Inhibition of microtubules and dynein rescues human immunodeficiency virus type 1 from owl monkey TRIMCyp-mediated restriction in a cellular context-specific fashion

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IFN-induced restriction factors can significantly affect the replicative capacity of retroviruses in mammals. TRIM5α (tripartite motif protein 5, isoform α) is a restriction factor that acts at early stages of the virus life cycle by intercepting and destabilizing incoming retroviral cores. Sensitivity to TRIM5α maps to the N-terminal domain of the retroviral capsid proteins. In several New World and Old World monkey species, independent events of retrotransposon-mediated insertion of the cyclophilin A (CypA)-coding sequence in the trim5 gene have given rise to TRIMCyp (also called TRIM5-CypA), a hybrid protein that is active against some lentiviruses in a species-specific fashion. In particular, TRIMCyp from the owl monkey (omkTRIMCyp) very efficiently inhibits human immunodeficiency virus type 1 (HIV-1). Previously, we showed that disrupting the integrity of microtubules (MTs) and of cytoplasmic dynein complexes partially rescued replication of retroviruses, including HIV-1, from restriction mediated by TRIM5α. Here, we showed that efficient restriction of HIV-1 by omkTRIMCyp was similarly dependent on the MT network and on dynein complexes, but in a context-dependent fashion. When omkTRIMCyp was expressed in human HeLa cells, restriction was partially counteracted by pharmacological agents targeting MTs or by small interfering RNA-mediated inhibition of dynein. The same drugs (nocodazole and paclitaxel) also rescued HIV-1 from restriction in cat CRFK cells, although to a lesser extent. Strikingly, neither nocodazole, paclitaxel nor depletion of the dynein heavy chain had a significant effect on the restriction of HIV-1 in an owl monkey cell line. These results suggested the existence of cell-specific functional interactions between MTs/dynein and TRIMCyp.

INTRODUCTION

The replication of retroviruses and retrotransposons is inhibited by a diverse family of type I IFN-stimulated gene products commonly called restriction factors (Harris et al., 2012; Malim & Bieniasz, 2012; Zheng et al., 2012). The replication of human immunodeficiency virus type 1 (HIV-1) is reduced by IFN treatment of cells in vitro (Ho et al., 1985; Yamamoto et al., 1986) and in vivo (Skillman et al., 1996). At least some of the restriction factors that mediate the IFN-induced restriction of HIV-1 have been uncovered. For instance, the IFN-induced transmembrane proteins 2 and 3 (IFITM2 and IFITM3) can affect the entry or immediate post-entry steps of HIV-1 replication (Lu et al., 2011). If the virus reaches the cytoplasm, it can then be inhibited by myxovirus resistance protein 2 (Mx2, also called MxB) (Goujon et al., 2013; Kane et al., 2013). Mx2 binds capsid (CA) proteins that are part of the ‘intact’ (not-yet-disassembled) HIV-1 core and interferes with core disassembly (uncoating) (Fricke et al., 2014; Liu et al., 2013). TRIM5α (tripartite motif protein 5, isoform α) is another IFN-inducible factor that intercepts incoming retroviruses in the cytoplasm of infected cells (Stremlau et al., 2004). Restriction is host- and virus-specific, and HIV-1 is generally insensitive to human TRIM5α, as its CA is not recognized by the human version of the protein (Hatziioannou et al., 2004; Keckesova et al., 2004; Sebastian & Luban, 2005; Stremlau et al., 2004, 2006; Yap et al., 2004). However, HIV-1 is efficiently inhibited by TRIM5α expressed in several monkey species (up to ~100-fold in single-cycle assays), including macaques and the African green monkey (Hatziioannou et al., 2003). TRIM5α belongs to the tripartite motif (TRIM) family of proteins – a very large family (close to 100 members) of ubiquitin ligases that function in various pathways, including antiviral defence

Two supplementary figures are available with the online Supplementary Material.
(Han et al., 2011; Reymond et al., 2001). TRIM proteins contain, starting at the N-terminal domain, a RING domain, one or two B-boxes and a coiled-coil domain. Most of them also have an additional functional domain at the C terminus (Sardiello et al., 2008); in the case of TRIM5α, this additional domain is a PRYSPRY motif, also called SPRY or B30.2 (Song et al., 2005). The PRYSPRY domain is responsible for the specificity of restriction, by mediating direct interactions with surface patches present in the N-terminal region of retroviral CA proteins (Biris et al., 2013; Sebastian & Luban, 2005; Yang et al., 2014). In several independent instances, retrotransposition events have led to the insertion of the cyclophilin A (CypA)-coding sequence into the trim5 locus of New World or Old World primate species, leading to the expression of various TRIMCyp hybrid proteins. TRIMCyp expressed in the owl monkey (Aotus trivirgatus; omkTRIMCyp) very efficiently decreases HIV-1 infectivity (~100- to 500-fold in single-cycle assays) (Nisole et al., 2004; Sayah et al., 2004). Amongst the other TRIMCyp hybrid proteins discovered, those expressed in pig-tailed macaques (Macaca nemestrina) and in some rhesus macaques (Macaca mulatta) do not restrict HIV-1, but target other lentiviruses such as HIV-2 (Brennan et al., 2008; Newman et al., 2008; Virgen et al., 2008; Wilson et al., 2008).

Restriction of HIV-1 by omkTRIMCyp seems to occur by a mechanism similar to that of TRIM5α and is initiated by the direct binding of the CypA domain of TRIMCyp to CA proteins that are part of incoming cores (Black & Aiken, 2010; Price et al., 2009; Shi et al., 2013). This interaction occurs relatively quickly, within 1 h following virus entry into the cytoplasm (Hulme et al., 2011), and results in the destabilization of the retroviral core – a process sometimes referred to as premature decapsidation (Bérubé et al., 2007; Diaz-Griffero et al., 2006b, 2007). Over-expression of omkTRIMCyp in human cells results in an HIV-1 inhibition as potent as that observed in owl monkey cell lines such as owl monkey kidney (OMK) cells, showing that the HIV-1 restriction-null endogenous human TRIM5α does not interfere with restriction in this context (Berthoux et al., 2005a). Interestingly, TRIM5 proteins are expressed at very low levels endogenously in the absence of IFN (Carthagena et al., 2008), as illustrated by the difficulty in detecting them by immunoblotting or immunofluorescence microscopy. Moreover, restriction by TRIM5α or TRIMCyp involves their aggregation or multimerization on the targeted retroviral core, as seen in various imaging studies (Campbell et al., 2008; Ganser-Pornillos et al., 2011; Zhao et al., 2011). Taken together, these observations raise an intriguing question: how can a sparsely expressed restriction factor quickly and efficiently intercept incoming retroviral cores in a process that may require hundreds or thousands of TRIM5 molecules? To address this question, we have hypothesized that TRIM5 proteins associate with cytoskeleton components used by retroviruses for their transport toward the nucleus, which would dramatically enhance the probability of successful interception. omkTRIMCyp, like TRIM5α, forms cytoplasmic bodies (CBs) whose sizes are dependent on expression levels (Perez-Caballero et al., 2005). TRIM5α CBs co-localize with ubiquitin (Campbell et al., 2008) and proteasomal subunits (Danielson et al., 2012; Lukic et al., 2011), which supports a functional role for CBs as the proteasome is involved in TRIM5α-mediated restriction (Anderson et al., 2006). However, CBs and proteasome were found to be dispensable for HIV-1 restriction by omkTRIMCyp (Perez-Caballero et al., 2005), pointing to the possibility of differences between the precise mechanisms of action for these two restriction factors. TRIM5α CBs associate with microtubules (MTs) and their movements along these filaments have been reported (Campbell et al., 2007). HIV-1 and other viruses are thought to recruit dynein motor complexes translocating on MTs for their retrograde transport (toward the nucleus) (reviewed by Dodding & Way, 2011; Hsieh et al., 2010; Mouland & Milev, 2012). In a previous report, we showed that restriction of HIV-1 and N-tropic murine leukemia virus (N-MLV) by TRIM5α could be significantly attenuated by pharmacological treatments that affect the integrity and dynamics of MTs (Pawlica et al., 2014). TRIM5α-mediated restriction was also inhibited by pharmacological or RNA interference-mediated disruption of dynein complexes (Pawlica et al., 2014). Owing to the reported differences between TRIM5α and omkTRIMCyp in restricting HIV-1, we investigated whether the MT network and dynein complexes were relevant to omkTRIMCyp-mediated restriction.

RESULTS

Size and localization of TRIMCyp CBs are modulated by MTs and cytoplasmic dynein

Previously, we showed that treatment of HeLa cells with nocodazole – a drug that inhibits MT polymerization (Luđuena & Roach, 1991) – increased the size of TRIM5α CBs and caused them to localize closer to the nucleus, on average (Pawlica et al., 2014). Depleting dynein heavy chain (DHC) also caused TRIM5α CBs to be larger and they were found to localize closer to the plasma membrane, on average (Pawlica et al., 2014). We used the same approach to analyse the effect of these two interventions on omkTRIMCyp CBs. HeLa cells stably expressing omkTRIMCyp or transduced with the empty vector were stained with a monoclonal anti-FLAG antibody. Cells were treated with nocodazole at a concentration (2 μM) previously determined to result in efficient MT disassembly (Pawlica et al., 2014) or left untreated (Fig. 1a). Alternatively, cells were transfected with a DHC small interfering RNA (siRNA) under conditions leading to an ~85% decrease in DHC protein expression levels (Pawlica et al., 2014) or were transfected with a control (luciferase-targeting) siRNA (Fig. 1b). No staining was detected in the empty vector-transduced cells under the conditions used (Fig. 1a, b). The size of omkTRIMCyp CBs, as represented by the area on the two-dimensional images.
recorded, was calculated, along with their relative localization, as described previously (Pawlica et al., 2014). In the absence of drug and in the cells transfected with the luciferase-targeting siRNA, omkTRIMCyp CBs had a median area of ~0.12 μm², whilst the mean area was ~0.13 μm² (Fig. 1c). Treatment with nocodazole increased the median and mean size of CBs by 16.7 and 20.3%, respectively (Fig. 1c, left panel). DHC depletion increased the median size of omkTRIMCyp by 16.7%, but had a bigger effect on their mean size, compared with the control siRNA (a 67.0% increase, indicating that the effect was much more pronounced on a minority of CBs). The effect was significant in both experiments (P=0.01 and P=0.002, respectively), showing that the dynamics of TRIMCyp bodies are affected by treatments that disrupt MTs or interfere with cytoplasmic dynein function, similar to TRIM5α (Diaz-Griffero et al., 2006a; Pawlica et al., 2014).

The relative intracytoplasmic position of omkTRIMCyp CBs was determined according to their closest distance to the nuclear membrane and the cytoplasmic membrane (Pawlica et al., 2014). Treatment with nocodazole caused omkTRIMCyp CBs to localize closer to the nucleus, relative to the untreated control (Fig. 1d, left panel). This effect was highly significant (P=0.001) and mirrored our previous observations with TRIM5α (Pawlica et al., 2014). In contrast, DHC depletion had no significant effect on omkTRIMCyp relative localization (Fig. 1c, right panel). This stands in contrast to what was observed with TRIM5α CBs, which were found to be closer to the cell’s edge upon DHC depletion (Pawlica et al., 2014). Altogether, results in Fig. 1 show that the dynamics of omkTRIMCyp are sensitive to perturbation of MTs and cytoplasmic dynein, but the specific effects are different from what is observed with TRIM5α, hinting at differences in their interactions with the cytoskeleton.

Nocodazole and paclitaxel partially rescue HIV-1 from restriction by omkTRIMCyp in HeLa cells

The fact that nocodazole treatment altered the dynamics of omkTRIMCyp CBs prompted us to analyse whether disrupting MTs would affect restriction. In addition to nocodazole, we also tested the effect of paclitaxel – a drug that prevents the disassembly of MTs (Jordan et al., 1992; Schiff et al., 1979). HeLa cells stably expressing omkTRIMCyp or transduced with the parental construct were treated with two different concentrations of nocodazole (Fig. 2a) or paclitaxel (Fig. 2b) and then infected with increasing amounts of an HIV-1 vector, HIV-1\textsubscript{CMV-GFP}. We previously showed that nocodazole and paclitaxel at these concentrations had the expected effects on the MT network in HeLa cells and were not strongly cytotoxic (Pawlica et al., 2014). In the absence of drug, HIV-1\textsubscript{CMV-GFP} infectivity was restricted 300- to 400-fold by omkTRIMCyp (Fig. 2a, b), which is comparable with previous observations (Bérubé et al., 2007). Nocodazole increased infectivity of HIV-1\textsubscript{CMV-GFP} in HeLa cells expressing omkTRIMCyp by ~50-fold, whilst only slightly increasing infectivity in the permissive cells transduced with the empty vector (Fig. 2a). Thus, nocodazole counteracted omkTRIMCyp-mediated restriction, and this effect was seen at all virus doses tested and at both drug concentrations. Likewise, paclitaxel increased HIV-1\textsubscript{CMV-GFP} infectivity by ~30-fold in HeLa cells expressing omkTRIMCyp, whilst slightly decreasing infectivity in the empty vector-transduced control cells (Fig. 2b).

In the reciprocal experiment, we infected the same cell lines with HIV-1\textsubscript{CMV-GFP} in the presence of increasing nocodazole concentrations and using fixed virus doses. Nocodazole increased infectivity in HeLa-omkTRIMCyp cells by up to ~80-fold, compared with the untreated control, and this effect was observed at a wide range of nocodazole concentrations (Fig. 2c; see non-normalized data in Fig. S1a, b, available in the online Supplementary Material). As before, nocodazole slightly increased HIV-1\textsubscript{CMV-GFP} infectivity in the control cells, by up to about twofold at 1 μM (Fig. 2c). We performed a similar experiment with increasing concentrations of paclitaxel and fixed virus doses. Paclitaxel treatment increased HIV-1\textsubscript{CMV-GFP} infectivity in omkTRIMCyp-expressing cells by up to 15-fold compared with the untreated control (Fig. 2d; see non-normalized data in Fig. S1d). The magnitude of this effect was identical at all the drug concentrations tested. In contrast, paclitaxel decreased HIV-1\textsubscript{CMV-GFP} infectivity in the empty vector-transduced control cells by up to 2.5-fold (Fig. 2d; see non-normalized data in Fig. S1c). If the effect of nocodazole and paclitaxel was calculated relative to the effect seen in treated control vector-transduced cells, rather than relative to the untreated control, then both drugs were found to stimulate infectivity by roughly the same magnitude, ~50-fold. This observation mirrored previously published findings that nocodazole and paclitaxel both rescue N-MLV and HIV-1 from restriction by endogenous human TRIM5α and exogenous rhesus TRIM5α, respectively, in HeLa cells, and the effects of the two drugs were also of similar magnitude (Pawlica et al., 2014).

Depletion of DHC partially rescues HIV-1 from omkTRIMCyp-mediated restriction in HeLa cells

Previously, we found that DHC depletion partially suppressed the restriction of HIV-1 by rhesus TRIM5α expressed in HeLa cells (Pawlica et al., 2014). However, DHC depletion had a milder effect on the size and position of omkTRIMCyp CBs, compared with rhesus TRIM5α CBs (Fig. 1). To investigate whether the presence of DHC would be important to restriction mediated by TRIMCyp, we used an siRNA to suppress its expression in HeLa cells that were transduced with omkTRIMCyp or the empty vector. DHC knockdown was efficient (>90%), as shown in Fig. 3(a). Then, cells were challenged with HIV-1\textsubscript{CMV-GFP} using virus doses leading to ~0.49% infected cells (empty vector-transduced) and 0.17% infected cells (omkTRIMCyp-transduced). When cells were transfected with the DHC-targeting siRNA, infectivity in omkTRIMCyp-expressing cells increased by 34.8 ± 1.3-fold compared with cells transfecte
TRIMCyp antiviral activity is modulated by the cytoskeleton

Fig. 1. DHC depletion and MT perturbation alter the size and/or distribution of omkTRIMCyp CBs. (a, b) Immunofluorescence microscopy. HeLa cells transduced with FLAG-tagged omkTRIMCyp and control cells transduced with the empty vector were seeded on glass coverslips and 24 h later treated with 2 μM nocodazole (Noc) or not (Ctl) for 6 h (a), or transfected with 40 nM siRNAs targeting DHC (siDHC) or luciferase (siLuc) and 48 h later seeded on glass coverslips and incubated for an additional 24 h (b). Cells were fixed and immunostained for FLAG (red). DNA was stained with Hoechst 33342 (blue). Representative images are shown with outlined cell edges. (c) Sizes of CBs. All CBs were outlined in cells from a minimum of five randomly chosen fields and their area was calculated using image analysis software (AxioVision). P values were calculated using Student’s t-test analysis. (d) The relative localization of all TRIMCyp CBs from a minimum of five randomly chosen cells was calculated using the formula \( x / (x + y) \), where \( x \) is the shortest distance to the nucleus and \( y \) is the shortest distance to the cell’s edge. Red bars show the mean ± SEM and green bars show the medians.

with a control siRNA against luciferase (Fig. 3b; see non-normalized data in Fig. S2a). In contrast, the DHC-targeting siRNA had no effect in control cells transduced with the empty vector. Thus, DHC depletion counteracted omkTRIMCyp-mediated restriction, and the magnitude seen was comparable with what was observed with nocodazole and paclitaxel (Fig. 2). To determine whether the pharmacological disruption of MTs (using nocodazole or paclitaxel) and the siRNA-mediated disruption of cytoplasmic dynein counteracted omkTRIMCyp through the same mechanism or by acting independently, HeLa cells expressing omkTRIMCyp were infected with HIV-1CMV-GFP in the presence of an siRNA targeting DHC or luciferase and in presence or not of nocodazole or paclitaxel (Fig. 3c; see non-normalized data in Fig. S2b). As before, paclitaxel (alone or in combination with DHC knockdown) slightly decreased HIV-1CMV-GFP infectivity (~1.5-fold), whilst nocodazole and DHC depletion had no significant effect (Fig. 3c). Compared with the untreated control, treatment with nocodazole and paclitaxel increased infectivity in omkTRIMCyp-expressing cells by 27.0 ± 2.4- and 59.2 ± 3.7-fold, respectively. DHC depletion alone increased infectivity by 16.9 ± 0.7-fold in omkTRIMCyp-expressing cells. Combining DHC depletion with nocodazole and paclitaxel increased infectivity by 32.3 ± 6.9- and 53.9 ± 4.98-fold, respectively (Fig. 3c). Thus, the effects were almost identical to those obtained with the drugs alone,
strongly suggesting that DHC depletion and MT perturbation rescued HIV-1 from omkTRIMCyp by interfering with the same pathway. Of note, in this experiment, only 0.14% of omkTRIMCyp-expressing cells were infected in the absence of drug, which implies that we would have been able to observe 100-fold increases (or more) in infectivity if they had occurred. Thus, the absence of an additive effect in Fig. 3(c) was not due to saturated infection.

**Overexpression of p50/dynamitin partially counteracts omkTRIMCyp-mediated restriction**

As an alternative approach to analyse the importance of dynein-mediated transport in omkTRIMCyp-mediated restriction, we overexpressed p50/dynamitin in control and omkTRIMCyp-expressing HeLa cells (Fig. 4a). p50 is a subunit of the dynactin complex that is responsible for binding cargos to the dynein complex. Its overexpression results in the disassembly of dynactin complexes, thereby disrupting cargo binding to dynein (Burkhardt et al., 1997; Melkonian et al., 2007). Overexpression of p50 increased HIV-1<sub>CMV-GFP</sub> infectivity in TRIMCyp-expressing cells by 2.3 ± 0.35-fold, whilst replication in the control permissive cells was not affected (Fig. 4b; see non-normalized data in Fig. S2c). Altogether, the data in Figs 3 and 4 strongly suggest that functional dynein complexes are important, but not absolutely necessary, to omkTRIMCyp-mediated restriction.
Nocodazole and paclitaxel partially rescue HIV-1 from omkTRIMCyp-mediated restriction in cat cells

The experiments in Figs 1, 2, 3 and 4 were all performed in HeLa cells. To analyse whether nocodazole and paclitaxel could also interfere with omkTRIMCyp function in another cell line, we used CRFK cat cells stably expressing omkTRIMCyp or transduced with the empty vector as a control.

Cats, like other feline species, apparently do not have a TRIM5α or TRIMCyp orthologue (McEwan et al., 2009) and thus using this cell line also ensured that endogenous TRIM5α did not play a role in the effects seen in HeLa cells. CRFK cells were first transfected with a construct expressing GFP-α-tubulin and then treated with 40 nM siRNA against DHC or Luc, then treated or not with either 0.2 μM nocodazole (Noc) or 0.1 μM paclitaxel (Pxl) and infected with HIV-1CMV-GFP. Infection yields were analysed as above. The ratios of infected cells in the absence of drug were 0.14 (TRIMCyp) and 5.77 % (empty vector). **P<0.005 and ***P<0.0001 compared with the untreated omkTRIMCyp-transduced cells, as calculated using Student’s t-test. Error bars represent SD.

Disrupting MT dynamics or dynein function does not counteract restriction of HIV-1 in OMK cells

Finally, we tested whether MTs and cytoplasmic dynein were important to omkTRIMCyp function when expressed...
Fig. 4. Overexpression of p50/dynamitin decreases omkTRIMCyp-mediated restriction of HIV-1 in HeLa cells. (a, b) HeLa cells transduced with omkTRIMCyp or the empty vector were transfected with p50/dynamitin-HA or an irrelevant control plasmid. (a) Detection of p50 in the transfected cells by Western blotting. (b) Cells were infected in triplicates with HIV-1CMV-GFP 48 h after transfection. Infected cells were counted by flow cytometry 2 days post-infection and results are presented as fold changes in infectivity relative to the relevant mock-transfected control. Higher amounts of input virus were used in omkTRIMCyp-expressing cells than in the control cells, so that all values fall within the dynamic range for this assay. The ratios of infected cells in the mock-transfected cells were 0.25% (for TRIMCyp) and 0.42% (for the empty vector). **P≤0.005 in Student’s t-test. Error bars represent SD.

endogenously in owl monkey cells. For this, we used OMK cells, which are kidney epithelial cells known to be very poorly permissive to HIV-1 (Sayah et al., 2004; Towers et al., 2003). GFP-α-tubulin transfection allowed us to analyse the effect of nocodazole and paclitaxel on the MT network in this cell line (Fig. 6a). Similar to what was seen in CRFK cells, treatment with 2 μM nocodazole resulted in GFP-α-tubulin being more diffuse in the cytoplasm of cells (Fig. 6a), consistent with inhibition of MT polymerization by this drug. Conversely, treatment with 1 μM paclitaxel caused the formation of abnormal MT bundles that were found close to the cell’s edge (Fig. 6a). OMK cells were then infected with HIV-1NL43-GFP, a vesicular stomatitis virus (VSV) G-pseudotyped HIV-1 vector devoid of an HIV-1 envelope and expressing GFP in place of Nef (He et al., 1997). As a non-restricted control, we used SIVmac-GFP, a similarly constructed vector derived from the omkTRIMCyp-insensitive simian immunodeficiency virus (SIV) strain mac239 (Berthoux et al., 2005b). OMK cells were infected with multiple doses of HIV-1NL43-GFP and SIVmac-GFP, in absence of drug or in the presence of nocodazole (Fig. 6b) or paclitaxel (Fig. 6c). After normalization of the virus amounts used according to their titres in the non-restrictive CRFK cells, we found that, as expected, HIV-1NL43-GFP infectivity was ~500-fold smaller than that of SIVmac-GFP in these cells (Fig. 6b, c). Addition of nocodazole at 0.1 or 1 μM slightly increased HIV-1NL43-GFP infectivity, but this drug also had a small positive effect on SIVmac-GFP infectivity (Fig. 6b).

Likewise, treatment with paclitaxel slightly increased infectivity of SIVmac-GFP and also that of HIV-1NL43-GFP at least at some virus doses (Fig. 6c). Thus, HIV-1NL43-GFP was not rescued from TRIMCyp-mediated restriction in OMK cells by nocodazole or paclitaxel treatment. The discrepancy between this result and what was observed in HeLa and CRFK cells could possibly be due to differences in restriction sensitivity between the vectors used, HIV-1CMV-GFP and HIV-1NL43-GFP. To exclude that possibility, we infected OMK cells with several doses of HIV-1CMV-GFP in the absence or presence of nocodazole or paclitaxel. As shown in Fig. 6(d), neither drug could rescue this vector from restriction by TRIMCyp in OMK cells. As additional evidence that TRIMCyp was expressed and functional in these cells, we tested whether inhibition of its interaction with HIV-1 CA would rescue HIV-1 infectivity (Sayah et al., 2004). Treatment with 5 μM cyclosporin A during infection and introducing the G89V mutation in HIV-1 CA both increased HIV-1 infectivity by ~200-fold in OMK cells (Fig. 6e), thus completely disrupting restriction.

It is conceivable, albeit unlikely (as dynein complexes translocate on MTs), that cytoplasmic dynein could have a role in TRIMCyp function in OMK cells even if the MT network does not. To test this possibility, we depleted DHC in OMK cells using the same siRNA that was used in human cells. We found that DHC knockdown by this siRNA was as efficient in OMK cells as in human cells (>90%; Fig. 6f). DHC depletion had no significant effect on HIV-1NL43-GFP, even decreasing infectivity at one of the virus doses compared with the cells transfected with the control (luciferase-targeting) siRNA (Fig. 6g). SIVmac-GFP infectivity was similarly not significantly affected by DHC depletion (Fig. 6g). Thus, HIV-1 infectivity in OMK cells was not rescued by interfering with the integrity of the MT network or by targeting DHC, suggesting the existence of cell context specificity for the involvement of MTs and cytoplasmic dynein in the restriction process.

DISCUSSION

Previously, we showed that full restriction by TRIM5α required the presence of a functional MT network, as well as functional dynein complexes (Pawlica et al., 2014). This was true whether TRIM5α was expressed endogenously or overexpressed by retroviral transduction. Moreover, the phenotype was observed in human cells and in rhesus macaque cells, and whether the restricted virus was a lentivirus (HIV-1) or an oncoretrovirus (N-MLV). Here, we show that the antiretroviral function of TRIMCyp – a TRIM5 protein bearing a CypA domain instead of a PRYSPRY domain – can be similarly sensitive to inhibition of MTs or dynein complexes. All viruses used in this study...
were VSV G-pseudotyped and we cannot totally exclude that such pseudotyping modulates the involvement of MTs or dynein in omkTRIMCyp-mediated restriction. However, this seems unlikely considering that the mode of virus entry was previously shown to be irrelevant for the involvement of MTs in TRIM5α-mediated restriction of HIV-1 (Pawlica et al., 2014). Interestingly, the MT/dynein dependency of omkTRIMCyp varied between the cell lines used, being high in HeLa cells, relatively less so in CRFK cells and absent in OMK cells. Endogenous TRIMCyp in OMK cells is expressed at much lower levels compared with overexpression in HeLa or CRFK cells, but this is unlikely to explain the differences seen here, considering that (i) the magnitude of omkTRIMCyp-mediated HIV-1 restriction is similar in OMK cells and in TRIM5α-transduced HeLa cells (100-fold or more), and (ii) MTs and dynein were shown to be important for restriction mediated by both endogenous and overexpressed TRIM5α (Pawlica et al., 2014). However, we cannot totally exclude this possibility. Of note, none of the experimental approaches used here had a significant effect on omkTRIMCyp expression levels in HeLa cells (not shown), ruling out such a putative unspecific effect as the cause for the loss of restriction. As cytoplasmic dynein complexes translocate on MTs (Höök & Vallee, 2006), it appears likely that paclitaxel and nocodazole inhibit TRIM5α and TRIMCyp indirectly, by preventing dynein-mediated transport. In support of this conclusion is the observation that combining DHC depletion with paclitaxel or nocodazole treatment has no additional effect on restriction in cells, and this has been shown for rhesus TRIM5α (Pawlica et al., 2014) and now for omkTRIMCyp as well. The fact that neither TRIM5α nor TRIMCyp is completely dependent on the presence of an intact MT network or functional dynein, regardless of the cellular context, leads us to conclude that dynein complexes have a function in TRIM5α/TRIMCyp-mediated restriction that is not absolutely required or, possibly, this function can be accomplished by another transporter complex that does not translocate on MTs. This hypothesis helps make sense of the fact that neither DHC depletion nor pharmacological disruption of MTs could rescue HIV-1 infection of OMK cells. We speculate that the

Fig. 5. Pharmacological perturbation of MTs partially counteracts HIV-1 restriction by omkTRIMCyp in CRFK cells. (a) Immunofluorescence microscopy analysis of MTs. CRFK cells were transfected with GFP-α-tubulin and 2 days later were left untreated or subjected to 2 h treatments using either 2 μM nocodazole (Noc) or 1 μM paclitaxel (Pxl) and then fixed. GFP fluorescence was observed by immunofluorescence microscopy, along with DNA that was stained using Hoechst 33342 (blue). A representative image from each condition is presented. (b, c) Effect of nocodazole and paclitaxel on restriction. CRFK cells transduced with omkTRIMCyp or with the empty vector were infected with multiples doses of HIV-1(GFP-CMV) in the presence or absence of 2 μM nocodazole (b) or 1 μM paclitaxel (c). Infections were performed for 16 h and infected cells were detected by flow cytometry 2 days later.
Fig. 6. Pharmacological perturbation of MTs and DHC depletion do not rescue HIV-1 from TRIMCyp-mediated restriction in OMK cells. (a–d) Effect of nocodazole (Noc) and paclitaxel (Pxl) on restriction. (a) Immunofluorescence microscopy analysis of MTs. OMK cells were transfected with GFP-α-tubulin and 2 days later were left untreated or subjected to 2 h treatments using nocodazole (Noc) or paclitaxel (Pxl). (b) Nocodazole treatment. (c) Paclitaxel treatment. (d) HIV-1 restriction analysis. (e) WT HIV-1 and G89V HIV-1 restriction analysis. (f) siRNA targeting DHC and Luc. (g) HIV-1 restriction analysis with siRNA targeting DHC.
either 2 μM nocodazole or 1 μM paclitaxel and then fixed. GFP fluorescence was observed by immunofluorescence microscopy, along with DNA that was stained using Hoechst 33342 (blue). A representative image from each condition is presented. (b–d) OMK cells were infected with multiple doses of HIV-1NL43-GFP (b, c), SIVmac-GFP (b, c) or HIV-1CMV-GFP (d) in the presence or absence of nocodazole (b, d) or paclitaxel (c, d) at the indicated concentrations. Infections were performed for 16 h and infected cells were detected by flow cytometry 2 days later. (e) Abrogation of restriction by cyclosporin A (CsA) treatment or CA mutation. OMK cells were infected with increasing amounts of WT HIV-1CMV-GFP or the G89V CA mutant and in presence or absence of cyclosporin A (5 μM). The x-axis shows the amounts of virus used for both G89V and WT virus as determined by their titres in CRFK cells in the absence of drug. The percentage of GFP-positive cells was determined by flow cytometry 2 days post-infection. (f) Western blot analysis of DHC expression in OMK cells 48 h after transfection of the siRNAs targeting DHC (siDHC) or, as a control, luciferase (siLuc). Actin was analysed as a loading control. (g) OMK cells were transfected with the indicated siRNAs and infected 72 h later with multiple doses of either HIV-1NL43-GFP or SIVmac-GFP for 16 h. Infected cells were detected by flow cytometry 2 days post-infection.

endogenous omkTRIMCyp expressed in these cells does not require MTs or dynein to make initial contact with the incoming virus CA core and elucidating the molecular basis for this behaviour will require additional investigations. Alternatively, the discrepancy of results between the cell lines used in this study could stem from different effects of inhibiting MTs and dynein on virus uncoating in these cell lines. Targeting MTs and dynein may delay uncoating, as has been shown recently by us (Pawlica & Berthoux, 2014; Pawlica et al., 2014) and by others (Lukic et al., 2014), and perhaps this has a greater impact on restriction in some cell lines than in others. Interestingly, Lukic et al. (2014) also included data showing that nocodazole treatment did not rescue HIV-1 from restriction by rhesus TRIM5α or omkTRIMCyp expressed in HeLa cells, apparently conflicting with our previous report (Pawlica et al., 2014) and the present report. However, HeLa cells have been shown to be a highly heterogeneous cell line (Carson & Pirruccello, 2013), including clones with various permissiveness toward HIV-1 infection (De Iaco & Luban, 2014), and it is also known that the magnitude of restriction by TRIM5α and TRIMCyp proteins can vary widely between cell lines (Bérubé et al., 2007; Gong et al., 2011). Altogether, it appears likely that a cellular context effect explains the different results obtained by Lukic et al. (2014). In conclusion, the MT network and dynein complexes can potentiate the antiretroviral activity of not only TRIM5α, but also TRIMCyp, but is not strictly required.

**METHODS**

**Cells, pharmaceuticals and antibodies.** Human embryonic kidney 293T (HEK 293T) cells, human epithelial carcinoma HeLa cells, feline renal CRFK cells and OMK cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose, supplemented with 10% FBS and antibiotics at 37 °C, 5% CO2. All cell culture reagents were from HyClone (Thermo Scientific). Nocodazole, paclitaxel and cyclosporin A were from Sigma. HeLa and CRFK cells were transfected using FuGENE HD (Promega) and Flag epitope was detected using the M2 antibodies against DHC and p50/dynamitin were from Santa Cruz and Millipore, respectively. The FLAG epitope was detected using the M2 antibodies used as secondary antibodies in Western blots were from Santa Cruz.

**Plasmid DNAs and retrovirus production.** p50/dynamitin-HA was a gift from Tina Schroer (Schrader et al., 2000). To produce viral vectors, 10 cm culture dishes of subconfluent HEK 293T cells were co-transfected using polyethyleneimine (molecular mass 25 000; Polyscience) with the appropriate plasmids, as follows: for the viral vector HIV-1CMV-GFP, pTRIPTRIP-CMV-GFP (10 μg), pPAR8.9 (WT or G89V; 10 μg) and pMD-G (5 μg); for HIV-1NL43-GFP, pNL-GFP (10 μg) and pMD-G (5 μg); for SIVmac-GFP, pSIVmac-GFP (10 μg) and pMD-G (5 μg) (Berthoux et al., 2003; He et al., 1997; Zuffery et al., 1997). Media were changed 16 h post-transfection and virus-containing supernatants were collected after an additional 1.5 days of culture. Viral stocks were clarified by centrifugation for 5 min at 400 g.

**Viral challenges.** Cells were seeded in 24-well plates at 104 cells well-1 well (CRFK and HeLa cells) or 5 × 104 cells per well (OMK cells) and challenged the next day with the appropriate GFP-expressing viral vectors. When applicable, cells were pre-treated for 15 min with nocodazole, paclitaxel or cyclosporin A and supernatants were replaced with fresh medium 16 h post-infection. Cells were tetrasyprined 48 h post-infection and fixed in 2% formaldehyde (Fisher Scientific). The percentages of GFP-positive cells were then determined by analysing 104–105 cells on a FC500 Mpl cytometer (Beckman Coulter) using CXP Software (Beckman Coulter).

**siRNA and plasmid transfections.** For the siRNA treatments, 106 cells were seeded in a 10 cm dish in Opti-MEM (Gibco) and transected the next day with 40 nM siRNA using DharmaFECT 1 (Dharmacon). The siRNA targeting the sequence 5′-GATGACGGAATT-3′ of the DHC has been described previously (Lehmann et al., 2009; Pawlica et al., 2014) and was purchased from Qiagen (Venlo). A control siRNA (5′-CGTACGCGGAATCTTCGAGTT-3′) targeting the luciferase mRNA (Pawlica et al., 2014) was purchased from Dharmacon. At 48 h post-transfection, cells were seeded in 24-well plates they were infected the next day with HIV-1 vectors as described above. For p50 overexpression, 106 cells seeded in a 10 cm dish were polyethyleneimine-transfected with 5 μg p50/dynamitin-HA or an irrelevant plasmid (pMiP). Cells were seeded in 24-well plates 24 h later and challenged with viral vectors the next day.

**Immunofluorescence microscopy.** For siRNA treatments, HeLa cells in 3.5 cm wells were transfected 48 h prior to seeding as described above. For the cells expressing GFP-tubulin, 2 μg plasmid was transfected per well using polyethyleneimine 24 h prior to seeding on coverslips. Then, 2 × 104 cells were seeded on glass coverslips placed in 3.5 cm wells. After 24 h, the cells were treated or not with nocodazole for 2 h, and then fixed and processed for...
immunofluorescence staining. Fixation was done for 10 min in 4% formaldehyde/DEEM in 37°C, followed by three washes with ice-cold PBS. Cells were then permeabilized by treatment with 0.1% Triton X-100/0.1 mM sodium citrate for 1–2 min on ice. Cells were then washed three times with PBS and treated with 10% normal goat serum (Sigma) containing 0.3 M glycine (Sigma) for 30 min at room temperature. This was followed by a 4 h incubation with a murine antibody against the FLAG epitope diluted 1:400 in PBS containing 10% normal goat serum. Cells were washed five times and fluorescently stained with the Alexa 594-conjugated goat anti-mouse antibody (Molecular Probes) at a 1:200 dilution. Cells were washed five times in PBS before mounting in Vectashield (Vector Laboratories). Hoechst 33342 (0.8 μg ml⁻¹; Molecular Probes) was added along with the penultimate PBS wash to reveal DNA. Z-stacks were acquired on an AxioObserver microscope (Zeiss) equipped with an Apotome module and median Z-stacks were retained for analysis. For the analysis of TRIM5α CB sizes, FLAG foci in a given cell were manually outlined in the AxioVision software for calculation of the surface. A minimum of 80 and up to 180 CBs from 10 randomly chosen cells were included in the analysis. For the analysis of TRIM5α CB localization, the cell’s edge was outlined, and for each FLAG foci in a given cell we measured the closest distance to the nuclear membrane and the closest distance to the plasma membrane using AxioVision. A minimum of 170 and up to 320 CBs from a minimum of five randomly chosen cells were included in the analysis.

Statistical analysis. All statistical analyses were carried out in Prism 5 (GraphPad).

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