The Interferon Response Inhibits HIV Particle Production by Induction of TRIM22

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Abstract

Treatment of human cells with Type 1 interferons restricts HIV replication. Here we report that the tripartite motif protein TRIM22 is a key mediator. We used transcriptional profiling to identify cellular genes that were induced by interferon treatment and identified TRIM22 as one of the most strongly up-regulated genes. We confirmed, as in previous studies, that TRIM22 over-expression inhibited HIV replication. To assess the role of TRIM22 expressed under natural inducing conditions, we compared the effects of interferon in cells depleted for TRIM22 using RNAi and found that HIV particle release was significantly increased in the knockdown, implying that TRIM22 acts as a natural antiviral effector. Further studies showed that TRIM22 inhibited budding of virus-like particles containing Gag only, indicating that Gag was the target of TRIM22. TRIM22 did not block the release of MLV or EIAV Gag particles. Inhibition was associated with diffuse cytoplasmic staining of HIV Gag rather than accumulation at the plasma membrane, suggesting TRIM22 disrupts proper trafficking. Mutational analyses of TRIM22 showed that the catalytic amino acids Cys15 and Cys18 of the RING domain are required for TRIM22 antiviral activity. These data disclose a pathway by which Type 1 interferons obstruct HIV replication.

Introduction

The interferon system is a well studied branch of the innate immune system active against viruses. Infection of vertebrate cells by many viruses provokes synthesis and secretion of interferons (IFNs), which mediate induction of a cellular antiviral state that obstructs further viral spread. Type I IFNs (α and β) are produced by many cell types, while Type II IFN (gamma) is produced by immune cells. IFN-induced signaling pathways begin with IFN binding to IFN receptors at the cell surface. This results in signal transduction via the Jak/Stat pathway, which leads to activation of interferon-responsive genes and synthesis of effector proteins, including PKR, RNaseL, Mx, and many others (reviewed in [1,2,3,4]).

For HIV, previous studies have implicated IFNs in blocking both early and late stages of the HIV-1 lifecycle [5,6,7,8,9,10]. In many cell types, the inhibition of later steps in the life cycle following integration seems to be most potent (reviewed in [2]). Although the effector mechanisms acting late have not been fully clarified, one report suggested that the IFN-inducible protein ISG15 interfered with the endosomal trafficking pathway used by HIV-1 to exit 293T cells by blocking the interaction of TSG101 with HIV-1 Gag [11].

Many tripartite motif (TRIM) proteins may function in innate immunity to restrict viral replication. TRIM5α from rhesus macaque (RhTRIM5α) blocks early replication steps of HIV-1 and other retroviruses (reviewed in [12]); in addition, one group has proposed that RhTRIM5α acts late during HIV replication as well [13]. The early target of RhTRIM5α is the capsid (CA) protein, which forms the protein shell of the viral core. Anti-HIV-1 activity has also been reported for other TRIM family members. TRIM1 has been shown to target the CA protein at an early stage pre-reverse transcription, TRIM22 has been suggested to affect HIV transcription and TRIM19 and TRIM32 have been suggested to affect trafficking of viral proteins (reviewed in [14]). Recently, TRIM28 was shown to repress transcription from a retroviral promoter by binding to proviral DNA [15].

TRIM proteins contain a highly conserved RBCC motif comprised of a RING domain, one or two B-box domains, and a predicted coiled-coil region (reviewed in [14]). The RING domain contains a specialized zinc finger [16] and has been shown to possess E3 ubiquitin ligase activity [17,18,19,20,21,22]. Little is known about the B-box domain, which is unique to TRIM proteins. The coiled-coil domain is believed to promote protein oligomerization [23]. Several TRIM proteins also contain a C-terminal SPRY domain that is proposed to be involved in protein-protein interactions and RNA binding [24,25].

Many TRIM family members are inducible by IFNs, providing candidate mediators of IFN inhibition. In an effort to identify TRIM proteins mediating anti-retroviral activity, we carried out transcriptional profiling of IFN-treated human osteosarcoma (HOS) cells and found that TRIM22 was the most up-regulated TRIM queried on the microarray used. HOS-CD4/CXCR4 cells were studied because they support robust HIV replication and show a strong antiviral response to IFNβ. Human TRIM22 (also known as Staf-50) was previously identified from a search for IFN-
Author Summary

Interferons are produced by cells in response to challenge by foreign pathogens such as viruses. The molecular mechanisms by which Type I interferons (e.g., IFNβ) inhibit the replication of HIV-1 are not fully clarified. We identified a gene called TRIM22 that belongs to the tripartite motif (TRIM) family that was strongly induced by IFNβ. Using RNA interference to reduce the expression of TRIM22, we showed that TRIM22 is a key mediator of the IFNβ response when expressed at natural levels. We demonstrate that TRIM22 blocks the intracellular trafficking of the viral structural protein Gag to the surface of the cell, and that the antiviral activity of TRIM22 is dependent on two cysteine residues (Cys15 and Cys18) that are critical for the E3 ligase activity of RING-containing proteins. This report describes a mechanism by which Type I interferons block HIV-1 replication.

Results

TRIM22 restricts HIV-1 replication in HOS-CD4/CXCR4 cells

Human osteosarcoma cells modified to express CD4 and CXCR4 (HOS-CD4/CXCR4) support robust HIV-1 replication, which is potently restricted by pre-treatment with IFNβ. We wished to identify restriction factors induced by IFNβ, and so carried out transcriptional profiling of HOS-CD4/CXCR4 cells before and after IFNβ treatment. As expected, a large number of genes were induced (52 total with a false discovery rate of 7%; Table S1), including the well-known antiviral genes OAS1 (~253-fold), MX1/2 (~24-fold), ISG20 (~37-fold), ISGF3G (~12-fold), IRF7 (~13-fold) and TRIM19zeta (~18-fold).

We focused our study on TRIM22, which was induced at least 86-fold after IFNβ-treatment. Other TRIM genes that were notably up-regulated included TRIM19 variants (2.8 to 18-fold), TRIM21 (2.6-fold), TRIM34 variant 1 (2.4-fold) and TRIM5delta (2.4-fold), consistent with previous studies [26,36,37,38,39]. Gene chip data on TRIM22 induction was confirmed using Northern blot analysis (Figure 1A). These results support previous studies of other cell types, although we observed a higher level of IFNβ-induced TRIM22 expression than previously reported [4,27].

To analyze TRIM22 inhibition of HIV-1 replication, we generated HOS-CD4/CXCR4 cells that stably express hemagglutinin (HA)-tagged TRIM22 protein (Figure 1B). There was no significant difference between the doubling times of the empty vector control cells (24.4+/−2.4 hours) and the cells expressing TRIM22 (25.5+/−1.8 hours) (P = 0.7, Mann-Whitney Test), indicating TRIM22 expression was not detectably toxic. Cells stably expressing TRIM22 were then infected with increasing amounts of replication-competent HIV-1. Virus released into the
supernatant after several rounds of replication was detected using p24CA antibodies in a Western blot (Figure 1C). Cells expressing TRIM22 released substantially less virus than the control cells. Similar results were obtained using another HIV-1 clone LAI (data not shown). These findings parallel a previous report [27].

TRIM22 blocks late events of the HIV-1 lifecycle

We next analyzed the step in the HIV-life cycle affected by over-expression of TRIM22. To test for inhibition of early steps (entry through integration), we infected cells with HIV-1 pseudotyped with the VSV-G envelope and transducing GFP. We infected two isolates of the HOS-CD4/CXCR4 cell lines expressing TRIM22 and found no reduction in the fraction of cells positive for GFP (Figure 1D), indicating that TRIM22 did not strongly affect HIV/VSV-G early steps.

To test whether TRIM22 interferes with late events in the HIV lifecycle, we co-transfected plasmids encoding TRIM22 and a replication-competent HIV provirus. Forty-eight hours after transfection, Western blots of cell lysates and extracellular supernatants were probed with an antibody against the p24 capsid component of the Gag polyprotein (Figure 2). Co-expression of TRIM22 blocked the release of virions from HOS-CD4/CXCR4 cells, despite the production of substantial amounts of intracellular Gag (Figure 2). Gag also failed to accumulate in the extracellular supernatant in transfected U2OS, 143B and HeLa cells.

The nature of the blocks differed among the cell types studied. After correcting with an internal transfection control (peGFP), Gag proteins accumulated to a reduced extent in U2OS and 143B cells in the presence of TRIM22, while Gag accumulated inside cells to near wild-type levels in HOS-CD4/CXCR4 and HeLa cells but were not released (Figure 2 and data not shown). In all cell types, however, accumulation of extracellular Gag was abolished by co-expression with TRIM22.

Knocking down TRIM22 using RNA interferences boosts HIV particle production in the presence of IFNβ

The above data and results in [27] indicated that ectopic expression of TRIM22 can interfere with HIV replication, but from over-expression data alone it is uncertain whether expression at physiological levels would result in antiviral activity. For this reason, we tested whether depletion of TRIM22 using short hairpin ribonucleic acid (shRNA) in the context of an interferon response allowed increased production of extracellular HIV particles. HOS-CD4/CXCR4 cells were transiently transfected for 24 hours with either pLKO.1/TRIM22shRNA1 or the empty vector control (pLKO.1). In control cells, TRIM22 RNA accumulation increased in the presence of increasing concentrations of IFNβ. In the cells expressing the TRIM22 shRNA, TRIM22 RNA levels were significantly reduced (Figure 3A and B). As a control, expression of the related TRIM34 gene was tested and found to be unaffected by the TRIM22 shRNA (Figure 3A).

The knockdown cells and the control cells were then tested for their ability to release HIV-1 virus after transfection with a plasmid encoding replication-competent HIV-1 (pR9). Accumulation of HIV Gag in cells and supernatants was monitored by Western blot using p24CA antibodies. Cells knocked down for TRIM22, when treated with IFN, released substantially more virus than the control cells. Similar results were obtained using another HIV-1 clone LAI (data not shown). These findings parallel a previous report [27].

Cells knocked down for TRIM22, when treated with IFN, released substantially more virus into the cell supernatant than control IFN-treated cells (Figure 3C, 500 and 1000 units/ml of IFNβ). Figure 3D compares the fold decrease in particle release after treatment with 1000 units/ml of IFNβ in cells transfected with the empty vector control, an irrelevant target (eGFP) control (pLKO.1/eGFPshRNA), a scrambled shRNA control (pLKO.1/scramshRNA) and two different TRIM22 shRNAs targeting a different region of the TRIM22 3′ UTR. Gag release was inhibited by IFN more strongly in cells transfected with each of the control shRNAs than in cells transfected with TRIM22 shRNA. Over 14 control experiments, the fold-inhibition values for 1000 units/ml IFNβ ranged from 3.0-fold to 7.4-fold. Over 8 experiments with two different TRIM22 shRNAs and 1000 u
IFNβ inhibition ranged from 1.2-fold to 1.6-fold. The difference between the means of the fold-inhibition values of TRIM22 shRNA (1.4-fold) and the pooled controls (4.5-fold) was highly significant (P = 0.0002 Mann-Whitney) (Figure 3E). These data indicate that TRIM22 is an active antiviral effector in the context of the normal response to IFNβ.

TRIM22 inhibits budding of HIV-1 Gag-only particles

The HIV Gag polyprotein is transported to the plasma membrane after translation where it assembles into particles and buds from the cell surface. Gag protein alone is able to bud from cells when expressed in the absence of the other HIV-1 proteins (reviewed in [40]). We asked whether TRIM22 acted on the Gag...
polyprotein by testing inhibition of budding by Gag-only virus-like particles.

We co-transfected a codon-optimized HIV Gag expression plasmid in the presence or absence of TRIM22 and measured the accumulation of Gag proteins in the cells and in culture supernatants by Western blot (Figure 4A). In the absence of TRIM22, Gag protein was detected in the cell lysate and efficiently released into the supernatant. In the presence of TRIM22, Gag was readily detected in the cell lysate, but not the supernatant. This effect parallels the effect of IFNβ on Gag-only particle release in HOS-CD4/CXCR4 cells (Figure 4B).

To assess the specificity of TRIM22, we assayed effects on murine leukemia virus (MLV) and equine infectious anemia virus (EIAV) Gag. Co-transfection of TRIM22 with either MLV Gag-Pol or EIAV Gag-Pol failed to restrict the release of Gag-containing particles into the supernatant, whereas TRIM22 restricted the release of HIV-1 Gag (Figure 4C). Together these data indicate that TRIM22 acts selectively on HIV-1 Gag.

**TRIM22 alters Gag trafficking to the plasma membrane**

We next investigated whether TRIM22-mediated inhibition was associated with altered Gag trafficking by monitoring the subcellular localization of HIV Gag fused to GFP (pGag-GFP) [41,42,43,44]. We transfected pGag-GFP in the presence or absence of pTRIM22, then 3 hours later treated the cells with cycloheximide for 3 hours. Forty-eight hours after release of the cycloheximide block, the localization of the Gag-GFP protein was visualized by fluorescence microscopy by taking optical slices through the center of cells (Figure 5A). In the control cells, 63% of GFP-positive cells had punctate fluorescence at or near the plasma membrane and 35% of the cells showed diffuse cytoplasmic fluorescence. In contrast, in cells expressing TRIM22, 12% of the cells had punctate fluorescence at or near the plasma membrane and 88% of the cells yielded diffuse cytoplasmic localization without visible puncta (Figure 5B). The observed difference in proportions was highly significant (P<0.0001, Chi-square test).

To test whether IFNβ altered Gag localization, as with TRIM22 expression alone, we compared the effects of pre-treating cells with 1000 units/ml of IFNβ followed by transfection with Gag-GFP and microscopy. In the absence of IFNβ, 64% of GFP-positive cells had punctate fluorescence at or near the plasma membrane and 36% of the cells showed diffuse cytoplasmic fluorescence. In contrast, 22% of the IFNβ-treated cells had punctate fluorescence at or near the plasma membrane and 78% of the cells yielded diffuse cytoplasmic localization without visible puncta (Figures 5A and 5B). The difference in the number of cells exhibiting punctate localization at the cell surface before and after IFNβ-treatment was highly significant (P<0.0001, Chi-Square) and paralleled the result with the expression of TRIM22 alone.

To determine whether the diminished accumulation of Gag at the plasma membrane is due to accelerated degradation of Gag, we performed pulse-chase analysis on intracellular Gag levels in HOS-CD4/CXCR4 cells (Figure 5C). The half life of Gag was 4.4 hours in cells transfected with the empty vector control (pcDNA) and 4.5 hours when TRIM22 was co-transfected. In addition, Western analysis also yielded similar levels of intracellular Gag protein in the presence and absence of TRIM22 (data not shown). These data indicate that inhibition of particle release by TRIM22 is associated with diminished accumulation of Gag at the plasma membrane and is not likely the result of increased turnover of Gag in HOS-CD4/CXCR4 cells.

The myristoylation of Gag is an important requirement for targeting Gag to the plasma membrane and for virus assembly and release [45,46,47,48,49]. We asked whether the TRIM22-mediated disruption of Gag trafficking is a result of a defect in Gag myristoylation. We co-expressed Gag with or without TRIM22 in the presence of [3H]-myristic acid and immunoprecipitated Gag from the cellular extract with anti-p24CA. Gag myristoylation was readily detected both in the absence and presence of TRIM22, indicating that TRIM22 does not block the myristoylation of Gag (Figure 5D). A fraction of the immunoprecipitated protein was subjected to Western blot analysis using anti-p24CA, confirming the presence of comparable amounts of Gag protein in both of the samples.

The **E3 ligase catalytic site of TRIM22 is required for antiviral activity**

The modification of proteins by E3 ligases is known to be associated with altered endocytic trafficking [reviewed in [50,51]]. Since TRIM22 alters Gag trafficking and the RING domain of
TRIMs have homology with E3 ligases, we tested whether the E3 ligase active site, present in the RING domain, is required for the antiviral effects of TRIM22. We mutated two conserved cysteine residues (C15A/C18A) in the RING domain that have been shown to inactivate the ubiquitin ligase activity in other TRIMs [17,52,53].

We found that cells co-transfected with pR9 and either the empty vector control or the pTRIM22 C15A/C18A mutant did not block the release of virus, though wildtype TRIM22 blocked quite strongly (Figure 6). Gag accumulated inside cells to near wild-type levels (Figure 6, second panel) and the expression levels of wildtype TRIM22 and the C15A/C18A mutant were similar (Figure 6, second panel). These data implicate the catalytic cysteine residues at position 15 and 18 of the RING domain as important for the observed antiviral effects of TRIM22.

We next asked whether we could detect an interaction between TRIM22 and Pr55Gag. We immunoprecipitated a Gag-GFP fusion protein expressed in the presence of FLAG-tagged TRIM22 with anti-GFP antibodies and then performed a Western blot with anti-Flag antibodies and detected co-precipitation of FLAG-TRIM22 (Figure 7). Reverse immunoprecipitation using anti-FLAG pulled down Gag-GFP only when FLAG-TRIM22 was co-expressed. Treatment of the samples with RNaseA prior to immunoprecipitation did not interfere with the association of TRIM22 with Gag, indicating that an RNA bridge did not mediate the interaction. TRIM22 did not co-immunoprecipitate

Figure 5. TRIM22 alters the sub-cellular localization of Gag protein. A) Analysis of Gag localization by fluorescence microscopy. HOS-CD4/CXCR4 cells were co-transfected with pTRIM22 and pGag/GFP for 3 hours, washed and incubated with 100 μM cycloheximide for 3 hours. IFNβ pre-treatment was done for 16 hours prior to transfection with pGag/GFP. Cells were washed and incubated in fresh media for a further 48 hours. Gag-GFP localization was assessed using fluorescence microscopy. High magnification images of the white squares are shown to the right. B) Quantifications of images as in A from three independent experiments. GFP-positive cells were classified as showing either punctate fluorescence at the cell periphery or diffuse cytoplasmic fluorescence. The number of cells counted was: pcDNA, n = 332; TRIM22, n = 386; −IFNβ, n = 497; +IFNβ, n = 398.

C) HOS-CD4/CXCR4 cells were co-transfected with pGag in the absence or presence of pTRIM22 and pulse-chase analysis of intracellular Gag protein was done. D) Detection of 3H-myristic acid-labeled Gag. Cells were co-transfected with pGag in the absence or presence of pTRIM22 for 24 hours and then labeled with 3H-myristic acid overnight. Gag was immunoprecipitated and resolved on a SDS-PAGE gel before exposure to film ("Labeled Gag") or Western blot analysis using anti-p24CA ("Total Gag").

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with either MLV or EIAV Gag (data not shown). Thus TRIM22 binds specifically to HIV Gag.

Discussion

Here we present data that TRIM22 is an interferon inducible effector that is responsible at least in part for the late IFNβ block to HIV-1 replication. We observed, as have others, that over-expression of TRIM22 in cells obstructed HIV replication. We show here that knockdown of TRIM22 in HOS-CD4/CXCR4 cells in the context of a natural IFN response abrogated the late block to HIV replication, implicating TRIM22 as an antiviral effector. TRIM22 expression was able to block release of HIV-1 Gag-only virus-like particles, but not MLV or EIAV Gag particles, indicating that TRIM22 acts specifically on the HIV Gag protein. In binding studies, TRIM22 associated with HIV-1 Gag but not MLV or EIAV Gag particles, indicating that TRIM22 acts specifically on the HIV Gag protein. In addition, TRIM22 inhibited LTR-driven transcription. A similar mechanism may explain the rapid degradation of Gag in 293T cells [63]. We found no evidence that TRIM22 drastically altered the stability of Gag protein levels in HOS-CD4/CXCR4 or HeLa cells, however it is possible that altered Gag sorting results in faster degradation in U2OS and 143B cells than seen in HOS-CD4/CXCR4 and HeLa cells.

Against this background of cell type specific effects, comparisons of our results to previous reports are quite tentative. In one study, addition of 500 units/ml of IFNα to 293T cells was reported to inhibit HIV particle release about two-fold, and an siRNA against the interferon-induced ubiquitin-like molecule ISG15 blocked IFN inhibition [64,65,66]. In HOS-CD4/CXCR4 cells treated with IFNβ at 1000 units/ml, the inhibition of particle release was stronger (3.0-fold to 7.4-fold), but the shRNA against TRIM22 did not completely reverse IFN inhibition. One possibility is that TRIM22 and ISG15 both act in the same pathway. For example, TRIM22 might act as an E3 ligase for ISG15. Further experiments will be needed to assess the relationship of inhibition by TRIM22 to that of ISG15.

Another recent report suggested that IFNα treatment caused HIV-1 virions to become tethered to cell surfaces, resulting in eventual re-uptake by endocytosis [67]. The viral Vpu protein was reported to overcome this block in some cell types. It is possible that the mis-sorting of Gag observed here in HOS-CD4/CXCR4 cells results from virions trafficking to the membrane followed by re-uptake, though in HOS-CD4/CXCR4 cells there was no evidence for Gag accumulating at membranes at early times as might be expected from the re-uptake model. Also, in the HOS-CD4/CXCR4 cell model, expression of Gag in the presence or absence of Vpu did not noticeably affect particle accumulation in the supernatant. It will be useful to identify the cell type or experimental differences responsible for these divergent observations.

How human TRIM22 alters intracellular Gag trafficking in HOS-CD4/CXCR4 cells is unknown but a fascinating problem for further study. One candidate model would be that TRIM22 acts by interfering with Gag binding to membranes via the linked myristate switch [68,69,70,71]. We found that Gag was myristoylated normally in the presence of TRIM22 (Figure 5C) but TRIM22 might affect Gag multimerization and resulting exposure of the myristate residue. We also found that the E3 ligase activity of TRIM22...
is important for restricting viral particle release, which implies that TRIM22 probably disrupts Gag trafficking by transferring ubiquitin (or an ubiquitin-related moiety) to one or more targeted proteins. Although TRIM22 interacts with Gag, we have yet to detect a convincing effect of TRIM22 on the levels of Gag ubiquitylation or a size-altering modification of Gag (data not shown), raising the possibility that a cellular protein involved in Gag trafficking is the target. Thus studies of TRIM22 link E3 ligases, IFN, and HIV late replication steps, providing a new experimental route to investigating these issues.

**Materials and Methods**

**Cell lines, plasmids and antibodies**

293T, HOS-CD4/CXCR4, HeLa, 143B, and U2OS were maintained in standard growth media (Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 100 U/ml Penicillin and 100 μg/ml Streptomycin) at 37°C with 5% CO2. HOS-CD4/CXCR4 cells stably expressing TRIM22 was generated by transfecting the cells with pTRIM22 followed by selection with 2 mg/ml of Genetecin. pTRIM22 was generated by cloning the TRIM22 coding region (GenBank Accession X82200) into pcdNA3.1HA using MfeI and Xhol restriction sites to generate pTRIM22. pFLAG/TRIM22 was generated by cloning the same TRIM22 coding region into p3xFLAG-CMV-10 (Sigma) using HindIII and XbaI restriction sites. pT22-C15A/C18A was generated using the QuikChange Site Directed Mutagenesis kit (Stratagene) according to manufacturer’s directions. pTRIM22 was used as the template and the oligos used were 5’ gag aag gag 3’ (forward) and 5’ gga ggt tga ggt gac 3’ (reverse). PEG-precipitated virus and sucrose-purified Gag particle pellets were collected using standard protocols [82]. Prior to infection, virus aliquots were digested with DNasel (0.2 units/μl) for 1 hour at 37°C. Cells were infected with varying amounts of virus for 3 hours at 37°C in a minimal volume of media, after which the media containing virus was replaced with fresh media and the cells were then incubated for the desired time.

**Quantitation of virus and Gag particle release**

Virus released into the supernatant was pelleted and lysed as described in [82]. Briefly, 1ml viral supernatants were clarified by centrifugation at 350 g for 10 mins at 4°C and passed through a 0.45 μm filter. Virus was precipitated overnight on wet ice at 4°C using 0.5× volumes of PEG solution (30% PEG 8000, 0.4 M NaCl). Precipitated virus was pelleted by centrifugation at 800 g for 45 minutes at 4°C. Gag particles were prepared as previously described [42,44]. Briefly, 800 μl of supernatant containing Gag particles were pelleted by centrifugation at 20,000 g for 90 mins at 4°C over a 500 μl cushion of 20% sucrose.

PEG-precipitated virus and sucrose-purified Gag particle pellets were lysed with 75 μl and 30 μl of lysis buffer respectively and 2 μl 5 μl of the lysed pellets was used for Western blotting. Lysis buffer consisted of a fresh mix of Buffer A and Buffer B (2:1 ratio respectively). Buffer A contained 12.5 ml of 1 M Tris (pH 7.8), 1.25 ml of 0.1 M EDTA (pH 8.0), 1.25 ml of 10% Triton X-100, 250 ml of Glycerol, 0.77 g of DTT and 5.72 g of KCl with the volume brought up to 500 ml with ddH2O. Buffer B contained 45 ml of 10% Triton X-100 and 1.63 g KCl with the volume brought up to 500 ml with ddH2O. For analysis of cell lysates, cells were centrifuged at 350 g for 5 mins, washed twice with phosphate-buffered saline (PBS) and lysed with 30 μl of RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1× Complete Protease Inhibitor (Roche), 1% Triton X-100, 0.1% SDS). Two micrograms of the lysed cell pellets were used for Western blotting.

**Immunoprecipitation and Western blotting**

Cells were lysed with cold non-denaturing lysis buffer for 20 mins (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.5% Igepal CA-630 (Sigma), 1× Complete EDTA-free protease inhibitor (Roche)) and pre-cleared by centrifugation at 16,000 g× for 15 mins. Two micrograms of primary antibody was diluted in 500 μl of cold PBS with 0.01% Triton X-100 and mixed with 15 μl of a 50% protein A-Sepharose at 4°C for 1 hour with gentle rocking. Beads were washed 3 times with PBS and once with lysis buffer. 5 μl of 10% BSA was added to the beads and mixed with 600 μg–1 mg of pre-cleared cell lysate in a 500 μl–1 ml volume at 4°C for 2 hours with gentle rocking. Beads were washed 3 times with cold lysis buffer and once with PBS.
Samples for Western blotting were mixed with loading buffer and separated on a 10% SDS-PAGE gel. Protein was transferred to PVDF membrane by wet or semi-dry transfer. Western blotting was carried out by blocking the membrane for 1 hour in 5% skim milk followed by a ~16 hour incubation with 1:1000 dilution of primary antibody. Detection was carried out using HRP-labeled secondary antibody (1:5000 for 30 mins) and the Odyssey Infrared Imaging System (LI-COR). Membranes were verified using an IRDye-labeled secondary antibody (1:20,000 for 30 mins) and the Odyssey Infrared Imaging System (LI-COR Biosciences) according to manufacturer’s directions.

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**Author Contributions**

Conceived and designed the experiments: SDB JRS FDB. Performed the experiments: SDB. Analyzed the data: SDB JRS FDB. Contributed reagents/materials/analysis tools: SDB JRS FDB. Wrote the paper: SDB JRS FDB.

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**Supporting Information**

Table S1 Top 52 probe sets affected by interferon beta treatment of HOS-CD4/CXCR4 cells. Found at: doi:10.1371/journal.ppat.1000007.s001 (0.02 MB XLS)
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