Cell Host & Microbe
Resource

Quantitative Phosphoproteomics Reveals Extensive Cellular Reprogramming during HIV-1 Entry

Jason A. Wojcechowskyj,[1] Chuka A. Didigu,[1] Jessica Y. Lee,[2] Nicholas F. Parrish,[3,4] Rohini Sinha,[3] Beatrice H. Hahn,[3,4] Frederic D. Bushman,[4] Shane T. Jensen,[5] Steven H. Seeholzer,[2] and Robert W. Doms[1,*]
[1]Department of Pathology and Laboratory Medicine
[2]Protein and Proteomics Core
Children's Hospital of Philadelphia Research Institute, Philadelphia, PA 19104, USA
[3]Department of Medicine, Perelman School of Medicine
[4]Department of Microbiology, Perelman School of Medicine
[5]Department of Statistics, The Wharton School
University of Pennsylvania, Philadelphia, PA 19104, USA
*Correspondence: domsr@email.chop.edu
http://dx.doi.org/10.1016/j.chom.2013.04.011

SUMMARY

Receptor engagement by HIV-1 during host cell entry activates signaling pathways that can reprogram the cell for optimal viral replication. To obtain a global view of the signaling events induced during HIV-1 entry, we conducted a quantitative phosphoproteomics screen of primary human CD4$^+$ T cells after infection with an HIV-1 strain that engages the receptors CD4 and CXCR4. We quantified 1,757 phosphorylation sites with high stringency. The abundance of 239 phosphorylation sites from 175 genes, including several proteins in pathways known to be impacted by HIV-receptor binding, changed significantly within a minute after HIV-1 exposure. Several previously uncharacterized HIV-1 host factors were also identified and confirmed through RNAi depletion studies. Surprisingly, five serine/arginine-rich (SR) proteins involved in messenger RNA splicing, including the splicing factor SRm300 (*SRRM2*), were differentially phosophorylated. Mechanistic studies with *SRRM2* suggest that HIV-1 modulates host cell alternative splicing machinery during entry in order to facilitate virus replication and release.

INTRODUCTION

Viruses rely not only on host molecules to successfully infect and replicate in new cells, but often directly manipulate cellular processes to facilitate more efficient virus replication. Vaccinia virus (Mercer and Helenius, 2008), herpes viruses (Soroceanu et al., 2008), and enteroviruses (Coyne and Bergelson, 2006) induce signaling cascades resulting from viral engagement of cell-surface receptors that can induce the internalization of viral particles and facilitate viral replication. Many studies have contributed to the growing list of cellular host factors that are required for viral replication; however, relatively little is known of the full extent to which viruses can facilitate their entry and replication by manipulating host signal-transduction pathways.

HIV-1 primarily infects CD4$^+$ T cells in vivo. The gp120 subunit of the viral envelope protein first engages CD4, then binds to one of two chemokine receptors, CCR5 or CXCR4 (Wilen et al., 2012). These interactions enable the viral envelope protein to undergo the conformational changes needed to elicit fusion between the viral and host cell membranes, and also result in signal transduction via CD4 and the CCR5 or CXCR4 coreceptors that can enhance T cell activation and virus replication (Briant et al., 1996; Davis et al., 1997). More specifically, HIV-regulated phosphorylation of cofilin (Yoder et al., 2008), moesin (Barrero-Villar et al., 2009), filamin (Jiménez-Baranda et al., 2007), and LARG (Hodges et al., 2007) during entry can modulate various early steps of viral replication. Whether these or other virus-induced signaling events enhance later steps of the HIV-1 life cycle is not well understood.

Given the growing list of virus-induced cellular signaling events that can influence entry and replication, we hypothesized that uncovering signaling events resulting from HIV-host cell interactions would reveal additional HIV-1 host factors. Using mass spectrometry (MS)-based phosphoproteomics in combination with stable isotope labeling by amino acids in cell culture (SILAC), we quantified 1,757 phosphorylation sites with high stringency in primary human CD4$^+$ T cells during entry of an HIV-1 strain that engages CD4 and CXCR4. We found that 239 phosphorylation sites from 175 genes significantly changed in abundance within 1 min of exposure to HIV-1, including several proteins in signaling pathways that are known to be impacted by HIV-receptor binding. Unexpectedly, we found that five serine/arginine-rich (SR) proteins involved in messenger RNA (mRNA) splicing were differentially phosophorylated in T cells upon HIV-receptor engagement. These included nine phosphorylation sites in the splicing factor SRm300 (*SRRM2*), a cellular protein without previously described functional links to HIV-1 replication. We found that suppression of *SRRM2* with RNAi in Jurkat and MAGI cell lines enhanced HIV-1 gene expression only when virions containing Env proteins that engage CXCR4 were used. In addition, *SRRM2* modulated alternative splicing of HIV-1 transcripts and was required for efficient virion release. These findings show that HIV-1 induced signaling events are far more extensive than previously suspected and that by inducing signaling pathways, HIV-1 can reprogram cells to not only enhance entry, but

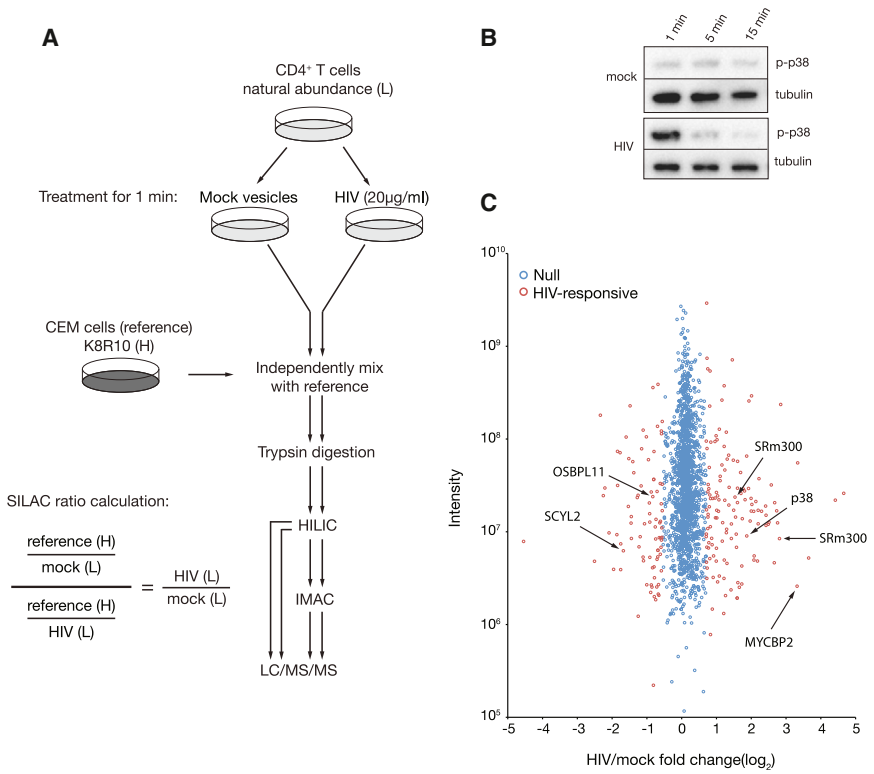

Figure 1. Phosphoproteomics Workflow

(A) Phosphoproteomics workflow for T cell stimulations, processing, and fractionation. Virus- and mock-treated samples were independently processed following mixing with a common reference lysate. HIV and mock phosphorylation site fold changes were calculated by dividing the ratio of reference/mock by reference/HIV phosphorylation site fold changes. The abbreviations used are as follows: light, L; heavy, H; hydrophilicity interaction liquid chromatography, HILIC; immobilized metal affinity chromatography, IMAC; and liquid chromatography tandem mass spectrometer, LC/MS/MS.

(B) Primary CD4$^+$ T cells were incubated with purified HIV-1 or a mock vesicle preparation for various lengths of time at 37°C and probed for phosphorylated p38 (pT180/pY182) by immunoblot for determining the optimal kinetics for large-scale phosphoproteomics stimulations.

(C) Distribution of phosphorylation site fold changes. HIV and mock phosphorylation site fold changes are shown according to the mixture-model designation of HIV responsive (red) or HIV nonresponsive, i.e., null (blue). For clarity, only a subset of SRm300-responsive phosphorylation sites is indicated.

See also Figure S1.

to make later stages of the virus-replication cycle, such as splicing, more efficient as well.

RESULTS

SILAC-Based Quantitative Phosphoproteomics in Nondividing Primary CD4$^+$ T Cells

To identify signaling events induced soon after HIV-1 engagement of cell-surface receptors, we titrated highly purified HIV-1 MN—a virus strain that utilizes CD4 and CXCR4—on unstimulated primary human CD4$^+$ T cells. A mock virus preparation, produced from the same cells and in the same manner as the virus, was used as a negative control. We reasoned that the relatively quiescent state of these cells makes it possible to monitor virus-induced signaling events that might otherwise be masked following the potent in vitro stimulation regimes that are commonly used to maximize virus infection. To synchronize virus binding, we added a high concentration of virus at 37°C in order to obtain a sufficient number of binding events within a very short time frame. Using cells from multiple donors, we found that the addition of 20 μg/ml of viral p24 (~15 nM gp120) to unstimulated CD4$^+$ T cells for 1 min yielded optimal phosphorylation of mitogen-activated protein kinase (MAPK) p38, a known HIV-responsive phosphorylation site (data not shown and Figure 1B) (Furler and Uittenbogaart, 2010). We used these conditions to study early signaling events induced by HIV-1 upon binding to the cell surface.

To quantify relative fold changes of phosphorylation sites with MS, we used SILAC. Given that unstimulated T cells do not have sufficient metabolic activity for complete isotope labeling, we labeled the human CD4$^+$ T cell line CEM with media containing ^{13}C and ^{15}N arginine and lysine so as to construct a "heavy" isotope reference proteome (Geiger et al., 2011). Lysates from mock or virus-treated T cells were then independently mixed with this common reference lysate, making it possible to measure the fold change in abundance of any phosphopeptide that was identified in the mock, virus-treated, and reference proteomes (Figure 1A). Importantly, the distribution of SILAC ratios for each reference mixture was unimodal, and over 90% of ratios were 7.2-fold or less, suggesting that the reference phosphoproteome sufficiently represented the primary cell phosphoproteome (Figure S1 available online). Following several filtering steps, 1,757 phosphorylation sites (from 799 genes) were quantified with high stringency in the mock-reference and HIV-reference samples (Figure S1; Tables S1–S3). At a 1% false discovery rate threshold, a total of 239 phosphorylation sites (from 175 genes) were deemed HIV responsive, with 144 increasing and 95 decreasing in abundance upon HIV-1 treatment (Figure 1C; Tables S3 and S4).

HIV-Induced Phosphorylation of the Transcription Factor ETS

We took several approaches to validate the SILAC phosphorylation site ratios, with the most direct being the use of phospho-specific antibodies. Unfortunately, only two antibodies for the identified HIV-responsive phosphorylation sites, p38 (pT180/pY182) and ETS-1 (pS282/pS285), worked sufficiently well by immunoblot to make this analysis possible. We used each to assess changes in their respective phoshosites via immunoblot following the addition of purified HIV-1 to CD4$^+$ T cells. Enhanced p38 phophorylation (pT180/pY182) upon the addition of HIV-1 for 1 min was confirmed (Figure 2A), as was HIV-induced

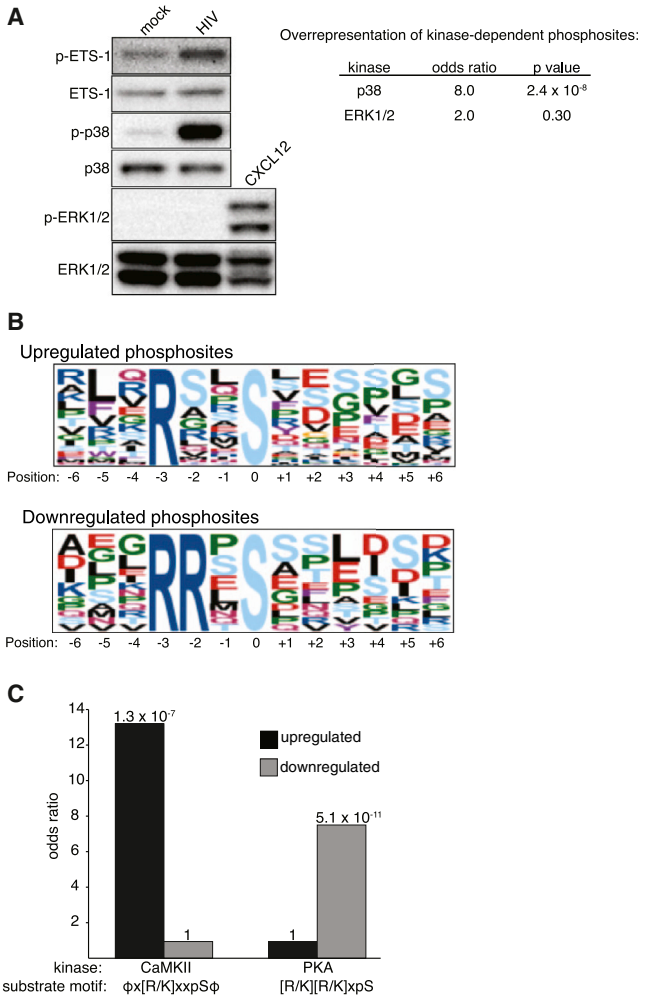

Figure 2. Immunoblot and Bioinformatic Validation of HIV-Responsive Phosphorylation Site Ratios

(A) Primary CD4$^+$ T cells were incubated with purified HIV-1 or a mock vesicle preparation and probed for phosphorylated p38 (pT180/pY182), ETS-1 (pS282/pS285), and ERK1/2 (pT202/pY204) by immunoblot. The chemokine CXCL12 (SDF-1) served as a positive control for pERK1/2. OR and p values were calculated with Fisher's exact text with Benjamini and Hochberg corrections.

(B) Putative kinase substrate motifs were searched for within up- or downregulated HIV-responsive phosphorylation sites. The amino-acid-position-weighted matrix, centered on phosphorylated serine (position 0), is shown for each top-scoring motif.

(C) Overrepresentation of the CaMKII and PKA motifs within HIV up- and downregulated phosphorylation sites was calculated with the Fisher's exact test; each p value is indicated above each bar. ϕ, L/V/I/M/Y/F; pS, phosphoserine.

See also Table S4.

phosphorylation of ETS-1 (pS282/pS285) (Figure 2A). We also confirmed that the kinase ERK1/2 was not phosphorylated in an HIV-dependent manner, though it was responsive to CXCL12.

Phosphoproteome-wide Validation of HIV-Induced MAPK Activity

To further validate the HIV-responsive phosphorylation site ratios, we took advantage of the fact that differential phosphorylation is in part regulated by changes in the catalytic activity of upstream kinases. Because we observed robust phosphorylation of p38 (pT180/pY182), but not of ERK1/2 (pT202/pY204), upon addition of HIV-1 to CD4$^+$ T cells (Figure 2A), we hypothesized that HIV-responsive phosphorylation sites should contain an overrepresentation of p38-dependent, but not ERK1/2-dependent, phosphorylation sites. To examine this, we took advantage of p38 and ERK1/2-responsive phosphorylation sites as defined by Pan et al. (2009). We then measured the overrepresentation of these p38 or ERK1/2-dependent phosphorylation sites within HIV-responsive phosphorylation sites with the Fisher's exact test. Only p38-dependent phosphorylation sites were significantly overrepresented among HIV-responsive phosphorylation sites (odds ratio [OR]: 6.7, $p = 2.0 \times 10^{-6}$ versus OR: 1.5, $p = 0.52$) (Figure 2A; Table S4), supporting the fact that, in both immunoblot and SILAC analysis, the addition of HIV-1 to CD4$^+$ T cells leads to rapid phosphorylation of p38 phosphorylation sites that are known to enhance its kinase activity.

Identification of Overrepresented Kinase Substrate Motifs

To determine whether HIV-1 activated additional kinases, we took advantage of the fact that the phosphorylation of many kinase substrates is highly influenced by the amino acids that immediately surround the phosphorylation site. Therefore, we surveyed the amino acid residues surrounding each HIV-responsive phosphorylation site using the Group-based Prediction System (GPS) (Xue et al., 2011) and motif extractor (motif-x) tool (Schwartz and Gygi, 2005). The GPS uses a hierarchical algorithm to rank the likelihood that a particular kinase or kinase family phosphorylates a given phosphorylation site, whereas motif-x measures the overrepresentation of amino acid sequence patterns, providing an unbiased list of potential kinase substrate motifs. With the GPS tool, substrates for MAPKAPK were significantly overrepresented according to the Fisher's exact test (OR: 7.3, $p = 6.1 \times 10^{-6}$) (Table S4). Members of the MAPKAPK family are known substrates for p38 (Cargnello and Roux, 2011), which is rapidly activated upon HIV-1 binding to CD4$^+$ T cells (Figure 1B).

The motif-x tool revealed that the [R/K]xxS motif, which displayed a bias toward hydrophobic amino acids at the P − 5 and P + 1 positions (ϕx[R/K]xxSϕ), was significantly overrepresented among upregulated HIV-responsive phosphorylation sites (OR: 13.2, $p = 1.1 \times 10^{-7}$) (Figures 2B and 2C; Table S4). This motif is one of many that are targets for calmodulin-dependent kinase II (CaMKII) (Schwartz and Gygi, 2005). CaMKII activity is regulated by fluxes in free Ca^{2+} levels, and HIV-1 gp120 induces a transient Ca^{2+} flux in primary CD4$^+$ T cells (Weissman et al., 1997). These data, combined with the fact that the HIV-responsive phosphorylation site ETS-1 (pS282/pS285) is phosphorylated by CaMKII (Fisher et al., 1994), support an increase in CaMKII activity during HIV-1 entry. For the downregulated HIV-responsive phosphorylation site motifs, RRxS matches the consensus motif for protein kinase A (PKA), [R/K][R/K]xS (OR: 7.5, $p = 5.1 \times 10^{-11}$) (Figures 2B and 2C; Table S4). PKA kinase activity is regulated by intracellular levels of the second messenger, cyclic AMP (cAMP) (Mosenden and Taskén, 2011). The observed decrease in phosphorylated PKA substrates is supported by the observation that gp120 treatment of primary

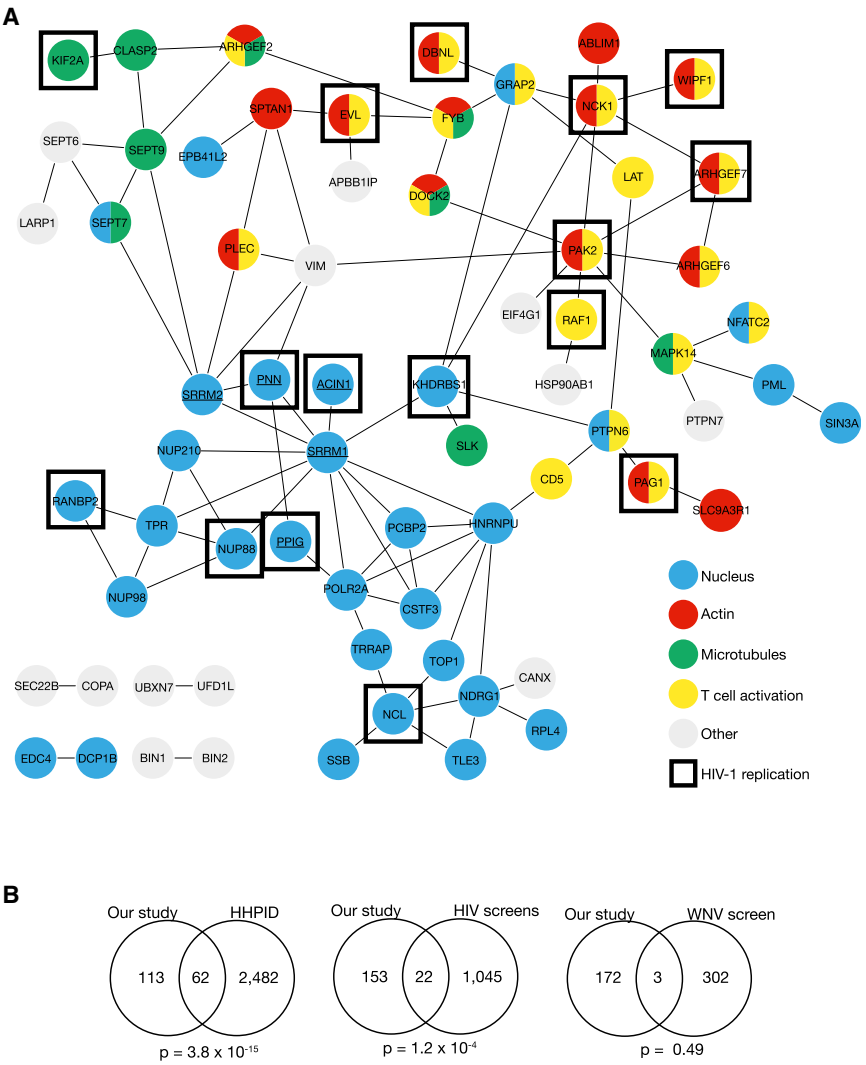

Figure 3. Bioinformatic Analysis of Genes from HIV-Responsive Phosphorylation Sites

(A) Interaction network of HIV-responsive phosphoproteins. Functional or physical interactions (see Experimental Procedures) between genes from HIV-responsive phosphosites were extracted from the STRING database (version 9.0). Underlined genes indicate SR proteins. Genes indicated as "HIV-1 replication" were taken from Table 1.

(B) HIV-1 host factors are overrepresented within HIV-responsive phosphoproteins. Overlaps of genes from HIV-responsive phosphoproteins and genes from the HHPID, HIV-1 RNAi screens, and West Nile virus (WNV) siRNA screen were calculated with the hypergeometric test using the human genome as a background (n = 20,402). See also Table S6.

CD4$^+$ T cells decreases cAMP levels within minutes and that cAMP is a negative regulator of HIV-1 replication in primary cells (Navarro et al., 1998).

Cellular Pathways

To gauge which cellular pathways HIV-1 may activate during entry, we tested whether HIV-responsive phosphoproteins contained an overrepresentation of gene ontology or cellular pathway terms from manually curated databases. The most notable ontologies and pathways involved various aspects of GTPase biology, actin, and T cell activation (Table S5). Strengthening the association with T cell activation, upregulated T cell receptor-responsive phosphorylation sites from both Jurkat (Mayya et al., 2009) (OR: 19.2, p = 0.039) and primary CD4$^+$ T cells (Ruperez et al., 2012) (OR: 4.3, p = 8.7 × 10^{-5}) were overrepresented among HIV-responsive phosphorylation sites (Table S4). We also generated an interaction network using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (Figure 3A) and found a striking segregation between cytoplasmic- and nuclear-localized genes and discreet functional modules of genes that regulate microtubules, actin, and T cell activation—all processes that have clear links to HIV-1 biology (Figure 3A) (Liu et al., 2009; Stevenson et al., 1990). The fact that the majority of genes that regulate T cell activation also regulate actin further strengthens the association between these processes and HIV-induced signaling (Liu et al., 2009).

Because our hypothesis is that HIV-responsive phosphoproteins are important for HIV-1 infection and replication, we measured whether there was an overrepresentation of previously defined HIV-1 host factors within our data set. The HIV-1 human protein interaction database (HHPID) is a manually curated list of both physical and functional interactions between HIV-1 and human proteins (Fu et al., 2009). The overlap with the HHPID data set was highly significant (hypergeometric test, p = 3.8 × 10^{-15}) (Figure 3B; Table S6). In addition, the overlap with genes from the four published HIV-1 RNAi screens (Brass et al., 2008; König et al., 2008; Krishnan et al., 2008; Yeung et al., 2009) was also significant (hypergeometric test, p = 1.3 × 10^{-4}), but the overlap with a small interfering RNA (siRNA) screen conducted with West Nile virus was not significant (Krishnan et al., 2008) (p = 0.49) (Figure 3B; Table S6). These overlaps, combined with literature mining, resulted in a total of 84/175 genes from HIV-responsive phosphorylation sites having been previously described as HIV-1 host factors (Table S6), supporting our hypothesis that HIV-responsive phosphoproteins are involved in HIV-1 infection.

Role of HIV-Responsive Phosphoproteins during HIV-1 Infection

To examine whether HIV-responsive phosphoproteins regulate HIV-1 infection, we depleted 69 of the 175 genes from HIV-responsive phosphosites using siRNAs (four per gene) in MAGI cells and measured the infection levels of three full-length

Table 1. HIV-Responsive Phosphoproteins that Impact HIV-1 Replication in MAGI Cells

Gene Symbol	Cellular Pathway	Previously Linked to HIV?[a]
ACIN1	apoptosis	yes
AHNAK	T cell activation	
ANXA2	actin	yes (+)
ARHGEF18	actin	
ARHGEF7	actin	yes
BUD13	splicing	
CRTC3	cyclic AMP	yes (+)
DBN1	actin	yes (+)
DBNL	actin	
DIDO1	apoptosis	
DNM1L	apoptosis, T cell activation	yes
DOCK11	actin	
EVL	actin, T cell activation	yes
FKBP15	actin	
GORASP2	vesicle trafficking	yes (+)
KHDRBS1	splicing, NF-κB	yes (+)
KIAA1967	chromatin	yes
KIF2A	microtubules	
LSM14A	splicing	
MAP4	microtubules	yes (+)
MTDH	NF-κB	yes
NCK1	actin, T cell activation	yes
NCL	ribosome synthesis	yes (+)
NUP88	nuclear transport	yes
PAG1	T cell activation	
PAK2	actin	yes
PNN	splicing	yes
PPIG	transcription	
RAF1	NF-κB	yes (+)
RANBP2	HIV integration site selection	yes (+)
RAPGEF6	NF-κB	yes (+)
REPS1	vesicle trafficking	
RNF31	transcription	
SH3BP1	actin	
TBC1D10B	vesicle trafficking	
TBC1D5	vesicle trafficking	
WIPF1	actin	

A subset (n = 69) of HIV-responsive phosphoproteins was depleted in MAGI cells, then infected with replication-competent HIV-1. Genes are shown whose depletion led to an at least 2-fold decrease in infection with three out of four siRNAs tested.
[a](+) indicates reported functional link between HIV-1 replication and gene. See also Table S8.

infectious molecular clones (IMCs): NL4.3, 89.6, and SF162 (Tables S7 and S8). We deemed a gene important for HIV-1 infection if at least three out of four siRNAs decreased MAGI cell β-galactosidase reporter activity by 2-fold (compared to a nontargeting siRNA) in at least one IMC; 37 genes satisfied this criteria (Table 1). Although roughly half of these genes have been previously linked to HIV-1 biology in some way, 27 have not yet been functionally linked to HIV-1 to our knowledge (Tables 1 and S8). Cellular pathways such as actin, microtubules, T cell activation, and various nuclear processes such as splicing were heavily represented, further linking known aspects of HIV-1 signaling and our phosphoproteomics data set (Figure 3A; Table 1).

To address whether HIV-responsive phosphoproteins are important for HIV-1 infection, we selected four HIV-responsive phosphoproteins, *MYCPB2*, *OSBPL11*, *SCYL2*, and *SRRM2*, on the basis of an identical phosphorylation site that was both HIV and CXCL12 reponsive (Wojcechowskyj et al., 2011). CXCL12 is the chemokine ligand for CXCR4, which is the receptor used by the HIV-1 strain in our study. We depleted each protein with four individual siRNAs per gene in MAGI cells (Figure 4B) and with pools of siRNAs in Jurkat cells (Figure 4C). We then infected depleted cells with a single-cycle HIV-1 reporter virus bearing either an X4 - or R5-tropic Env or the glycoprotein of vesicular stomatitis virus G protein (VSV-G). As expected, treatment with CD4-specific siRNAs inhibited infection by HIV-1, but not VSV; the siRNAs to CXCR4 only inhibited infection by the X4 virus strain; and the siRNAs to *IGSF3* (an irrelevant gene for HIV-1 infection) had no effect on any virus (Figure 4A). Among the experimental samples, only suppression of *SRRM2* consistently enhanced HIV-1 reporter gene expression in both MAGI and Jurkat cells (Figures 4A and 4D). We also measured the fraction of virus-infected MAGI cells via intracellular p24 staining of NL4.3 and 89.6 IMC or GFP expression and found that suppression of *SRRM2* had no consistent effect on the permissivity of cells to HIV-1 infection (Figure 4E). Therefore, we conclude that suppression of *SRRM2* results in higher levels of HIV-1 gene expression per infected cell; thus, we focused on the possible role of *SRRM2* in HIV-1 infection in subsequent experiments.

Regulation of HIV-1 Alternative Splicing and Infection by *SRRM2*

SRRM2 encodes SRm300, an SR protein that contains two characteristic arginine-serine (RS) domains (Figure S2). SR proteins are central regulators of cellular splicing whose activity can be modulated by phosphorylation of RS domains (Long and Caceres, 2009). Of the 37 unique phosphorylation site ratios in SRm300 that we were able to quantify, nine were HIV responsive (Figure S2; Table S3). During HIV-1 infection, dozens of fully and partially spliced viral mRNAs are generated through a complex interplay between viral and cellular elements, of which many of the latter are cellular SR proteins (Stoltzfus, 2009). To address whether *SRRM2* regulates HIV-1 alternative splicing, we specifically amplified fully spliced transcripts from mRNA isolated from *SRRM2*-depleted MAGI cells that were infected with LAI or VSV-G pseudovirions or with the primary IMC, 89.6 (Figure 5A). A representative gel is shown for 89.6, with the corresponding exon composition of each transcript. For all viruses, depletion of *SRRM2* led to an approximately 2-fold increase in the ratio of tat-2/tat-1 and nef-3/nef-2 transcripts (Figure 5B). The ratio of tat-3/tat-1 and nef-4/nef-2 also increased with 89.6, yet we were not able to clearly resolve tat-3 and nef-4 with the pseudovirions. We conclude that *SRRM2* regulates the inclusion of exons 2 and 3, perhaps by modulating the strength of the splice acceptors A1 and A2. Unexpectedly, LAI pseudovirions had an average of 1.8 times less nef-3 relative to nef-2 than VSV-G

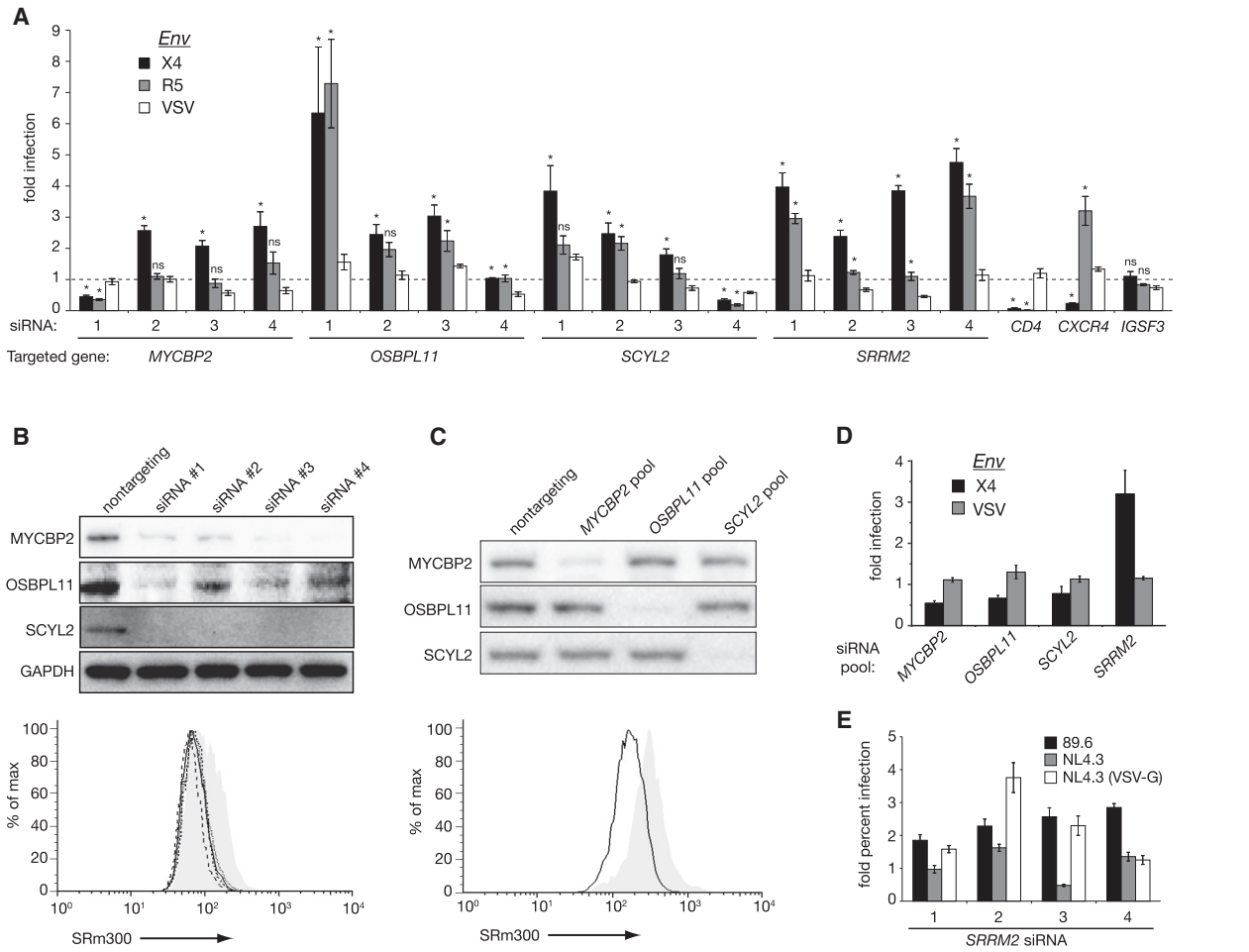

Figure 4. RNAi-Mediated Knockdown of HIV-Responsive Phosphoproteins

(A) MAGI cells were transfected with individual siRNAs, then infected with NL4.3 pseudovirions with an X4-tropic Env, R5-tropic Env, or VSV-G. Infection values (luciferase activity) were normalized to a nontargeting control siRNA.

(B) Lysates from depleted MAGI cells were analyzed by immunoblot (top panel). SRm300 protein levels were monitored by FACS (bottom panel): gray area, nontargeting control; solid line, siRNA#1; dotted line, siRNA#2; short dashed line, siRNA#3; long dashed line, siRNA#4.

(C) Jurkat cells were nucleofected with indicated pools of siRNA and analyzed by immunoblot (top panel). SRm300 protein levels were monitored by FACS (bottom panel): gray area, nontargeting control; solid line, SRm300 pool.

(D) Nucleofected Jurkat cells were infected as in (A). Bars represent SEM; n = 3.

(E) Relative percent infection of SRm300-depleted MAGI cells with replication-competent 89.6, NL4.3, and GFP-expressing VSV-G pseudovirions. Bars represent SEM; n = 4; *p < 0.05 Mann-Whitney; ns, not significant (p > 0.05).

pseudovirions did, regardless of SRm300 levels (Figure 5B), suggesting that signals induced by HIV-1 Env may themselves influence alternative splicing.

The balance of HIV-1 splicing products is finely tuned and highly dependent on an overlapping, interconnected network of diverse of *cis* and *trans* factors, which in turn can affect HIV-1 replication (Stoltzfus, 2009). Consistent with reports that increased splicing at A1 and A2 leads to decreased virus production (Jacquenet et al., 2005), we observed up to a 6- and 30-fold reduction in p24 release upon infection with 89.6 and NL4.3, respectively, in three out of four siRNAs targeting *SRRM2* (Figure 5C). We also tested four additional siRNAs against *SRRM2* and observed the same increase in tat and nef alternatively-spliced transcripts and the same impact on virion release (Figure S4). When the fold-change magnitudes of tat and nef transcripts were correlated with p24 release for all siRNAs tested (n = 8), the correlation with nef-4 was statistically significant (p = 0.022, Spearman's correlation coefficient) (Table S9), suggesting that *SRRM2*-dependent dysregulation of nef-4 transcript abundance is most tightly linked to productive HIV-1 infection.

Regulation of HIV-1 Alternative Splicing and Infection by HIV-Responsive SR Proteins

Five other SR proteins, *PNN*, *PPIG*, *TRA2A*, *ACIN1*, and *SRRM1*, were also HIV-responsive phosphoproteins, leading us to hypothesize that they too are important for HIV-1 splicing. We depleted each with pools of siRNAs in MAGI cells and measured fully spliced HIV-1 transcripts (Figure 6A). Depletion of all SR proteins except *PPIG* led to a unique HIV-1 splicing pattern. Depletion of *PNN* led to increases in all exon 2- and 3-containing tat

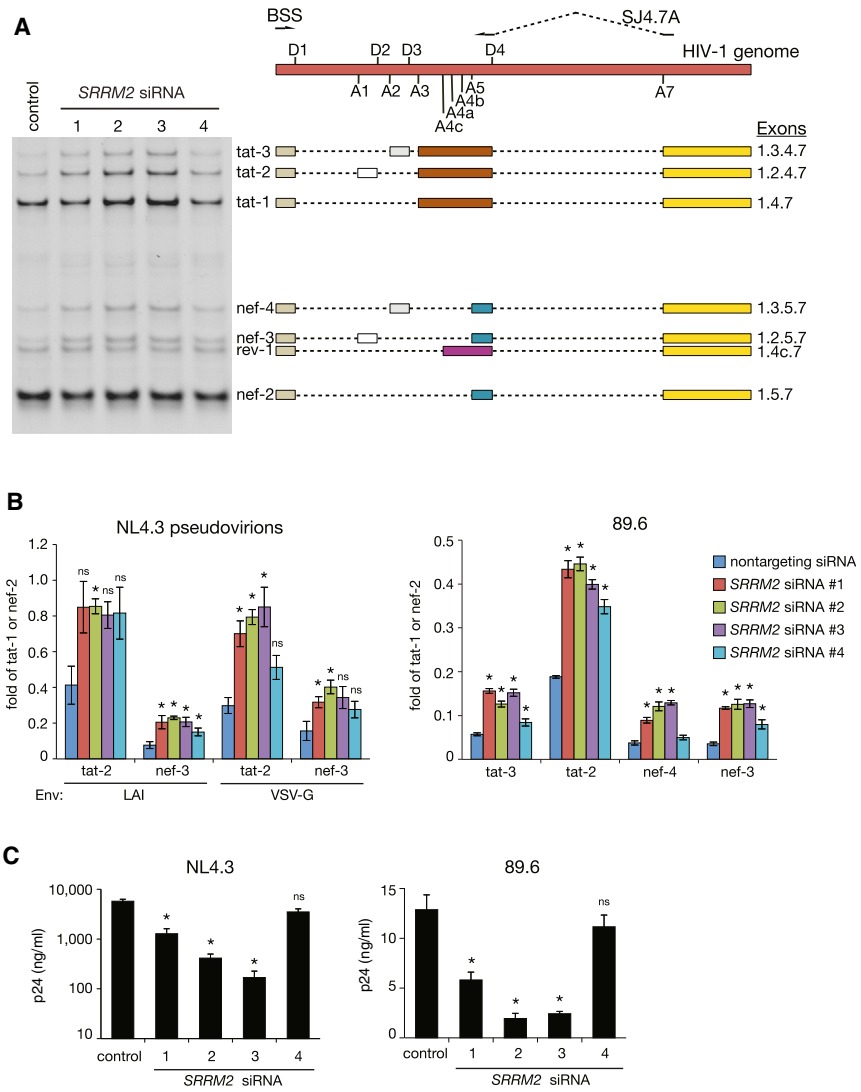

Figure 5. *SRRM2* Regulates Alternative Splicing of HIV-1

(A) RT-PCR analysis of HIV-1 transcripts. Exon compositions of sequence-confirmed transcripts are indicated to the right of a representative 89.6 DNA gel, positioned with respect to the HIV-1 genome above. D1–D4: 5′ splice sites. A1–A7: 3′ splice sites.

(B) Relative quantification of HIV-1 transcripts. Band intensities of corresponding RT-PCR products of tat and nef alternatively spliced products were divided by tat-1 and nef-2, respectively. Comparisons were made between each *SRRM2* siRNA and the nontargeting control siRNA.

(C) HIV-1 release following depletion of *SRRM2* as measured by p24 collected on day 5 post infection. Comparisons were made between each *SRRM2* siRNA and the nontargeting control siRNA. Bars represent SEM; n = 4; *p < 0.05 Mann-Whitney; ns, not significant (p > 0.05).

See also Figure S2.

transcripts, yet only decreased levels of nef-4, suggesting that *PNN* regulates the inclusion of exons 2 and 3 only in the context of tat transcription (Figure 6B). A similar pattern of tat transcripts was seen with *TRA2A* (Figure 6B). Depletion of *ACIN1* led to a dramatic increase in tat-4 and tat-3 transcripts and a corresponding decrease in all nef transcripts, making quantification of nef transcripts difficult (Figure 6B). Depletion of *SRRM1*, a binding partner of *SRRM2*, led to increases in tat-2 and nef-3, but not tat-3 and nef-4 (Figure 6B). This suggests that *SRRM1* regulates the activity of site A1, but not A2, in both tat and nef transcripts and that *SRRM1* and *SRRM2* may modulate HIV-1 splicing in both overlapping and distinct protein complexes.

DISCUSSION

Viral infection is intimately tied to interactions with host proteins and pathways that facilitate virtually every step of the virus life cycle. By binding to and crosslinking cell-surface molecules, viruses can induce intracellular signaling cascades that can enhance the permissiveness of the host cell to infection. The receptors used by HIV-1 to enter cells are well-known signaling molecules and important immune modulators. Binding of Env to these receptors results in downstream phosphorylation events and the modulation of various intracellular second messengers; however, the breadth of HIV-induced signaling cascades, their temporal relationship to virus infection, and the roles that these signaling events play in specific aspects of virus infection are far from well characterized.

We employed unstimulated CD4$^+$ T cells, because these most closely reflect the major cell type encountered by HIV-1 in vivo and because a growing body of evidence indicates that virus-induced signaling events play a significant role in virus infection of primary T cells. We used an X4-tropic strain of HIV-1 in part because of the fact that virus engagement of CXCR4 induces signals that lead to cytoskeleton reorganization in primary T cells and more efficient virus infection (Balabanian et al., 2004; Barrero-Villar et al., 2009; Jiménez-Baranda et al., 2007; Yoder et al., 2008). Thus, at a minimum, we anticipated finding changes in the phosphorylation status of proteins linked to cytoskeletal rearrangements upon binding of HIV-1 to host cells. Our findings not only supported this supposition, but also showed that HIV-receptor interactions rapidly modulate phosphorylation of numerous host cell proteins with the potential to impact multiple steps of viral replication. Of 1,757 quantified phosphorylation sites, 14% proved to be HIV responsive. Although some fraction of these HIV-responsive phosphorylation sites may prove to be false positives, our analysis only detected a small fraction of total cellular phosphorylation sites, and we only examined a single early time point. Thus, it is probable that binding of HIV-1 to its cell-surface receptors leads to altered phosphorylation of at least hundreds of host proteins.

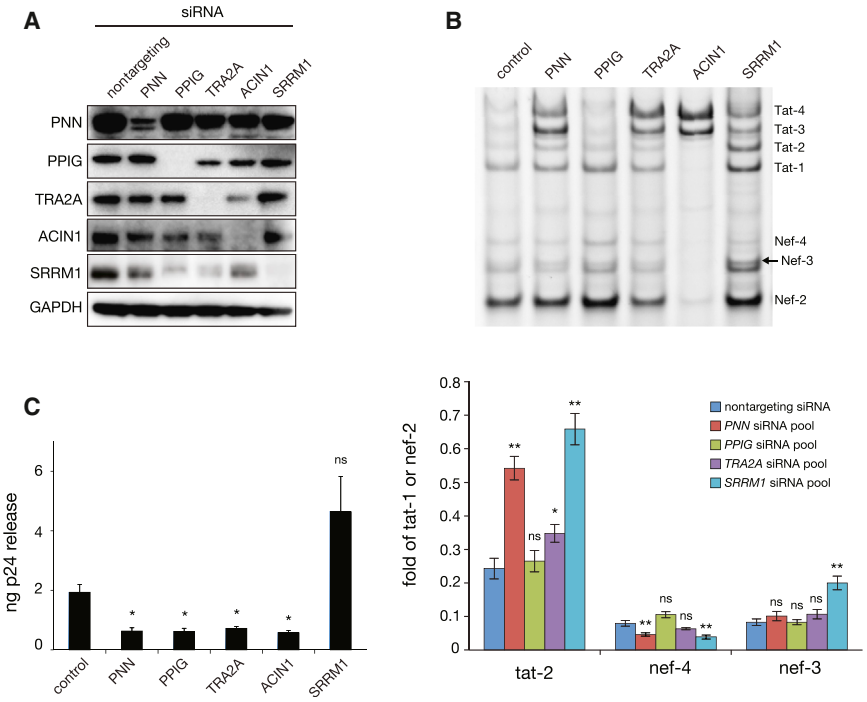

Figure 6. HIV-Responsive SR Proteins Regulate HIV-1 Splicing and Virion Release

(A) Lysates from depleted MAGI cells were analyzed by immunoblot with each antibody.

(B) Relative quantification of HIV-1 transcripts. Band intensities of tat and nef alternatively spliced products (top panel) were divided by tat-1 and nef-2, respectively, for quantification (bottom panel). n = 6.

(C) HIV-1 release following depletion of SR proteins as measured by p24 collected on day 5 post infection. Comparisons were made between each *SRRM2* siRNA and the nontargeting control siRNA. Bars represent SEM; n = 4; *p < 0.05, **p < 0.01 Mann-Whitney; ns, not significant (p > 0.05). See also Figure S3.

Several complementary lines of evidence, including western blots, analysis of experimentally and computationally derived kinase substrates, and bioinformatics of cellular gene ontologies, support the validity of the MS-derived phosphorylation site ratios described here. Fluxes of intracellular calcium were one of the first and most consistently observed examples of HIV-induced cellular signaling (Kornfeld et al., 1988; Liu et al., 2000; Weissman et al., 1997). Calcium activates the CaMK family, and we detected a strong overrepresentation of CaMKII substrates among upregulated HIV-responsive phosphorylation sites along with phosphorylation of the transcription factor ETS-1 (pS282/pS285), a well-characterized substrate of CaMKII that has been linked to HIV-1 gene expression (Sieweke et al., 1998; Soderling, 1999; Yang et al., 2009). In addition, overexpression of a splice variant of ETS-1 lacking exon VII reactivated latent HIV-1 (Yang et al., 2009). Interestingly, exon VII contains serines 282 and 285, and the ETS-1 isoform lacking this exon binds to DNA more strongly than wild-type ETS-1 independently of CaMKII activity (Fisher et al., 1994).

In a previous study, we characterized the CXCL12-responsive phosphoproteome in a T cell line (Wojcechowskyj et al., 2011), and the overlap of significantly changed phosphorylation sites between these data sets was statistically significant (OR: 3.1, p = 0.039) (Table S4). However, based on the unique nature of each study and the modest overlap of quantified phosphorylation sites (~50%), it is difficult to determine which of the HIV-responsive sites were dependent on CXCR4 signaling. To increase the likelihood of capturing relevant signaling events, we focused on proteins that contained both HIV- and CXCL12-responsive phosphorylation sites. Suppression of only *SRRM2* showed a consistent phenotype on virus replication in both MAGI and Jurkat cells, whereas *MYCBP2*, *OSBPL11*, and *SCYL2* did show effects with some siRNAs. The most consistent of these was with *MYCBP2*, a negative regulator of cAMP (Scholich et al., 2001). Interestingly, our data support the observations of others that the role of cAMP during HIV-1 replication is cell type dependent. In HeLa cells, components of PKA signaling enhance HIV-1 replication (König et al., 2008; Lemay et al., 2008; Zhou et al., 2008), whereas the opposite is true in Jurkat and primary T cells (Navarro et al., 1998). Finally, a recent study showed that *SCYL2* limits the release of HIV-1 from cells—something that we did not measure (Miyakawa et al., 2012).

HIV-1 can disseminate among T cells either through cell-free or cell-cell routes, and this data set may help elucidate components of the cell-cell transmission of HIV-1. The contact between infected and uninfected CD4 T cells forms structures, termed "virological synapses" (VSs), that resemble immunological synapses (ISs) (Haller and Fackler, 2008). We found several HIV-responsive phosphoproteins that are known to participate in either the VS or IS, such as *LAT*, *FYB* (ADAP), *GRAB2* (Gads), *NCK1*, and *PTPN6* (SHIP-1). The HIV-responsive phosphoproteins *APBB1IP* (RIAM), *RAPGEF6* (rap1 GEF), and *SIPA1* (rap1 GAP) are also components of integrin "inside-out" signaling, a feature of both the VS and IS (Hioe et al., 2011).

Our application of quantitative MS-based phosphoproteomics made it possible to explore the possibility that HIV-1 may induce cellular signals that not only enhance virus entry, but that render the cell more permissive to later steps in the viral-replication cycle as well. Six SR proteins and five other genes that can regulate cellular splicing were among the 175 phosphoproteins that were HIV responsive, raising the possibility that HIV-induced signaling during entry may influence the splicing of incoming viral transcripts. Indeed, signaling events can regulate the localization, intrinsic splicing activity, protein-protein interactions, or protein-RNA interactions of various splicing factors. Tools for firmly establishing this link are crude, yet this hypothesis is supported by the fact that five SR proteins and three others were important for HIV-1 infection and that altered ratios of nef transcripts correlated with p24 release levels. It is also possible that HIV-dependent regulation of these SR proteins is indirect, i.e., that they may affect the alternative splicing of additional HIV-1 host factors. In

fact, it is well known that cellular alternative splicing is altered during T cell activation (Martinez et al., 2012). Further complicating matters, SR proteins are heavily phosphorylated—over 600 unique phosphorylation sites have been cataloged for SRm300 alone, making the task of assigning roles for each phosphorylation site a daunting one.

At first glance, the kinetic and spatial disconnect between the plasma membrane and the nucleus in regulating downstream stages of the HIV-1 life cycle may seem improbable. However, phosphorylation of cellular proteins during HIV-1 entry can promote viral gene expression from the provirus (Gringhuis et al., 2010), and epidermal growth factor induces the phosphorylation of 62 nuclear phosphorylation sites in HeLa cells within 1 min (Olsen et al., 2006). In addition, the transcription factors NF-κB (Briant et al., 1996), NFAT (Cicala et al., 2006), and AP-1 (Briant et al., 1996; Chirmule et al., 1995), all of which bind to the HIV-1 long terminal repeat (LTR) and facilitate transcription, localize into the nucleus upon HIV-1 binding to the cell surface.

The screening approach taken here suggests that the interplay between HIV-1 and its receptors results in a much broader array of signaling events than has been suspected and that through these interactions the virus can modulate the host cell environment in ways that impact virus replication at multiple steps, including splicing of viral transcripts. Examining the status of SRm300 phosphorylation and that of other splicing factors and phosphoproteins identified in this study over the course of virus infection in the presence or absence of agents that inhibit virus-receptor signaling should more fully reveal how the virus can reprogram cells to make them optimal hosts.

EXPERIMENTAL PROCEDURES

Cell Lines and Viruses

Jurkat E6-1 cells (ATCC) were propagated in RPMI supplemented with 10% (v/v) fetal bovine serum (FBS). MAGI cells, which are a clone of HeLa cells stably expressing CD4, CCR5, and β-galactosidase under the control of the HIV-1 LTR, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS. CEM cells were grown in DMEM medium (AthenaES) with 10% dialyzed fetal calf serum, and lysine and arginine were replaced by [$^{13}C_6$, $^{15}N_2$]-lysine and [$^{13}C_6$, $^{15}N_4$]-arginine. Protein isolates from these cells served as the stable-isotope-labeled reference material for the SILAC quantitative analyses. The HIV-1 MN strain was used for primary cell stimulations and was grown in SupT1 cells, purified, concentrated with sucrose gradients, and inactivated with aldrithiol-2 (AT-2) (Chertova et al., 2003; Rossio et al., 1998). A mock preparation was produced from uninfected cells using the same procedures. Replication-incompetent HIV-1 pseudovirions (pNL43-ΔEnv-vpr$^+$-luc$^+$) were generated as previously described (Parrish et al., 2012). The HIV-1 Env strains used for pseudovirion generation were the lab-adapted X4 strain LAI and the Clade B-transmitted/founded R5 strain REJO.D12. Replication-competent 89.6 and NL4.3 strains of HIV-1 were grown in CEMx174 cells and SupT1 cells, respectively.

Primary Human CD4$^+$ T Cell Stimulations

Primary human CD4$^+$ T cells from healthy donors were obtained from the Center for AIDS Research Human Immunology Core at the University of Pennsylvania and purified by negative selection against HLA-DR, CD21, CD16, CD11b, CD14, and CD8. Cells rested overnight in 10% FBS containing RPMI at 37°C and were serum starved for 90 min before stimulation. AT-2-treated HIV-1 preparations or (volume-matched) mock vesicles were added to 4.5 × 10^8 cells (5 × 10^6 cells/ml) for a final concentration of 20 μg/ml p24. This concentration of p24 corresponds to approximately 15 nM gp120 and a multiplicity of infection of 100, a concentration that is commonly used for biochemical measurements of virus-induced signaling events. After 1 min incubation at 37°C, two volumes of ice-cold PBS containing phosphatase inhibitors (Sigma-Aldrich) were added to each culture of cells, which were then spun at 450 × *g* at 4°C. Supernatant was aspirated, and cell pellets were frozen in liquid nitrogen.

MS and Data Processing

Details of sample preparation and enrichment, MS, sequence database searching, mixture-model calculations, and statistical analyses are included in Supplemental Experimental Procedures.

Immunoblot Analysis

Cell pellets were lysed in 1% Triton X-100 with phosphatase (Sigma-Aldrich P5726 and P0044) and protease (Roche 1836170) inhibitors for 5 min on ice, then centrifuged at 20,000 × *g* for 10 min. Samples were denatured and reduced, incubated at 70°C for 10 min, then run on 10% Bis-Tris gels. Gels were transferred to polyvinylidene fluoride membranes and blocked for 30 min in evaporated milk. Blots were incubated at 4°C overnight with a 1:1,000 dilution of primary antibodies. Antibodies for ERK1/2 (pT202/pY204), ERK1/2, α-tubulin, p38 (pT180/pY182), p38, and PPIG were obtained from Cell Signaling Technology; ETS-1 (pS282/pS285) and ETS-1 from Invitrogen; MYCPB2, SCYL2, PNN, and ACIN1 from Abcam; OSBPL11 and TRA2A from Sigma-Aldrich; GAPDH from Calbiochem; SRRM1 from Proteintech; and SRm300 (B4A11) was a generous gift from Jeff Nickerson.

RNA Interference

Jurkat cells were transfected with an Amaxa nucleofector (Lonza) as follows: 2 × 10^6 cells were resuspended in 100 μl solution V containing 100 μM siRNA, electroporated with program X-001, then incubated in 4 ml of complete RPMI for 48 hr before harvesting for immunoblot or infection with HIV-1. MAGI cells were transfected with Lipofectamine RNAiMax Reagent (Invitrogen). We reverse-transfected 2.5 × 10^3 cells in a 96-well plate with 50 nM (final) siRNA according to the manufacturer's instructions. Cells were then harvested for immunoblot or infected with HIV-1 72 hr later. siRNA sequences are listed in Tables S7 and S9.

HIV-1 Infections

Approximately 5 × 10^5 nucleofected Jurkat cells were spinoculated with pseudovirions at 1,200 × *g* for 2 hr at room temperature. Cells were harvested for firefly luciferase expression 48 hr post infection (hpi). For infection of MAGI cells for RT-PCR splicing analysis, 100 ng p24 of either NL4.3 or 89.6 was added per 1 × 10^6 cells, rinsed following a 2 hr incubation at 37°C, then harvested for fluorescence-activated cell sorting (FACS) or RNA 48 hpi. For β-galactosidase-based infections of MAGI cells, siRNA-treated cells in 96 wells were infected with 2.5 ng p24 per well of replication-competent IMC of NL4.3, 89.6, and SF162, and β-galactosidase activity was measured 48 hr later. For measurement of percent infected cells, infected MAGI cells were stained for intracellular p24 and analyzed by FACS. For p24 release, siRNA-treated MAGI cells were infected with NL4.3 and 89.6 as above, media replaced, and collected 5 days later. Supernatant p24 was measured using AlphaLISA (PerkinElmer) in 384-well plates.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, R code for the mixture model, three figures, and ten tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.04.011.

ACKNOWLEDGMENTS

We especially thank Jim Riley and the Penn Immunology Core for human primary CD4$^+$ T cells, Lynn Spruce and the Children's Hospital of Philadelphia Research Institute Protein and Proteomics Core for expert technical assistance, Julian Bess and Jeffrey Lifson for AT-2-treated HIV-1 virions, and Jeffrey Nickerson for the SRm300 antibody. We would also like to thank Matt Pace, Rebecca Custers-Allen, Karen Ocwieja, Gary Koretzky, Vijaya Ramesh, Vesa Olkkonen, Bruce Freedman, Alexander Babich, Janis Burkhardt, James Murray, Robert Siliciano, Erika Holzbaur, Jim Alwine, Farida Shaheen, and Steven Bryan for various reagents and technical assistance and Logan Everett

and Craig Wilen for helpful discussions. These experiments utilized regents provided by the AIDS and Cancer Virus Program, SAIC-Frederick./National Cancer Institute (Frederick, MD, USA) under contract HHSN261200800001E, and by the Penn Center for AIDS Research (P30 AI 045008). J.A.W. was supported by T32-AI007632 and R01 AI40880. J.A.W., S.H.S., and R.W.D. conceived and designed the experiments. J.A.W., C.A.D., J.Y.L., and N.F.P. performed the experiments. J.A.W., S.T.J., S.H.S., and R.W.D. analyzed the data. N.F.P., R.S., B.H.H., F.D.B., S.T.J., and S.H.S. contributed reagents and analysis tools. J.A.W., S.T.J., S.H.S., and R.W.D. wrote the paper.

Received: November 8, 2012
Revised: February 11, 2013
Accepted: April 22, 2013
Published: May 15, 2013

REFERENCES

Balabanian, K., Harriague, J., Décrion, C., Lagane, B., Shorte, S., Baleux, F., Virelizier, J.L., Arenzana-Seisdedos, F., and Chakrabarti, L.A. (2004). CXCR4-tropic HIV-1 envelope glycoprotein functions as a viral chemokine in unstimulated primary CD4+ T lymphocytes. J. Immunol. *173*, 7150–7160.

Barrero-Villar, M., Cabrero, J.R., Gordón-Alonso, M., Barroso-González, J., Alvarez-Losada, S., Muñoz-Fernández, M.A., Sánchez-Madrid, F., and Valenzuela-Fernández, A. (2009). Moesin is required for HIV-1-induced CD4-CXCR4 interaction, F-actin redistribution, membrane fusion and viral infection in lymphocytes. J. Cell Sci. *122*, 103–113.

Brass, A.L., Dykxhoorn, D.M., Benita, Y., Yan, N., Engelman, A., Xavier, R.J., Lieberman, J., and Elledge, S.J. (2008). Identification of host proteins required for HIV infection through a functional genomic screen. Science *319*, 921–926.

Briant, L., Coudronnière, N., Robert-Hebmann, V., Benkirane, M., and Devaux, C. (1996). Binding of HIV-1 virions or gp120-anti-gp120 immune complexes to HIV-1-infected quiescent peripheral blood mononuclear cells reveals latent infection. J. Immunol. *156*, 3994–4004.

Cargnello, M., and Roux, P.P. (2011). Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol. Mol. Biol. Rev. *75*, 50–83.

Chertova, E., Crise, B.J., Morcock, D.R., Bess, J.W., Jr., Henderson, L.E., and Lifson, J.D. (2003). Sites, mechanism of action and lack of reversibility of primate lentivirus inactivation by preferential covalent modification of virion internal proteins. Curr. Mol. Med. *3*, 265–272.

Chirmule, N., Goonewardena, H., Pahwa, S., Pasieka, R., Kalyanaraman, V.S., and Pahwa, S. (1995). HIV-1 envelope glycoproteins induce activation of activated protein-1 in CD4+ T cells. J. Biol. Chem. *270*, 19364–19369.

Cicala, C., Arthos, J., Censoplano, N., Cruz, C., Chung, E., Martinelli, E., Lempicki, R.A., Natarajan, V., VanRyk, D., Daucher, M., and Fauci, A.S. (2006). HIV-1 gp120 induces NFAT nuclear translocation in resting CD4+ T-cells. Virology *345*, 105–114.

Coyne, C.B., and Bergelson, J.M. (2006). Virus-induced Abl and Fyn kinase signals permit coxsackievirus entry through epithelial tight junctions. Cell *124*, 119–131.

Davis, C.B., Dikic, I., Unutmaz, D., Hill, C.M., Arthos, J., Siani, M.A., Thompson, D.A., Schlessinger, J., and Littman, D.R. (1997). Signal transduction due to HIV-1 envelope interactions with chemokine receptors CXCR4 or CCR5. J. Exp. Med. *186*, 1793–1798.

Fisher, R.J., Fivash, M., Casas-Finet, J., Erickson, J.W., Kondoh, A., Bladen, S.V., Fisher, C., Watson, D.K., and Papas, T. (1994). Real-time DNA binding measurements of the ETS1 recombinant oncoproteins reveal significant kinetic differences between the p42 and p51 isoforms. Protein Sci. *3*, 257–266.

Fu, W., Sanders-Beer, B.E., Katz, K.S., Maglott, D.R., Pruitt, K.D., and Ptak, R.G. (2009). Human immunodeficiency virus type 1, human protein interaction database at NCBI. Nucleic Acids Res. *37*(Database issue), D417–D422.

Furler, R.L., and Uittenbogaart, C.H. (2010). Signaling through the P38 and ERK pathways: a common link between HIV replication and the immune response. Immunol. Res. *48*, 99–109.

Geiger, T., Wisniewski, J.R., Cox, J., Zanivan, S., Kruger, M., Ishihama, Y., and Mann, M. (2011). Use of stable isotope labeling by amino acids in cell culture as a spike-in standard in quantitative proteomics. Nat. Protoc. *6*, 147–157.

Gringhuis, S.I., van der Vlist, M., van den Berg, L.M., den Dunnen, J., Litjens, M., and Geijtenbeek, T.B. (2010). HIV-1 exploits innate signaling by TLR8 and DC-SIGN for productive infection of dendritic cells. Nat. Immunol. *11*, 419–426.

Haller, C., and Fackler, O.T. (2008). HIV-1 at the immunological and T-lymphocytic virological synapse. Biol. Chem. *389*, 1253–1260.

Hioe, C.E., Tuen, M., Vasiliver-Shamis, G., Alvarez, Y., Prins, K.C., Banerjee, S., Nádas, A., Cho, M.W., Dustin, M.L., and Kachlany, S.C. (2011). HIV envelope gp120 activates LFA-1 on CD4 T-lymphocytes and increases cell susceptibility to LFA-1-targeting leukotoxin (LtxA). PLoS ONE *6*, e23202.

Hodges, A., Sharrocks, K., Edelmann, M., Baban, D., Moris, A., Schwartz, O., Drakesmith, H., Davies, K., Kessler, B., McMichael, A., and Simmons, A. (2007). Activation of the lectin DC-SIGN induces an immature dendritic cell phenotype triggering Rho-GTPase activity required for HIV-1 replication. Nat. Immunol. *8*, 569–577.

Jacquenet, S., Decimo, D., Muriaux, D., and Darlix, J.L. (2005). Dual effect of the SR proteins ASF/SF2, SC35 and 9G8 on HIV-1 RNA splicing and virion production. Retrovirology *2*, 33.

Jiménez-Baranda, S., Gómez-Moutón, C., Rojas, A., Martínez-Prats, L., Mira, E., Ana Lacalle, R., Valencia, A., Dimitrov, D.S., Viola, A., Delgado, R., et al. (2007). Filamin-A regulates actin-dependent clustering of HIV receptors. Nat. Cell Biol. *9*, 838–846.

König, R., Zhou, Y., Elleder, D., Diamond, T.L., Bonamy, G.M., Irelan, J.T., Chiang, C.Y., Tu, B.P., De Jesus, P.D., Lilley, C.E., et al. (2008). Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. Cell *135*, 49–60.

Kornfeld, H., Cruikshank, W.W., Pyle, S.W., Berman, J.S., and Center, D.M. (1988). Lymphocyte activation by HIV-1 envelope glycoprotein. Nature *335*, 445–448.

Krishnan, M.N., Ng, A., Sukumaran, B., Gilfoy, F.D., Uchil, P.D., Sultana, H., Brass, A.L., Adametz, R., Tsui, M., Qian, F., et al. (2008). RNA interference screen for human genes associated with West Nile virus infection. Nature *455*, 242–245.

Lemay, J., Maidou-Peindara, P., Cancio, R., Ennifar, E., Coadou, G., Maga, G., Rain, J.C., Benarous, R., and Liu, L.X. (2008). AKAP149 binds to HIV-1 reverse transcriptase and is involved in the reverse transcription. J. Mol. Biol. *383*, 783–796.

Liu, Q.H., Williams, D.A., McManus, C., Baribaud, F., Doms, R.W., Schols, D., De Clercq, E., Kotlikoff, M.I., Collman, R.G., and Freedman, B.D. (2000). HIV-1 gp120 and chemokines activate ion channels in primary macrophages through CCR5 and CXCR4 stimulation. Proc. Natl. Acad. Sci. USA *97*, 4832–4837.

Liu, Y., Belkina, N.V., and Shaw, S. (2009). HIV infection of T cells: actin-in and actin-out. Sci. Signal. *2*, pe23.

Long, J.C., and Caceres, J.F. (2009). The SR protein family of splicing factors: master regulators of gene expression. Biochem. J. *417*, 15–27.

Martinez, N.M., Pan, Q., Cole, B.S., Yarosh, C.A., Babcock, G.A., Heyd, F., Zhu, W., Ajith, S., Blencowe, B.J., and Lynch, K.W. (2012). Alternative splicing networks regulated by signaling in human T cells. RNA *18*, 1029–1040.

Mayya, V., Lundgren, D.H., Hwang, S.I., Rezaul, K., Wu, L., Eng, J.K., Rodionov, V., and Han, D.K. (2009). Quantitative phosphoproteomic analysis of T cell receptor signaling reveals system-wide modulation of protein-protein interactions. Sci. Signal. *2*, ra46.

Mercer, J., and Helenius, A. (2008). Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. Science *320*, 531–535.

Miyakawa, K., Sawasaki, T., Matsunaga, S., Tokarev, A., Quinn, G., Kimura, H., Nomaguchi, M., Adachi, A., Yamamoto, N., Guatelli, J., and Ryo, A. (2012). Interferon-induced SCYL2 limits release of HIV-1 by triggering PP2A-mediated dephosphorylation of the viral protein Vpu. Sci. Signal. *5*, ra73.

Mosenden, R., and Taskén, K. (2011). Cyclic AMP-mediated immune regulation—overview of mechanisms of action in T cells. Cell. Signal. *23*, 1009–1016.

Navarro, J., Punzón, C., Jiménez, J.L., Fernández-Cruz, E., Pizarro, A., Fresno, M., and Muñoz-Fernández, M.A. (1998). Inhibition of phosphodiesterase type IV suppresses human immunodeficiency virus type 1 replication and cytokine production in primary T cells: involvement of NF-kappaB and NFAT. J. Virol. *72*, 4712–4720.

Olsen, J.V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006). Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell *127*, 635–648.

Pan, C., Olsen, J.V., Daub, H., and Mann, M. (2009). Global effects of kinase inhibitors on signaling networks revealed by quantitative phosphoproteomics. Mol. Cell. Proteomics *8*, 2796–2808.

Parrish, N.F., Wilen, C.B., Banks, L.B., Iyer, S.S., Pfaff, J.M., Salazar-Gonzalez, J.F., Salazar, M.G., Decker, J.M., Parrish, E.H., Berg, A., et al. (2012). Transmitted/founder and chronic subtype C HIV-1 use CD4 and CCR5 receptors with equal efficiency and are not inhibited by blocking the integrin $\alpha 4\beta 7$. PLoS Pathog. *8*, e1002686.

Rossio, J.L., Esser, M.T., Suryanarayana, K., Schneider, D.K., Bess, J.W., Jr., Vasquez, G.M., Wiltrout, T.A., Chertova, E., Grimes, M.K., Sattentau, Q., et al. (1998). Inactivation of human immunodeficiency virus type 1 infectivity with preservation of conformational and functional integrity of virion surface proteins. J. Virol. *72*, 7992–8001.

Ruperez, P., Gago-Martinez, A., Burlingame, A.L., and Oses-Prieto, J.A. (2012). Quantitative phosphoproteomic analysis reveals a role for serine and threonine kinases in the cytoskeletal reorganization in early T cell receptor activation in human primary T cells. Mol. Cell. Proteomics *11*, 171–186.

Scholich, K., Pierre, S., and Patel, T.B. (2001). Protein associated with Myc (PAM) is a potent inhibitor of adenylyl cyclases. J. Biol. Chem. *276*, 47583–47589.

Schwartz, D., and Gygi, S.P. (2005). An iterative statistical approach to the identification of protein phosphorylation motifs from large-scale data sets. Nat. Biotechnol. *23*, 1391–1398.

Sieweke, M.H., Tekotte, H., Jarosch, U., and Graf, T. (1998). Cooperative interaction of ets-1 with USF-1 required for HIV-1 enhancer activity in T cells. EMBO J. *17*, 1728–1739.

Soderling, T.R. (1999). The Ca-calmodulin-dependent protein kinase cascade. Trends Biochem. Sci. *24*, 232–236.

Soroceanu, L., Akhavan, A., and Cobbs, C.S. (2008). Platelet-derived growth factor-alpha receptor activation is required for human cytomegalovirus infection. Nature *455*, 391–395.

Stevenson, M., Stanwick, T.L., Dempsey, M.P., and Lamonica, C.A. (1990). HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO J. *9*, 1551–1560.

Stoltzfus, C.M. (2009). Chapter 1. Regulation of HIV-1 alternative RNA splicing and its role in virus replication. Adv. Virus Res. *74*, 1–40.

Weissman, D., Rabin, R.L., Arthos, J., Rubbert, A., Dybul, M., Swofford, R., Venkatesan, S., Farber, J.M., and Fauci, A.S. (1997). Macrophage-tropic HIV and SIV envelope proteins induce a signal through the CCR5 chemokine receptor. Nature *389*, 981–985.

Wilen, C.B., Tilton, J.C., and Doms, R.W. (2012). HIV: cell binding and entry. Cold Spring Harb. Perspect. Med. *2*, a006866.

Wojcechowskyj, J.A., Lee, J.Y., Seeholzer, S.H., and Doms, R.W. (2011). Quantitative phosphoproteomics of CXCL12 (SDF-1) signaling. PLoS ONE *6*, e24918.

Xue, Y., Liu, Z., Cao, J., Ma, Q., Gao, X., Wang, Q., Jin, C., Zhou, Y., Wen, L., and Ren, J. (2011). GPS 2.1: enhanced prediction of kinase-specific phosphorylation sites with an algorithm of motif length selection. Protein Eng. Des. Sel. *24*, 255–260.

Yang, H.C., Shen, L., Siliciano, R.F., and Pomerantz, J.L. (2009). Isolation of a cellular factor that can reactivate latent HIV-1 without T cell activation. Proc. Natl. Acad. Sci. USA *106*, 6321–6326.

Yeung, M.L., Houzet, L., Yedavalli, V.S., and Jeang, K.T. (2009). A genome-wide short hairpin RNA screening of jurkat T-cells for human proteins contributing to productive HIV-1 replication. J. Biol. Chem. *284*, 19463–19473.

Yoder, A., Yu, D., Dong, L., Iyer, S.R., Xu, X., Kelly, J., Liu, J., Wang, W., Vorster, P.J., Agulto, L., et al. (2008). HIV envelope-CXCR4 signaling activates cofilin to overcome cortical actin restriction in resting CD4 T cells. Cell *134*, 782–792.

Zhou, H., Xu, M., Huang, Q., Gates, A.T., Zhang, X.D., Castle, J.C., Stec, E., Ferrer, M., Strulovici, B., Hazuda, D.J., and Espeseth, A.S. (2008). Genome-scale RNAi screen for host factors required for HIV replication. Cell Host Microbe *4*, 495–504.