

# HIV-1 Vpr Induces Defects in Mitosis, Cytokinesis, Nuclear Structure, and Centrosomes

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Human immunodeficiency virus type 1 (HIV-1) Vpr is a 15-kDa accessory protein that contributes to several steps in the viral replication cycle and promotes virus-associated pathology. Previous studies demonstrated that Vpr inhibits G2/M cell cycle progression in both human cells and in the fission yeast *Schizosaccharomyces pombe*. Here, we report that, upon induction of *vpr* expression, fission yeast exhibited numerous defects in the assembly and function of the mitotic spindle. In particular, two spindle pole body proteins, *sad1p* and the polo kinase *plp1p*, were delocalized in *vpr*-expressing yeast cells, suggesting that spindle pole body integrity was perturbed. In addition, nuclear envelope structure, contractile actin ring formation, and cytokinesis were also disrupted. Similar Vpr-induced defects in mitosis and cytokinesis were observed in human cells, including aberrant mitotic spindles, multiple centrosomes, and multinucleate cells. These defects in cell division and centrosomes might account for some of the pathological effects associated with HIV-1 infection.

## INTRODUCTION

Lentiviruses possess a number of accessory genes in addition to the *gag*, *pol*, and *env* genes common to all retroviruses. Human immunodeficiency virus type 1 (HIV-1), the lentivirus most commonly associated with the acquired immunodeficiency syndrome, carries six accessory genes that are required either for full viral replication capacity or for pathogenicity. Vpr is a 15-kDa accessory protein that is virion-associated (Cohen *et al.*, 1990; Yuan *et al.*, 1990; Paxton *et al.*, 1993) and transported to the nucleus as part of the viral preintegration complex (Heinzinger *et al.*, 1994; Fassati and Goff, 2001). All primate immunodeficiency viruses possess either Vpr or the closely related protein Vpx (Tristem *et al.*, 1992). The functional importance of Vpr is demonstrated by the fact that an intact *vpr* open reading frame is selected for in vivo: repair of a disrupted *vpr* open reading frame was observed in chimps, or the rare human, in which there was documented infection with a provirus bearing *vpr* mutations (Goh *et al.*, 1998). Vpr variants have been correlated with long-term survival in HIV-1-infected individuals (Zhao *et al.*, 2002). Vpr increases virus replication rates in macrophages in standard tissue culture systems (Connor *et al.*, 1995; Vodicka *et al.*, 1998) and in tonsil histoculture (Eckstein *et al.*, 2001). Increased virus replication rates in macrophages may be related to effects of Vpr on nuclear transport of preintegration complexes upon entry into these nondividing cells (Heinzinger *et al.*, 1994). Many laboratory strains of HIV viruses lack *vpr*, because Vpr inhibits growth of cells in

culture (Rogel *et al.*, 1995). Vpr thus contributes to HIV replication and pathogenesis.

Numerous effects of Vpr on the cell have been reported, including disruption of cell cycle control, enhancement of transcription, and induction of apoptosis. One relatively well characterized function is the arrest of proliferating CD4<sup>+</sup> T cells in the G2 stage of the cell cycle (He *et al.*, 1995; Jowett *et al.*, 1995; Re *et al.*, 1995; Rogel *et al.*, 1995; Bartz *et al.*, 1996). This provides a replication advantage for the virus because proviral transcription is highest during G2 (Forget *et al.*, 1998; Goh *et al.*, 1998). In human or fission yeast cells expressing *vpr*, *cdc2p*, the cyclin-dependent kinase that regulates entry into mitosis, is in the inactive phosphorylated state (He *et al.*, 1995; Re *et al.*, 1995; Zhao *et al.*, 1996; Zhang *et al.*, 1997). Vpr does not seem to be a stoichiometric inhibitor of the *cdc2p*-cyclin B complex, but rather, may exert effects on regulators of *cdc2p*, such as *cdc25C*, *Wee1*, or *PP2A* (He *et al.*, 1995; Re *et al.*, 1995; Zhao *et al.*, 1996; Elder *et al.*, 2000, 2001; Masuda *et al.*, 2000).

A second major function of Vpr is on nuclear transport. Vpr promotes nuclear entry of the viral preintegration complex and colocalizes with nuclear pores on the nuclear envelope (Heinzinger *et al.*, 1994; Vodicka *et al.*, 1998). Recent studies show that Vpr expression in mammalian cells causes herniations and large holes in the nuclear envelope, which may facilitate nuclear entry of viral preintegration complexes and disrupt the localization of cell cycle regulatory proteins (de Noronha *et al.*, 2001).

Here, we have characterized additional effects of Vpr in both fission yeast and human cells. Vpr caused multiple defects in cell division, including effects on the mitotic spindle, centrosome/spindle pole body (SPB), nuclear envelope, and cytokinesis. Because the centrosome/SPB acts not only as a microtubule-organizing center but also as a repository for many cell cycle regulatory factors (Lange, 2002), defects

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in the SPB/centrosome might explain some of the multiple effects of Vpr on the cell division cycle.

## MATERIALS AND METHODS

### Plasmid DNAs

HIV-1<sub>NL4-3</sub> *vpr* was polymerase chain reaction (PCR) amplified using Pfu DNA polymerase (Stratagene, La Jolla, CA) and the following primers: GCGCGGATCCGAGAGATGGAAACAAGCCCCAGAAAGAC and GCGCGGATCCCTAGGATCTACTGGCTCCATTTC. To construct a hemagglutinin (HA) epitope tagged *vpr*, HIV-1<sub>NL4-3</sub> *vpr* was PCR amplified using Pfu DNA polymerase (Stratagene) and the following primers: 5'-GCGCGGATCCGC-CACCATGGGTTACCCATACGATGTCCAGATTACGCTGAACAAGCCC-CAGAAGACCAAGG-3' (HA sequence underlined) and 5'-GCGCGGATCCCTAGGATCTACTGGCTCCATTTC-3'. The PCR products were digested with *Bam*HI, cloned into the *Bam*HI site of pBluescript II KS<sup>-</sup> (Stratagene) and sequenced. For thiamine-regulated expression in *Schizosaccharomyces pombe*, *Bam*HI fragments containing either *vpr* or *HA-vpr* sequences were then cloned into the *Bam*HI site of pREP1N (Maundrell, 1993). For expression in mammalian cells, a *Spe*I/*Xho*I fragment containing *HA-vpr* was inserted into the *Nhe*I and *Xho*I sites of pCep4 (Invitrogen, Carlsbad, CA). The bicistronic vector pVpr-IRES-EGFP was constructed by inserting the *Bam*HI fragment containing the *vpr* sequence into the *Bgl*III site of pIRES2-EGFP (BD Biosciences Clontech, Palo Alto, CA).

### Fission Yeast Methods

Standard methods for *S. pombe* media, genetic manipulations, immunofluorescence, and staining are described at <http://www.bio.uva.nl/pombe/handbook/>. *S. pombe* strains used were FC16 (*h-* *leu1-32*) and FC840 (*h-* *leu1-32* *plol1-GFP-kanMX*) (Bahler *et al.*, 1998). Yeast transformations were performed using a Frozen EZ yeast transformation kit (Zymo Research, Orange, CA). Fresh *pnmt-vpr* transformants were characterized within 1 or 2 wk after transformation, because cells that had been passaged repeatedly or frozen previously exhibited less consistent phenotypes. For induction of the *nmt1* promoter, exponential phase liquid cultures of cells in EMM with thiamine were filtered, washed in water, and then resuspended in prewarmed Edinburg minimal media (EMM) without thiamine and grown at 30°C.

To monitor nuclear envelope defects, we used a *S. pombe* strain in which an SV40 NLS-GFP-LacZ nuclear reporter and an Nsp1p-GFP nucleoporin that localizes to the nuclear envelope are expressed by the *nmt1* gene promoter from constructs integrated into the chromosome (Yoshida and Sazer, 2004). For immunofluorescence, methanol-fixed cells were stained with a monoclonal anti-tubulin antibody TAT1 (Woods *et al.*, 1989) (gift from K. Gull, University of Oxford, Oxford, United Kingdom) and rabbit anti-sad 1p antibody (Hagan and Yanagida, 1995) (gift from I. Hagan, University of Manchester, Manchester, United Kingdom) at a 1/100 dilution. Rhodamine-phalloidin (Molecular Probes, Eugene, OR) staining was performed as described previously (Chang *et al.*, 1996a) using a 4-min formaldehyde fixation period.

### Culture and Transfection of Human Cells

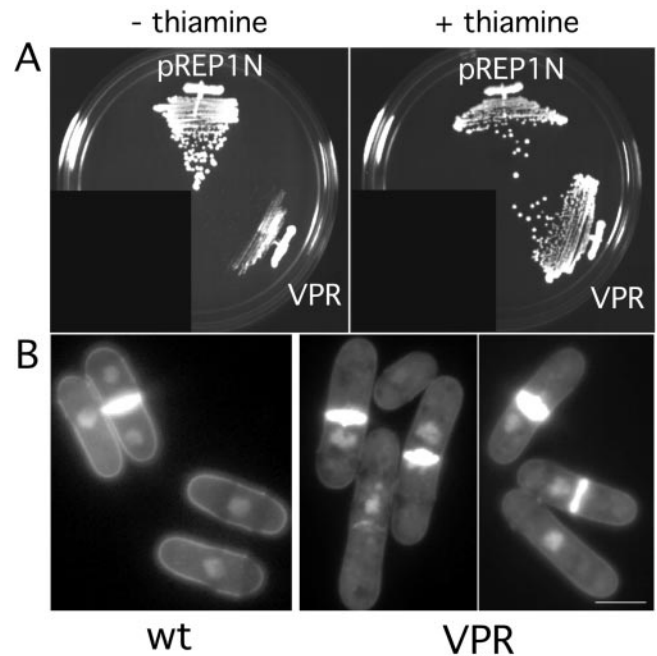
HeLa cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM with 10% fetal calf serum. Pooled human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex Bio Science Walkersville (San Diego, CA). HUVECs were grown on gelatin-coated petri dishes and maintained <10 passages in human endothelial-SFM basal growth medium (Invitrogen) supplemented with 20% fetal calf serum (Invitrogen), 10 ng/ml human recombinant epidermal growth factor (Intergen, Purchase, NY), 15 ng/ml human recombinant basic fibroblast growth factor (Intergen), and 1  $\mu$ g/ml heparin (Sigma-Aldrich). For transfection, HeLa and HUVECs were briefly trypsinized, washed in DMEM with 10% fetal calf serum, and resuspended in ice-cold phosphate-buffered saline (PBS) with 1.25% dimethyl sulfoxide at a concentration of  $2 \times 10^7$  cells/ml. Then, 300  $\mu$ l of the cell suspension was electroporated with 30  $\mu$ g of plasmid DNA at 250 V, 960  $\mu$ F by using an electroporator (Bio-Rad, Hercules, CA). Hygromycin concentrations used for plasmid selection were 750  $\mu$ g/ml for HeLa cells and 100  $\mu$ g/ml for HUVECs. DNA content was measured by flow cytometry after stained with propidium iodide as described previously (Re *et al.*, 1995).

### Microscopy

Microscopy was performed using a wide field fluorescence microscope as described previously (Pelham and Chang, 2001) by using Open Lab 2 software (Improvision, Lexington, MA) for acquisition and analysis. In some cases, multiple focal planes were acquired and used to assemble deconvolved maximum projections.

### Microscopic Examination of Human Cells

HeLa cells grown on glass coverslips were fixed in PBS with 4% paraformaldehyde for 30 min, permeabilized in PBS with 0.1% Triton X-100, and mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 1



**Figure 1.** Nuclear division defects in *S. pombe* expressing *vpr*. (A) Fission yeast cells carrying pREP1N vector or pREP-VPR were streaked onto minimal plates without thiamine (*nmt1* promoter on) or with thiamine (*nmt1* promoter off). (B) Cells stained with DAPI to visualize DNA and calcofluor to visualize septum. Left, wild-type cells. Right, cells with pREP-VPR 17 h after shift into media lacking thiamine. These panels show septated cells that have a failure in nuclear division.

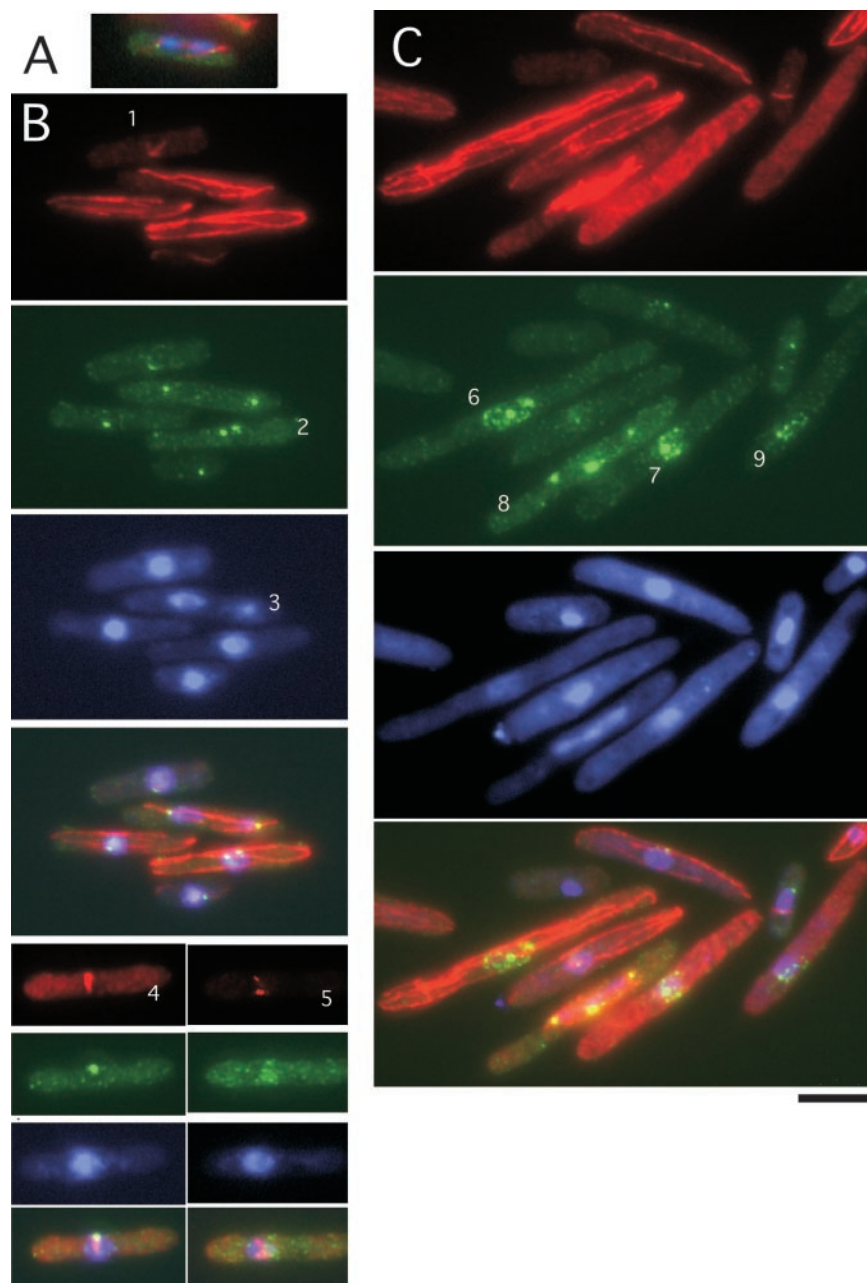
$\mu$ g/ml 4,6-diamidino-2-phenylindole (DAPI). HUVECs were photographed on an inverted phase-contrast microscope. For immunofluorescence staining, cells were fixed in methanol at  $-20^{\circ}\text{C}$  for 20 min and permeabilized in PBS/0.1% Tween 20. Coverslips were incubated with primary antibody in PBS/3% bovine serum albumin for 1 h, washed three times, and incubated with secondary antibodies in PBS/3% bovine serum albumin/0.1% Tween 20 for 30 min. The following antibodies and dilutions were used: anti- $\gamma$ -tubulin GT335, mouse IgG, 1:1000; and anti Centrin-2, rabbit serum, 1:2000. They were both kindly provided by Anne Paoletti (Institute Curie, Paris, France). The following secondary antibodies were used: Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes) and phycoerythrin-conjugated goat anti-rabbit. Both were used at 1:1000 dilution. For images of mitotic spindles, images were acquired in multiple focal planes and then were deconvolved and maximum projected using OpenLab 3 image analysis software (Improvision, Lexington, MA).

## RESULTS

### *vpr* Expression Causes Mitotic Spindle Defects

The effects of HIV-1 Vpr on *S. pombe* were examined using a vector in which expression was under the control of the thiamine-repressible *nmt1* promoter. In the presence of thiamine, cells showed little or no inhibition in growth rate (Figure 1A). In the absence of thiamine, *vpr* expression caused strong inhibition of colony formation (Figure 1A).

Fission yeast expressing Vpr were analyzed in time courses 17–24 h after removal of thiamine. Previous studies showed that Vpr expression in *S. pombe* is associated with an accumulation of elongated cells that are delayed or arrested at the G2/M cell cycle transition (Zhao *et al.*, 1996; Zhang *et al.*, 1997). We found that cells began to exhibit elongated cell shapes by 19 h and lengthened progressively by 24 h. We noticed that after 17 h in thiamine-free media, before significant cell elongation, 95% of cells with a septum (7% of total

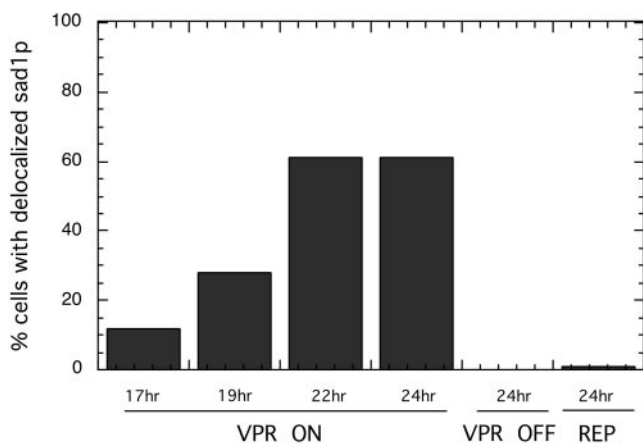


**Figure 2.** Mitotic spindle defects in *S. pombe* expressing *vpr*. Cells carrying pREP1N (A) or pREP-VPR (B and C) were grown under inducing conditions for 17 h (B) or 24 h (A and C) and then fixed with methanol and stained by immunofluorescence for tubulin, *sad1p*, and DNA. Presented are projections of multiple focal planes. Panels are organized in sets of four, with the top (red) for tubulin, second (green) for *sad1p*, third (blue) for DNA, and fourth (multicolor) for merged image. Examples of particular phenotypes are indicated with numbers as follows: 1, abnormal V-shaped spindle; 2, interphase cell with two separate spindle pole bodies; 3, cell in anaphase with unequal separation of the chromosomes; 4, monopolar spindle; 5, cell in which spindle microtubules do not connect and spindle pole body is delocalized; 6, interphase cell with delocalized *sad1p* in a perinuclear pattern; 7 and 9, mitotic cells with perinuclear *sad1p*; and 8, mitotic cell with a double spindle.

cells;  $n = 100$ ) exhibited a “cut” phenotype, in which the nucleus failed to divide and was sometimes cut by the septum or was dispersed unevenly between the two daughter cells (Figure 1B). Because this phenotype is indicative of mitotic defects, this finding suggested that Vpr may affect nuclear division before development of a G2 arrest.

To characterize these mitotic defects, we visualized mitotic spindles by staining cells for tubulin, DNA, and the spindle pole body marker *sad1p*. In cells carrying the vector only, bipolar spindles were organized from two discrete spindle pole bodies located at the nuclear membrane (Figure 2A). In *vpr*-expressing cells, many types of mitotic defects were seen, including monopolar spindles, V-shaped spindles, and lagging chromosomes during anaphase (Figure 2, B and C). At 19 h after thiamine removal, 20% of the cells were in mitosis (by tubulin staining), and of these ( $n = 113$  mitotic cells), 59% exhibited an abnormal spindle, with 51%

showing some defect in mitotic spindle assembly. Twenty-eight percent of mitotic cells had a monopolar spindle, in which a dot or a single bar of microtubules emanated from one *sad1p*-staining dot, which can be caused by a defect in spindle pole body duplication or separation. Twenty-three percent of the spindles were abnormal V-shaped spindles, in which two or more intranuclear microtubule bundles were associated with a single *sad1p*-staining dot, which can be caused by mitotic spindle collapse or by defects in spindle pole body separation. These patterns suggest defects in spindle pole body function, separation, and duplication. We also noted lagging chromosomes during anaphase, indicative of chromosomal segregation defects. There were also some SPB defects in interphase cells, notably premature duplication or separation of spindle pole bodies (Figure 2, cell 2). Similar defects in mitotic spindles were also seen after 17 h of induction (31% spindles were abnormal;  $n = 100$ ). At both of



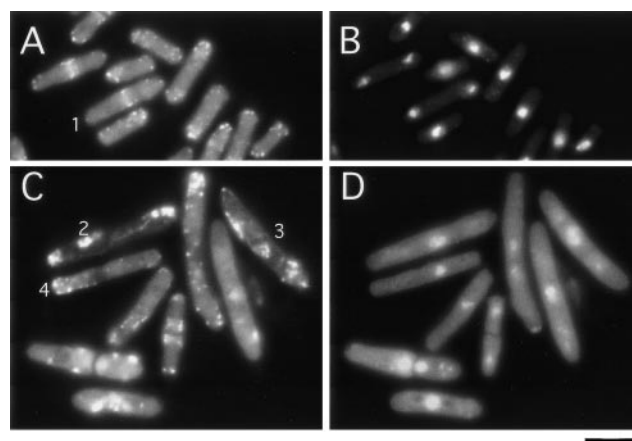
**Figure 3.** Progressive delocalization of the spindle pole body protein sad1p upon induction of *vpr* expression. Cells bearing pREP1 or pREP-VPR were grown under inducing conditions for 17, 19, 22, or 24 h. Cells were harvested, fixed, and examined by immunofluorescence for Sad1p, and imaged in three-dimensional projections; 78–200 cells were examined for each time point.

these early time points, mitotic spindle abnormalities were present in cells before cell elongation, suggesting that G2 arrest did not contribute to these phenotypes.

One striking defect was that sad1p, a nuclear envelope transmembrane protein that is usually concentrated in one or two discrete dots (the spindle pole body) on the nuclear envelope, was sometimes delocalized in a punctuate pattern around the nuclear envelope (Figure 2C, cells 6, 7, and 9). This perinuclear distribution was present in 12% of cells at 17 h, and by 22 and 24 h, in >60% of cells (Figure 3). This sad1p pattern was seen in mitotic as well as interphase cells and occurred in nonelongated cells in the early time points. However, this phenotype was especially prevalent in elongated cells at the later time points: 92% of the elongated cells (>13  $\mu\text{m}$ ) at 24 h exhibited perinuclear sad1p. Many mitotic cells exhibited perinuclear sad1p staining even with a bipolar spindle (30–40% cells with a bipolar spindle at 17–19 h), suggesting that these additional sad1p dots did not represent extrafunctional spindle pole bodies. Cells in both interphase and mitosis exhibited this perinuclear pattern. Cells carrying the vector only or the *vpr* plasmid under repressing conditions did not exhibit these defects in mitosis or sad1p distribution. In contrast to sad1p, the distribution of the  $\gamma$ -tubulin complex protein alp4-GFP was not disrupted in Vpr-expressing cells, even at later time points, suggesting that only a subset of spindle pole body components were affected. Thus, Vpr disturbs mitosis in part by affecting the integrity of a portion of the spindle pole body.

#### Vpr Causes Cytokinesis Defects

Cell division in *S. pombe* involves the formation of an actin-based contractile ring in early mitosis and then contraction of the ring and formation of the cell wall septum in late mitosis. At 17 h, *vpr*-expressing cells formed fairly normal septa and actin rings (Figure 1C). However, at later time points, severe defects in actin ring formation and septum formation were apparent (Figure 4; our unpublished observations). In some mitotic cells (cells with condensed chromosomes shown by DAPI staining or in cells with chromosomes in an anaphase configuration), F-actin was located in abnormal aggregates instead of a discrete ring (Figure 4, cells 2 and 3) (see also Chen *et al.*, 1999). In some mitotic



**Figure 4.** Vpr causes defects in the contractile actin ring. Cells bearing pREP-VPR were grown under inducing conditions for 21 h, fixed, and stained with Alexa Fluor-phalloidin (A and C) and DAPI (B and D). Cell 1 exhibits a normal actin ring. Cells 2 and 3 are mitotic cells (as evidenced by condensed chromatin) and exhibit very abnormal actin organization. Cell 4 has normal interphase actin distribution.

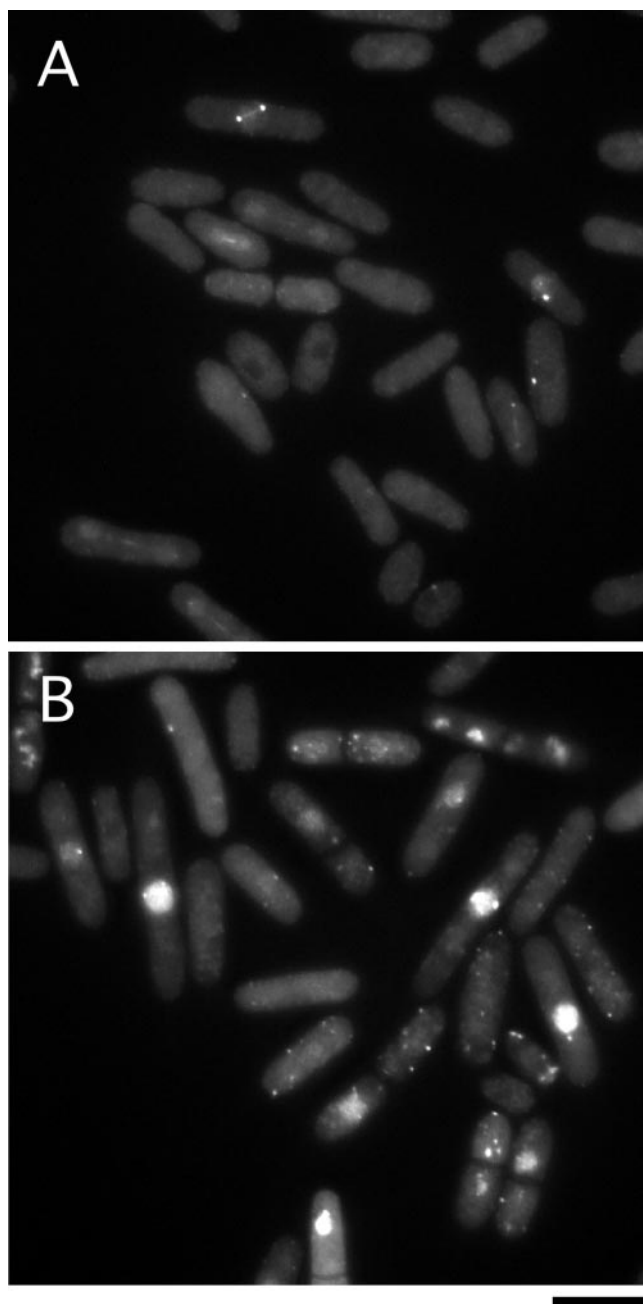
cells, actin was present as a disorganized medial web of actin. Some cells maintained normal interphase actin distribution of polarized actin patches (Figure 4, cell 4), suggesting that Vpr inhibits actin rings specifically and not other actin structures. These actin ring defects were accompanied by similar severe defects in the organization of the septum (Chen *et al.*, 1999; our unpublished observations). Because actin ring assembly is not dependent on spindle assembly (Chang *et al.*, 1996b), these types of cytokinesis defects were probably not a secondary effect of the mitotic defects.

#### Vpr Causes Mislocalization of Polo Kinase

The polo kinase plo1p is a conserved regulator of multiple aspects of cell division, including mitotic entry, mitotic spindle assembly, centrosome maturation, mitotic exit, and cytokinesis (Ohkura *et al.*, 1995; Glover *et al.*, 1998; Bahler *et al.*, 1998). Because many of the phenotypes of Vpr expression are similar to those of plo1-loss of function mutants, we tested the effect of Vpr on plo1-GFP localization. This plo1-GFP fusion is functional and expressed from the *plo1+* chromosomal locus at endogenous levels (Bahler *et al.*, 1998). In cells carrying the control vector, plo1-GFP was located on the spindle pole body, spindle and weakly in the nucleus during mitosis, with no detectable fluorescence in interphase cells (Figure 5A). In Vpr-expressing cells, plo1-GFP was delocalized in multiple, discrete cytoplasmic dots in some cells or concentrated in the nucleus (Figure 5B). Because abnormal plo1-GFP distribution occurred even in nonelongated cells, this effect is not secondary to G2 arrest. These results indicate that at least some of the effects of Vpr may be caused by abnormal localization of polo kinase.

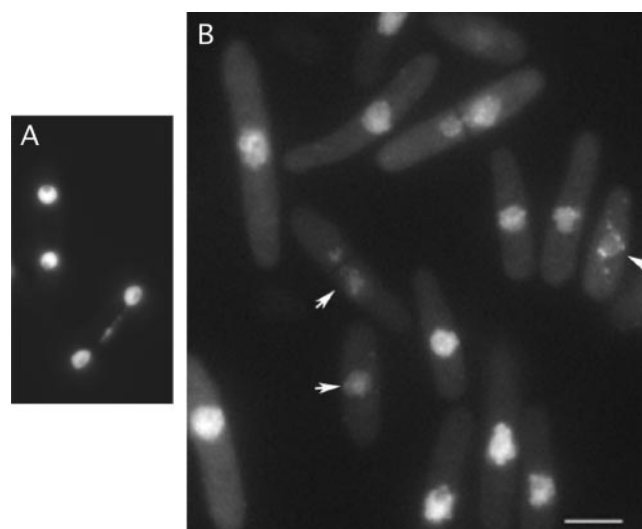
#### Vpr Causes Defects in the Nuclear Envelope

A recent study in mammalian tissue culture cells suggested that Vpr expression causes large herniations and holes in the nuclear envelope (de Noronha *et al.*, 2001). We tested whether Vpr affects nuclear structure in fission yeast. We examined a strain that expressed both a nucleoporin-GFP, which marks the nuclear envelope, and a GFP-lacZ fusion containing a nuclear localization signal (NLS), a soluble



**Figure 5.** Vpr causes mislocalization of the polo kinase *plp1p* in *S. pombe*. Cells with a chromosomal *plp1p*-GFP construct and containing either pREP1 (A) or pREP-VPR (B) plasmids were grown under inducing conditions for 21 h and imaged for GFP fluorescence.

marker that enters the nucleus from constructs integrated into the genome to ensure uniform expression throughout the population (Yoshida and Sazer, 2004). Expression of these fusions themselves does not affect nuclear structure or cell viability. In cells with broken nuclear envelopes, the soluble NLS-GFP leaks out of the nucleus, so that the nucleus is dimmer and exhibits the nucleoporin GFP outlining the nuclear envelope (Yoshida and Sazer, 2004). In wild-type cells, the NLS-GFP-lacZ was strongly concentrated in the nucleus (Figure 6A). In cells expressing Vpr, we observed multiple types of nuclear defects. First, the majority of cells

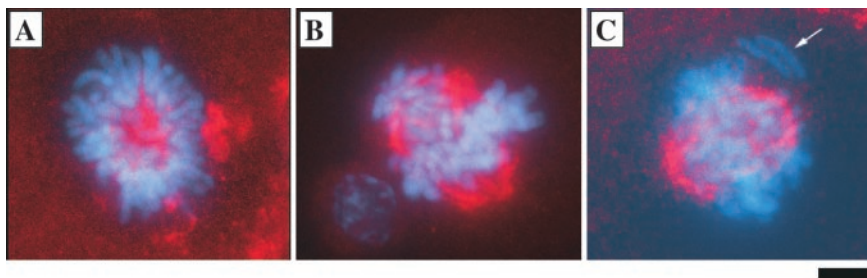


**Figure 6.** Vpr causes defects in nuclear structure in *S. pombe* cells. Nuclei in cells carrying a pREP1N (A) or pREP-VPR (19-h induction time) (B) were imaged by fluorescence microscopy by coexpression of an NLS-GFP-lacZ, which assays for nuclear transport and breakage, and a nucleoporin-GFP fusion protein, which labels the nuclear envelope. (A) Wild-type nuclei just after or during nuclear division. The dots between one pair of nuclei represent nuclear pores that are present on nuclear envelope that surrounds the elongating mitotic spindle. (B) Dim nuclei (arrows) represent ones that lack NLS-GFP-lacZ staining and thus may have broken.

exhibited odd nuclear shape: nuclei were often larger and were no longer round and smooth but had a lumpy shape with multiple small herniations or projections (Figure 6B) (84% abnormal nuclei at 19 h, 90% at 21 h, and 93% at 24 h;  $n \geq 85$ ). Second, a subset of nuclei was no longer stained brightly with NLS-GFP-lacZ. Some of these cells also had an uneven distribution of the GFP-labeled nuclear pores at the nuclear envelope (45% of nuclei at 21 h;  $n = 96$ ) (Figure 6B, cells marked with arrows). In addition, in many of these cells, (11% cells at 21 h of induction), dots of nucleoporin staining were detected in the cytoplasm, consistent with a structural defect in nuclear envelope components (Figure 6B, cell on right with arrow). Some of these defective nuclei were cut by the septum, but others were in interphase cells without a septum. The loss of NLS-GFP-lacZ staining was consistent with breaks in the nuclear envelope or defects in nuclear transport. Time-lapse movies (>30–50 min) generally did not show sudden changes in the NLS-GFP intensity or large changes in nuclear shape. During the course of these movies, we documented nuclear breakage events (by the sudden loss of NLS-GFP-lacZ fluorescence) in <1% of cells, suggesting that in contrast to findings in mammalian cells, frequent breakage, and resealing of the nuclei did not occur. Thus, these images suggest that as in mammalian cells, Vpr induced nuclear envelope defects in fission yeast.

#### *Vpr Causes Mitotic and Cytokinesis Defects in Human Cells*

These results in fission yeast prompted reevaluation of the phenotype of human cells expressing Vpr. First, we examined the effect of Vpr expression in HeLa cells (Figures 7 and 8). To analyze the effect of *vpr* expression within hours of transfection, we electroporated HeLa cells with a bicistronic vector that expresses both *vpr* and GFP sequences. GFP



**Figure 7.** Expression of *vpr* induces mitotic defects in HeLa cells. HeLa cells were electroporated with pVpr-IRES-EGFP. After 6 h, EGFP expressing cells were isolated by FACS sorting and replated on coverslips. Eighteen hours later, cells were fixed and stained with anti- $\gamma$ -tubulin antibodies and DAPI. Shown are representative cells exhibiting monopolar spindle (A), multipolar spindle (B), or misaligned chromosomes (C).

positive cells (which can be presumed to express Vpr) were purified by flow cytometry and analyzed for nuclear abnormalities by DAPI staining. By 6 h posttransfection GFP expression was detectable. Staining of the mitotic apparatus in *vpr*-expressing cells with anti-tubulin antibodies and DAPI revealed abnormal mitotic spindles (Figure 7), such as monopolar spindles (6.4% of mitotic cells), multipolar spindles (26.8% of mitotic cells) and misaligned or lagging chromosomes (8% of mitotic cells). These defects were not apparent in cells transfected with the GFP expression vector alone (100 mitotic cells examined).

After 3 d of Vpr expression (Figure 8F), large cells were apparent and the incidence of mitotic cells decreased. By 5 d posttransfection, the proportion of large, multinucleate cells steadily increased to 31% of *vpr*-expressing cells versus 3% of control cells (Figure 8F). Multinucleate *vpr*-expressing HeLa cells often contained micronuclei and incompletely separated nuclei still connected by bridges of DNA, resembling a “cut” phenotype indicative of a defect in nuclear division (Figure 8, B–E). Thus, as in fission yeast cells, *vpr*-expressing human cells also exhibited defects in nuclear division and cytokinesis.

To determine whether Vpr induces centrosome abnormalities, we stained *vpr*-expressing cells with centriole markers pericentrin,  $\gamma$ -tubulin, and Cen2 (Figure 8; our unpublished data). Most cells, even ones with a single nucleus, contained multiple pairs of centrioles scattered in the pe-

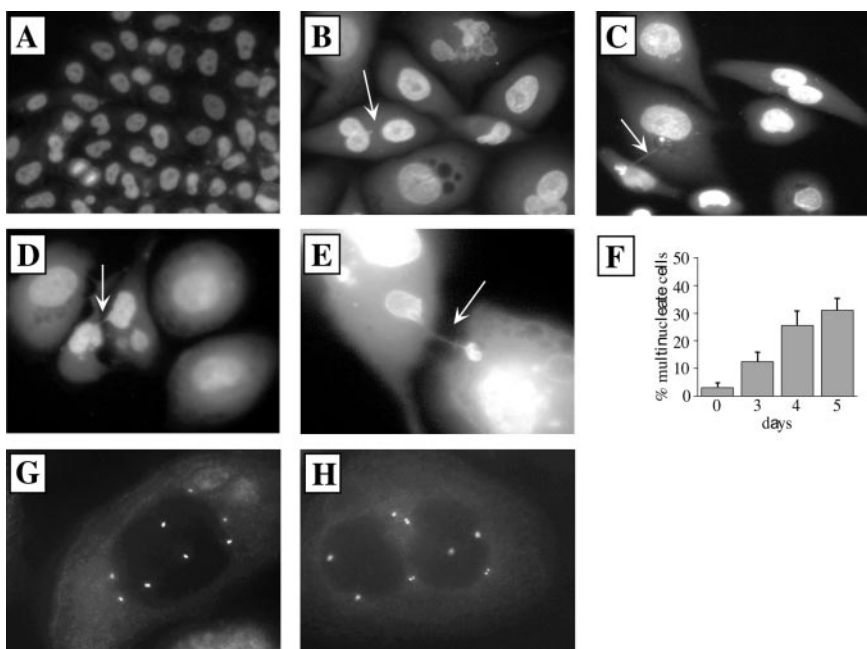
rinuclear region (Figure 8, G and H). Thus, Vpr causes the accumulation of multiple centrosome-like structures.

Next, we examined the effect of Vpr expression on primary HUVECs. Cells were electroporated with a *vpr*-expression plasmid or a control vector. After 48 h under selection, cells were trypsinized and replated. Five days postelectroporation, the morphology of cells was analyzed by phase contrast microscopy, and the DNA content was measured by flow cytometry. Cells electroporated with empty vector were observed to grow during this time period. No multinucleate cells were evident (Figure 9A, top), and FACS analysis showed the expected DNA profile of an asynchronously proliferating population (Figure 9A, bottom; 67.3% of the cells had 2N DNA and 16.9% had 4N DNA). In contrast, cells electroporated with the *vpr*-expression vector increased in size, did not divide, and became multinucleate (Figure 9B, top). DNA profiles showed increased number of cells with 4N (36.6% cells) and 8N (21.7% cells) DNA contents compared with the population control cells (Figure 9B, bottom). These findings indicate that Vpr also induces cell division defects in primary human cells.

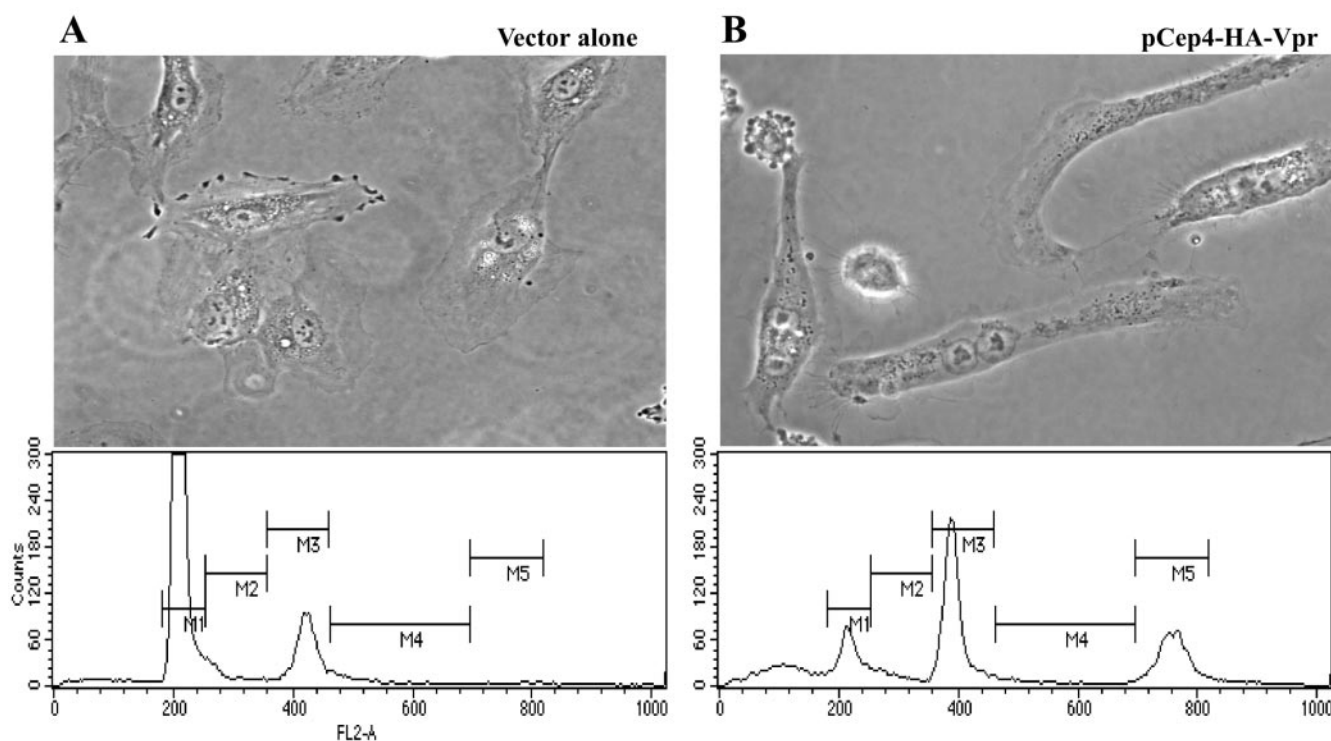
## DISCUSSION

### *Multiple Effects of Vpr on Cell Division*

Here, we reexamined the effect of Vpr expression in both fission yeast and human cells and found that, in addition to



**Figure 8.** Expression of *vpr* induces cytokinesis defects in HeLa cells. HeLa cells were electroporated with pCEP4 (A) or pCEP4-HA-Vpr (B–E, G, and H). After 48 h of selection in hygromycin-containing medium, cells were trypsinized and replated. Five days after electroporation, cells were fixed and stained with DAPI (A–E) or with antibodies against pericentrin (G) or  $\gamma$ -tubulin (H) and examined by UV-fluorescence microscopy. Arrows indicate bridges of DNA that seem to connect separate nuclei within a given cell (B) or nuclei between two daughter cells (C–E). (F) Kinetics of the appearance of multinucleated cells in cultures electroporated with pCEP4-HA-Vpr.



**Figure 9.** Expression of *vpr* induces G2 arrest and cytokinesis defects in primary human cells. HUVEC were electroporated with pCEP4 (A) or pCEP4-HA-Vpr (B). After 48 h of selection in hygromycin-containing medium, cells were trypsinized and replated. Five days after electroporation, cells were examined by phase-contrast microscopy at 400 $\times$  magnification (top). Arrows indicate multinucleate cells. Bottom, flow cytometric analysis after staining with propidium iodide. M1 indicates 2N DNA content. M3 indicates 4N DNA content. M5 indicates 8N DNA content.

cell cycle arrest, Vpr also induces defects in nuclear morphology, mitotic spindle assembly and function, and actin ring assembly in cytokinesis. These additional effects are not secondary to cell cycle arrest. Spindle and nuclear abnormalities were clearly apparent well before development of elongated cells in fission yeast. Also, previous fission yeast studies have established that cell cycle delay in G2 phase alone (for instance in a *cdc25* temperature-sensitive mutant) does not produce defects in mitosis or cytokinesis as severe as those reported here (Hagan *et al.*, 1990; Naqvi *et al.*, 1999).

Several different models can be imagined to explain how Vpr has so many effects on the cell division cycle. Vpr might disrupt multiple targets, each of which controls a different process or pathway. Alternatively, Vpr may target a single protein or organelle that affects these interdependent processes.

#### *Vpr Effects on Centrosomes*

Vpr seemed to disturb centrosome/SPB integrity and function. In fission yeast, characterization of mitotic defects indicated that SPB function or replication of the spindle pole body was disrupted. Furthermore, the distributions of *sad1p* and polo kinase *plp1p*, but not  $\gamma$ -tubulin complex, were perturbed, suggesting that only part of the SPB structure was affected. In human cells, Vpr expression led to the accumulation of multiple centrosome-like structures, as shown by pericentrin and  $\gamma$ -tubulin staining, although it is not yet clear whether the integrity of the centrosome was disrupted.

The centrosome abnormalities that we observed are unlikely to be artifacts of our experimental system because

multiple centrosomes have been observed in cells infected with wild-type HIV-1 but not in cells infected with HIV-1 bearing a *vpr* mutation (Watanabe *et al.*, 2000). Similar multiple centrosome phenotypes are commonly found in tumor cells, perhaps arising from multiple centrosome duplication cycles without proper cell division (Meraldi *et al.*, 2002) or from abnormal de novo synthesis of centrosome-like structures after the centrosome laser ablation (Khodjakov *et al.*, 2002). Vpr might cause the multiple centrosome phenotype by perturbing cytokinesis or via direct effects on the regulation of centrosome duplication or integrity. Once established, the multiple centrosome phenotype could then contribute to further abnormalities in mitosis and cytokinesis.

#### *Vpr Effects on the Nuclear Envelope*

Vpr has been shown to disrupt the integrity of the nuclear envelope in mammalian cells, causing the formation of large holes in the interphase nuclear envelope that may facilitate the entry of viral particles into the nucleus (de Noronha *et al.*, 2001). One proposed candidate target for Vpr are nuclear lamins, which are responsible for nuclear structure and integrity. In fission yeast, we found that Vpr also causes abnormal nuclear morphology and DNA staining (Zhao *et al.*, 1998). In addition, as with Vpr in mammalian cells, a GFP-Vpr fusion protein is localized in a perinuclear distribution in fission yeast (Chen *et al.*, 1999). Thus, it is likely that the effect of Vpr on the nucleus is conserved. Because fission yeast do not contain nuclear lamins, Vpr must affect a nuclear envelope target separate from nuclear lamins.

Vpr at the nuclear envelope can potentially affect mitosis and centrosomes in multiple ways. First, both fission yeast

SPB and mammalian centrosomes are often tightly associated with nuclear envelopes, but the possible effects of nuclear envelope integrity on the SPB and centrosome assembly and function are not yet known. Second, Vpr has been shown to bind to the nuclear transport factor importin  $\alpha$  (Popov *et al.*, 1998; Vodicka *et al.*, 1998), which with ran GTPase, regulates both nuclear transport and mitotic spindle assembly (Dasso, 2001). Disruption of the Ran GTPase system in fission yeast also causes nuclear envelope fragmentation (Demeter *et al.*, 1995) and spindle assembly defects (Fleig *et al.*, 2000; Salus *et al.*, 2002).

### Vpr and Cytokinesis

Vpr induced strong defects in actin ring formation, and thus may affect actin indirectly or directly. In T cells, Vpr has been shown to modulate cell adhesion and actin organization (Matarrese *et al.*, 2000), and it has been found to bind to F-actin directly (D. Schafer, personal communication). Centrosome defects can lead also to cytokinesis defects, although in fission yeast, mitotic spindle defects do not necessarily disrupt contractile actin ring formation (Chang *et al.*, 1996a). Thus, Vpr may target directly or indirectly one or more of the proteins involved in actin ring assembly, such as actin.

### Ways to Arrest the Cell Cycle

Much prior work has focused on the role of Vpr on its effect on the cell cycle. Nuclear envelope defects may affect cell cycle regulators by affecting their nuclear trafficking (de Noronha *et al.*, 2001). Our findings lead to additional possible mechanisms for how Vpr causes cell cycle arrest. Disturbances in centrosome integrity caused by Vpr are likely to affect the function of many regulators of the cell cycle and cytokinesis present on the centrosome. Removal of the centrosome causes cells to go through M phase, elicits cytokinesis defects in a fraction of the cells, and then arrests cells with a 4N DNA content in the next cell cycle before S phase (Hinchcliffe *et al.*, 2001; Khodjakov and Rieder, 2001). The centrosome/SPB contains many regulators of cell cycle progression (Doxsey, 2001), including factors required for G2/M transition (*cdc2/cyclin B*), mitotic spindle assembly (*plp1p*), contractile ring assembly (*plp1p*, *rng2p*), septation, and exit from mitosis (*sid* components). Here, we examined the effect of Vpr on one of these regulators, *plp1p* polo kinase. Polo kinase has multiple conserved functions in mitotic spindle function, cytokinesis, and G2/M transition through interactions with multiple proteins (Glover *et al.*, 1998). In human cells, overexpression of polo kinase causes cell division defects and accumulation of multiple centrosomes (Meraldi *et al.*, 2002). In *S. pombe*, loss of function of the polo kinase *plp1p* can lead to similar cell division defects as seen in *vpr*-expressing cells (Ohkura *et al.*, 1995). The *plp1p*-GFP fusion protein normally localizes to the SPB and spindle but was grossly mislocalized in *vpr*-expressing cells. However, we currently have no evidence suggesting that Vpr interacts directly with *plp1p*.

### CONCLUSION

This work leads to a new view that Vpr affects multiple aspects of cell division, including mitotic spindle function and cytokinesis. Interestingly, proteins encoded by other viruses have also recently been shown to affect centrosomal function (Duensing *et al.*, 2000; Ploubidou *et al.*, 2000; Gailard *et al.*, 2001). How these multiple Vpr effects relate to HIV infection in vivo is not yet clear, but it is likely that these effects contribute to cell killing or possibly to intracellular

trafficking of viral components (McDonald *et al.*, 2002). Genetic studies in *S. pombe* may allow the identification of Vpr cellular target(s), a prerequisite for understanding Vpr function in HIV-1 replication and pathogenesis.

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