphase plate. These analyses confirmed the effects of DAB in mitosis, in a manner comparable to those that can be achieved with low doses of paclitaxel (10 nM) (Figure 4C). Based on these findings, we hypothesized that low doses of paclitaxel could also be used to select for cells with lower ploidy. Indeed, a low dose of paclitaxel selected for haploid cells in mixed cultures of haploid and diploid HAP1 cells, and for diploid cells in mixed cultures of diploid and tetraploid DLD-1 cells (Figures 4D and 4E).

We here present our work on a chemical screen to identify chemicals that facilitate the maintenance of haploidy in mammalian cells. The paclitaxel precursor DAB fulfills this objective, providing a useful and facile manner to overcome the progressive loss of haploidy in mammalian haploid cultures. While other compounds such as Regorafenib or Nilotinib could also moderately diminish diploidization rates, these are drugs that reduce cellular growth rates in multiple cell types, so that their effects can be explained by a slowdown in the overgrowth of haploid of cells by diploids, which we recently showed is the basis of the observed diploidization in HAP1 cells and mhaESCs (Olbrich et al., 2017). Accordingly, ''diploidization'' rates are much faster in haploid ESCs than in HAP1 or KBM7 cells due to the faster proliferation of stem cell cultures. Strategies that select for haploid cells, chemical or genetic, should facilitate further research in the biology of haploid mammalian cells, and might enable us to overcome some of the remaining challenges such as the generation of additional primary differentiated haploid cell lines different to ESCs. Of note, and while we here focused on compounds that stabilize haploidy, it is remarkable that all chemicals having an opposite effect, namely that they accelerate the loss of haploid cells, were statins, broadly used to lower cholesterol levels in humans. Even if at this point we lack an understanding for this observation, it is interesting that recent studies have identified a particular dependency of aneuploid cells on the biosynthesis of sphingolipids (Hwang et al., 2017; Tang et al., 2017). To what extent lipid biosynthesis is particularly relevant for cells with an altered ploidy deserves further attention.

In regard to the mechanism of action of DAB, this compound is normally used as a precursor in the synthesis of paclitaxel and was not known to have biological activity. In fact, and while the compound was included as part of an FDA-approved library, it is not approved per se and has no medical use. Nevertheless, we here show that DAB, while significantly less potent than paclitaxel, does have effects on microtubule dynamics and mitotic progression. Of note, while we previously observed that mhaESCs are more sensitive than diploid mESCs to high doses of paclitaxel (Olbrich et al., 2017), it is well documented that the suppression of microtubule dynamics with paclitaxel occurs at much lower concentrations than doses needed to exert toxicity (Jordan et al., 1993). Accordingly, the effects of DAB in selecting for cells with lower ploidy can be mimicked by low doses of paclitaxel. Interestingly, the ploidy dependent mitotic arrest that is observed with DAB or low-dose paclitaxel is not detected with other chemicals that arrest cells in mitosis such as Nocodazole or the Eg5 kinesin inhibitor Monastrol, arguing that this effect is not just a consequence of increasing the time cells spend in mitosis but is specific to reagents that specifically decrease microtubule dynamics and therefore stabilize microtubule-kinetochore attachments (Figure S4D).

Based on all of the above, our model shows that while mild perturbations of the mitotic spindle might be tolerated in cells with fewer chromosomes, as all chromosomes could finally find their way to the metaphase plate, these problems become progressively acute as the chromosome number increases, leading to an irreversible mitotic arrest and cell death. In support of this concept, components of the spindle assembly checkpoint have been shown to be dispensable in organisms with few chromosomes such as flies (Buffin et al., 2007), as well as in HAP1 cells (Raaijmakers et al., 2018). Furthermore, a genome-wide screening performed in yeast revealed that mitotic spindle disturbances are preferentially essential for triploid or tetraploid cell (Storchová et al., 2006). In conclusion, in addition to its usefulness for studies using haploid cells, we should note that the capacity of DAB or low-dose paclitaxel to select for cells with lower ploidy in mixed cultures of mammalian cells might also be relevant in the context of cancer, as an average of 37% of all tumors have been found to present whole-genome duplication events in recent genomic studies (Zack et al., 2013). Given that taxanes are widely used medical compounds, we propose to focus on low doses of paclitaxel for further studies in this direction. To what extent this approach can be used as a strategy to selectively target polyploid and aneuploid cells in cancer, and whether this is actually beneficial or damaging, remains to be seen.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.06.060.

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AUTHOR CONTRIBUTIONS

O.F.-C. supervised the project; T.O., M.V.-S., and M.M. performed research; T.O., G.d.C., M.M., S.O., S.R., and O.F.-C. defined the experiments and analyzed the data; and T.O. and O.F.-C. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Monoclonal anti-p53	Cell Signaling	2524; AB_331743
Rabbit Polyclonal anti-CDK2	Santa Cruz	Sc-163; AB_631215
Mouse Monoclonal anti- phospho-histone H2A.X	Millipore	05-636; AB_309864
Mouse Monoclonal anti-α-Tubulin	Sigma	T9026; AB_477593
Polyclonal anti-phospho-histone H3-Ser10 (pH3)	Millipore	05-636; AB_310177
anti-centromeric antibody (ACA)	Kind gift of Marcos Malumbres, CNIO, Madrid, Spain	N/A
Chemicals, Peptides, and Recombinant Proteins		
FDA-approved compounds (977)	Selleckchem	L1300
Deacetylbaccatin III	Selleckchem	S2409
Paclitaxel	Sigma	T7402
Nocodazole	Sigma	M1404
Hoechst 33342	Thermo Fisher Scientific	62249
Propidium Iodide (PI)	Sigma	P4170
Critical Commercial Assays		
Amaxa® Cell Line Optimization Nucleofector® Kit	Lonza	N/A
Lipofectamine 2000 (Invitrogen)	Invitrogen	11668019
CellTiter-Glo® Luminiscent Cell Viability Assay	Promega	G7570
Click-iT™ EdU Alexa Fluor®	Invitrogen	C10086
Experimental Models: Cell Lines		
HAP1 cells	Kind gift of Thijn Brummelkamp, NKI, Amsterdam	N/A
DLD1 cell lines	Kind gift of Spiros Linardopoulos, ICR, London, UK	N/A
HEK293T	American Type Culture Collection	CRL-3216
U2OS CyclinB1-164-mCherry	Kind gift of Marcos Malumbres, CNIO, Madrid, Spain	N/A
Mouse ESCs	Olbrich et al., 2017	N/A
Recombinant DNA		
SV40-T121	Kind gift of Manuel Serrano, IRB, Barcelona, Spain	N/A
pX330-U6-Chimeric_BB-CBh-hSpCas9	Cong et al., 2013	Addgene #42230
pLenti-H2B-RFP	Kind gift of Marcos Malumbres, CNIO, Madrid, Spain	N/A
pLVTHM	Wiznerowicz and Trono, 2003	Addgene #12247
pHIV-tdTomato	Kind gift of Bryan Welm	Addgene #21374
pKLV-U6gRNA(BbsI)-PGKpuro2ABFP	Addgene	Addgene #50946
pVSVg	Kind gift of Oded Singer, The Salk Institute, La Jolla, USA	N/A
pMDLg/RRE	Dull et al., 1998	Addgene #12251
pRSV-Rev	Dull et al., 1998	Addgene #12253
Oligonucleotides		
pX330-CRISPRp53- F: CACCGTGAAGCTCC CAGAATGCCAG;	Olbrich et al., 2017	N/A
pX330-CRISPR-p53-R: AAACCTGGCATTCT GGAGCTTCAC	Olbrich et al., 2017	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and Algorithms		
GraphPad Prism	GraphPad Software Inc	http://www.graphpad.com/scientific-software/prism/
Definiens Developer XD™ software	Definiens Inc.	https://www.definiens.com/
Fiji softwares	Schindelin, et al., 2012	https://imagej.net/Fiji
Flow Jo 10™ software	Flowjo, LLC	https://www.flowjo.com/

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Oscar Fernandez-Capetillo (ofernandez@cnio.es).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Culture

HAP1 cells (kind gift of Dr. Thijn Brummelkamp, NKI, Amsterdam) were grown in IMDM (Invitrogen) supplemented with 15% FBS (Sigma), 1% P/S and 1% Glutamine (34). Mouse embryonic fibroblasts (MEFs) were obtained from 13.5 dpc embryos using standard methods and cultured in DMEM (Invitrogen), 15% FBS and 0.1 mM non-essential amino acids in low-oxygen conditions. Feeder layers were generated from MEFs grown at early passages and growth arrested by ionizing irradiation (IR) with 80 Gy for 30 min. MEFs were immortalized by lentiviral expression of the SV40-T121 antigen following a standard protocol. Diploid and tetraploid DLD1 cell lines were a kind gift of Spiros Linardopoulos (ICR, London, UK). Cells were cultured in RPMI, 10% FBS and 1% P/S. HEK293T (American Type Culture Collection) cells and the CyclinB1-164-mCherry (Addgene #26063) expressing U2OS cell line were cultured in DMEM (Invitrogen), 10% FBS and 1% P/S. The following compounds were used to treat the cells: DAB (Deacetylbaccatin III, S2409, Selleckchem), Paclitaxel (T7402, Sigma), Nocodazole (M1404, Sigma). A total of 5x105 cells were transfected with 10 μg of pX330-sgRNA-p53 plasmid (sequences provided below) using the Amaxa® Nucleofector kit (Reactive L, X-001 program) to generate P53-deficient HAP1 cells.

METHODS DETAILS

Chemical Screen

HAP1 haploid cells expressing tdTomato and HAP1 diploid cells expressing EGFP were mixed in a ratio 4:1 and seeded in 96-well plates (a total of 2500 cells per well). Cells were treated individually with a chemical library containing 977 bioactive compounds, most of which are FDA-approved (Z145127, Selleckchem) (Table S1). The screen was carried out in duplicate. Fresh compounds were added twice per week and cells passaged once per week. After 21 days of treatment, cells were trypsinized and 14 analyzed for the expression of tdTomato and EGFP by high throughput flow cytometry (BD FACS Canto II™, BD Biosciences).

Derivation and Culture of mhaESCs

mhaESCs were generated as described recently (Leeb and Wutz, 2011) and cultured on feeder layers in gelatin-coated plates at 37°C in N2B27-based medium plus 1000 U/ml LIF, PD0325901 (1 μM), CHIR99021 (3 μM) and supplemented with 15% knockout serum replacement (Invitrogen), 0.1 mM nonessential amino acids and 0.35% BSA fraction V.

Plasmids

The plasmid pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene, 42230) (Cong et al., 2013) was used in cells for gene editing. The sequences of the sgRNAs (pX330-CRISPRp53- F: CACCGTGAAGCTCCCAGAATGCCAG; pX330-CRISPR-p53-R: AAACCTGG CATTCTGGAGCTTCAC) used were designed and cloned as described by using the MIT CRISPR design tool (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design). Only sgRNAs with the higher scores and lower probabilities of generating off-target effects were selected. pLenti-H2B-RFP was obtained from Dr. Marcos Malumbres (Cell Division and Cancer Group, CNIO, Madrid). The lentiviral plasmids pLVTHM (Addgene, 12247) (Wiznerowicz and Trono, 2003), pHIV-tdTomato (Addgene, 21374), pKLV-U6gRNA(BbsI)-PGKpuro2ABFP (Addgene, 50946) (Koike-Yusa et al., 2014) were used to express EGFP, tdTomato or BFP fluorescent proteins after infection. All de-novo generated constructs were sequenced entirely to rule out the presence of mutations.

Lentiviral Production

Lentiviral vectors were individually co-transfected in HEK293T cells using Lipofectamine 2000 (Invitrogen) with 3rd generation packaging vectors (pMDLg/RRE, Addgene #12251; pRSV-Rev, Addgene #12253 and pCMV-VSVg, kind gift of Dr. Oded Singer) to

generate viral supernatants (Dull et al., 1998). Lentiviral supernatants were collected 36 hours after transfection, pooled and passed through a 0.45 μM filter to eliminate cellular debris.

Flow Cytometry

We routinely analyzed the cell cycle profiles after cell sorting and when indicated by flow cytometry. Briefly, cells were trypsinized, stained with 10 μg/ml Hoechst 33342 for 30 minutes at 37°C and the analytic flow profiles of the DNA content was recorded on a BD Fortessa™ (BD Biosciences). At the same time or independently, we monitored and recorded the percentage of the corresponding tdTomato+, EGFP+ and BFP+ in the HAP1, DLD1 or immortalized MEF cell populations. For the evaluation of DAB toxicity, cells were fixed in suspension with 70% ethanol and stained with PI (100 μg/ml) for DNA content analyses.

Flow Activated Cell Sorting (FACs)

HAP1, immortalized MEFs and mhaESCs were trypsinized followed by Hoechst staining (10 μg/ml Hoechst 33342, Thermo Fisher Scientific) for 30 minutes at 37°C. Sorting for specific haploid, diploid or tetraploid cell populations was based on DNA content and/or expression of tdTomato, EGFP or BFP on a BD Influx™ cell sorter (BD Biosciences). In a mix culture of haploid/diploid cells, haploids were sorted based on the G1 haploid (1n) and diploids on the G2/M diploid (4n) peaks, respectively. In a mix culture of diploid/tetraploid cells, diploids were sorted based on the G1 diploid (2n) and G2/M tetraploid (8n) peak, respectively. After each sort the purity of the sorted cells was carefully checked by flow cytometry analysis.

Western Blot

HAP1 cell pellets were lysed in 50mM Tris, 150mM NaCl, 1% Triton X-100 or in 50mM Tris pH 7.9/8M Urea/1%Chaps followed by 30 min of incubation time shaking at 4°C. 15-25 μg of supernatants were run on precast gels and transferred for protein detection by using the following antibodies: p53 (1:1000, Cell Signaling, #2524); CDK2 (1:2000; sc-163, Santa Cruz).

Immunofluorescence

Cells were fixed with 4% PFA followed by permeabilization with 0.1% Triton X-100. Antibodies against γH2AX (1:1000, Millipore 05-636), αTubulin (1:1000, Sigma #T9026), phospho-histone H3-Ser10 (pH3) (1:100, Millipore 06-570), anti-centromeric antibody (ACA, 1:500, obtained from Marco Malumbres, Cell Division and Cancer Group, CNIO, Madrid) were used. Images were acquired using a Leica TCS-SP5 equipped with a 0.7 NA 20x oil or 1.4 NA 63x oil (HCX plan Apo CS) objective and LAS AF 2.6 software.

High-Throughput Microscopy (HTM)

High-throughput microscopy analyses were carried out as described in (Lopez-Contreras et al., 2013). 10000 HAP1 cells were seeded per well on μClear® bottom 96-well plates (Greiner Bio-One). Immunofluorescence staining with the indicated antibodies (γH2AX or phospho-H3) was performed (see above) and images from each well were automatically acquired using an Opera High-Content Screening System (Perkin Elmer). The images were taken at non-saturating settings and segmented using the DAPI staining to generate masks matching cell nuclei. The levels of EdU incorporation was measured by adding EdU to the media at a final concentration of 20 μM for 30 minutes. For EdU detection the Click-iT™EdU Alexa Fluor® imaging kit (Invitrogen™) was used.

Metaphase Spreads

HAP1 cells were arrested at mitosis with overnight treatment with 100 ng/ml Colcemide (GIBCO/BRL). Cells were then collected, incubated in a hypotonic buffer (0.075 mM KCl) for 15 min at 37°C and fixed with Carnoy's buffer (methanol-glacial acetic acid, 3:1). To obtain metaphase spreads, cells were dropped on slides and stained with Giemsa solution. Images from metaphases were captured and a minimum of 19 metaphases were analyzed.

Live Cell Imaging

To evaluate mitosis entry and duration, HAP1 cells were infected with lentiviruses encoding the histone H2B-RFP and seeded on 8 wells μ-Slide (Ibidi, 80826). The day after, the cells were treated with the indicated compounds and imaged every 4 minutes for a total of 16h with a 20x objective in a Leica DMI 6000 B system. For a more careful evaluation of mitosis, SAC-dependent time was defined as the time from nuclear envelope breakdown (NEBD) until the observation of a metaphase plate and SAC-independent time from metaphase plate to chromosome decondensation. At least, 35 cells were followed to evaluate the time spent in mitosis and interphase as well as cell fate for each individual cell. To evaluate cyclin B degradation, U2OS cells expressing a CyclinB-mCherry fusion protein were seeded on 8 wells μ-Slide (Ibidi, 80826). The following day, cells were treated with the indicated compounds and imaged every 4 minutes for a total of 16h in a Leica DMI 6000 B system. Cyclin B degradation was evaluated in cells from the NEBD, with the maximum level of cyclin B expression, until the start of anaphase, where mCherry signal was lost entirely.

Cold Shock Treatment

U2OS cells were pretreated with 10 μM DAB for 1 hour at 37°C. Then, cells were placed on ice and put in ice-cold media supplemented with HEPES (pH 7.4) for 30 min to allow microtuble depolymerization. Finally, cells were either fixed with 4% PFA or placed back to regular media with or without 10 μM DAB at 37°C and fixed after 3 min. Immunofluorescence staining was performed as described above.

QUANTIFICATION AND STATISTICAL ANALYSIS

Where indicated, data are represented as means ± standard error of the mean. Differences between groups were evaluated by two-way analysis of variance (ANOVA) and P values are indicated when differences between two groups were statistically significant (< 0.05). Statistical analyses were performed using Prism 8 (GraphPad La Jolla, CA).