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The Reversibility of Mitotic Exit in Vertebrate Cells

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Abstract

A guiding hypothesis for cell cycle regulation asserts that regulated proteolysis constrains the directionality of certain cell cycle transitions[1, 2]. We have tested this hypothesis for mitotic exit, which is regulated by degradation of the Cdk1 activator, cyclin B[3-5]. Application of chemical Cdk1 inhibitors to cells in mitosis induces cytokinesis and other normal aspects of mitotic exit, including cyclin B degradation. However, chromatid segregation fails, resulting in entrapment of chromatin in the midbody. If cyclin B degradation is blocked with proteasome inhibitor or by expression of non-degradable cyclin B, Cdk inhibitors will nonetheless induce mitotic exit and cytokinesis. If after mitotic exit, the Cdk1 inhibitor is washed free from cells where cyclin B degradation is blocked, cells can reverse back to M phase. This reversal is characterized by chromosome recondensation, nuclear envelope breakdown, assembly of microtubules into a mitotic spindle, and in most cases, dissolution of the midbody, reopening of the cleavage furrow, and realignment of chromosomes at the metaphase plate. These findings demonstrate that proteasome-dependent protein degradation provides directionality for the M phase to G1 transition.

Cdk1, the major regulator of mitotic progression is activated through binding of cyclin A or B. Cyclin A is degraded during prometaphase when chromosomes move to align at the metaphase plate[6, 7]. Cyclin B degradation begins at metaphase and continues during chromatid segregation in anaphase and exit from M phase[5]. Cytokinesis is initiated shortly after anaphase onset. Cdk1 inactivation and dephosphorylation of Cdk1 substrates during mitotic exit likely serve as timing mechanisms to ensure that cytokinesis occurs after chromatid separation[8-12]. For example prior to anaphase, high Cdk1 activity blocks the accumulation of the cytokinetic regulators Aurora B and MKLP1 at the cleavage furrow and on the microtubules of the spindle midzone[13-15].

Flavopiridol is a potent inhibitor of Cdk1[16]. We found that treatment of vertebrate cells in mitosis with Flavopiridol resulted in premature mitotic exit accompanied by cytokinesis (Fig 1a and Supplementary Video 1). Similar results were recently found for the Cdk inhibitor, BMI-1026[17]. Flavopiridol induced the microtubule network to undergo changes characteristic of anaphase and mitotic exit. The spindle poles moved apart, and microtubule bundles formed in the spindle midzone and at the equatorial cortex. Even though chromatid separation did not occur, cytokinetic furrows formed and ingressed to completion. The cleavage furrow trapped chromosomes in the midbody resulting in a “cut” phenotype. Nevertheless, the chromosomes decondensed and nuclear envelopes reformed. Eventually cytoplasmic contractile activity

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diminished as cells flattened fully onto the substratum, and the microtubule array established an interphase pattern.

During normal mitotic exit, Cdk1 activity is reduced by ubiquitylation and proteasome-mediated degradation of cyclin B[3, 5]. Proteasome inhibitors such as MG132 induce mitotic cells to arrest at metaphase. We found that Flavopiridol treatment overrode the metaphase arrest induced with MG132, causing mitotic exit and cytokinesis that was accompanied by chromosome decondensation and reformation of the nuclear envelope (Fig 1b and Supplementary Video 2). The proteolysis of cyclin B at mitotic exit is thought to ensure the uni-directionality of the M phase to G1 transition[2]. In cells where the proteasome was inhibited, we found that Flavopiridol-induced mitotic exit was reversible. Upon its removal, cells that had exited mitosis could return to metaphase (Fig. 1c and Supplementary Videos 3 and 4). The microtubules, having assumed an interphase configuration after Flavopiridol-induced mitotic exit, reassembled a mitotic spindle when Flavopiridol was removed. The midbody disappeared and the cytokinetic furrow retracted. The newly formed nuclear envelope dissolved. The chromosomes recondensed, attached to spindle microtubules, and realigned at the metaphase plate. This main finding is summarized in Supplementary Fig. 1. We found that cells induced to reverse back to metaphase could subsequently undergo a second, normal mitotic exit including chromatid separation and movement, as well as a second cytokinesis if the proteasome inhibitor was subsequently washed away (Supplementary Video 5). We used Flavopiridol for the majority of our experiments because of it high potency in inhibiting Cdk1 (Supplementary Fig 2). However, certain other chemical Cdk inhibitors also produced similar phenotypes (see Supplementary Information).

In Xenopus S3 cells arrested at metaphase with MG132, Aurora B kinase was concentrated at centromeres and MKLP1 was localized diffusely in the cytoplasm (Fig. 1d). Upon treatment of cells with Flavopiridol, Aurora B and MKLP1 rapidly accumulated at the equatorial cell cortex associating with the nascent cleavage furrows and at the midzone overlaying the microtubule bundles. Eventually the proteins became highly concentrated at the midbody. These changes were consistent with the typical relocation of Aurora B and MKLP1 in normal anaphase. We found that these protein translocation events were also reversible. In cells that were induced through mitotic exit then allowed to reverse back metaphase, Aurora B and MKLP1 returned to their typical metaphase distributions (Fig 1d, 60 min).

Metaphase cells treated with Flavopiridol in the presence of proteasome inhibitor advanced through mitotic exit and cytokinesis, reached the midbody stage with decondensed chromosomes and reassembled nuclear envelopes by 25 minutes post treatment (Fig 1b). Reversibility of mitotic exit was dependent on the duration of exposure to Flavopiridol. Treatment at 5 μM for 17 to 30 min resulted in the majority of cells (81 of 106) returning to metaphase upon Flavopiridol release (Table 1). With longer exposure to Flavopiridol the proportion of cells that underwent reversal declined. However, a few cells (3 of 24) still reversed when Flavopiridol was removed 80 min after its addition. We detected no reversal among cells treated for 90 min (n = 26). Reversibility was entirely dependent on the presence of the proteasome inhibitor. In medium without proteasome inhibitor, premature mitotic exit and cytokinesis induced with Flavopiridol did not reverse in any of 13 prometaphase cells when the Flavopiridol was removed at 17 – 20 min.

Previous studies have shown that several early mitotic regulators (e.g. cyclin A) are normally degraded during prophase and prometaphase and, as well, during the prometaphase arrest induced with microtubule drugs[6, 7]. Xenopus S3 cells were arrested with the microtubule drug, nocodazole, to allow degradation of early mitotic regulators. Cells were then released from nocodazole to medium containing proteasome inhibitor. The cells assembled spindles and arrested at metaphase. These cells were fully capable of undergoing Flavopiridol-induced

mitotic exit and reversal (Fig. 2a and Supplementary Video 6). This result was consistent with the interpretation that mitotic exit reversal in cells treated with proteasome inhibitor was due to the protection of cyclin B from degradation and not due to the preservation of early mitotic regulators.

Cell cycle markers were measured during induced mitotic exit and reversal. Nocodazole-arrested Hela cells were treated with Flavopiridol according to the scheme depicted in Fig 2b. Mitotic indices were calculated from the percentage of cells with condensed chromosomes (Fig 2c, top), and samples were blotted for various cell cycle markers (Fig 2c, bottom). While the level of Cdk1 protein remained constant, Flavopiridol induced mitotic exit and degradation of cyclins B1 and B2 in the absence of the proteasome inhibitor, MG132 (Group 3, −MG132). In medium containing MG132, Flavopiridol induced mitotic exit, but cyclin B1 and B2 were preserved (Group 3, +MG132). Flavopiridol removal at 25 min caused reversal back to M phase in the presence of MG132 (Group 5, +MG132, asterisk), but not in its absence (Group 5, −MG132). As expected, Cyclin A, prominent when the initial population of mitotic cells was analyzed −45 min (Group 1) was substantially degraded during the subsequent 45 min incubation before Flavopiridol addition (Group 2). When the Flavopiridol was removed at 25 min for the mitotic exit reversal experiments, cyclin A was very low (Group 3) and thus was unlikely to contribute significantly to the reactivation of Cdk1. At longer times after treatment with Flavopiridol, some resynthesis of cyclin A protein appeared to occur in cells with MG132 (Group 4 and 5, +MG132). In summary, reversal of mitotic exit after removal of Flavopiridol correlates with the preservation of cyclin B1 and B2.

Flavopiridol was an effective inhibitor when purified Cdk1/CyclinB was used to phosphorylate histone H1 in vitro showing higher potency than certain commonly used Cdk1 inhibitors such as olomoucine and roscovitine (Supplementary Fig. 2). In living cells Flavopiridol resulted in the loss of known Cdk1 target phosphorylations. In both the presence and absence of MG132, Flavopiridol eliminated labeling with the TG-3 antibody that recognizes a Cdk1-catalyzed phosphoepitope on the protein Nucleolin[18] (Group 3). Cdh1, an activator of the mitotic ubiquitin ligase APC/C, is inhibited by Cdk1 phosphorylation during M phase[19]. Flavopiridol treatment induced an increase the electrophoretic mobility of Cdh1 but, for yet unknown reasons, only in the absence of MG132. Cdh1 dephosphorylation may be involved the ubiquitylation and breakdown of cyclin B after Flavopiridol treatment in the absence MG132 (Group 3, −MG132).

Like Xenopus S3 cells, Hela cells cultured in MG132 exhibited a defined period after Flavopiridol-induced mitotic exit when removal of Flavopiridol led to reversal back to metaphase. When Flavopiridol was removed 25 min after its addition, 10 of 10 cells (100%) reversed back to metaphase. When removed at 60 min, only 2 of 12 cells (17%) underwent reversal. To test whether preservation of cyclin B alone was sufficient for reversal of mitotic exit and cytokinesis, a series of experiments were performed using non-degradable cyclin B1 fused to green fluorescent protein (R42A-CyclinB1-GFP) in place of the proteasome inhibitor. Most Hela cells expressing non-degradable cyclin B arrested at metaphase as was previously shown[12]. When these metaphase-arrested cells were treated with Flavopiridol, they completed cytokinesis and exited mitosis. In some cells, Flavopiridol treatment also induced chromatids to separate and to move to the poles before mitotic exit and cytokinesis. When the Flavopiridol was removed 25 min after its addition, 17 of 37 transfected cells underwent reversal back to M phase. In all 17 cells that underwent mitotic exit reversal, the chromosomes recondensed within 10 min after Flavopiridol removal. Cells that had not separated their chromatids opened their cleavage furrows rapidly (average time = 13 min after Flavopiridol removal) and reversed back to metaphase within 30 min (Fig. 3a and Supplementary Video 7). Flavopiridol addition induced chromatid separation in 7 of the 17 cells that subsequently underwent mitotic exit reversal. In these cells the chromatids recondensed rapidly (within 10 minutes after

Flavopiridol removal) but opening of the cleavage furrow was somewhat delayed (average time = 45 min after Flavopiridol removal) (Figure 3b and Supplementary Video 8). The separated chromatids remained near the center of the cell but did not realign to form a metaphase plate. Two of the 7 cells with separated chromatids did not exhibit evidence of reopening their cleavage furrows at their last observation time of 90 min after Flavopiridol removal. Thus cells with separated chromatids can undergo reversal of mitotic exit and cytokinesis but, predictably, the chromatids cannot realign at the metaphase plate if sister chromatids have lost cohesion. Moreover while cytokinesis is reversible after chromatid segregation, reopening of the cleavage furrow generally took longer to initiate. Possibly the trapped chromatin physically facilitates reopening of the furrow, or cells that separate their chromatids may have biochemical differences that restrict reopening of the furrow. Mitotic cells expressing high levels of wild type cyclin B fused to GFP underwent mitotic exit and cytokinesis upon Flavopiridol treatment, but as expected, removing Flavopiridol at 25 min never resulted in reversal of mitotic exit (n = 6) (Fig. 3c and Supplementary Video 9).

Cells expressing high levels of non-degradable cyclin B, as measured by GFP fluorescence, were more likely to undergo reversal (Fig. 3d). Previous work suggests that cell cycle regulation in M phase exhibits the property of hysteresis because entry into M phase from G2 requires higher Cdk1 activity than does maintaining M phase once it is achieved[20, 21]. This hysteresis may explain why many cells expressing relatively low levels of non-degradable cyclin B arrested at metaphase before Flavopiridol treatment but were unable to undergo reversal when Flavopiridol was removed. In addition, in confirmation of our previous conclusion that the reversal of mitotic exit and the reestablishment of high Cdk1 activity levels in proteasome inhibitor are likely driven by cyclin B and not cyclin A, we found cells arrested at metaphase by expression of non-degradable cyclin B had nearly undetectable levels of cyclin A (Fig. 3e).

The studies above were completed using immortalized cell lines. To determine whether these findings were broadly applicable to non-immortalized cells, we also tested primary human keratinocytes. We found that these cells, cultured in medium containing MG132, underwent mitotic exit and cytokinesis in response to Flavopiridol treatment, and reversed back to metaphase when Flavopiridol was removed (Fig. 4 and Supplementary Video 10).

The importance of regulated proteolysis for guiding the directionality of cell cycle transitions is a central tenet of modern cell cycle theory extended from the founding work on the proteolysis of cyclins in early embryos[1-4, 22]. Here we have tested this idea, showing that if cyclin B is stabilized, the events of mitotic exit and cytokinesis are immediately reversible even in cells that have progressed through chromosome decondensation, reconstitution of the interphase nucleus, and mitotic spindle disassembly and cytokinesis to the stage of midbody formation. However, with increasing time after Flavopiridol-induced mitotic exit, reversibility wanes. Thus cyclin degradation does provide directionality during mitotic exit but this function is later supplemented by other events that inhibit backtracking. These events are likely to involve other biochemical changes to Cdk1 including phosphorylation changes or the binding of small molecule inhibitors. The ability to regulate mitotic exit in the absence of cyclin degradation in vertebrate cells will facilitate elucidating these downstream regulators of the M phase to G1 phase transition in vertebrate cells.

METHODS

Cell culture and live cell imaging

Xenopus S3 cells stably expressing GFP-α-tubulin were grown at 23°C in 70% L-15 medium supplemented with 15% FBS. For control images of these cells transiting M phase see Supplementary Video 12. HeLa cells were grown in DMEM with 10% FBS in 5% CO_2 at 37° C. Normal human epidermal keratinocytes (Clonectics) were grown in Defined Keratinocyte

SFM (InVitrogen). Time-lapse phase contrast and fluorescence images were collected from cells grown on glass coverslips using with a Zeiss Axiovert 200M microscope equipped with a Hamamatsu ORCA camera. When applicable, cells were incubated for 30 - 60 min with 25 μM MG132 (Calbiochem) or with 100ng/ml nocodazole (Sigma). Flavopiridol was provided by the National Cancer Institute (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis), and used at 5 - 10μM. In cells expressing Cyclin B1 fused to GFP, levels of expression were quantified using Metamorph software (Molecular Devices).

Immunofluorescence and Immunoblotting

Cells on coverslips were fixed in 2 – 3% formaldehyde in 60mM PIPES, 25Mm HEPES (pH 6.8), 10mM EDTA, 4mM $MgCl_2$ (PHEM) for 15 minutes, then permeabilized with 0.5% or 1% Triton X-100. Hela cells in suspension were fixed in PHEM buffer containing 1.5% formaldehyde and 0.5% Triton X-100. Antibodies to Xenopus MKLP1 and Aurora B were prepared in rabbits. Mouse antibody to human cyclin A was obtained from J. Gannon and T. Hunt or obtained commercially (Abcam). Fluorescence images were gathered with a Zeiss Axioplan II microscope with a Hamamatsu ORCA-II camera. Samples were treated with DAPI and mounted in Vectashield containing 10 mM $MgSO_4$. Cells were lysed in NuPAGE (Invitrogen) western blotting sample buffer containing 50 mM DTT. Samples were electrophoresed on 4% - 12% gradient gels, transferred to PVDF membranes, and probed with primary antibodies overnight at 4°C. Blots were treated with appropriate HRP-conjugated secondary antibodies and were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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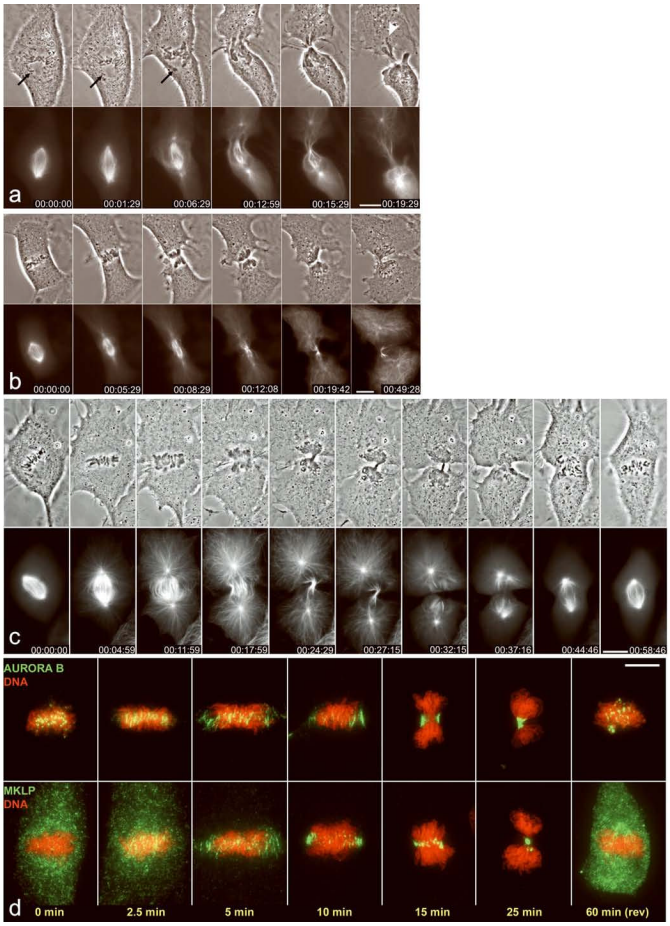

Figure 1.

The Cdk inhibitor Flavopiridol induces reversible mitotic exit and cytokinesis if proteasome activity is inhibited. a, Treatment of mitotic cells with Flavopiridol induces premature mitotic exit and cytokinesis without chromatid separation. A Xenopus S3 cell expressing GFP-α-tubulin was treated with 5 μM Flavopiridol at time 0. The black arrow indicates a chromosome not yet at metaphase when Flavopiridol was added. The white arrowhead indicates the reassembled nuclear envelope. The complete video sequence is available as Supplementary Video 1. Time is indicated as hours:minutes:seconds (a.- c.). **b,** Flavopiridol induces mitotic exit in cells arrested at metaphase with MG132. An MG132-arrested cell was treated with 10 μM Flavopiridol at time 0. The complete video sequence is found as Supplementary Video 2. **c,** Flavopiridol-induced mitotic exit and cytokinesis are reversible if the proteasome is inhibited. An MG132-arrested cell was treated with 5 μM Flavopiridol at time 0. The Flavopiridol was removed at 25 min. The complete video sequence is available as Supplementary Video 3. **d,** Flavopiridol induces normal mitotic exit changes in the distributions of Aurora B kinase and MKLP1 that are reversible. Xenopus S3 cells were incubated in medium containing MG132 and were fixed before and after treatment with 5 μM Flavopiridol. Samples were labeled for Aurora B kinase (upper panels) or MKLP1 (lower panels). Initial and final images (0 min and 60 min) were scaled identically for brightness and contrast. Others were scaled individually. Bars = 10 μm.

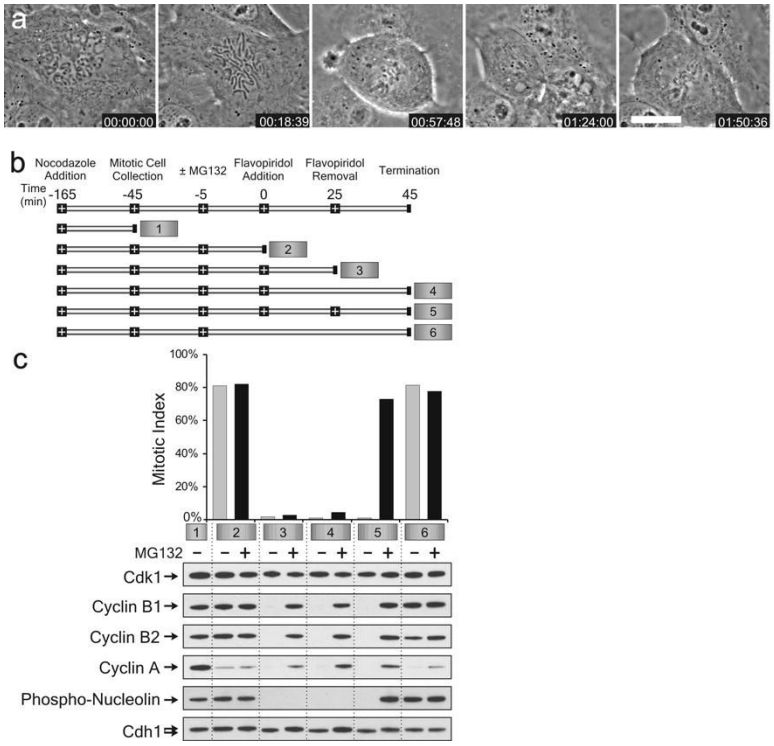

Figure 2.

Reversibility of mitotic exit requires preservation of Cyclin B. a, A Xenopus S3 cell treated with 100 ng/ml nocodazole at time 0. At 30 min after nuclear envelope breakdown, nocodazole was removed and the cell was arrested at metaphase with MG132. Flavopiridol at 5 μM was added at 1:04:00 and removed 25 min later after mitotic exit. The complete video sequence is available as Supplementary Video 6. Bar = 10 μm. **b,** Experimental protocol employed for producing samples from Hela cells at various stages of induction and reversal of mitotic exit. **c,** Samples were analyzed for mitotic index and blotted for the indicated proteins.

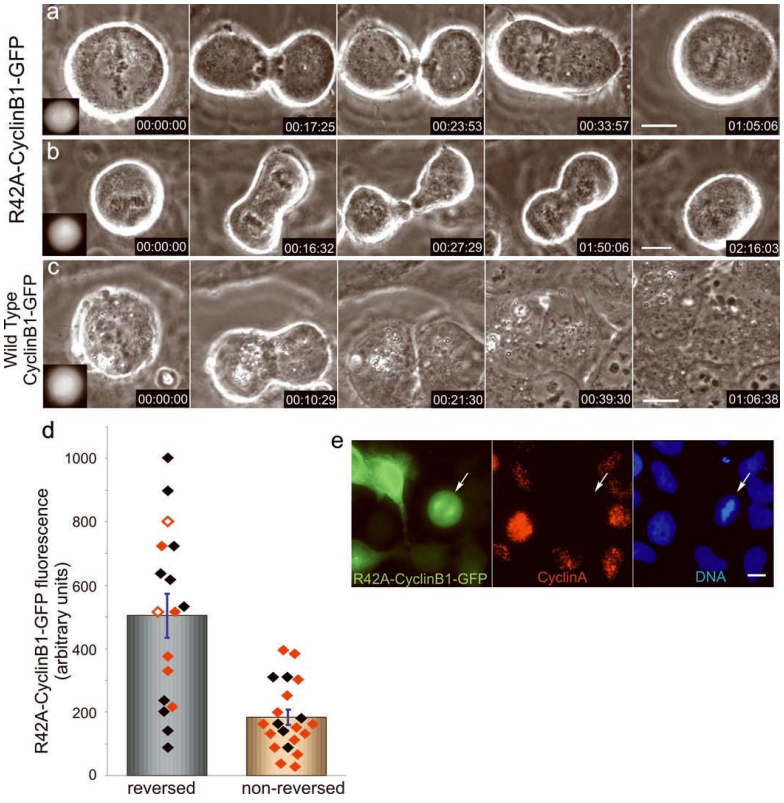

Figure 3.

Cells expressing non-degradable Cyclin B1 undergo mitotic exit reversal. a, Hela cells expressing non-degradable cyclin B1 can undergo mitotic exit reversal without segregating chromosomes. Flavopiridol was added at time 0 and removed at 25 min. The complete video sequence is available Supplementary Video 7. **b,** Hela cells expressing non-degradable cyclin B1 can undergo mitotic exit reversal after chromatid separation. Flavopiridol added and removed as in (a). The complete video sequence is available as Supplementary Video 8. **c,** Hela cells expressing wild type Cyclin B1 do not undergo reversal of mitotic exit. Flavopiridol added and removed as in (a). The complete video sequence is available as Supplementary Video 9. Insets show GFP fluorescence images at time 0. Level of the wild type Cyclin B1 in (c) was approximately twice that of the non-degradable Cyclin B1 expressed in (a) and (b). **d,** Cells expressing high levels of non-degradable Cyclin B1 were more likely to undergo mitotic exit reversal. Cells that did not separate chromatids upon treatment with Flavopiridol are depicted by black symbols. Those that separated chromatids are depicted in red. Two cells that recondensed their chromosomes but had not opened their cleavage furrows by 90 min after Flavopiridol removal are depicted by hollow red symbols. Error bars show S.E.M. **e,** A mitotic Hela cell at metaphase (arrow) expressing non-degradable Cyclin B1, shows low levels of Cyclin A expression by immunofluorescence. Bars = 10μm.

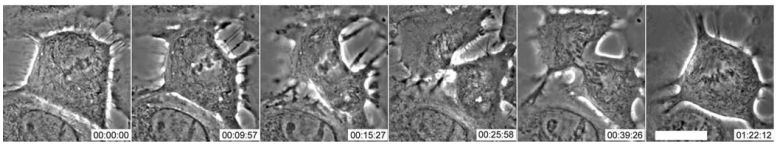

Figure 4.

Primary cultures of human cells can undergo mitotic exit reversal. An MG132-arrested metaphase cell was imaged by phase contrast microscopy after addition of 7.5 μM Flavopiridol at time 0. At 26 min, the Flavopiridol was removed. The complete video sequence is available as Supplementary Video 10. Bar = 10 μm.

Table 1

Reversal of mitotic exit in Xenopus S3 cells upon removal of Flavopiridol

Duration of Flavopiridol treatment (min)	# cells imaged	# cells reversed through mitotic exit (% reversed)
17	29	28 (97%)
20	27	25 (93%)
25	27	17 (63%)
30	23	11 (48%)
33 - 37	17	8 (47%)
40	22	6 (27%)
50	19	4 (21%)
60	17	1 (06%)
70	24	1 (04%)
80	24	3 (12%)
90	26	0 (0%)