Vpr drives massive cellular proteome remodelling in HIV-1 infection
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Abstract
Viral infection causes global remodelling of the cellular proteome. We previously mapped the temporal changes in abundance of thousands of proteins in HIV-1 infected cells (Greenwood & Matheson, 2016). While a small proportion of these changes were attributable to specific HIV-1 accessory proteins, most were unexplained. Here, we use complementary unbiased mass spectrometry-based approaches to demonstrate that a single viral protein, Vpr, is both necessary and sufficient to cause the vast majority of these changes. This global protein regulation requires substrate binding and degradation via DCAF1, but is mostly independent of Vpr-mediated cell cycle arrest. Combined approaches of pulsed-Stable Isotope Labelling with Amino Acids in Cell Culture (pulsed-SILAC) and immunoprecipitation-mass spectrometry (IP-MS) identified at least 38 such cellular proteins directly targeted for degradation by Vpr. Thus, whilst other HIV-1 accessory proteins downregulate a small number of host factors, Vpr degrades multiple protein targets, causing systems-level remodelling of the cellular proteome.

Impact statement
HIV infection causes massive changes to the cellular proteome and a single HIV protein, Vpr, is necessary and sufficient to drive almost all these changes by degrading multiple host proteins.

Introduction
The HIV-1 ‘accessory proteins’ Vif, Vpr, Nef and Vpu deplete host proteins in infected cells, and are required for viral replication \textit{in vivo} [1-3]. We previously used unbiased quantitative proteomics to map temporal changes in cellular protein abundance during HIV infection of CEM-T4 T-cells, and identify novel targets of Vpu (SNAT1), Nef (SERINC3/5) and Vif (PPP2R5A-E) [4, 5]. Nonetheless, known accessory protein targets only account for a tiny fraction of all protein changes observed during HIV infection [5]. Since a single accessory protein, Vif, is sufficient to drive global remodelling of the cellular phosphoproteome [5], we hypothesised that undiscovered targets of the accessory protein, Vpr, might explain a proportion of the remaining protein-level changes.

Results
Vpr is required for global proteome remodelling in HIV infected cells.
We compared total proteomes of uninfected cells with cells infected with either WT HIV or an HIV Vpr deletion mutant (HIV ΔVpr) at an infectious MOI of 1.5 (Figure 1A), resulting in approximately 75% infection (Figure 1B). Data from this experiment are available, together with the other proteomics datasets presented here, in a readily searchable interactive format in Figure 1—source data 1. Amongst the 7,774 quantitated proteins, we observed widespread changes in cells infected with wild-type HIV (Figure 1C left panel). Together with known Nef, Vpu and Vif, targets, we saw
depletion of previously reported Vpr targets including HLTF [6, 7], ZGPAT [8], MCM10 [9], UNG [10], TET2 [11], MUS81 and EME1 [12, 13]. DCAF1, part of the ligase complex used by Vpr to degrade targets was also depleted, consistent with a previous report [14].

In HIV ΔVpr infection (Figure 1C, right panel), depletion of Nef, Vpu and Vif targets was maintained. Remarkably, as well as abolishing depletion of known Vpr targets, almost all of the previously uncharacterised protein changes were also reduced or abolished in HIV ΔVpr infection. Whilst 1,944 proteins changed significantly (q<0.01) in WT HIV-infected cells, only 41 protein changes (2%) retained significance when cells were infected with HIV ΔVpr. Indeed, principle component analysis showed that cells infected with HIV ΔVpr virus share more similarity on the proteome level with uninfected cell than with cells infected with WT virus (Figure 1D).

Incoming Vpr protein alone drives massive cellular proteome remodelling.

Since Vpr enhances expression of other viral proteins [15, 16] (Figure 1C), differences between WT and ΔVpr viruses could potentially be explained by secondary changes in expression levels of other proteins, or different rates of progression of WT and ΔVpr viral infections. To eliminate these potential confounders, we therefore examined the effect of Vpr alone. Further, unlike other HIV-1 accessory proteins, Vpr is specifically packaged into nascent viral particles, and as such, we wanted to examine the effect of this delivered protein. We therefore repeated our proteomic analysis using cells exposed to lentiviral particles lacking or bearing Vpr in the presence of reverse transcriptase inhibitors (RTi). We therefore excluded all de novo viral protein expression and examined the changes induced by Vpr-containing viral particles delivered directly from the virion (Figure 2A). Cells were exposed to these viral particles at an infectious MOI of 0.5 (determined in the absence of RTi).

Strikingly, changes induced by Vpr-containing viral particles phenocopied the Vpr-dependent proteome remodelling seen in HIV infection (Figure 2B), with a high degree of correlation (r² = 0.67; Figure 2C). Taking both experiments together, Vpr is both necessary and sufficient to cause the significant (q<0.01) depletion of at least 302 proteins, and the upregulation of 413 – highlighted in blue and red, respectively (Figure 2c). This is a stringent false discovery rate and, in practice, the number of Vpr-dependent changes is almost certainly even higher. Where antibodies were available, we confirmed a proportion of these changes by immunoblot (Figure 2 – figure supplement 1).

Cellular proteome remodelling requires interaction with DCAF1 and cellular substrates.

While the function or functions of Vpr remain controversial, in all phenotypic descriptions, Vpr activity is dependent on the interaction between Vpr and the DCAF1/DDB/Cul4 ligase complex, recruitment of which results in ubiquitination and degradation of known Vpr targets through the ubiquitin-proteasome system. Therefore, in addition to testing the effect of wild type Vpr protein delivered in viral particles to the cellular proteome, we tested a number of previously described mutant Vpr variants (See schematic in Figure 2A and results in Figures 2D,E and additional information in Figure 2 - figure supplement2)

Since the Q65 residue of Vpr is required for the interaction with DCAF1 [17], we compared proteome changes caused by a Q65R Vpr mutant with WT Vpr. As predicted, the Q65R Vpr was almost completely inactive (Figure 2D,E). We recapitulated this finding by comparing the effects of WT Vpr in control cells, or cells depleted of DCAF1 (Figure 2 – figure supplement 3). ShRNA mediated depletion of DCAF1 resulted in an approximately 50% reduction in protein abundance of DCAF1
(Figure 2 – figure supplement 3B). Depletion of DCAF1 alone did not phenocopy the Vpr mediated proteome remodelling, and the widespread effects of Vpr are therefore unlikely to be only dependent on the Vpr mediated sequestration and/or depletion of DCAF1. As a proportion of cellular DCAF1 was still expressed, known Vpr effects including degradation of HLTF and upregulation of CCNB1 was partially rather than completely inhibited (Figure 2 – figure supplement 3C). Consistent with this, Vpr mediated changes were broadly reduced in magnitude in the DCAF1 knockdown cells (Figure 2 – figure supplement 3D). Thus, as with depletion of known Vpr targets, extensive Vpr-dependent proteomic remodelling is dependent on Vpr’s interaction with its cognate DCAF1/DDB/Cul4 E3 ligase.

Residues E24, R36, Y47, D52 and W54 of Vpr are also required for the recruitment and degradation of previously described Vpr targets, and are reported to form the substrate-binding surface [7, 18, 19]. Y47, D52 & W54 make up the proposed DNA mimicking motif by which Vpr binds the cellular target UNG2 [18]. In agreement, the Vpr<sub>E24R</sub>,<sub>R36P</sub> and Vpr<sub>W54R</sub> mutants showed attenuated remodelling of the proteome, while a triple mutant, Vpr<sub>E24A,D52A,W54R</sub>, was defective for almost all Vpr-dependent protein changes (Figure 2D,E). Global protein remodelling therefore depends on both the substrate binding surfaces of Vpr, and the recruitment of DCAF1, suggesting that this process is mediated by recruiting Vpr substrates to the DCAF1/DDB1/CUL4 E3 ligase complex, and their subsequent degradation.

Vpr causes G2/M arrest in cycling cells, but the mechanism is contentious [9, 13, 20-26], as is the connection to any replicative advantage Vpr provides in vivo. To investigate this important issue, we took advantage of previously characterised Vpr mutants. Residue S79 of Vpr is required for Vpr-dependent cell cycle arrest [27] (Figure 2D and Figure 2 – figure supplement 1). Of the other mutants we tested, Vpr<sub>Q65R</sub> and Vpr<sub>E47A,D52A,W54R</sub> mutants are also unable to cause G2/M arrest, while Vpr<sub>E24R,R36P</sub> has an intermediate phenotype, and Vpr<sub>W54R</sub> caused G2/M arrest at wild type levels (Figure 2D and Figure 2 – figure supplement 1). Strikingly, most Vpr-dependent protein changes were also observed with the Vpr<sub>S79A</sub> mutant (Figure 2D, E), and are therefore independent of G2/M cell cycle arrest. The presence or absence of G2/M arrest was also a poor correlate of proteomic remodelling across the entire panel of mutants. Cell cycle arrest therefore only explains a minority of Vpr-dependent changes (Figure 2D,E).

Further, previous datasets/reports investigating gene and protein expression during the cell cycle have identified proteins increased or depleted during different phases of the cell cycle, or in chemically G2/M arrested cells [28, 29] (Figure 2 – figure supplement 4). Cells arrested in G2 using a PLK1 inhibitor show similar regulation of the cyclin family of proteins (Figure 2 - figure supplement 4B), but there is little other correlation between these datasets. Thus, whilst some changes in protein levels induced by Vpr may be explained by the effects of cell cycle arrest, proteins regulated by cell cycle in these datasets only account for a small fraction of Vpr-dependent changes (Figure 2 - figure supplement 4A,C,D).

Within this minority of changes that correlate with Vpr mediated G2/M arrest, depleted proteins may either represent secondary effects of alteration to the cell cycle, or may correspond to proteins depleted by Vpr, resulting in G2/M arrest. For example, MCM10 has previously been identified as a direct target for Vpr mediated depletion, and it was also reported that depletion of MCM10 by Vpr is at least partly responsible for Vpr mediated G2/M arrest [9]. We found that depletion of MCM10
correlated with the degree of G2/M arrest mediated by the different Vpr variants tested in this experiment (Figure 2 – figure supplement 5A), and confirmed that RNAi-mediated depletion of MCM10 could partly phenocopy Vpr by inducing G2/M arrest, (Figure 2 – figure supplement 5), as shown previously. Thus, a proportion of proteins whose depletion correlates with G2/M arrest in this system may be causes, rather than consequences of Vpr mediated G2/M arrest.

Identifying direct targets for Vpr-mediated degradation.
Vpr has a nuclear localisation and all reported direct Vpr targets are nuclear proteins. Primary Vpr targets are therefore predicted to be nuclear. Conversely, secondary effects resulting from, for example, transcriptional changes, can be distributed across the cell. Analysis of the 302 proteins depleted by Vpr revealed a profound enrichment for proteins that reside in the nucleus (>80%) (Figure 3A). This raised the possibility that a large proportion of the proteins depleted by Vpr are direct targets, as secondary effects should not be limited to the nucleus. Consistent with this, proteins upregulated by Vpr, which are all predicted to be secondary effects, were distributed across multiple compartments. Furthermore, proteins depleted by Vpr were enriched (>70%) for nucleic acid binding activity (Figure 3B). Vpr associates with DNA binding proteins such as UNG via a substrate-binding surface that mimics DNA [18]. Thus, rather than targeting a small number of cellular proteins for degradation, Vpr may have a much wider range of direct cellular targets, and the structure of the substrate binding surface suggests a possible this mechanism for recruitment of multiple cellular factors.

To identify those proteins targeted directly by Vpr, we first adopted a co-immunoprecipitation approach (Figure 4A). Cells were transduced with a 3xHA tagged Vpr lentivirus in the presence of an shRNA to DCAF1 and the pan-cullin inhibitor MLN4924, to minimise substrate degradation and allow successful co-immunoprecipitation. Factors specifically co-immunoprecipitated in the presence of Vpr are expected to include direct Vpr targets and, accordingly, were enriched for proteins depleted (rather than increased) in the presence of Vpr (Figure 4B,D,E). However, the co-IP was dominated by DCAF1, a stable binding partner of Vpr, identified with a signal intensity 2 orders of magnitude greater than all other proteins (Figure 4C). This is despite the knockdown of DCAF1 in these cells, which reduces the DCAF1 protein abundance by approximately 50% (Figure 2 – figure supplement 3B). In addition, at least 13 of the proteins co-immunoprecipitating with Vpr are reported to physically interact with DCAF1 alone [30–32], of which 11 are not regulated by Vpr, and two, CEP78 and IQGAP2, are upregulated by Vpr, explaining their presence in this list of proteins.

This mismatch between the high abundance of DCAF1 and the relatively low abundance of direct Vpr targets for degradation is reminiscent of previous reports, which have also found that MS-IP based techniques are ideal for the identification of the cellular machinery co-opted by viral proteins, but often struggle to identify cellular targets, which interact transiently and in competition with each other [33, 34]. We therefore adopted an alternative, pulsed-Stable Isotope Labelling with Amino Acids in Cell Culture (pulsed-SILAC) approach to identify host proteins specifically destabilised within 6 hrs of exposure to Vpr (Figure 5A). This technique is directly analogous to a traditional pulse-chase experiment using radiolabelled methionine/cysteine, but allows a global, unbiased analysis of potential cellular targets [35]. Since proteins are fully labelled prior to exposure to Vpr, differences in abundances of labelled proteins between conditions exclusively reflect changes in protein degradation rates.
Six hours after exposure to Vpr, the stability of most proteins was unchanged (Figure 5B, top panel). However, a subset of proteins depleted by Vpr were already destabilised, consistent with Vpr-dependent proteasomal degradation. These 27 proteins, including HLTF, are therefore very likely to represent direct targets for Vpr-mediated depletion (Figure 5C). After 24 hours of exposure to Vpr, changes in protein stability reflected overall changes in protein abundance caused by Vpr in other experiments (Figure 5B, lower panel), including proteins with increased as well as decreased stability. These changes are therefore indicative of both direct and indirect Vpr targets.

Combining all our orthogonal approaches - TMT-based whole cell proteomics to identify proteins depleted by Vpr in the context of viral infection or Vpr protein alone delivered in viral particles, MS co-IP with epitope tagged Vpr, and pulsed-SILAC based identification of proteins post-translationally degraded by Vpr - we have identified 38 direct targets for Vpr mediated degradation (Table 1). Vpr is both necessary and sufficient for depletion of these proteins, which are either bound by Vpr, or destabilised within 6 hours of Vpr exposure (or both). This list very likely underestimates the true number of direct Vpr targets, as several known targets of Vpr behaved appropriately, but beyond the statistical cut-offs used to derive this table (Table 1 – table supplement 1). It is also limited to proteins expressed in this T-cell model.

**Novel Vpr target involvement in G2/M arrest**

Several cellular phenotypes have been described for Vpr, including G2/M arrest, transactivation of the HIV LTR, and modulation of cellular signalling pathways such as NFκB and NFAT arrest [21, 22, 36-40]. The mechanisms responsible for these phenotypes are controversial. The wide-scale proteome remodelling by Vpr, and the direct targeting of multiple proteins, suggests a model in which Vpr interacts with diverse cellular proteins and pathways, perhaps resulting in cumulative or redundant effects on cellular phenotypes. This model does not contradict any single mechanism, but suggests that several are involved. To test this, we investigated the best described phenotype for Vpr, cell cycle arrest at the G2/M phase. As noted, MCM10 degradation correlated with the extent of G2/M arrest in the panel of Vpr mutants tested, and that RNAi for MCM10 results in G2/M arrest, as previously described [9]. We therefore interrogated the Vpr mutant panel dataset (Figure 2) for other direct targets of Vpr mediated degradation described in Table 1 that correlated with the extent of G2/M arrest. 14 proteins with a significant relationship (a linear regression analysis with p < 0.05) (Figure 6A) were identified. We tested if their shRNA-mediated depletion could phenocopy Vpr by inducing cell cycle arrest at G2/M (Figure 6A). Depletion of 3 Vpr targets: SMN1, CDCA2 and ZNF267 caused G2/M arrest, and these phenotypes were confirmed with a second shRNA (Figure 6C). We suggest that depletion of multiple cellular Vpr targets contribute to the Vpr-mediated G2/M arrest, consistent with our proposed model.

**Conservation of Vpr targets across primate lentiviruses.**

Targeting of key cellular proteins such as BST2 or the APOBEC3 family is conserved across multiple lentiviral lineages, demonstrating the in vivo selective advantage of these interactions. We therefore tested a diverse panel of lentiviral Vpr proteins to determine if they shared activity with the NL4-3 Vpr variant used in all the experiments above. We included Vpr variants from primary isolates of HIV-1 from two distinct cross-species transmissions from apes to humans (Group M and Group O), in addition to a closely related SIVcpz variant. We also tested Vpr variants from divergent primate lineages, including HIV-2, SIVsmm, SIVagm and SIVrcm (Figure 7A and Figure 7 – figure supplement 1). In addition to Vpr, which is present in all primate lentiviruses, viruses of some lineages also bear...
Vpx, a gene duplication of Vpr. Since depletion of some substrates switches between Vpr and Vpx in lineages bearing this accessory gene, we also included a Vpx variant from HIV-2.

Extensive Vpr-dependent remodelling of the cellular proteome was conserved across the HIV-1/SIVcpz lineage (Figure 7B – top row). Vpr variants from other lineages showed a narrower set of changes, but the depletion of some proteins, particularly those most heavily depleted by HIV-1, was conserved by Vpr variants across multiple lineages (Figure 7B,C). Depletion of selected proteins for which commercial antibody reagents were available was readily confirmed by immunoblot of cells transduced with an overlapping panel of Vpr variants (Figure 7D). Conserved targets of direct Vpr-mediated degradation (highlighted in Figure 7C and Figure 7 – figure supplement 1) are therefore likely to provide an in vivo replicative advantage for all primate lentiviruses.

While none of the identified HIV-1 Vpr targets were degraded by the HIV-2 Vpx (HIV-2R00) tested, we noted a shared ability of HIV-2 Vpx and SIVagm Vpr to deplete TASOR, a critical component of the Human Silencing Hub (HuSH) transcription repressor complex we recently described [41] (Figure 8). The HuSH complex mediates position-dependent transcriptional repression of a subset of lentiviral integrations, and antagonism of HuSH is able to potentiate HIV reactivation in the J-LAT model of latency [41]. As predicted, Vpx-VLPS phenocopied the effect of RNAi-mediated TASOR depletion on reactivation of the HuSH-sensitive J-LAT clone A1 (Figure 8B). With some exceptions, most primate lentiviruses can be categorised into 5 lineages (Figure 8C), of which two encode Vpx. The previously described canonical function of Vpx is the degradation of SAMHD1 [42, 43]. Two lentiviral lineages lack Vpx, but use Vpr to degrade SAMHD1, while the HIV-1/SIVcpz lineage lacks SAMHD1 antagonism [44]. We considered that TASOR antagonism may follow the same pattern, and thus tested Vpx proteins from both Vpx bearing lineages, and representative Vpr variants from lineages that use Vpr to degrade SAMHD1. All of these proteins were able to deplete TASOR in Vpx/Vpr transduced cells (Figure 8D). Antagonism of SAMHD1 and TASOR therefore follow the same pattern. Whilst this manuscript was in preparation, two other groups independently discovered Vpx-mediated depletion of TASOR [45, 46], and the role of HuSH antagonism in lentiviral infection is the subject of ongoing investigation.

While the targeting of some substrates was conserved across Vpr variants from multiple lineages, we did not observe broad proteome remodelling outside the HIV-1/SIVcpz lineage. However, in the experiment described in Figure 7 an HIV-1 based lentiviral transduction system was used. Of the Vpr and Vpx variants tested, only Vpr protein from only the HIV-1/SIVcpz alleles are efficiently packaged. In these cases, cells receive both incoming and de novo synthesized Vpr, while in the case of other variants tested, only de novo synthesized Vpr is present. There is therefore a time-lag of at 18-24 hours for viral entry, reverse transcription, integration and de novo synthesis of protein to begin. As such, the more limited proteome remodelling seen in Vpr variants may reflect a lack of time for such changes to occur.

In order to account for this, we carried out an experiment in which cells were transduced with a Vpr or Vpx from the primary HIV-2 isolate 7312a and assayed 48 h to 96 hours post transduction (Figure 9A), allowing time for additional changes to develop after de novo Vpr/Vpx synthesis. Even at 96 hours post transduction, HIV-2 Vpr showed very limited changes. The majority of these changes consisted of the depletion of proteins described above as being also targeted by HIV-1 Vpr (Figure 9B left panel). Widespread proteome remodelling was not observed, and global regulation of the
proteome is therefore unique to the HIV-1/SIVcpz lineage. As the HIV-1/SIVcpz lineage appears to be more pathogenic than other primate lentiviral lineages [47, 48] this activity of HIV-1 Vpr may be significant, particularly given the potential to drive these changes in Vpr exposed but uninfected bystander cells.

Curiously, 7312a HIV-2 Vpx depleted several proteins we have identified as being modulated by HIV-1 Vpr (Figure 9B – right panel), including direct targets for HIV-1 Vpr mediated degradation, BBX, HASPIN and ARHGAP11A. Some proteins were degraded by both HIV-2 Vpr and Vpx, while others were only degraded by the Vpx of this isolate. In lentiviral strains and/or lineages that encode both Vpr and Vpx, responsibility for degrading key targets of HIV-1 Vpr is therefore shared between Vpr and Vpx, further emphasising their in vivo importance.

**Discussion**

Proteomic analyses of cells infected with viruses from different orders have revealed widespread and varied changes to the cellular proteome [5, 49-51]. While these changes are presumed to be multifactorial, in the case of HIV-1 infection, the majority can be attributed to the action of a single viral protein, Vpr. We propose that this massive cellular proteome remodelling consist of firstly, the direct targeting of multiple cellular proteins for proteosomal degradation via the DCAF1/DDB1/CUL4A E3 ligase complex, followed by resulting secondary effects on other proteins.

While many of the changes caused by Vpr are secondary, these changes occur within 48 hours of infection or exposure to Vpr, the physiological timeframe of productively infected T-cell [52, 53], and are therefore relevant to our understanding of the HIV-1 infected cell.

The list of direct targets for Vpr mediated depletion was already extensive. We have confirmed here the Vpr mediated depletion of previously described Vpr targets HLT [6, 7], ZGPAT [8], MCM10 [9], UNG2 [10], TET2 [11], MUS81 and EME1 [12, 13], while other previously described Vpr targets SMUG1 [10] and PHF13 [54] were not detected in this cell line. Based on our knowledge of the activity of other HIV accessory proteins, it might have been predicted that this list of Vpr targets was nearly complete. Rather, we show here that it is only the tip of the iceberg. Indeed, even with the large number of direct targets identified or confirmed in this work, we do not propose that the list of 38 proteins identified here as direct targets here is comprehensive.

While surprising, the ability of Vpr to degrade multiple cellular factors can perhaps be explained by elements of the biology of this small protein. Mechanistically, depletion of multiple proteins with nucleic acid binding properties is consistent with known structural determinants of Vpr substrate recruitment. From the functional viewpoint, while HIV-1 has three accessory proteins (Vpu, Nef and Vif) to aid viral replication and counteract host defences in the late stages of the viral life cycle, Vpr is the only HIV-1 accessory protein specifically packaged in virions. Multiple targets may therefore be required both to protect incoming virions from cellular factors, and to prime newly infected cells for productive viral replication.

This work does not represent the first attempt to use unbiased proteomics analysis to characterise Vpr function. In general, previous studies have used one proteomics based experiment or method to identify candidate proteins that either interact with, or are depleted by, Vpr, with individual proteins subsequently followed up using targeted methods with immunoreagents. For example, proteins identified as binding Vpr by co-immunoprecipitation MS were identified by Jager et al [33] and
Hrecka et al [7]. Of proteins co-immunoprecipitating with Vpr, Jager et al did not determine whether any proteins were depleted following binding Vpr. Hrecka et al [7] also used an MS-IP and confirmed identified the single target protein, HLTFT, as depleted by Vpr, thus satisfying the criteria to be identified as a Vpr target. Conversely, Lahuassa et al [6] used a SILAC based approach to quantify protein changes in cells exposed to viral particles bearing Vpr, identifying 8 proteins which were depleted by at least 20%. Of these, only one, HLTFT was subsequently found to also bind Vpr, and thus again by identified as a direct Vpr target. As expected, the lists of ‘candidate’ Vpr proteins, identified, but not pursued, in the above studies overlaps with this work, and in some examples we have confirmed these candidates as direct targets for Vpr mediated degradation. For example, SMN1 has been identified as binding Vpr by Jager et al, and as being degraded by Vpr by Lahuassa et al in independent proteomics experiments. ESCO2 was identified as binding Vpr by Hrecka et al, but the depletion was not confirmed by immunoblot – most likely due to poor performance of the commercial antibody used. We have confirmed that both of these proteins are direct targets for Vpr mediated degradation.

By contrast to previous studies, rather than using a targeted approach to follow up only a small number of potential Vpr targets we have combined complementary proteomic analyses to describe (i) the global proteome remodelling caused by Vpr, and (ii) multiple direct substrates for Vpr mediated depletion. The two established criteria for Vpr targets, that Vpr both directly binds and depletes a given protein are satisfied by several of the proteins identified here, including ESCO2, SMN1, BBX and KIF18A. Thus, these proteins are not candidate Vpr targets, but bone fide Vpr targets, proven to the same standard of evidence as previously described direct substrates.

However, in our proposed model, Vpr binds and degrades multiple cellular proteins, with the total pool of Vpr shared over multiple targets. The identification of cellular targets by co-IP is thus technically problematic and more prone to false negatives compared to other proteins that establish interactions with a small number of binding partners. We therefore propose an alternative method of identifying direct targets for Vpr mediated depletion – proteins that are post-translationally degraded within 6 hours of treatment with Vpr. This strategy was more successful at capturing the known effects on cellular Vpr targets than the MS-IP approach, with the degradation of HLTFT and ZGPAT being demonstrated within 6 hours of Vpr exposure. We are confident that the other proteins identified in this fashion also represent direct targets for Vpr mediated degradation, as secondary effects are excluded by both intrinsic elements of the technique, and the short time frame allowed.

A comparison of this work with the recent publication by Lapek et al [14] is required, given the major differences between these studies, despite apparent similarities in the approach. Lapek et al use an inducible HIV-1 provirus and quantify changes to the cellular proteome up to 24 hours post activation. They compare a wild type and a Vpr deficient provirus but find fewer differences than in this study – indeed, they identify only nine proteins with significant difference between wild type and dVpr, none of which are previously defined Vpr targets – although DCAF1 is significantly reduced in the presence of Vpr. The difference most likely relates to differences between the systems used. In our model system, as in physiological conditions, Vpr is delivered with the virus particle at ‘0 h’ but de novo production of Vpr occurs late in the virus lifecycle. In Lapek et al, The experiment is concluded 24 hours after induction of the provirus. As production of de novo Vpr is Rev-dependent, Vpr production must occur after a significant delay within that 24-hour period. Thus, while some limited primary effects are evident such as the depletion of DCAF1, other primary and secondary
effects may not have had time to occur. Aside from the matter of timing, it is also worth noting that
that in the Lapek et al system, all Vpr is produced concurrently with Gag. The interaction between
Vpr and Gag, and the recruitment of a proportion of Vpr into nascent viral particles, may inhibit
nuclear localisation and substrate degradation.

Despite the importance of Vpr in vivo, an in vitro viral replication phenotype is often absent in T-cell
infection models [55]. Nonetheless, expression of Vpr in T-cells causes cell cycle arrest [36-38], cell
death [37], transactivation of the viral LTR [36], enhancement or antagonism of crucial signalling
pathways including NFAT [21] and NF-κB [22, 39, 40], disruption of PARP1 localisation [21, 40],
defects in chromatin cohesion [56] and induction of the DNA damage response [57, 58]. At least
some of these phenotypes can be segregated [21, 37]. The molecular mechanisms underpinning
these phenomena have remained controversial. In our model, we propose that these multiple
phenotypes can be explained by Vpr targeting multiple cellular proteins and pathways, with
potential for redundant or cumulative effects.

Here, we have considered the most well described cellular phenotype for Vpr, G2/M cell cycle arrest,
with findings compatible with this model. In addition to confirming the previously described effect of
Vpr mediated MCM10 degradation, we have identified three other proteins that are directly
targeted by Vpr, show depletion correlating with the extent of G2/M mediated arrest in a panel of
Vpr mutants and result in arrest at G2/M when depleted through RNAi. Notably, depletion of two of
these proteins, SMN1 and CDC2 (Repo-Man), has been shown to activate the DNA damage
response and stimulate ATM/ATR kinase activity [59, 60], demonstrated by many groups to be a
critical step towards the G2/M arrest caused by Vpr [20, 23, 61]. The contribution of depletion of
multiple cellular targets towards difference cellular phenotypes is also illustrated by another
example of a Vpr mediated, premature chromatid segregation (PCS). While this phenotype was not
investigated here, the RNAi mediated knockdown of any of three proteins we describe as novel
targets for Vpr mediated depletion, ESCO2, CDC5 (Sororin) or HASPIN results in this phenotype [62-
64].

In conclusion, Vpr degrades multiple cellular targets, resulting in global remodeling of the host
proteome, and labyrinthine changes to different cellular pathways. This explains why its effects on
cellular phenotypes and viral replication are complex and remain poorly understood, why the
functional consequences of individual Vpr targets identified and studied in isolation have proved
elusive, and why the search for a single, critical Vpr target has been problematic.

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Data availability
In addition to Figure 1—source data 1, which includes data from all of the proteomics experiments
carried out here, proteomics data have been deposited to the ProteomeXchange Consortium via the
PRIDE [65] partner repository with the dataset identifier PXD01029.
Experimental Procedures

NL4-3 molecular clones

pNL4-3-de-EGFP (derived from the HIV-1 molecular clone pNL4-3 but encoding Enhanced Green Fluorescent Protein (EGFP) in the env open reading frame (ORF), rendering Env non-functional) was obtained through the AIDS Reagent Program, Division of AIDS, NIAD, NIH: Drs Haili Zhang, Yan Zhou, and Robert Siliciano [66] and the complete sequence verified by Sanger sequencing. The ΔVpr mutant was generated by cloning three stop codons into the Vpr open reading frame, immediately after the overlap with Vif to prevent interference with that gene:

WT Vpr ORF sequence
ATGGAACAAGCCCCAGAAGACCAAGGGCCACAGAGGGAGCCATACAATGAATGGACACTAGAGCTTTTAA

ΔVpr ORF sequence
ATGGAACAAGCCCCAGAAGACCAAGGGCCACAGAGGGAGCCATACAATGAATGGACACTAGAGCTTAATAGTAA

Viral stocks

VSVG-pseudotyped NL4-3-de-EGFP HIV viral stocks were generated by co-transfection with pMD.G (VSVG) as previously described [5]. NL4-3-de-EGFP HIV viral stocks were titred by infection/transduction of known numbers of relevant target cells under standard experimental conditions followed by flow cytometry for GFP and CD4 at 48 hr to identify % infected cells.

Single Vpr and Vpx proteins were expressed in a modified dual promotor pHRSIN [67] vector in which Vpr or Vpx expression is driven by the rous sarcoma virus (RSV) promotor, and Emerald GFP expression is driven by the ubiquitin promotor. Virus was generated by co-transfection with p8.91 and pMD.G in 293T as previously described [5]. Infectious MOI was normalised by infection in the absence of reverse transcription (RTi) inhibitors, which were included where specified (see below).

The panel of Vpr and Vpx proteins used are detailed in Table 2. Amino acid sequences were codon optimized and synthesized as double stranded DNA (IDT), inserted into the empty vector construct by Gibson assembly, and confirmed by sanger sequencing.

CEM-T4 T-cell infections

CEM-T4 T-cells were infected with concentrated NL4-3-de-EGFP or pHRSIN lentiviral stocks by spinoculation at 800 ×g for 1 h in a non-refrigerated benchtop centrifuge in complete media supplemented with 10 mM HEPES. Where reverse transcription treatment was specified (RTi), cells were incubated with zidovudine (10 μM) and efavirenz (100 nM) (AIDS Reagent Program, Division of AIDS, NIAD, NIH) for 1 hr prior to spinoculation, and inhibitors maintained at these concentrations during subsequent cell culture. For MS experiments, cells were subject to dead cell removal (magnetic dead cell removal kit, Miltenyi). Subsequent sample preparation, TMT labelling, and MS are described below, at the end of this section.

Pulsed Stable isotope labelling with amino acids in cell culture (Pulsed-SILAC)

For SILAC labelling, CEM-T4 T-cells were grown for at least 7 cell divisions in SILAC RPMI lacking lysine and arginine (Thermo Scientific, Thermo Fisher Scientific, UK) supplemented with 10% dialysed FCS (Gibco, Thermo Fisher Scientific), 100 units/ml penicillin and 0.1 mg/ml streptomycin, 280 mg/L proline (Sigma, UK) and medium (K4, R6; Cambridge Isotope Laboratories, Tewksbury, MA) or heavy (K8, R10; Cambridge Isotope Laboratories) 13C/15N-containing lysine (K) and arginine (R) at 50 mg/L. At 0 h, cells were washed in media containing only light (12C/14N) lysine and arginine and were
maintained in this media for the duration of the experiment. Additional detail on MS methods is available at the end of this section.

**Lentivector for shRNA expression**

For lentiviral shRNA-mediated knockdown of DCAF1 hairpins were cloned into pHRIREN-PGK-hygro, with transduced cells selected for hygromycin resistance.

The following oligonucleotide was inserted using BamHI-EcoRI (only top oligonucleotide shown), identified from the Broad Institute GPP Web portal. Gene specific target sequence (forward and reverse complement) is underlined.

**DCAF1**

GATCCGCTGAATACTCTCTCAAGAATTCAAGAGATTTCTGAAAGATTTTCTCAAGCCTTTTTG

The same methodology was used to clone other shRNA hairpins, the forward orientation targeting sequence used for each is shown below.

- **MCM10-1**
  
  AGATGCAGGACGCCTACTTTG

- **MCM10-2**
  
  GACGGCGACGGTAACTTAT

- **CCNT1**
  
  GCCTGCATTGGACCACATT

- **PINX1**
  
  CCTTCAGCAAGAGGATTTAAT

- **ZNF267-1**
  
  TACTCGTTCCTCCAATCTTAT

- **ZNF267-2**
  
  ATCAATATAGGAAGGTCCTTTA

- **MBD1**
  
  CACCAACCTCAGAATGTAAA

- **KIF20A**
  
  CCGATGACGATGTCGTAGTTT

- **ZNF316**
  
  CTTCACAGAGAAGATGGCA

- **DDX20**
  
  CGAGAAACAGAAACGTAAGAA

**Mass spectrometry Immunoprecipitation (MS-IP)**

CEM-T4 T-cells were lysed in 1 % NP-40. Lysates were pre-cleared with IgG-Sepharose (GE Healthcare, UK) and incubated for 3 hr at 4°C with anti-HA coupled to agarose beads (Sigma EZview Red Anti-HA Affinity Gel). After washing in 0.5% NP-40, samples were eluted with 0.5 mg/ml HA peptide at 37°C for 1 hr. Additional detail on MS methods is available at the end of this section.
Proteins defined as co-immunoprecipitating with Vpr were detected with at least 3 peptides in the Vpr condition, not identified in the control condition, and were present in <20% of MS-iP available in the Crapome [68] database http://crapome.org/.

**Antibodies**

Antibodies against the following proteins were used for immunoblot, listed by manufacturer:
- Atlas antibodies: TASOR (HPA006735).
- Bethyl: BBX (A303-151A), HLTF (A300-230A), RALY (A302-070A), ZNF512B (A303-234A).
- Cell signalling: SMN1/2 (2F1).
- Novus: ESCO2 (NB100-87021), RALY (A302-070A), ZNF512B (A303-234A).
- Origine: UNG2 (2C12).
- Proteintech: Vpr (51143-I-AP).
- Sigma: β-actin (AC74).
- Abcam: p24 (ab9071), VCP (ab11433).

The following secondary antibodies were used: goat anti-mouse-HRP and anti-rabbit-HRP (immunoblot, Jackson ImmunoResearch, West Grove, PA).

**J-LAT reactivation experiments.**

JLAT A1 cells were transduced with Cre recombinase, NL4-3 Vpr or HIV-2 ROD Vpx within a pHRSIN IRES NGFR vector[69]. 24 h after transduction, cells were treated with 2 ng/ml TNFα (PeproTech, 300-01A). After an additional 24 h cells were stained with anti-NGFR APC and analysed for APC and GFP expression by flow cytometry. Cells were gated for NGFR+ cells to exclude non-transduced cells.

Flow cytometry data was acquired on a BD FACSCalibur.

**Statistical analysis**

Anova and Fisher’s exact test analysis were carried out as described in figure legends using Graphpad Prism (v7.04). TMT multiplexed proteomics datasets were analysed using a moderated T-test analysis with Benjamini-Hochberg correction [70], carried out using R (v3.3.1) [71]. Significance B values for pulsed-SILAC analysis were calculated using Perseus [72].

**Gene ontology enrichment analysis**

Analysis was carried out using Panther [73], using the web interface at: http://pantherdb.org/. Lists of proteins highly significantly depleted or increased by Vpr were compared to a background list of proteins quantified in the two experiments used to define those lists.

**7-AAD staining**

Cells were fixed in ice cold 70% ethanol for at least 30 minutes, washed with PBS and stained in 25ug/ml 7-AAD for 15 minutes before acquisition on a BD FACSCalibur. Analysis was carried out in Flowjo, v.10.5.2. Dead cells and doublets were excluded by gating on forward scatter, side scatter, and fluorescent area and height. Univariate cell cycle modelling was carried out using the Watson pragmatic method.

**ShRNA knockdown confirmation by RT-PCR**

Total RNA was extracted using the RNeasy Plus Mini kit (Qiagen). Total RNA (250 ng) was reverse transcribed into cDNA using a poly(d)T primers and SuperScript III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Real-time qRT–PCR was performed using the ABI 7500 Real-Time PCR system (Applied Biosystems) and SYBR Green PCR Master Mix (Applied Biosystems), with cycling parameters of 50°C for 2 min and 95°C for 5 min, followed by 40 cycles of 95°C for 15 s
and 60°C for 1 min. The gene-of-interest specific primer pairs were predesigned and purchased from Sigma-Aldrich as KiCqStart primers, with the following sequences: MCM10_FOR 5′-CTTATACAGAAGGCTGATG and MCM10_REV 5′-GTGAATTCTCTTTGGAGGAG and MCM10_REV 5′-GATGGTTTGTGTTCAGGAG; CDCA2_FOR 5′-AGGAAAGCTCATCATCTACC and CDCA2_REV 5′-AGAATCTGGACATATGGGAG; SMN1_FOR 5′-GGAAAGCTAGGTCTAAAATTC and SMN1_REV 5′-AGAATCTGGACATATGGGAG. The difference in the amount of input cDNA was normalized to an internal control of GAPDH, using the following primers: GAPDH_FOR: 5′ ATGGGGAAGGTGAAGGTCG and GAPDH_REV: 5′-CTCCACGACGTACTCAGCG.

Phylogenetic tree of Vpr sequences.

An existing nucleic acid sequence alignment of 191 representative Vif sequences from HIV-1, HIV-2 and SIVs from 22 primate species was downloaded from: https://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html A tree was generated using the percentage identity average distance in jalview [74] and visualised in Figtree: http://tree.bio.ed.ac.uk/software/figtree/

10 sequences from 5 primate species not falling into the 5 highlighted lineages are not shown.

Sample Preparation for Mass spectrometry

Samples were prepared using three different methods depending on the experiment. Initial infection experiment was by SDC-FASP. VLP and shRNA experiments were by PreOmics NHS-iST sample preparation Kit. pSILAC and MS-IP experiments were by SP3.

SDC-FASP

Samples prepared essentially according to the protocol in Leon et al[75]. Briefly samples were lysed in 50mM TEAB (pH8.5) 2% SDS, reduced and alkylated with TCEP/Iodoacetamide, quantified by BCA assay. 50ug of each sample was diluted with TEAB/8M urea for loading onto 30kDa ultrafiltration devices. Samples were washed 3 times with 500uL urea buffer and 3 times with digestion buffer (TEAB/0.5% sodium deoxycholate) before resuspending in 50uL digestion buffer containing 1ug trypsin and incubating overnight at 37 degrees. After digestion samples were spun through the filters and filters washed with 50uL TEAB. SDC was removed by acidification and two phase partitioning with ethyl acetate before vacuum drying and labelling with TMT reagents according to the manufacturer’s instructions.

PreOmics iST

Samples lysed in kit lysis buffer and quantified by BCA assay. 25ug of each sample was digested essentially according to manufacturer’s instructions, scaling volumes for a digestion of 25ug total protein.

SP3

Samples were lysed in 50mM TEAB (pH8.5) 2% SDS (MS-IPs were adjusted to 2% SDS), reduced and alkylated with TCEP/Iodoacetamide before digestion using the SP3 method[76]. Briefly, carboxylate modified paramagnetic beads are added to the sample and protein is bound to the beads by acidification with formic acid and addition of acetonitrile (ACN, final 50%). The beads are then washed sequentially with 100% ACN, 70% Ethanol (twice) and 100% ACN. 10-20uL TEAB (Triethylammonium
bicarbonate) pH 8 and 0.1% Sodium deoxycholate (SDC) is then added to the washed beads along with trypsin. Samples were then incubated overnight at 37 degrees with periodic shaking at 2000 rpm. After digestion, peptides are immobilised on beads by addition of 200-400 uL ACN and washed twice with 100 uL ACN before eluting in 19 uL 2% DMSO and removing the eluted peptide from the beads.

Off-line high pH reversed-phase (HpRP) peptide fractionation

For whole cell proteome samples HpRP fractionation was conducted on an Ultimate 3000 UHPLC system (Thermo Scientific) equipped with a 2.1 mm × 15 cm, 1.7 μm Acquity BEH C18 column (Waters, UK). Solvent A was 3% ACN, Solvent B was 100% ACN, solvent C was 200 mM ammonium formate (pH 10). Throughout the analysis solvent C was kept at a constant 10%. The flow rate was 400 μL/min and UV was monitored at 280 nm. Samples were loaded in 90% A for 10 min before a gradient elution of 0–10% B over 10 min (curve 3), 10-34% B over 21 min (curve 5), 34-50% B over 5 mins (curve 5) followed by a 10 min wash with 90% B. 15 s (100 μL) fractions were collected throughout the run.

Peptide containing fractions were orthogonally recombined into 24 fractions (i.e. fractions 1, 25, 49, 73, 97 combined) and dried in a vacuum centrifuge. Fractions were stored at −80°C prior to analysis.

Mass spectrometry

Data were acquired on an Orbitrap Fusion mass spectrometer (Thermo Scientific) coupled to an Ultimate 3000 RSLC nano UHPLC (Thermo Scientific). HpRP fractions were reuspended in 20 μl 5% DMSO 0.5% TFA and 10 uL injected. Fractions were loaded at 10 μl/min for 5 min on to an Acclaim PepMap C18 cartridge trap column (300 um × 5 mm, 5 um particle size) in 0.1% TFA. After loading a linear gradient of 3–32% solvent B was used for sample separation over a column of the same stationary phase (75 μm × 50 cm, 2 μm particle size) before washing at 90% B and re-equilibration.

Solvents were A: 0.1% FA and B:ACN/0.1% FA. 3h gradients were used for whole cell proteomics samples, 1h gradients for MS-IPs.

An SPS/MS3 acquisition was used for TMT experiments and was run as follows. MS1: Quadrupole isolation, 120’000 resolution, 5e5 AGC target, 50 ms maximum injection time, ions injected for all parallelisable time. MS2: Quadrupole isolation at an isolation width of m/z 0.7, CID fragmentation (NCE 35) with the ion trap scanning out in rapid mode from m/z 120, 8e3 AGC target, 70 ms maximum injection time, ions accumulated for all parallelisable time. In synchronous precursor selection mode the top 10 MS2 ions were selected for HCD fragmentation (6SNCE) and scanned out in the orbitrap at 50’000 resolution with an AGC target of 2e4 and a maximum accumulation time of 120 ms, ions were not accumulated for all parallelisable time. The entire MS/MS/MS cycle had a target time of 3 s. Dynamic exclusion was set to +/-10 ppm for 90 s, MS2 fragmentation was triggered on precursor ions 5e3 counts and above. For MS-IPs, MS2 instead used HCD fragmentation (NCE 34) and a maximum injection time of 250ms and had a target cycle time of 2s. For pSILAC MS1 was acquired at 240’000 resolution.

Data processing and analysis

For TMT labelled samples data were searched by Mascot within Proteome Discoverer 2.1 in two rounds of searching. First search was against the UniProt Human reference proteome (26/09/17), the HIV proteome and compendium of common contaminants (GPM). The second search took all unmatched spectra from the first search and searched against the human trEMBL database (Uniprot, 26/09/17). The following search parameters were used. MS1 Tol: 10 ppm, MS2 Tol: 0.6 Da. Enzyme:
Trypsin (/P). MS3 spectra were used for reporter ion based quantitation with a most confident centroid tolerance of 20 ppm. PSM FDR was calculated using Mascot percolator and was controlled at 0.01% for ‘high’ confidence PSMs and 0.05% for ‘medium’ confidence PSMs. Normalisation was automated and based on total s/n in each channel. Protein/peptide abundance was calculated and output in terms of ‘scaled’ values, where the total s/n across all reporter channels is calculated and a normalised contribution of each channel is output. Proteins/peptides satisfying at least a ‘medium’ FDR confidence were taken forth to statistical analysis in R. This consisted of a moderated T-test (Limma) with Benjamini-Hochberg correction for multiple hypotheses to provide a q value for each comparison [70]. MS-IPs were submitted to a similar search workflow with quantitative data being derived from MS1 spectra via proteome discover minora feature detector node. For pSILAC experiments data were processed in MaxQuant and searched using Andromeda with similar search parameters [77]. MaxQuant output was uploaded into Perseus for calculation of significance B [72]. Where conditions were not carried out in triplicate, downstream analysis was limited to proteins identified with at least 3 unique peptides.

References

1. Matheson, N.J., E.J. Greenwood, and P.J. Lehner, Manipulation of immunometabolism by HIV-accessories to the crime? Curr Opin Virol, 2016. 19: p. 65-70.
2. Sugden, S.M., et al., Remodeling of the Host Cell Plasma Membrane by HIV-1 Nef and Vpu: A Strategy to Ensure Viral Fitness and Persistence. Viruses, 2016. 8(3).
3. Simon, V., N. Bloch, and N.R. Landau, Intrinsic host restrictions to HIV-1 and mechanisms of viral escape. Nat Immunol, 2015. 16(6): p. 546-53.
4. Matheson, N.J., et al., Cell Surface Proteomic Map of HIV Infection Reveals Antagonism of Amino Acid Metabolism by Vpu and Nef. Cell Host Microbe, 2015. 18(4): p. 409-23.
5. Greenwood, E.J., et al., Temporal proteomic analysis of HIV infection reveals remodelling of the host phosphoproteome by lentiviral Vif variants. Elife, 2016. 5.
6. Lahouassa, H., et al., HIV-1 Vpr degrades the HLTF DNA translocase in T cells and macrophages. Proc Natl Acad Sci U S A, 2016.
7. Hrecka, K., et al., HIV-1 and HIV-2 exhibit divergent interactions with HLTF and UNG2 DNA repair proteins. Proc Natl Acad Sci U S A, 2016. 113(27): p. E3921-30.
8. Maudet, C., et al., HIV-1 Vpr induces the degradation of ZIP and sZIP, adaptors of the NuRD chromatin remodeling complex, by hijacking DCAF1/VprBP. PLoS One, 2013. 8(10): p. e77320.
9. Romani, B., et al., HIV-1 Vpr Protein Enhances Proteasomal Degradation of MCM10 DNA Replication Factor through the Cul4-DD81[VprBP] E3 Ubiquitin Ligase to Induce G2/M Cell Cycle Arrest. J Biol Chem, 2015. 290(28): p. 17380-9.
10. Schrofelbauer, B., et al., Human immunodeficiency virus type 1 Vpr induces the degradation of the UNG and SMUG uracil-DNA glycosylases. J Virol, 2005. 79(17): p. 10978-87.
11. Lv, L., et al., Vpr Targets TET2 for Degradation by CRL4(VprBP) E3 Ligase to Sustain IL-6 Expression and Enhance HIV-1 Replication. Mol Cell, 2018. 70(5): p. 961-970 e5.
12. Zhou, X., M. DeLucia, and J. Ahn, SLX4-SLX1 Protein-independent Down-regulation of MUS81-EME1 Protein by HIV-1 Viral Protein R (Vpr). J Biol Chem, 2016. 291(33): p. 16936-16947.
13. Laguette, N., et al., Premature activation of the SLX4 complex by Vpr promotes G2/M arrest and escape from innate immune sensing. Cell, 2014. 156(1-2): p. 134-45.
14. Lapek, J.D., Jr., et al., Quantitative Temporal Viromics of an Inducible HIV-1 Model Yields Insight to Global Host Targets and Phospho-Dynamics Associated with Protein Vpr. Mol Cell Proteomics, 2017. 16(8): p. 1447-1461.
15. Goh, W.C., et al., HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. Nat Med, 1998. 4(1): p. 65-71.
16. Forget, J., et al., Human immunodeficiency virus type 1 vpr protein transactivation function: mechanism and identification of domains involved. J Mol Biol, 1998. 284(4): p. 915-23.

17. Le Rouzic, E., et al., HIV-1 Vpr arrests the cell cycle by recruiting DCAF1/VprBP, a receptor of the Cul4-DDB1 ubiquitin ligase. Cell Cycle, 2007. 6(2): p. 182-8.

18. Wu, Y., et al., The DDB1-DCAF1-Vpr-UNG2 crystal structure reveals how HIV-1 Vpr steers human UNG2 toward destruction. Nat Struct Mol Biol, 2016. 23(10): p. 933-940.

19. Selig, L., et al., Uracil DNA glycosylase specifically interacts with Vpr of both human immunodeficiency virus type 1 and simian immunodeficiency virus of sooty mangabeys, but binding does not correlate with cell cycle arrest. J Virol, 1997. 71(6): p. 4842-6.

20. Fregoso, O.I. and M. Emerman, Activation of the DNA Damage Response Is a Conserved Function of HIV-1 and HIV-2 Vpr That Is Independent of SLX4 Recruitment. MBio, 2016. 7(5).

21. Hohne, K., et al., Virion encapsidated HIV-1 Vpr induces NFAT to prime non-activated T cells for productive infection. Open Biol, 2016. 6(7).

22. Liang, Z., et al., HIV-1 Vpr protein activates the NF-kappaB pathway to promote G2/M cell cycle arrest. Virol Sin, 2015. 30(6): p. 441-8.

23. Berger, G., et al., G2/M cell cycle arrest correlates with primate lentiviral Vpr interaction with the SLX4 complex. J Virol, 2015. 89(1): p. 230-40.

24. Belzile, J.P., et al., HIV-1 Vpr induces the K48-linked polyubiquitination and proteasomal degradation of target cellular proteins to activate ATR and promote G2 arrest. J Virol, 2010. 84(7): p. 3320-30.

25. Terada, Y. and Y. Yasuda, Human immunodeficiency virus type 1 Vpr induces G2 checkpoint activation by interacting with the splicing factor SAP145. Mol Cell Biol, 2006. 26(21): p. 8149-58.

26. Re, F., et al., Human immunodeficiency virus type 1 Vpr arrests the cell cycle in G2 by inhibiting the activation of p34cdc2-cyclin B. J Virol, 1995. 69(11): p. 6859-64.

27. Zhou, Y. and L. Ratner, Phosphorylation of human immunodeficiency virus type 1 Vpr regulates cell cycle arrest. J Virol, 2000. 74(14): p. 6520-7.

28. Fischer, M., et al., Integration of TP53, DREAM, MMB and RB-E2F target gene analyses identifies cell cycle gene regulatory networks. Nucleic Acids Res, 2016. 44(13): p. 6070-86.

29. Ly, T., A. Endo, and A.I. Lamond, Proteomic analysis of the response to cell cycle arrests in human myeloid leukemia cells. Elife, 2015. 4.

30. Hossain, D., et al., Cep78 controls centrosome homeostasis by inhibiting EDD-DYRK2-DDB1(Vpr)(BP). EMBO Rep, 2017. 18(4): p. 632-644.

31. Coyaud, E., et al., Global Interactomics Uncovers Extensive Organellar Targeting by Zika Virus. Mol Cell Proteomics, 2018. 17(11): p. 2242-2255.

32. Guo, Z., et al., DCAF1 controls T-cell function via p53-dependent and -independent mechanisms. Nat Commun, 2016. 7: p. 10307.

33. Jager, S., et al., Global landscape of HIV-human protein complexes. Nature, 2011. 481(7381): p. 365-70.

34. Luo, Y., et al., HIV-host interactome revealed directly from infected cells. Nat Microbiol, 2016. 1(7): p. 16068.

35. Boisvert, F.M., et al., A quantitative spatial proteomics analysis of proteome turnover in human cells. Mol Cell Proteomics, 2012. 11(3): p. M111 011429.

36. Gummuluru, S. and M. Emerman, Cell cycle- and Vpr-mediated regulation of human immunodeficiency virus type 1 expression in primary and transformed T-cell lines. J Virol, 1999. 73(7): p. 5422-30.

37. Bolton, D.L. and M.J. Lenardo, Vpr cytopathicity independent of G2/M cell cycle arrest in human immunodeficiency virus type 1-infected CD4+ T cells. J Virol, 2007. 81(17): p. 8878-90.

38. Rogel, M.E., L.I. Wu, and M. Emerman, The human immunodeficiency virus type 1 vpr gene prevents cell proliferation during chronic infection. J Virol, 1995. 69(2): p. 882-8.
39. Liu, R., et al., HIV-1 Vpr stimulates NF-kappaB and AP-1 signaling by activating TAK1. 
Retrovirology, 2014. 11: p. 45.

40. Muthumani, K., et al., The HIV-1 Vpr and glucocorticoid receptor complex is a gain-of-function interaction that prevents the nuclear localization of PARP-1. Nat Cell Biol, 2006. 8(2): p. 170-9.

41. Tchasovnikarova, I.A., et al., GENE SILENCING. Epigenetic silencing by the HUSH complex mediates position-effect variegation in human cells. Science, 2015. 348(6242): p. 1481-1485.

42. Hrecza, K., et al., Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein. Nature, 2011. 474(7353): p. 658-61.

43. Laguette, N., et al., SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. Nature. 2014. 477(7353): p. 654-7.

44. Lim, E.S., et al., The ability of primate lentiviruses to degrade the monocyte restriction factor SAMHD1 preceded the birth of the viral accessory protein Vpx. Cell Host Microbe, 2012. 11(2): p. 194-204.

45. Chougui, G., et al., HIV-2/SIV viral protein X counteracts HUSH repressor complex. Nat Microbiol, 2018.

46. Yurkovetskiy, L., et al., Primate immunodeficiency virus proteins Vpx and Vpr counteract transcriptional repression of proviruses by the HUSH complex. Nat Microbiol, 2018.

47. Greenwood, E.J., et al., Simian Immunodeficiency Virus Infection of Chimpanzees (Pan troglodytes) Shares Features of Both Pathogenic and Non-pathogenic Lentiviral Infections. PLoS Pathog, 2015. 11(9): p. e1005146.

48. Keele, B.F., et al., Increased mortality and AIDS-like immunopathology in wild chimpanzees infected with SIVcpz. Nature, 2009. 460(7254): p. 515-9.

49. Ersing, I., et al., A Temporal Proteomic Map of Epstein-Barr Virus Lytic Replication in B Cells. Cell Rep, 2017. 19(7): p. 1479-1493.

50. Weekes, M.P., et al., Quantitative temporal viromics: an approach to investigate host-pathogen interaction. Cell, 2014. 157(6): p. 1460-72.

51. Diamond, D.L., et al., Temporal proteome and lipidome profiles reveal hepatitis C virus-associated reprogramming of hepatocellular metabolism and bioenergetics. PLoS Pathog, 2010. 6(1): p. e1000719.

52. Murray, J.M., A.D. Kelleher, and D.A. Cooper, Timing of the components of the HIV life cycle in productively infected CD4+ T cells in a population of HIV-infected individuals. J Virol, 2011. 85(20): p. 10798-805.

53. Perelson, A.S., et al., HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. Science, 1996. 271(5255): p. 1582-6.

54. Hofmann, S., et al., Dual role of the chromatin-binding factor PHF13 in the pre- and post-integration phases of HIV-1 replication. Open Biol, 2017. 7(10).

55. Guenzel, C.A., C. Herate, and S. Benichou, HIV-1 Vpr-a still "enigmatic multitasker". Front Microbiol, 2014. 5: p. 127.

56. Shimura, M., et al., Epigenetic displacement of HP1 from heterochromatin by HIV-1 Vpr causes premature sister chromatid separation. J Cell Biol, 2011. 194(5): p. 721-35.

57. Vassena, L., et al., The human immunodeficiency virus type 1 Vpr protein upregulates PVR via activation of the ATR-mediated DNA damage response pathway. J Gen Virol, 2013. 94(Pt 12): p. 2664-9.

58. Richard, J., et al., HIV-1 Vpr up-regulates expression of ligands for the activating NKG2D receptor and promotes NK cell-mediated killing. Blood, 2010. 115(7): p. 1354-63.

59. Kannan, A., et al., Combined deficiency of Senataxin and DNA-PKcs causes DNA damage accumulation and neurodegeneration in spinal muscular atrophy. Nucleic Acids Res, 2018. 46(16): p. 8326-8346.

60. Peng, A., et al., Repo-man controls a protein phosphatase 1-dependent threshold for DNA damage checkpoint activation. Curr Biol, 2010. 20(5): p. 387-96.

17
Roshal, M., et al., *Activation of the ATR-mediated DNA damage response by the HIV-1 viral protein R*. J Biol Chem, 2003. **278**(28): p. 25879-86.

Hou, F. and H. Zou, *Two human orthologues of Eco1/Ctf7 acetyltransferases are both required for proper sister-chromatid cohesion*. Mol Biol Cell, 2005. **16**(8): p. 3908-18.

Dai, J., B.A. Sullivan, and J.M. Higgins, *Regulation of mitotic chromosome cohesion by Haspin and Aurora B*. Dev Cell, 2006. **11**(5): p. 741-50.

Rankin, S., N.G. Ayad, and M.W. Kirschner, *Sororin, a substrate of the anaphase-promoting complex, is required for sister chromatid cohesion in vertebrates*. Mol Cell, 2005. **18**(2): p. 185-200.

Vizcaino, J.A., et al., *2016 update of the PRIDE database and its related tools*. Nucleic Acids Res, 2016. **44**(22): p. 11033.

Zhang, H., et al., *Novel single-cell-level phenotypic assay for residual drug susceptibility and reduced replication capacity of drug-resistant human immunodeficiency virus type 1*. J Virol, 2004. **78**(4): p. 1718-29.

van den Boomen, D.J., et al., *TMEM129 is a Derlin-1 associated ERAD E3 ligase essential for virus-induced degradation of MHC-I*. Proc Natl Acad Sci U S A, 2014. **111**(31): p. 11425-30.

Mellacheruvu, D., et al., *The CRAPome: a contaminant repository for affinity purification-mass spectrometry data*. Nat Methods, 2013. **10**(8): p. 730-6.

Matheson, N.J., A.A. Peden, and P.J. Lehner, *Antibody-free magnetic cell sorting of genetically modified primary human CD4+ T cells by one-step streptavidin affinity purification*. PLoS One, 2014. **9**(10): p. e111437.

Schwammle, V., I.R. Leon, and O.N. Jensen, *Assessment and improvement of statistical tools for comparative proteomics analysis of sparse data sets with few experimental replicates*. J Proteome Res, 2013. **12**(9): p. 3874-83.

R Core Team, *R: A language and environment for statistical computing*. Vienna, Austria. [http://www.R-project.org/](http://www.R-project.org/). 2013.

Tyanova, S., et al., *The Perseus computational platform for comprehensive analysis of (pro)teomics data*. Nat Methods, 2016. **13**(9): p. 731-40.

Mi, H., et al., *PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements*. Nucleic Acids Res, 2017. **45**(D1): p. D183-D189.

Waterhouse, A.M., et al., *Jalview Version 2—a multiple sequence alignment editor and analysis workbench*. Bioinformatics, 2009. **25**(9): p. 1189-91.

Leon, I.R., et al., *Quantitative assessment of in-solution digestion efficiency identifies optimal protocols for unbiased protein analysis*. Mol Cell Proteomics, 2013. **12**(10): p. 2992-3005.

Hughes, C.S., et al., *Ultrasensitive proteome analysis using paramagnetic bead technology*. Mol Syst Biol, 2014. **10**: p. 757.

Cox, J. and M. Mann, *MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification*. Nat Biotechnol, 2008. **26**(12): p. 1367-72.

Adachi, A., et al., *Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone*. J Virol, 1986. **59**(2): p. 284-91.

Rodenburg, C.M., et al., *Near full-length clones and reference sequences for subtype C isolates of HIV type 1 from three different continents*. AIDS Res Hum Retroviruses, 2001. **17**(2): p. 161-8.

Van Heuverswyn, F., et al., *Genetic diversity and phylogeographic clustering of SIVcpzPtt in wild chimpanzees in Cameroon*. Virology, 2007. **368**(1): p. 155-71.

Ahuka-Mundeke, S., et al., *Full-length genome sequence of a simian immunodeficiency virus (SIV) infecting a captive agile mangabey (Cercocebus agilis) is closely related to SIVrcm*
19

751 infecting wild red-capped mangabeys (Cercocebus torquatus) in Cameroon. J Gen Virol, 2010.
752
753 91(Pt 12): p. 2959-64.
754
755 82. Gnanadurai, C.W., et al., Genetic identity and biological phenotype of a transmitted/founder
756 virus representative of nonpathogenic simian immunodeficiency virus infection in African
757 green monkeys. J Virol, 2010. 84(23): p. 12245-54.
758
759 83. Lopker, M.J., et al., Derivation and Characterization of Pathogenic Transmitted/Founder
760 Molecular Clones from Simian Immunodeficiency Virus SIVsmE660 and SIVmac251 following
761 Mucosal Infection. J Virol, 2016. 90(19): p. 8435-53.
762
763 84. Guyader, M., et al., Genome organization and transactivation of the human
764 immunodeficiency virus type 2. Nature, 1987. 326(6114): p. 662-9.
765
766 85. Aghokeng, A.F., et al., Full-length sequence analysis of SIVmus in wild populations of
767 mustached monkeys (Cercopithecus cephus) from Cameroon provides evidence for two co-
768 circulating SIVmus lineages. Virology, 2007. 360(2): p. 407-18.
769
770 Figure legends
771
772 Figure 1. Proteomic analysis of the effect of Vpr in HIV infection.
773
774 A, Graphical summary of the HIV and ΔVpr HIV infection TMT experiment. Three replicates of
775 uninfected (mock), WT HIV infected and ΔVpr infected cells were prepared and analysed in parallel
776 using TMT labelling. B, FACS plots showing the quantification of infection in an example replicate for
777 each of the three conditions. Infected cells lose CD4 expression and become GFP positive. C,
778 Scatterplots displaying pairwise comparisons between WT, ΔVpr and mock-infected cells. Each point
779 represents a single protein, with HIV proteins and host proteins of interest highlighted with different
780 symbols (see key). D, Principle component analysis of the samples in this experiment, with WT
781 infected replicates in red, ΔVpr in blue and mock infected cells in grey.
782
783 Figure 1 – source data 1. Interactive database of protein changes in the datasets presented here.
784
785 Figure 2. Analysis of the nature of Vpr mediated proteome remodelling.
786
787 A, Graphical summary of the Vpr viral particle TMT experiment. Three replicates of cells exposed to
788 empty viral particles or Vpr bearing viral particles, along with single replicates of cells exposed to
789 viral particles bearing five different Vpr mutants were prepared and analysed in parallel using TMT
790 labelling. B, Scatterplot displaying pairwise comparison between cells exposed to empty or Vpr
791 bearing viral particles. C, Scatterplot comparing pairwise comparisons from two proteomics
792 experiments, demonstrating the effect of Vpr in the context of HIV-1 infection (x-axis, as shown in
793 Figure 1A) or through cellular exposure to Vpr protein alone (y- axis, as shown in Figure 2A). D,
794 scatterplots showing the pairwise comparison between each Vpr mutant tested and empty vector
795 control, with defined groups of 302 Vpr depleted and 413 increased proteins highlighted in blue and
796 red respectively. E, Heatmap showing the behaviour of the 100 proteins most depleted by Vpr
797 particles (blue) and increased (red) within the defined highly modulated subsets. Colour indicates
798 the log2 fold change of each protein in each condition compared to empty particle treatment. Genes
799
800 19
were clustered using uncentered Pearson correlation and centroid linkage, and conditions clustered by column means.

**Figure 2 – figure supplement 1. Example proteins modulated by Vpr**

A, Scatterplots displaying pairwise comparisons between WT, ΔVpr and mock-infected cells. Vpr depleted proteins confirmed by blot in this figure are highlighted in blue, UNG, ZGPAT and HLTF are previously identified targets for Vpr mediated degradation, shown in purple. CCNB1 is increased by Vpr (red). B, Scatterplot displaying pairwise comparison between cells exposed to empty or Vpr bearing viral particles. C, Scatterplot comparing pairwise comparisons from two proteomics experiments, demonstrating the effect of Vpr in the context of HIV-1 infection (as shown in Figure 1A, x-axis) or through cellular exposure to Vpr protein alone (Figure 2A, y-axis). D, Bar charts showing the Vpr mediated changes in protein abundance in both proteomics experiments of the proteins highlighted in this figure. E, Immunoblot of proteins highlighted in this figure.

**Figure 2 – figure supplement 2. Additional details of the Vpr WT and Mutant experiment in Figure 2.**

A, Immunoblot of purified virus preparations used to infect cells for the proteomics experiment displayed in Fig 2A. B, Bar chart showing the average scaled abundance of matrix, capsid and integrase peptides detected in the cell lysate by MS. Bars show mean and standard deviation. C, 7-AAD stain of cells exposed to empty vector, or Vpr wild type or Vpr mutants. Watson pragmatic modelling was used to identify cells in G1 (blue), S (grey) or G2/M (red) phase.

**Figure 2 - figure supplement 3. Quantifying the effect of Vpr under reduced DCAF1 conditions.**

A, Graphical summary of the DCAF1 KD experiment. B, Scatterplot displaying pairwise comparison between Sh-Control and Sh-DCAF1 cells at 0 h, with defined groups of 302 Vpr depleted (blue) and 413 increased (red) proteins highlighted. DCAF1 is highlighted in purple. C, Example time-course behaviour for one Vpr target (HLTF) and one secondary Vpr effect (CCNB1). D, Scatterplots showing the pairwise comparison between the 6 or 24 h timepoint with the 0 h condition for each sh-transduced cell line.

**Figure 2- figure supplement 4. Cell cycle regulation of Vpr modulated proteins.**

A, Ly, 2015 contains a proteomic analysis of NB4 cells treated with RO-3306 cells arrested in G2/M with the PLK1 inhibitor RO-3306. Scatterplot showing the pairwise comparison of abundance of proteins isolated from RO-3306 treated vs mock treated cells. Groups of proteins defined in the current study of 302 Vpr depleted (blue) and 413 increased (red) proteins are highlighted, indicating the behaviour of these proteins in NB4 cells arrested at G2/M.

B, Correlation of the Vpr mediated change in cyclin abundance in the present study (x-axis), with RO-3306 mediated changes in NB4 cells in Ly, 2015. Changes in cyclin abundance in HIV infection are assumed to be secondary to cell cycle arrest, and thus this correlation indicates the concordance between effects secondary to cell cycle arrest between the two datasets.

C, Pie charts showing the overlap between changes in the present study and changes induced by G2/M arrest in Ly, 2015. Left panel shows the behaviour of all proteins quantified in both the present study (Figure 1A and Figure 2A) and Ly, 2015, i.e. a total of 1643 proteins were quantified in both datasets, of which 1100 proteins did not significantly change in G2/M arrest, 260 proteins were significantly depleted in G2/M arrested cells, and 283 proteins were significantly increased in G2/M.
arrested cells. Middle panel shows the behaviour the defined group of 302 Vpr depleted proteins. I.e. of 302 proteins, a total of 90 proteins were detected in Ly, 2015, 58 of which did not significantly change in G2/M arrest, 18 were significantly depleted in G2/M arrest and 14 were significantly increased in G2/M arrest. † Indicates the fraction in each case where the change induced by cell cycle arrest is in the same direction as the effect of Vpr, i.e. the fraction for which cell cycle arrest could explain the Vpr mediated protein changes. Right panel shows the behaviour of the defined group of 413 Vpr increased proteins.

D, Fischer, 2016, define lists of 115 proteins whose expression peaks in G1/S phase and 174 proteins whose expression peaks in G2/M phase. Pie-charts show the overlap between these lists and (left) all proteins detected in the present study, (middle) proteins defined as being depleted by Vpr, and (right) proteins defined as being increased by Vpr. As in the proteomics dataset, there is some enrichment of proteins with peak expression in G2/M within Vpr increased proteins, and some enrichment of proteins with peak expression in G1/S in Vpr depleted proteins, consistent with some effects being secondary to cell cycle arrest, but these effects are in the minority.

Figure 2 – figure supplement 5. Role of MCM10 in Vpr mediated G2/M arrest.

A, Correlation between depletion of MCM10 by each Vpr mutant tested and the extent of G2/M arrest caused by that mutant, red line shows linear regression analysis. B, Example DNA staining showing G2/M arrest caused by shRNA mediated depletion of MCM10, representative of three independent experiments. Watson pragmatic modelling was used to identify cells in G1 (blue), S (grey) or G2/M (red) phase. C, Real-time qRT-PCR analysis of MCM10 mRNA abundance in cells transduced with control or MCM10 targeting shRNA. Values were generated using the ΔΔCT method, relative to GAPDH mRNA abundance and normalised to the control condition. Bars show mean values, error bars show SEM of three technical replicates.

Figure 3. Gene ontology enrichment analysis for proteins depleted or increased by Vpr

Defined groups of Vpr depleted and increased proteins were subject to gene ontology enrichment analysis, compared with a background of all proteins quantitated in these experiments. A, Cellular compartment analysis – GO enrichment analysis results were manually curated for 9 commonly used organelle level classifications, shown here. + / - indicates if the classification was enriched or de-enriched compared with the expected number of proteins expected by chance. Where significant, associated p value represents the results of a Fisher’s exact test with Bonferroni correction. This is highly conservative as it is corrected for all 1061 possible cellular compartment terms, not just those shown. B, Molecular function analysis. Plots show all significantly enriched (Fisher’s exact test with Bonferroni correction) molecular function terms with associated p values. In all cases, proteins with no available compartment or function classification were removed from the denominator.

Figure 4. MS-IP approach to identify direct targets for Vpr mediated degradation.

A, Graphical summary of the MS-IP experiment. All cells were stably transduced with an ShDCAF1 vector as described earlier. MLN4924 is a pan-Cullin inhibitor. B, 20 most abundant proteins identified by Co-IP determined by number of unique peptides, normalised as a proportion of the maximum possible peptide count for each protein, (Exponentially modified protein abundance index, emPAI). Proteins falling within the defined list of 302 Vpr depleted and 413 Vpr increased proteins are highlighted in blue and red respectively. C, The same 20 proteins with signal intensity rather than peptide number shown. D, Pie chart indicating the overlap between the proteins co-immunoprecipitated with Vpr and the defined list of 302 Vpr decreased (blue) and 413 Vpr increased
proteins (red), and proteins detected but falling into neither list (grey). E, Bar chart showing the enrichment of Vpr depleted and Vpr increased proteins within proteins co-immunoprecipitated with Vpr compared to the expected numbers of proteins that would be co-immunoprecipitated from each group by chance. p-value indicates a Fisher’s exact test of a 2x2 contingency table (Vpr depleted/increased, identified by co-IP/not identified), indicating that the two criteria are significantly linked.

**Figure 5. Pulsed SILAC method to identify direct targets for Vpr mediated decay.**

A, Graphical summary of the pulsed SILAC experiment. B, Scatterplots showing the changes to protein stability of proteins after 6 or 24 hours of exposure to Vpr bearing lentivirus compared to control lentivirus, with defined groups of 302 Vpr depleted (blue) and 413 increased (red) proteins highlighted. C, Expanded view of proteins degraded within 6 hours of Vpr exposure. Significantly degraded (Sig.B <0.01) proteins are highlighted in gold. Previously described Vpr targets HLTF, MUS81 and ZGPAT are shown in purple.

**Table 1. Direct targets for Vpr mediated degradation**

Summary of the direct targets for Vpr mediated degradation. Proteins highlighted in purple are previously described Vpr targets, proteins with red text were predicted as potential Vpr targets based on their temporal profile of depletion in Greenwood & Matheson, 2016 [5].

**Figure 6. Novel Vpr targets involved in G2/M arrest.**

A, Targeted shRNA screen of direct Vpr target proteins identified here, whose depletion correlated with G2/M arrest in Figure 2. Bars show averages of at least two replicates from more than three independent experiments, error bars showing SEM. Lines show control average +/- 3 standard deviations. Control condition contains combined data from three different control shRNA. B, Real-time qRT-PCR analysis of mRNA abundance in cells transduced with control or targeting shRNA. Values were generated using the ΔΔCT method, relative to GAPDH mRNA abundance and normalised to the control condition. Bars show mean values, error bars show SEM of three technical replicates. C, Example DNA staining showing G2/M arrest caused by shRNA mediated depletion of SMN1, CDCA2 and ZNF267, representative of at least two independent experiments.

**Figure 7. Identification of proteome changes conserved between human and primate lentiviral Vpr lineages.** A, Graphical summary of the TMT experiment testing conservation of Vpr functions. B, Scatterplots showing the pairwise comparison between each Vpr tested and empty vector control with defined groups of 302 Vpr depleted (blue) and 413 increased (red) proteins highlighted. C, Heatmap showing the behaviour of the 100 proteins most depleted by Vpr particles (blue) and increased (red) within the defined highly modulated subsets. Colour indicates the log2 fold change of each protein in each condition compared to empty vector transduction. D, Bar chart showing the significantly Vpr depleted group showing profound (more than 50%) depletion in more than one lineage, of the four lineages tested (HIV-1/SIVcpz, HIV-2/SIVsmm, SIVagm, SIVrcm). Gold bars indicate proteins identified as direct targets for Vpr mediated degradation, DCAF1, known to interact with multiple lineages, is highlight in purple. E, Immunoblot of example known, non-conserved and conserved targets of Vpr mediated depletion. N.B. The HIV-2 Vpr is a primary isolate HIV-2 Vpr (7312a), while the proteomics experiment included HIV-2 ROD Vpr.
Figure 7 – figure supplement 1. Additional information relating to expanded panel of primate lentiviral Vpr and Vpx varients.

A, %GFP positive (transduced) cells at harvest. Cells were transduced at an infectious MOI of 1.5 based on prior titration, with the actual resulting % transduction varying slightly across the samples.

B, Proportion of cells in G2/M at point of harvest based on 7-AAD staining and Watson pragmatic modelling. B, Scatterplots displaying pairwise comparisons between Vpr/Vpx transduced cells and empty vector. Labelled proteins are direct targets for Vpr mediated degradation that are depleted by Vpr varients from at least 3 different lineages.

Figure 8. Depletion of HuSH components by lentiviral Vpx and Vpr proteins.

A, Scatterplots showing the pairwise comparison between each Vpr tested and empty vector control with HuSH complex components highlighted. B, Bar graph of GFP percentage positive of JLAT-A1 cells after transduction with control (Cre recombinase), Vpr or Vpx proteins and treatment with TNFα. Mean and SEM of 3 biological replicates per condition are shown, representative of three independent similar experiments. p-values determined by ordinary one-way ANOVA with Bonferroni comparison between Vpr/Vpx treatment and control treated cells. C, Phylogenetic tree of primate lentiviruses based on an alignment of Vif nucleic acid sequences, with 5 major lineages of primate lentiviruses labelled. Information on Vpr and Vpx activity based on a selected number of isolates tested in each lineage in Lim, 2012 [44] D, Immunoblot of TASOR in cells transduced with a panel of Vpx and Vpr proteins.

Figure 9. Extended time-course analysis of HIV-2 primary isolate Vpr and Vpx proteins.

A, Graphical summary of the TMT experiment. B, Scatterplots displaying pairwise comparison between cells transduced with 7312a HIV-2 Vpr and Vpx for 96 h compared with those transduced with empty vector for 96 h. Blue and red dots represent the defined groups or proteins depleted or increased by NL4-3 Vpr respectively. Points ringed in gold indicate direct targets for NL4-3 Vpr mediated degradation listed in Table 1.

Table 2. Vpr and Vpx variants tested.
**Figure 1**

A. Mock 48 h → HIV WT 48 h → HIV ΔVpr 48 h

Digest proteins and label peptides with TMT reporters

Mix peptides, fractionate and analyse by LC/MS³

B. Mock

HIV WT

HIV ΔVpr

Env-GFP

Infected 77.3%

Infected 75.9%

C. HIV WT vs Mock

HIV ΔVpr vs Mock

Depleted in HIV infection

 Increased in HIV infection

Depleted in ΔVpr infection

Increased in ΔVpr infection

-Log₂(q value)

Log₂(fold change)

q<0.01

q>0.01

Published Vif/Vpu/Nef targets

Published Vpr targets

HIV Proteins

Other Proteins

D. Principle component analysis

PC 1 (43.2%)

PC 2 (21.2%)

Mock

ΔVpr

WT
**Figure 2**

**A**

- **Empty Vector** Particles
- Vpr Particles
- Vpr Mut. Particles

**B**

**Vpr particles vs empty particles**

- **Depleted by Vpr**
- **Increased by Vpr**

- Log$_2$ (fold change) vs Empty Particles

- Log$_2$ (HIV-1 WT / ΔVpr)

- q<0.01 both datasets, decreased by Vpr
- q<0.01 both datasets, increased by Vpr

- Log$_2$ (q value)

**C**

- R$^2$=0.67
- P<0.001

**D**

- **DCAF-1 binding mutant**
  - WT
  - Q65R
  - S79A
  - E24R, R36P
  - W54R
  - Y47A, D52A, W54R

- **G2 arrest mutant**
  - S79A

- **Substrate binding mutants**
  - E24R, R36P
  - W54R
  - Y47A, D52A, W54R

- **G2/M arrest**
  - +
  - -
  - +/−
  - +
  - -

**E**

- **Log$_2$ (fold change)**
  - -1.5
  - -1
  - -0.5
  - 0
  - 0.5
  - 1
  - 1.5

- **Color Key**

- **WT .MCS**
- **Q65R**
- **S79A**
- **HL TF**
- **UNG DNA**
**Antibody binding may be affected by amino acid changes.**

**Gag-Pol peptide abundance**

**DCAF-1 binding mutant**

**G2 arrest mutant**

**Substrate binding mutants**

|   | WT | Q65R | S79A | E26R, R36P | W54R | Y47A, D52A, W54R |
|---|----|------|------|-------------|------|------------------|
| G2/M | 8.3% | 30%  | 9.4% | 10%         | 19%  | 12%              |
Figure 2 - figure supplement 3

A

- sh-Cntrl Vpr viral particles
- shDCAF1 Vpr Viral particles

Digest proteins and label peptides with TMT reporters

Mix peptides, fractionate and analyse by LC/MS

B

Sh-DCAF1 vs Sh Cntrl (Time = 0 h)

Log intensity

Log₂ (fold change)

C

- HLTF
- CCNB1

Protein abundance (normalised to time 0 h for each line)

D

- 6 h
- 24 h

sh-Cntrl
sh-DCAF1
A. Ly, 2015, Proteomics in cells arrested at G2/M using PLK1 inhibitor RO-3306

B. Log2 (HIV-1 WT / ΔVpr)

C. All Quantified

D. Fischer, 2016 - Transcriptional analysis

T-tests were performed on all datasets, and genes were considered significant if their q-value was less than 0.05. The graphs show the fold change in gene expression levels between the RO-3306 and Mock conditions. The x-axis represents the log2 fold change, and the y-axis represents the log2 q-value. The data points are color-coded to indicate whether the gene is significantly depleted (orange), increased (green), or not significantly changed (gray) in G2/M arrested cells. The r^2 value for the linear regression is 0.81, and the p-value is 0.01.
Figure 2 - figure supplement 5

(A) Log₂ (fold change) of MCM10 with various mutations.

(B) Flow cytometry histograms showing cell cycle distributions for Sh-Control, Sh-MCM10 I, and Sh-MCM10 II.

(C) Bar graph showing normalized MCM10 mRNA abundance.

- **r² = 0.87, p = 0.007**
- Fold increase cells in G2/M
- Log₂ (fold change)
- MCM10
- Normalized MCM10 mRNA abundance
- Sh-Control
- Sh-MCM10 I
- Sh-MCM10 II

Mutation points:
- Q65R
- S79A
- Y47A, D52A, W54R
- E24R, R36P
- W54R
- W

Cell cycle percentages:
- G2/M 10%
- G2/M 18%
- G2 18%
Figure 3

GO: Cellular compartment (Organelles)

A

Vpr depleted

Vpr increased

B

GO: Molecular Function

Genes with annotation (%)

Genes with annotation (%)
**Figure 4**

A. shDCAF-1 cells with MLN4924

Harvest and HA immunoprecipitate

- 24 h
- Digest proteins and analyse by LC/MS²

+ 3XHA-Vpr viral particles

B.

![EMPAI graph](image)

C.

![Signal Intensity graph](image)

D.

![Pie chart](image)

E.

![Log2 enrichment](image)
Cells grown for multiple generations in 'medium' or 'heavy' K/R media.

At t = 0 h switched to 'light' K/R media for remainder of experiment.

Harvest and combine samples

Digest proteins, fractionate and analyse by LC/MS²

Log₁₀(intensity) Log₂(heavy / medium) Vpr / empty particles

Log₁₀(intensity) Log₂(heavy / medium) Vpr / empty particles

Cells grown for multiple generations in 'medium' or 'heavy' K/R media.

At t = 0 h switched to 'light' K/R media for remainder of experiment.

Harvest and combine samples

Digest proteins, fractionate and analyse by LC/MS²

Log₁₀(intensity) Log₂(heavy / medium) Vpr / empty particles

Log₁₀(intensity) Log₂(heavy / medium) Vpr / empty particles

BBX HASPN CCDC137 HLTF ARGHAP11A CDCA2 GNL3L ZNF136 ECT2 ZNF512B KIF20A NUSAP1 DNAJB6 CDCA5 NEPRO CWC25 MUS81 CCDC59 ZGPAT NOL7 UTP14A CCNT1 ZNF512 DNTTIP2 PINX1
Table 1

| Accession | Gene      | Vpr necessary | Incoming Vpr sufficient | Degraded within 6 h | Co-IP |
|-----------|-----------|---------------|-------------------------|---------------------|-------|
| Q56N19    | ESCO2     | Yes           | Yes                     | ND                  | No    |
| Q16637    | SMN1 / SMN2 | Yes           | Yes                     | NS                  | Yes   |
| Q145Z7    | HLTf      | Yes           | Yes                     | Yes                 | -     |
| Q56K6     | ZNF512B   | Yes           | Yes                     | No                  | -     |
| Q8WY36    | BBX       | Yes           | Yes                     | Yes                 | -     |
| A6NF13    | ZNF316    | Yes           | Yes                     | Yes                 | -     |
| Q8NF77    | KIF16A    | Yes           | Yes                     | ND                  | No    |
| Q8TF76    | HAPSN     | Yes           | Yes                     | Yes                 | -     |
| Q9Y486    | VPRBIP    | Yes           | Yes                     | NS                  | Yes   |
| Q96FF9    | CCDA5     | Yes           | Yes                     | Yes                 | -     |
| Q6PK4     | CCDC137   | Yes           | Yes                     | Yes                 | -     |
| Q9NXE8    | CWC25     | Yes           | Yes                     | Yes                 | -     |
| Q8YO91    | KIF18B    | Yes           | Yes                     | Yes                 | -     |
| Q5PF7     | ARHGP11A  | Yes           | Yes                     | Yes                 | -     |
| Q6NW34    | NEPRQ     | Yes           | Yes                     | Yes                 | -     |
| Q9BV6     | UTP14A    | Yes           | Yes                     | Yes                 | -     |
| Q9YN8     | GNL3L     | Yes           | Yes                     | Yes                 | -     |
| Q5QJE6    | DNTTIP2   | Yes           | Yes                     | Yes                 | -     |
| Q69YH5    | CCDA2     | Yes           | Yes                     | Yes                 | -     |

ND: Not detected in this experiment
NS: Degraded with Sig.B >0.01

| Accession | Gene     | Vpr necessary | Incoming Vpr sufficient | Degraded within 6 h | Co-IP |
|-----------|----------|---------------|-------------------------|---------------------|-------|
| Q96BK5    | PINX1    | Yes           | Yes                     | Yes                 | -     |
| Q8NS5A5   | ZGPAT    | Yes           | Yes                     | Yes                 | -     |
| Q13823    | GNL2     | Yes           | Yes                     | Yes                 | -     |
| Q9UH6H    | DDX20    | Yes           | Yes                     | Yes                 | NS    |
| Q96ME7    | ZNF512   | Yes           | Yes                     | Yes                 | -     |
| Q145Z8    | ZNF267   | Yes           | Yes                     | Yes                 | -     |
| Q9HI6     | ECT2     | Yes           | Yes                     | Yes                 | -     |
| P5788     | GEMIN4   | Yes           | Yes                     | Yes                 | NS    |
| P52701    | MSH6     | Yes           | Yes                     | Yes                 | NS    |
| Q75190    | DNAJ6    | Yes           | Yes                     | Yes                 | -     |
| Q9NY9     | MUS81    | Yes           | Yes                     | Yes                 | -     |
| Q65653    | CCNT1    | Yes           | Yes                     | Yes                 | -     |
| Q9PO31    | CCDC59   | Yes           | Yes                     | Yes                 | -     |
| Q9US9     | MBD1     | Yes           | Yes                     | NS                  | Yes   |
| Q69YH5    | CDCA2    | Yes           | Yes                     | Yes                 | -     |
| Q9BV6     | ZNF512   | Yes           | Yes                     | Yes                 | -     |
| Q9X86     | NUSAP1   | Yes           | Yes                     | Yes                 | -     |
| Q95235    | KIF20A   | Yes           | Yes                     | Yes                 | -     |
| P35Z51    | RFC1     | Yes           | Yes                     | Yes                 | NS    |

ND: Not detected in this experiment
NS: Degraded with Sig.B >0.01
| Accession | Gene | Vpr necessary | Incoming Vpr sufficient | Degraded within 6 h | Co-IP | In direct target list (Fig. 3d)? |
|-----------|------|---------------|-------------------------|---------------------|-------|---------------------------------|
| Q14527    | HLTF | Yes           | Yes                     | Yes                 | -     | Yes                             |
| Q7L590    | MCM10| Yes           | Yes                     | ND                  | -     | No - Not detected in 6 hour pulsed SILAC or IP |
| Q8N5A5    | ZGPAT| Yes           | Yes                     | Yes                 | -     | Yes                             |
| Q96AY2    | EME1 | Yes           | Yes                     | NS                  | -     | No - depleted but with a Sig.B value of >0.01 in the pulsed SILAC experiment, not detected in IP |
| Q96NY9    | MUS81| Yes           | Yes                     | Yes                 | -     | Yes                             |
| Q6N021    | TET2 | Yes           | ND                      | ND                  | -     | No - Not detected in incoming Vpr experiment (Fig. 2a) |
| P13051    | UNG  | Yes           | Yes                     | NS                  | -     | No - depleted but with a Sig.B value of >0.01 in the pulsed SILAC experiment. Detected in the Co-IP but with a single peptide |
Figure 6

A

B

C

7-AAD

sh-CDCA2 I  sh-CDCA2 II  sh-ZNF267 I  sh-ZNF267 II

G2/M  18%  G2/M  17%  G2/M  16%  G2/M  15%

sh-CCNT1  sh-ZNF267  sh-PINX1  sh-MBD1  sh-CWC25  sh-ZNF574  sh-SMN1  sh-CDCA2  sh-DDX20  sh-ZNF316  sh-MSH6  sh-RFC1  sh-CDCA5

Normalised mRNA abundance

SMN1  CDCA2  ZNF267

G2/M  7%  G2/M  30%  G2/M  19%

G2/M  16%  G2/M  15%
Digest proteins and label peptides with TMT reporters
Mix peptides, fractionate and analyse by LC/MS

Figure 7

A

|                      | Empty Vector | HIV-1/SIVcpz Vpr 48 h | HIV-2/SIV Vpr 48 h | HIV-2 Vpx 48 h |
|----------------------|--------------|-----------------------|--------------------|---------------|

B

- Log₁₀(intensity)
- Log₂(fold change) vs empty vector

C

Lines showing profound depletion

D

I.B. Empty Vector Clade C SIVcpz Ptt SIVrcm SIVagm SIVsmm HIV-2 HIV-2 Vpx

- HLTF
- UNG2
- SMN1
- ZNF512B
- KIF18A
- ESCO2
- VCP
- ACTB
Figure 7 - Supplement 1

A

% Transduced (GFP+)

Empty Vector
Empty Vector
NL4-3
Clade C
Group O
SIVrcm
SIVagm
SIVsmm
HIV-2
HIV-2 Vpx

B

% cells G2/M

Empty Vector
Empty Vector
NL4-3
Clade C
Group O
SIVrcm
SIVagm
SIVsmm
HIV-2
HIV-2 Vpx

CX

Log

2

Intens

Log

2

(fold change) vs empty vector

NL4-3
Clade C
Group O
SIVrcm
SIVagm
SIVsmm
HIV-2
HIV-2 Vpx

VPRBP
ESCO2
ZNF512B
ARGHAP11A
NEPRO
KIF18A

VPRBP
ESCO2
ZNF512B
ARGHAP11A
NEPRO
KIF18A

VPRBP
ESCO2
ZNF512B
ARGHAP11A
NEPRO
KIF18A

VPRBP
ESCO2
ZNF512B
ARGHAP11A
NEPRO
KIF18A

VPRBP
ESCO2
ZNF512B
ARGHAP11A
NEPRO
KIF18A

VPRBP
ESCO2
ZNF512B
ARGHAP11A
NEPRO
KIF18A

VPRBP
ESCO2
ZNF512B
ARGHAP11A
NEPRO
KIF18A

VPRBP
ESCO2
ZNF512B
ARGHAP11A
NEPRO
KIF18A

% Transduced (GFP+)

% cells G2/M

% Transduced (GFP+)

% cells G2/M

Log

2

(fold change) vs empty vector

NL4-3
Clade C
Group O
SIVrcm
SIVagm
SIVsmm
HIV-2
HIV-2 Vpx

VPRBP
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ZNF512B
ARGHAP11A
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(fold change) vs empty vector

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Figure 8

A

HIV-2<sup>ViPR</sup> Vpx

SIVagm Vpr

NL4-3 Vpr

No Vpx

Vpx does not degrade SAMHD1

Vpx+ Vpx degrades SAMHD1

B

Log<sub>2</sub>(fold change) vs empty vector

TASOR

MPHOSPH8

PPHLN1

HIV-2 ROD Vpx SIVagm Vpr NL4-3 Vpr

C

D

Log<sub>10</sub>(intensity)

TASOR

VCP

Empty Vector

SIVagm Vpr

SIVmus Vpr

HIV-2 Vpx

SIVrcm Vpx

SIVmnd-2 Vpx

Mock

% GFP+

Control

p=0.02

p<0.001

50

40

30

20

10

0

I.B.
Figure 9

A. Digest proteins and label peptides with TMT reporters.

B. Log$_2$ (fold change) of Vpr/Vpx from primary HIV-2 isolate 7312a.

- Log$_2$ (q value)
- Log$_2$ (fold change)
## Table 2

| Vpr Species       | Genbank Accession | Data presented in:                                      |
|-------------------|-------------------|--------------------------------------------------------|
| HIV-1 NL4-3       | AF324493.2[78]    | Figures 1-9.                                          |
| HIV-1 98BR004 (Clade C) | AAK31002.1[79] | Proteomics experiment & IB (Figure 9A-D)              |
| HIV-1 BCF09 (Group O) | CAA75954.1     | Proteomics experiment (Figure 9A-C)                   |
| SIVcpzPtt MB897   | ABU53019.1[80]    | Proteomics experiment & IB (Figure 9A-D)              |
| SIVrcm 02CM8081   | ADK78264.1[81]    | Proteomics experiment & IB (Figure 9A-D)              |
| SIVagm S2032018   | ADO34202.1[82]    | Proteomics experiment & IB (Figure 9A-D), I.B, (Figure 10D) |
| SIVsmm E660       | ANT86736.1[83]    | Proteomics experiment & IB (Figure 9A-D)              |
| HIV-2 ROD         | CAA28911.1[84]    | Proteomics experiment (Figure 9A-C)                   |
| HIV-2 7312a       | AAL31354.1        | I.B. (Figure 9D), Proteomics experiment, (Figure 11A-C) |
| SIVmus 01CM1239   | ABO61047.1[85]    | I.B. (Figure 9D), I.B. (Figure 10D)                  |

| Vpx Species       | Genbank Accession | Data presented in:                                      |
|-------------------|-------------------|--------------------------------------------------------|
| HIV-2 ROD         | AAB00766.1[84]    | Proteomics experiment (Figure 9A-C)                   |
| HIV-2 7312a       | AAL31353.1        | I.B. (Fig.9D), I.B. (Fig. 10D), Proteomics experiment, (Figure 11A-C) |
| SIVrcm Ng411      | AAK69676.1[86]    | I.B. (Fig. 10D)                                       |
| SIVmnd-2 5440     | AAO22477.1 [87]   | I.B. (Fig. 10D)                                       |