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Immunoproteasome activation enables human TRIM5 α restriction of HIV-1

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Abstract

Type 1 interferon (IFN) suppresses viral replication by upregulating the expression of interferon-stimulated genes (ISGs) with diverse anti-viral properties¹. The replication of human immunodeficiency virus type-1 (HIV-1) is naturally inhibited by IFN, with the steps between viral entry and chromosomal integration of viral DNA being notably susceptible^{2–5}. The ISG myxovirus resistance 2 (MX2) has been defined as an effective post-entry inhibitor of HIV-1, but is only partially responsible for IFN's suppressive effect^{6–8}. Using siRNA-based library screening in IFN α -treated cells, we sought to characterize further ISGs that target the pre-integration phases of HIV-1 infection, and identified human tri-partite-containing motif 5 α (TRIM5 α) as a potent anti-HIV-1 restriction factor. Human TRIM5 α , in contrast to many non-human orthologues, has not generally been ascribed significant HIV-1 inhibitory function, a finding attributed to ineffective recognition of cytoplasmic viral capsids by TRIM5 α ^{2,9,10}. Here, we demonstrate that IFN α -mediated stimulation of the immunoproteasome, a proteasome isoform mainly present in immune cells and distinguished from the constitutive proteasome by virtue of its different catalytic β -subunits as well as the proteasome activator (PA) 28 regulatory complex^{11–13}, and the associated accelerated turnover of TRIM5 α underpin the reprogramming of human TRIM5 α for effective capsid-dependent inhibition of HIV-1 DNA synthesis and infection. These observations identify a mechanism for regulating human TRIM5 α anti-viral function in human cells and rationalize how TRIM5 α participates in the immune control of HIV-1 infection.

IFN α mobilizes the expression of hundreds of ISGs, with the functions and viral substrates of many awaiting definition¹. To identify ISGs that suppress HIV-1 replication, we designed an siRNA library targeting 598 ISGs (plus two negative controls; Supplementary Table 1).

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Data availability. The data that support the findings of this study are available from the corresponding author upon request.

Author Contributions J.M.J.-G, L.A. and M.H.M. conceived the siRNA screen; J.M.J.-G and M.H.M. designed the study and wrote the manuscript with input from all co-authors; J.M.J.-G carried out the experiments and analyzed the data; L.A. and G.B. contributed to the execution of experiments and provided reagents; and, M.H.M. supervised all aspects of the project.

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Focusing on the early stages of infection (up to and including viral transcription), two cultures of IFN α -responsive U87-MG CD4⁺ CXCR4⁺ cells were transfected with each siRNA, with one being maintained with 500 U ml⁻¹ IFN α for 24 h and one without. All cultures were then challenged with HIV-1/Nef-internal ribosome entry signal (IRES)-Renilla, a modified replication-competent reporter virus, and infection quantified by measuring Renilla luciferase activity at 48 h (Fig. 1a; unadjusted levels of infection indicated in left hand panel, and folds of IFN α -mediated suppression indicated in right hand panel; Supplementary Fig. 1). Three genes of well-established relevance to HIV-1 infection and whose suppression corresponded with markedly increased levels of infection in the presence of IFN α were interferon regulatory factor 9 (*IRF9*), *MX2* and *TRIM5 α* (Supplementary Fig. 2 displays the 14 genes with the strongest effects). *IRF9*, a transcription factor required for ISG induction⁴, and *MX2*, an established HIV-1 inhibitory ISG^{6,7}, were anticipated finds, but *TRIM5 α* was completely unexpected. Indeed, human *TRIM5 α* has hitherto been regarded as being virtually inactive against HIV-1; in contrast, non-human *TRIM5 α* proteins, for example from rhesus macaque, are potent HIV-1 restriction factors that recognize post-entry viral capsids to induce their premature fragmentation and the inhibition of reverse transcription^{2,9}.

The restorative impact of each component *TRIM5 α* siRNA of the smartpool against IFN α suppressed HIV-1 infection was confirmed using a HIV-1/Nef-IRES-green fluorescent protein (GFP) reporter virus, with IFN α -mediated inhibition consistently decreasing by 2- to 4-fold relative to the non-silencing siControl (Fig. 1b) and *TRIM5 α* expression being reliably silenced (Fig. 1c). Similar levels of rescue of infection were also observed in a bulk cell population and two independent knock-out cell lines in which the endogenous *TRIM5 α* alleles had been inactivated using CRISPR-Cas9 genome editing (Fig. 1d and 1e; Supplementary Fig. 3); and across a range of IFN α doses (Supplementary Fig. 4). Importantly, re-introduction of a CRISPR-resistant human *TRIM5 α* (r*TRIM5 α*) cDNA into these cells completely restored IFN α -mediated suppression, while no changes in HIV-1 susceptibility were detected with an irrelevant control gene (luciferase) (Fig. 1f). shRNA-mediated silencing was then used to suppress *TRIM5 α* expression in primary human CD4⁺ T cells. Relative to donor matched control samples, reducing *TRIM5 α* levels significantly impeded the IFN α -mediated inhibition of HIV-1 infection, therefore confirming the importance of this IFN α -regulated anti-viral pathway in the principal *in vivo* cell target of HIV-1 infection (Fig. 1g and Supplementary Fig. 5). As noted earlier, it has been established that IFN α is a potent and early inhibitor of HIV-1 reverse transcription following entry into susceptible cells³: *TRIM5 α* 's substantial contribution to this effect was demonstrated by using quantitative PCR to measure the restoration of viral cDNA accumulation following *TRIM5 α* silencing in IFN α treated cells (~5-fold rescue, Fig. 1h). The broad sensitivity of diverse strains to IFN α -activated human *TRIM5 α* , was confirmed using siRNA-mediated silencing and ten HIV-1 isolates engineered to confer GFP expression following productive infection (extents of rescue ranging from 2- to 5-fold, Supplementary Fig. 6).

TRIM5 α -mediated inhibition is initiated through the recognition of viral capsids in the cytoplasm, leading to their fragmentation and the suppression of reverse transcription (viral DNA synthesis). Binding is both sequence- and structure-specific, and is dependent on the capsid lattice (which is composed of multiple hexagonal and pentagonal assemblies the viral

Capsid (CA) protein)¹⁴ and the carboxy-terminal SPRY (a sequence repeat in dual-specificity kinase SPIA and RYanodine receptors) domain of TRIM5 α ^{2,9}. The specificity of human TRIM5 α for HIV-1 CA was demonstrated by the reduced inhibitory effect of IFN α following TRIM5 α silencing that was seen for virus bearing wild-type HIV-1 CA (WT_{CA}), but not for a matched HIV-1-based chimeric virus carrying (non-recognized) CA derived from a simian immunodeficiency virus (SIV) (SIV_{CA}) (Fig. 2a). The suppression of HIV-1 by human TRIM5 α is not, therefore, the result of a generalized induction of TRIM5 α anti-lentiviral activity by IFN α .

The central importance of the SPRY domain for TRIM5 α function in the presence of IFN α was then confirmed in gene transduction-based experiments using U87-MG CD4⁺ CXCR4⁺ cells where endogenous TRIM5 α and MX2 expression were ablated using CRISPR-Cas9 genome editing (Fig. 2b; Supplementary Fig. 7 and 8): luciferase and CD8 served as negative controls for not affecting infection (~5-fold suppression of infection in the presence of IFN α), whereas a previously defined TRIM-cyclophilin (TRIMCyp) fusion protein and MX2 were positive controls^{6,7,10} whose inhibitory phenotypes do not depend on IFN α (Fig. 2b). Consistent with our findings with the endogenous gene, ectopically expressed human TRIM5 α inhibited HIV-1 infection very effectively when IFN α was added (total suppression of ~14-fold). Removing the SPRY domain abolished this effect (~5-fold suppression), whereas including mutations (R332G and R335G) that have previously been shown to confer anti-HIV-1 activity^{15,16} still did so to a minor extent and without compromising activation by IFN α (~14-fold total suppression).

TRIM5 α is an E3 ubiquitin (Ub) ligase that is polyubiquitinated by cellular E2 Ub conjugating enzymes as a pre-requisite for suppressing reverse transcription and infection^{17–19}. Consistent with previous results²⁰, the integrity of TRIM5 α 's ligase domain was confirmed as being necessary for viral inhibition through its inactivation by the H43Y mutation (Fig. 2b). Next, to determine the effects of IFN α on TRIM5 α ubiquitination and ensuing proteasome-mediated degradation, cells were co-transfected with vectors expressing FLAG-tagged TRIM5 α and HA-tagged Ub, and whole cell lysates or immunoprecipitated FLAG-tagged proteins analyzed by immunoblotting (Fig. 3a). IFN α did not substantially affect global levels of protein ubiquitination (lanes 1 to 8), but did induce a marked decrease in the level of ubiquitinated TRIM5 α (lanes 10 and 14). This decrease was reversed by addition of the broadly acting proteasome inhibitor MG132 or the immunoproteasome-specific inhibitor ONX-0914 (lanes 15 and 16, respectively).

Confirmation that IFN α specifically promotes the proteolytic turnover of TRIM5 α was obtained in three ways: first, analysis of cells co-expressing FLAG-tagged TRIM5 α and FLAG-tagged GFP as a control showed that IFN α induced the marked loss of TRIM5 α and that this was blocked by MG132 or ONX-0914 (Fig. 3b); second, levels of endogenous TRIM5 α in IFN α treated cells were increased by adding MG132 or ONX-0914 (Fig. 3c; of note, because TRIM5 α is an ISG itself its levels were modestly increased by IFN α addition compared to untreated cells²¹, despite its accelerated turnover); and third, metabolic-labelling and pulse-chase analysis of FLAG-TRIM5 α in transfected cells demonstrated a shortening in initial half-life from ~100 min to ~70 min (Supplementary Fig. 9). As expected for proteasome-mediated degradation²², blocking the formation of lysine-48-linked

polyubiquitin chains by co-expression of the K48R ubiquitin mutant inhibited IFN α -induced TRIM5 α proteolysis (Supplementary Fig. 10).

The importance of proteasomal activity for IFN α -mediated suppression was confirmed in viral challenge experiments, where both inhibitors significantly reversed the inhibitory influence of IFN α (~3-fold rescue, Fig. 3d; Supplementary Fig. 11). This effect was shown to be dependent on TRIM5 α function since, in the presence of IFN α , HIV-1 infection of cells lacking endogenous TRIM5 α was not improved by MG132 or ONX-0914 (Fig. 3e; Supplementary Fig. 12).

The immunoproteasome is predominantly expressed in cells of haematopoietic origin in response to inflammatory cytokines such as IFN, and differs from the constitutive proteasome with respect to the identity of its three proteolytic β -subunits and dependence upon the PA28 regulatory complex^{11,12,23} (Fig. 4a). Its functional divergence from the constitutive proteasome is incompletely understood, though roles in MHC class I antigen processing, T cell differentiation and cytokine modulation have been described^{24,25}. In light of the ability of ONX-0914, a selective inhibitor of β 5i/PSMB8, to block IFN α -induced TRIM5 α function (Fig. 3e), the expression patterns of the immunoproteasome β -subunits and the PA28A/PA28B subunits of the PA28 regulatory complex were analyzed by immunoblotting (Fig. 4b). With some variation, all five proteins accumulated to higher levels in the presence of IFN α .

These five immunoproteasome components, as well as TRIM5 α , were then individually silenced using siRNA (Fig. 4b) and the effects on IFN α -induced viral suppression determined (Fig. 4c; Supplementary Fig. 13). All knock-downs conferred a degree of relief from inhibition by IFN α , with PA28A silencing being the most effective to the extent that the magnitude of rescue matched that observed with silencing of TRIM5 α itself. Finally, the functional inter-dependence of the IFN α -activated anti-HIV-1 phenotypes of human TRIM5 α and the immunoproteasome was established by the substantial diminution in the level of rescue from IFN α inhibition that was observed following PA28A silencing in cells that lacked TRIM5 α (Fig. 4d; Supplementary Fig. 14).

TRIM5 α restriction has been widely perceived to be important for preventing zoonotic retroviral infections, while being ineffective in controlling viruses in their natural hosts^{2,9}. Though there have been sporadic reports of human TRIM5 α affecting HIV-1 infection either by suppression of certain HLA-associated CTL escape mutant viruses²⁶, or by inducing autophagy in Langerhans cells²⁷, our findings demonstrate broad, non-strain specific inhibition of HIV-1 infection by human TRIM5 α . Importantly, we have shown that TRIM5 α function is operative in CD4⁺ T cells, and is dependent on IFN α and activation of the immunoproteasome. Given that IFN levels are elevated during the acute and chronic phases of natural HIV-1 infection⁴, we surmise that TRIM5 α contributes to the immune control of HIV-1 in infected humans; a conclusion consistent with noted associations between favourable clinical outcomes and elevated TRIM5 α expression²⁸ or specific *TRIM5 α* alleles^{29,30}. Finally, these results further indicate that the functionality of a specific protein can be fine-tuned by the proteasomal landscape of a cell.

Methods

HIV-1 molecular clones and retroviral vectors

The NL4-3/Nef-IRES-Renilla and NL4-3/Nef-IRES-green fluorescent protein (GFP) reporter viruses, the HIV-1 based lentiviral vectors containing wild-type Capsid (WT_{CA}) or CA from SIV_{MAC} (SIV_{CA}), and the infectious provirus molecular clones HIV-1 NL4.3, THRO, WITO, CH106.c, REJO.c, SUMA, pAD17, WARO, R66201 and IDU034.2 have been described^{31,32,33,34,35}.

Cells

U87-MG CD4⁺ CXCR4⁺ cells, which stably express the CD4 and CXCR4 receptors have been described⁶, and 293T cells were obtained from the American Type Culture Collection (ATCC). All cell lines were maintained in complete Dulbecco's modified Eagle medium (DMEM)-GlutaMAX (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U ml⁻¹ penicillin and 100 g ml⁻¹ streptomycin. Human primary CD4⁺ T cells were isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors (approved by the Guy's Research Ethics Committee; Ref 03/02/06) obtained by density gradient centrifugation through LymphoPrep (Axis-Shield) and isolated by negative selection using the CD4⁺ T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Cells were activated using Dynabeads Human T-Activator CD3/CD28 (ThermoFisher) and 50 U ml⁻¹ recombinant IL-2 (rIL-2) (Roche) for 48 h in RPMI 1640-GlutaMAX medium containing 10% heat-inactivated autologous serum, 100 U ml⁻¹ penicillin and 100 U ml⁻¹ streptomycin. Cells were maintained after activation in medium containing 30 U ml⁻¹ of rIL-2. Cells were treated with IFN α -2b (INTRON A, Merck, Sharpe & Dohme Corp.) for 24 h prior to infection. U87-MG CD4⁺ CXCR4⁺ cells were treated with DMSO (Sigma), the proteasome inhibitor MG132 (0.2 μ M, Abcam, ab141003) or the immunoproteasome inhibitor ONX-0914 (1 μ M, ApexBio, A4011).

siRNA library screen

To identify interferon stimulated genes (ISGs) that modulate HIV-1 infection, we performed an ISGs siRNA screen using a custom siRNA library (Dharmacon) containing 600 siRNA targets (Supplementary Table 1). U87-MG CD4⁺ CXCR4⁺ cells were plated at 1.2×10^4 per well in a 96-well plate and were reverse transfected with siRNA (10 nM). 24 h later, cells were treated with or without IFN α (500 U ml⁻¹) and, after 12 h incubation, a second siRNA transfection was performed. 24 h after IFN α treatment, cells were challenged with NL4-3/Nef-IRES-Renilla (15 ng p24^{Gag}) and infectivity was determined 48 h later by measuring Renilla activity.

Plasmids

pEasiLV plasmids expressing luciferase, CD8, MX2 or TRIMCYP have been described⁶. cDNAs encoding TRIM5 α and TRIM5 α isoform X7 lacking the SPRY domain were amplified using the SuperScript III One-Step RT-PCR System with Platinum Taq polymerase (Invitrogen) from 20 ng RNA obtained from IFN α -treated (500 U ml⁻¹) U87-MG CD4⁺ CXCR4⁺ cells and inserted into pEasiLV-MCS6 between the AgeI and XhoI

restriction sites. The TRIM5 α R332G/R335G and H43Y mutants were obtained by site directed mutagenesis (SDM). CRISPR-resistant TRIM5 α constructs (rTRIM5 α) were produced by SDM using primers designed to silently mutate the guide RNA target sequence. pFLAG-TRIM5 α was generated by subcloning a cDNA encoding TRIM5 α with an N-terminal FLAG tag into pCAGGS (Addgene) using the EcoRI and XhoI sites. pCAGGS expressing FLAG-tagged GFP, pFLAG-GFP, was derived from a plasmid encoding HA-tagged GFP36. The vectors encoding HA-tagged ubiquitin and the K48R and K63R mutant derivatives have been described^{37 38}.

Knock-out cells

To generate CRISPR/Cas9 cells, sequences encoding specific guide RNAs targeting TRIM5 α , MX2 or red fluorescent protein (RFP), as a control, were cloned into BsmBI-linearized lentiviral vector pLentiCRISPRv239,40 using the oligonucleotides (forward/reverse) 5'-caccgCCTCCTCCTTTACATTAACC-3' and 5'-aaacGGTAAATGTAAAGGAGGAGGc-3' for TRIM5 α , (5'-caccgGACAACCAGCCCCGAGACAT-3' and 5'-aaacATGTCTCGGGGCTGGTTGTc-3') for MX2 or 5'-caccgCTCAGTTCCAGTACGGCTCCA-3' and 5'-aaacTGGAGCCGTACTGGAAGTACTGAGc-3' for RFP. Vectors expressing the indicated guide RNAs were produced by co-transfection of 293T cells with pLentiCRISPRv2, p8.91 and VSV-G vectors at a ratio of 1.5:1.5:0.375, respectively. U87-MG CD4⁺ CXCR4⁺ cells were transduced with the TRIM5 α targeting or RFP control lentiviral vectors and selected using 1 μ g ml⁻¹ puromycin for three days. Single-cell clones (TRIM5 α #1 and TRIM5 α #2) were derived from the bulk (TRIM5 α) population by single-cell sorting in 96-well plates and assayed for the loss of TRIM5 α by immunoblotting. TRIM5 α gene disruption was validated by PCR amplification and sequencing of the targeted genomic region. To generate TRIM5 α /MX2 double knock-out cells, the TRIM5 α #1 line (Fig. 1d) was further transduced with a high concentration of the MX2 targeting vector and the loss of MX2 expression in the bulk population was confirmed by immunoblotting.

Viral production

All viruses were generated by transfection of 10 cm 293T cultures using TransIT-2020 transfection reagent (Mirus), the medium was changed after 6 h and virus containing medium was filtered and collected 48 h after transfection. NL4-3/Nef-IRES-GFP and NL4-3/Nef-IRES-Renilla viral particles were produced by transfection of 10 μ g of proviral plasmid. For analysis of HIV-1 reverse transcription products and shRNA-mediated TRIM5 α silencing, virus containing supernatants were DNase (RQ1 RNase free DNase; Promega) treated for 1 h at 37 °C, and viruses were purified through a 20% (wt/vol) sucrose cushion at 145,370 \times g for 75 min at 4 °C, re-suspended in phosphate buffered saline (PBS) and stored in aliquots at -80 °C. Lentiviral vectors stocks were produced by co-transfection of 293T cells with plasmids encoding Gag-Pol (p8.91 or p8.91-SIV_{CA}), a mini viral genome bearing a CMV-GFP expression cassette (pRRL.sin.cPPT.CMV/eGFP), and the VSV-G envelope (pMD.G), at a ratio of 4:4:2, respectively³. To generate GFP encoding derivatives of full-length molecular clones NL4.3, THRO, WITO, CH106.c, REJO.c, SUMA, pAD17, WARO, R66201 and IDU034.2 provirus plasmids were co-transfected with the GFP reporter

vector pCSGW and VSV-G at a ratio of 4.5:3:3, respectively⁴¹. Virus titers were determined by challenging U87-MG CD4⁺ CXCR4⁺ cells with different viral dilutions and by quantifying the number of GFP-positive cells after 48 h by flow cytometry (FACSCalibur; BD Biosciences) (a typical example is displayed in Supplementary Fig. 15), and p24^{Gag} content was quantified using an HIV-1 p24 ELISA detection kit (Perkin-Elmer). To produce EasiLV particles, 293T cells were co-transfected with pEasiLV, p8.91, pptTRKrab and VSV-G at a ratio of 1:1:0.5:0.25, respectively⁶. 48 h after transfection, viral particles were harvested, filtered, and used directly to transduce U87-MG CD4⁺ CXCR4⁺ cells. 6 h after transduction, the medium was replaced with fresh medium containing 0.5 µg ml⁻¹ doxycycline (Sigma) to induce transgene expression. 72 h after transduction, the percentage of E2-Crimson-positive cells was scored (typically >80%) by flow cytometry.

shRNA-mediated TRIM5α silencing

TRIM5α silencing in primary human CD4⁺ T cells was achieved using a modified version of the HIV-1-based lentiviral vector pHR-SIREN-S-SBP- LNGFR-W42 where the selectable marker for antibody-free magnetic cell sorting sequence (SBP- LNGFR) was replaced by the E2-Crimson fluorescent reporter gene for flow cytometry analysis, generating the pHR-SIREN-S-E2-W vector. The shRNA targeting sequence for TRIM5α⁴³ (shTRIM5) was 5'-TGGCTTCTGGAATCCTGGTTAA-3' and the scrambled shRNA sequence⁴² (shControl) used as negative control was 5'-GTTATAGGCTCGCAAAGG-3'. Lentiviral vectors were produced by co-transfection of 293T cells with p8.91, VSV-G and the pHR-SIREN-S-E2-W vectors at a ratio of 1:0.5:1.5, respectively. Lentiviral particles were concentrated by ultracentrifugation prior to use for transduction of primary human CD4⁺ T cells at 2000 x g for 2 h. 48 h after transduction, E2-crimson-positive cells were sorted by flow cytometry (typically >90%) and cultured in fresh medium. When indicated, 2.5 x 10⁴ cells per well in 96-well plates were treated with IFNα (2000 U ml⁻¹) for 24 h before infection with NL4-3/Nef-IRES-Renilla (30 ng p24^{Gag}) at 2000 x g for 2 h and infectivity was measured 48 h after infection by measuring Renilla activity.

Quantification of mRNA expression

10⁶ activated primary human CD4⁺ T cells were collected with or without 24 h treatment with IFNα, and total RNA was extracted using the RNeasy Mini Kit (Qiagen). cDNA was generated using 500 ng RNA and the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher) and qPCR of selected genes was performed using the following TaqMan gene expression assays (ThermoFisher): TRIM5α (Hs01552559_m1), MX2 (Hs01550808_m1) and GAPDH (Hs99999905_m1). Expression levels of target genes were normalized to GAPDH.

Measurement of HIV-1 reverse transcription products

10⁵ U87-MG CD4⁺ CXCR4⁺ were reverse transfected with 10 nM of a smartpool siRNA targeting human TRIM5α or a non-targeting siRNA (Dharmacon). Cells were treated with or without 500 U ml⁻¹ IFNα and transfected again 12 h after treatment. 24 h after IFNα treatment, cells were challenged with NL4-3/Nef-IRES-GFP (20 ng p24^{Gag}) for 2 h and 48 h after infection, cells were collected and total DNA extraction was performed using the DNeasy kit (Qiagen). Strong-stop cDNA products were detected using primers that amplify

the regions between nucleotides 500 and 635 of the provirus: oHC64, (5'-TAAGTAGGGGAACCCACTGC) and oHC65 (5'-GCTAGAGATTTTCCACACTG) with probe oHC66 (5'-FAM-ACACAACAGACGGGCACACACTA-TAMRA), where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine. qPCR reactions were performed in triplicate using TaqMan Universal PCR master mix, 900 nM of each primer, and 250 nM of probe. After 10 min at 95 °C, reactions were cycled through 15 s at 95 °C, followed by 1 min at 60 °C for 40 repeats, carried out using the QuantStudio 5 Real-Time PCR System (Applied Biosystems). NL4-3/Nef-IRES-GFP plasmid was diluted in salmon sperm DNA solution (20 ng ml⁻¹) to create dilution standards to calculate relative cDNA copy numbers and confirm the linearity of the assays.

siRNA mediated knock-down

U87-MG CD4⁺ CXCR4⁺ cells were plated at 1.2×10^4 per well in a 96-well plate and were reverse transfected with 10 nM of smartpool siRNAs (Dharmacon) targeting TRIM5 α (M-007100-00-0005), PSMB8 (M-006022-01-0005), PSMB9 (M-006023-02-0005), PSMB10 (M-006019-01-0005), PA28A (M-012254-00-0005), PA28B (M-011370-00-0005) or individual siRNAs targeting TRIM5 α (D-007100-01-0002, D-007100-02-0002, D-007100-03-0002, D-007100-04-0002). A non-targeting siRNA (D-001210-03-05) was used as a negative control. 24 h after the first transfection, cells were treated with or without IFN α (500 U ml⁻¹) and a second siRNA transfection was performed 12 h after treatment. 24 h after IFN α treatment, cells were challenged with the indicated viruses and infectivity was determined 48 h later. To confirm knock-down efficiency, cell lysates were harvested 24 h after IFN α treatment and were subjected to SDS-PAGE and immunoblot assays.

Immunoblotting

Cells were washed with PBS, lysed in sample buffer, boiled for 3 min, resolved by SDS-PAGE and analyzed by immunoblotting using primary antibodies specific for human MX2 (sc-47197 (N-17), Santa Cruz Biotechnology), human TRIM5 α (ab4389, Abcam), human PSMB8 (ab3329, Abcam), human PSMB9 (ab3328, Abcam), human PSMB10 (ab77735, Abcam), human PA28A (ab155091, Abcam), human PA28B (ab183727, Abcam), FLAG (HRP-conjugated M2, Sigma), HA (HRP-conjugated 3F10, Sigma) or human α -tubulin (DM1A, Sigma), and detected using either horseradish peroxidase-conjugated secondary antibodies and chemiluminescence (ECL⁺ western blotting substrate, Pierce) or IRDye®-800CW-labelled secondary antibodies and the LI-COR infrared imaging technology (LI-COR UK LTD).

Immunoprecipitation

3×10^6 U87-MG CD4⁺ CXCR4⁺ cells were seeded in 10 cm tissue culture plates and transfected with the indicated plasmids expressing FLAG-TRIM5 α and/or HA-ubiquitin at a ratio of 1:0.5, using TransIT-2020 transfection reagent according to manufacturer's instructions. After 24 h, indicated cells were treated with fresh medium containing or not IFN α (500 U ml⁻¹) and DMSO, MG132 (0.2 μ M) or ONX-0914 (1 μ M). 24 h after treatment, cells were washed twice with PBS, harvested with lysis buffer (1x PBS, 0.5% Triton X-100 and protease inhibitor cocktail (Roche)) and disrupted by brief sonication. Lysates were cleared by centrifugation at $10000 \times g$ for 10 min at 4 °C and FLAG-TRIM5 α

was immunoprecipitated using anti-FLAG M2 magnetic beads (M8823-5ML, Sigma) for 2 h at 4 °C and the beads washed a further 4 times in lysis buffer before adding sample buffer (200 mM Tris-HCl pH 6.8, 5.2% SDS, 20% glycerol, 0.1% bromophenol blue, 5% β -mercaptoethanol). HA-tagged proteins (ubiquitinated proteins) were resolved by SDS-PAGE and detected by immunoblotting using HRP-conjugated anti-HA antibodies.

S³⁵ pulse-chase labelling and radio-immunoprecipitation

U87-MG CD4⁺ CXCR4⁺ cells were seeded at 2×10^5 per well in 6-well plates and transfected with pFLAG-TRIM5 α using TransIT-2020 transfection reagent according to the manufacturer's instructions. After 24 h, indicated cells were treated with fresh medium containing or not IFN α (500 U ml⁻¹). 24 h later, cells were washed twice with PBS and incubated for 20 min in cysteine/methionine depleted DMEM (Gibco) at 37°C. The medium was replaced by depletion medium containing 0.25 mCi ml⁻¹ S³⁵-labelled cysteine/methionine and the cells maintained for 10 min at 37°C. Cells were then washed twice with PBS and incubated in DMEM. The cells were harvested at various times with lysis buffer (1x PBS, 0.5% Triton X-100 and protease inhibitor cocktail), incubated on ice for 10 min and clarified by centrifugation at $10000 \times g$ for 10 min at 4 °C. FLAG-TRIM5 α was immunoprecipitated using anti-FLAG M2 magnetic beads, resolved by SDS-PAGE and exposed on a phosphor image screen over several days before development on a Typhoon Trio phosphorimager (GE Healthcare).

Statistical analysis

Results in bar charts are expressed as means \pm standard deviation (s.d.) for experimental replicates in each case. Differences between the experimental groups were evaluated, where indicated, by paired or unpaired two-tailed *t*-tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Schoggins JW, et al. A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature*. 2011; 472:481–485. [PubMed: 21478870]
2. Malim MH, Bieniasz PD. HIV Restriction Factors and Mechanisms of Evasion. *Cold Spring Harb Perspect Med*. 2012; 2:a006940. [PubMed: 22553496]
3. Goujon C, Malim MH. Characterization of the alpha interferon-induced postentry block to HIV-1 infection in primary human macrophages and T cells. *J Virol*. 2010; 84:9254–9266. [PubMed: 20610724]

4. Doyle T, Goujon C, Malim MH. HIV-1 and interferons: who's interfering with whom? *Nat Rev Microbiol.* 2015; 13:403–413. [PubMed: 25915633]
5. Cheney KM, McKnight A. Interferon-alpha mediates restriction of human immunodeficiency virus type-1 replication in primary human macrophages at an early stage of replication. *PLoS One.* 2010; 5:e13521. [PubMed: 20975956]
6. Goujon C, et al. Human MX2 is an interferon-induced post-entry inhibitor of HIV-1 infection. *Nature.* 2013; 502:559–562. [PubMed: 24048477]
7. Kane M, et al. MX2 is an interferon-induced inhibitor of HIV-1 infection. *Nature.* 2013; 502:563–566. [PubMed: 24121441]
8. Liu Z, et al. The interferon-inducible MxB protein inhibits HIV-1 infection. *Cell Host Microbe.* 2013; 14:398–410. [PubMed: 24055605]
9. Stremlau M, et al. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature.* 2004; 427:848–853. [PubMed: 14985764]
10. Sayah DM, Sokolskaja E, Berthoux L, Luban J. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. *Nature.* 2004; 430:569–573. [PubMed: 15243629]
11. Akiyama K, et al. Replacement of proteasome subunits X and Y by LMP7 and LMP2 induced by interferon-gamma for acquirement of the functional diversity responsible for antigen processing. *FEBS Lett.* 1994; 343:85–88. [PubMed: 8163024]
12. Ma CP, Slaughter CA, DeMartino GN. Identification, purification, and characterization of a protein activator (PA28) of the 20 S proteasome (macropain). *J Biol Chem.* 1992; 267:10515–10523. [PubMed: 1587832]
13. Groettrup M, Kirk CJ, Basler M. Proteasomes in immune cells: more than peptide producers? *Nat Rev Immunol.* 2010; 10:73–78. [PubMed: 20010787]
14. Wagner JM, et al. General Model for Retroviral Capsid Pattern Recognition by TRIM5 Proteins. *J Virol.* 2018; 92
15. Pham QT, Bouchard A, Grutter MG, Berthoux L. Generation of human TRIM5alpha mutants with high HIV-1 restriction activity. *Gene Ther.* 2010; 17:859–871. [PubMed: 20357830]
16. Yap MW, Nisole S, Stoye JP. A single amino acid change in the SPRY domain of human Trim5alpha leads to HIV-1 restriction. *Curr Biol.* 2005; 15:73–78. [PubMed: 15649369]
17. Fletcher AJ, et al. TRIM5alpha requires Ube2W to anchor Lys63-linked ubiquitin chains and restrict reverse transcription. *EMBO J.* 2015; 34:2078–2095. [PubMed: 26101372]
18. Campbell EM, et al. TRIM5alpha-Mediated Ubiquitin Chain Conjugation Is Required for Inhibition of HIV-1 Reverse Transcription and Capsid Destabilization. *J Virol.* 2016; 90:1849–1857. [PubMed: 26676782]
19. Diaz-Griffero F, et al. Rapid turnover and polyubiquitylation of the retroviral restriction factor TRIM5. *Virology.* 2006; 349:300–315. [PubMed: 16472833]
20. Sawyer SL, Wu LI, Akey JM, Emerman M, Malik HS. High-frequency persistence of an impaired allele of the retroviral defense gene TRIM5alpha in humans. *Curr Biol.* 2006; 16:95–100. [PubMed: 16401428]
21. Carthagena L, et al. Human TRIM gene expression in response to interferons. *PLoS One.* 2009; 4:e4894. [PubMed: 19290053]
22. Komander D, Rape M. The ubiquitin code. *Annu Rev Biochem.* 2012; 81:203–229. [PubMed: 22524316]
23. Preckel T, et al. Impaired immunoproteasome assembly and immune responses in PA28-/- mice. *Science.* 1999; 286:2162–2165. [PubMed: 10591649]
24. Kloetzel PM. Antigen processing by the proteasome. *Nat Rev Mol Cell Biol.* 2001; 2:179–187. [PubMed: 11265247]
25. Muchamuel T, et al. A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nat Med.* 2009; 15:781–787. [PubMed: 19525961]
26. Granier C, et al. Pressure from TRIM5alpha contributes to control of HIV-1 replication by individuals expressing protective HLA-B alleles. *J Virol.* 2013; 87:10368–10380. [PubMed: 23864638]

27. Ribeiro CM, et al. Receptor usage dictates HIV-1 restriction by human TRIM5alpha in dendritic cell subsets. *Nature*. 2016; 540:448–452. [PubMed: 27919079]
28. Sewram S, et al. Human TRIM5alpha expression levels and reduced susceptibility to HIV-1 infection. *J Infect Dis*. 2009; 199:1657–1663. [PubMed: 19388851]
29. Celerino da Silva R, et al. TRIM5 gene polymorphisms in HIV-1-infected patients and healthy controls from Northeastern Brazil. *Immunol Res*. 2016; 64:1237–1242. [PubMed: 27388872]
30. van Manen D, et al. The effect of Trim5 polymorphisms on the clinical course of HIV-1 infection. *PLoS Pathog*. 2008; 4:e18. [PubMed: 18248091]
31. Schindler M, et al. Down-modulation of mature major histocompatibility complex class II and up-regulation of invariant chain cell surface expression are well-conserved functions of human and simian immunodeficiency virus nef alleles. *J Virol*. 2003; 77:10548–10556. [PubMed: 12970439]
32. Naldini L, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*. 1996; 272:263–267. [PubMed: 8602510]
33. Cordeil S, et al. Evidence for a different susceptibility of primate lentiviruses to type I interferons. *J Virol*. 2013; 87:2587–2596. [PubMed: 23255800]
34. Ochsenbauer C, et al. Generation of transmitted/founder HIV-1 infectious molecular clones and characterization of their replication capacity in CD4 T lymphocytes and monocyte-derived macrophages. *J Virol*. 2012; 86:2715–2728. [PubMed: 22190722]
35. Parrish NF, et al. Phenotypic properties of transmitted founder HIV-1. *Proc Natl Acad Sci U S A*. 2013; 110:6626–6633. [PubMed: 23542380]
36. Dicks MD, et al. Oligomerization Requirements for MX2-Mediated Suppression of HIV-1 Infection. *J Virol*. 2015; 90:22–32. [PubMed: 26446602]
37. Treier M, Staszewski LM, Bohmann D. Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain. *Cell*. 1994; 78:787–798. [PubMed: 8087846]
38. Dart AE, et al. PAK4 promotes kinase-independent stabilization of RhoU to modulate cell adhesion. *J Cell Biol*. 2015; 211:863–879. [PubMed: 26598620]
39. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods*. 2014; 11:783–784. [PubMed: 25075903]
40. Shalem O, et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science*. 2014; 343:84–87. [PubMed: 24336571]
41. Schaller T, et al. Effects of Inner Nuclear Membrane Proteins SUN1/UNC-84A and SUN2/UNC-84B on the Early Steps of HIV-1 Infection. *J Virol*. 2017; 91
42. Matheson NJ, Peden AA, Lehner PJ. Antibody-free magnetic cell sorting of genetically modified primary human CD4+ T cells by one-step streptavidin affinity purification. *PLoS One*. 2014; 9:e111437. [PubMed: 25360777]
43. Pertel T, et al. TRIM5 is an innate immune sensor for the retrovirus capsid lattice. *Nature*. 2011; 472:361–365. [PubMed: 21512573]

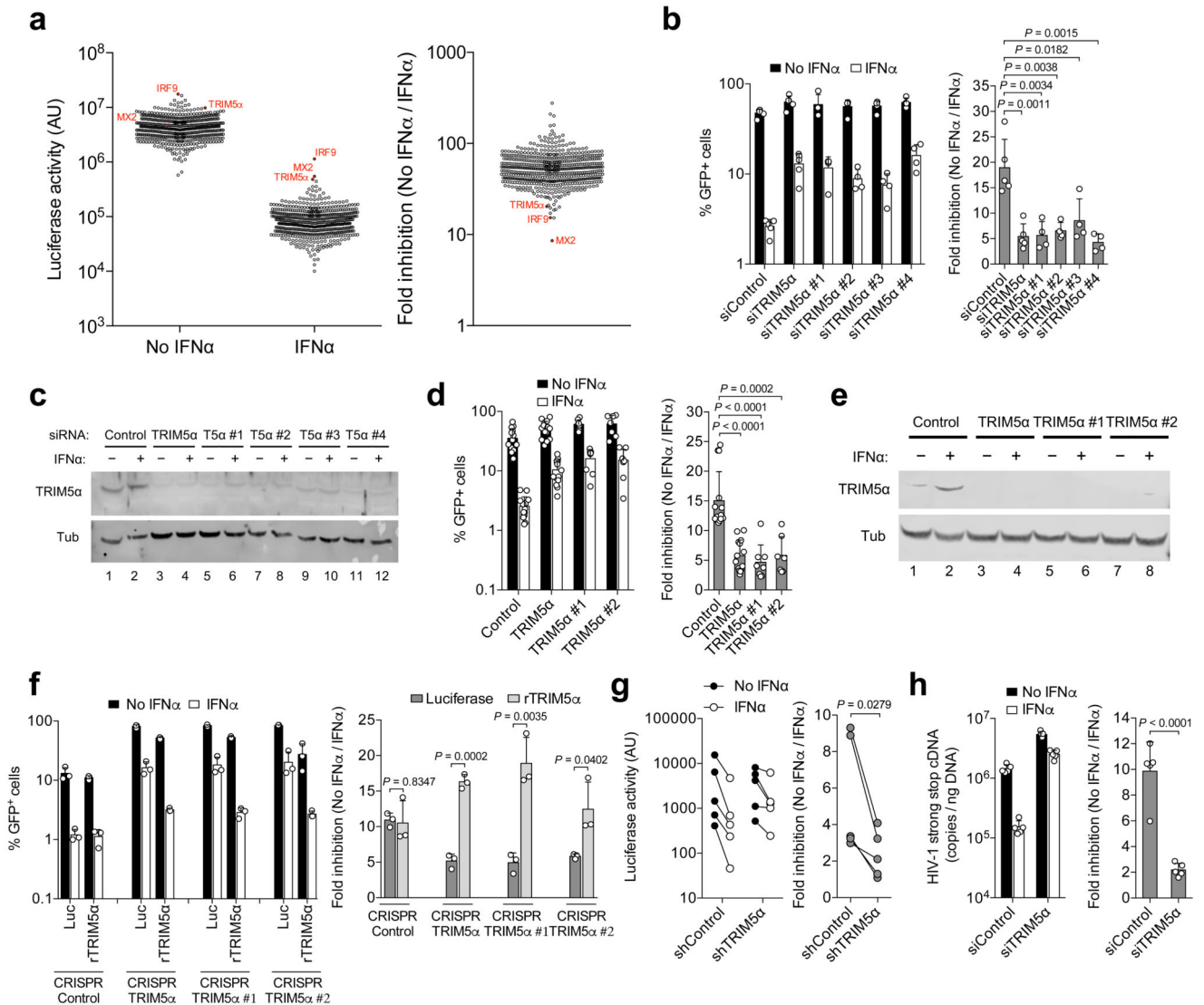


Figure 1. Human TRIM5 α is a key effector in the interferon-induced suppression of HIV-1 infection.

a, Dot plots of NL4-3/Nef-IRES-Renilla infectivity and IFN α -induced fold inhibition at 48 h post-infection in U87-MG CD4⁺ CXCR4⁺ cells doubly transfected with siRNAs targeting 598 ISGs and 2 negative controls with or without the addition of 500 U ml⁻¹ IFN α for 24 h. Three notably influential ISGs are indicated in red. **b**, Percentage of GFP-positive cells and IFN α -induced fold inhibition in U87-MG CD4⁺ CXCR4⁺ cells infected with NL4-3/Nef-IRES-GFP after TRIM5 α silencing using SMARTpool (n = 5) or individual siRNAs (n = 4) with or without added 500 U ml⁻¹ IFN α . **c**, Immunoblot analysis of TRIM5 α expression in U87-MG CD4⁺ CXCR4⁺ cells after siRNA transfection, α -tubulin served as a loading control. One representative immunoblot from two independent experiments is shown. **d**, NL4-3/Nef-IRES-GFP infection and IFN α -induced inhibition in U87-MG CD4⁺ CXCR4⁺ bulk [TRIM5 α (n = 14)] and clonal [TRIM5 α #1 (n = 8) and #2 (n = 7)] cell lines transduced to express TRIM5 α specific guide RNAs, with or without added 500 U ml⁻¹

IFN α . CRISPR/Cas9 control cells expressed an unrelated guide RNA (n = 14). **e**, Ablation of TRIM5 α expression in CRISPR/Cas9 engineered U87-MG CD4⁺ CXCR4⁺ cells was verified by immunoblotting, α -tubulin served as a loading control. One representative immunoblot from three independent experiments is shown. **f**, NL4-3/Nef-IRES-GFP infectivity and IFN α -induced inhibition at 48 h post-infection in TRIM5 α deficient U87-MG CD4⁺ CXCR4⁺ cells (CRISPR TRIM5 α , TRIM5 α #1 and TRIM5 α #2) or cells expressing an unrelated guide RNA (CRISPR control) transduced with EasiLV lentivirus vectors expressing luciferase (Luc) or a CRISPR-resistant TRIM5 α (rTRIM5 α) with or without added 500 U ml⁻¹ IFN α (n = 3). **g**, NL4-3/Nef-IRES-Renilla infectivity and IFN α -induced inhibition at 48 h post-infection in primary human CD4⁺ T cells transduced with shRNAs targeting TRIM5 α or a control shRNA, and treated with or without 2000 U ml⁻¹ IFN α for 24 h prior to infection (n = 5). **h**, U87-MG CD4⁺ CXCR4⁺ cells were transfected with control or TRIM5 α -specific siRNAs and treated or not with 500 U ml⁻¹ IFN α for 24 h before 2 h infections with NL4-3/Nef-IRES-GFP (corresponding to 20 ng p24^{Gag}). DNA was harvested at 48 h post-infection and early reverse transcription products (strong stop) and IFN α -induced inhibition were determined by qPCR (n = 5). Data are represented as the mean \pm s.d. *P* values (95% confidence interval) were determined using two-sided, unpaired (b, d, f and h) or paired (g) t-tests.

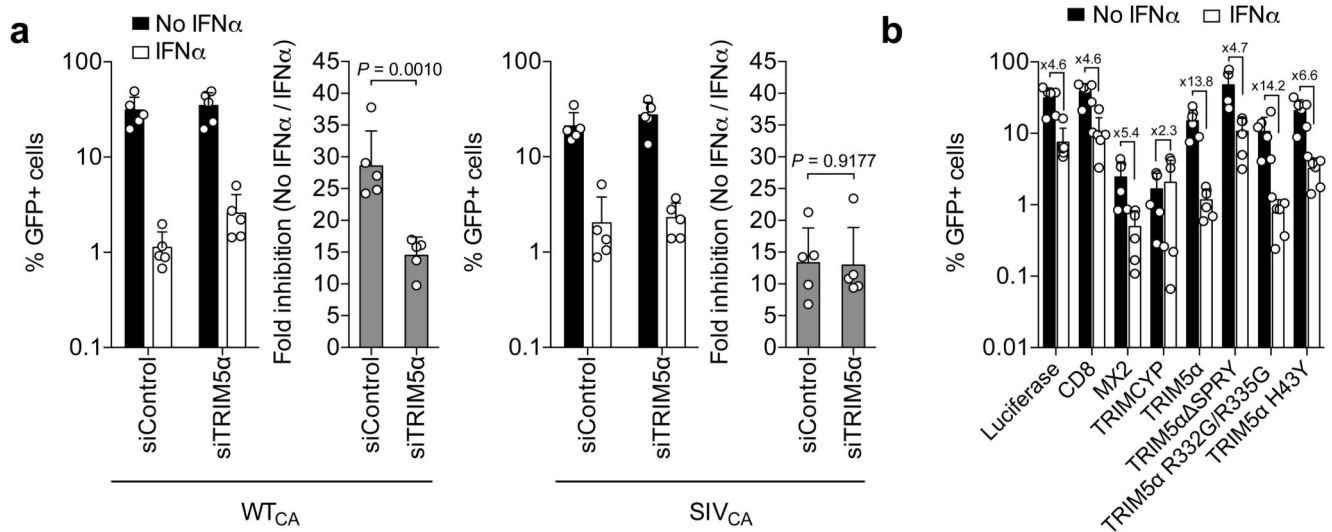


Figure 2. HIV-1 Capsid determines IFN α -induced restriction by human TRIM5 α .

a, Percentage of GFP-positive cells and IFN α -induced inhibition at 48 h post-infection in U87-MG CD4⁺ CXCR4⁺ cells challenged with GFP-encoding HIV-1-based vectors containing wild-type CA (WT_{CA}) or CA from SIV_{MAC} (SIV_{CA}). TRIM5 α was silenced using SMARTpool siRNAs and cells treated with or without 500 U ml⁻¹ IFN α before infection (n = 5). *P* values (95% confidence interval) were determined using two-sided, unpaired t-tests. **b**, NL4-3/Nef-IRES-GFP infectivity and IFN α -induced inhibition at 48 h post-infection in TRIM5 α /MX2 doubly deficient U87-MG CD4⁺ CXCR4⁺ cells (Supplementary Fig. 7) transduced with EasiLV lentivirus vectors expressing luciferase, CD8, CRISPR-resistant MX2, TRIMCYP or CRISPR-resistant TRIM5 α , TRIM5 α SPRY, TRIM5 α R332G/R335G or TRIM5 α H43Y with or without added 500 U ml⁻¹ IFN α (n = 6). Numbers represent fold inhibition of HIV-1 infection after IFN α treatment. Data are represented as the mean \pm s.d.

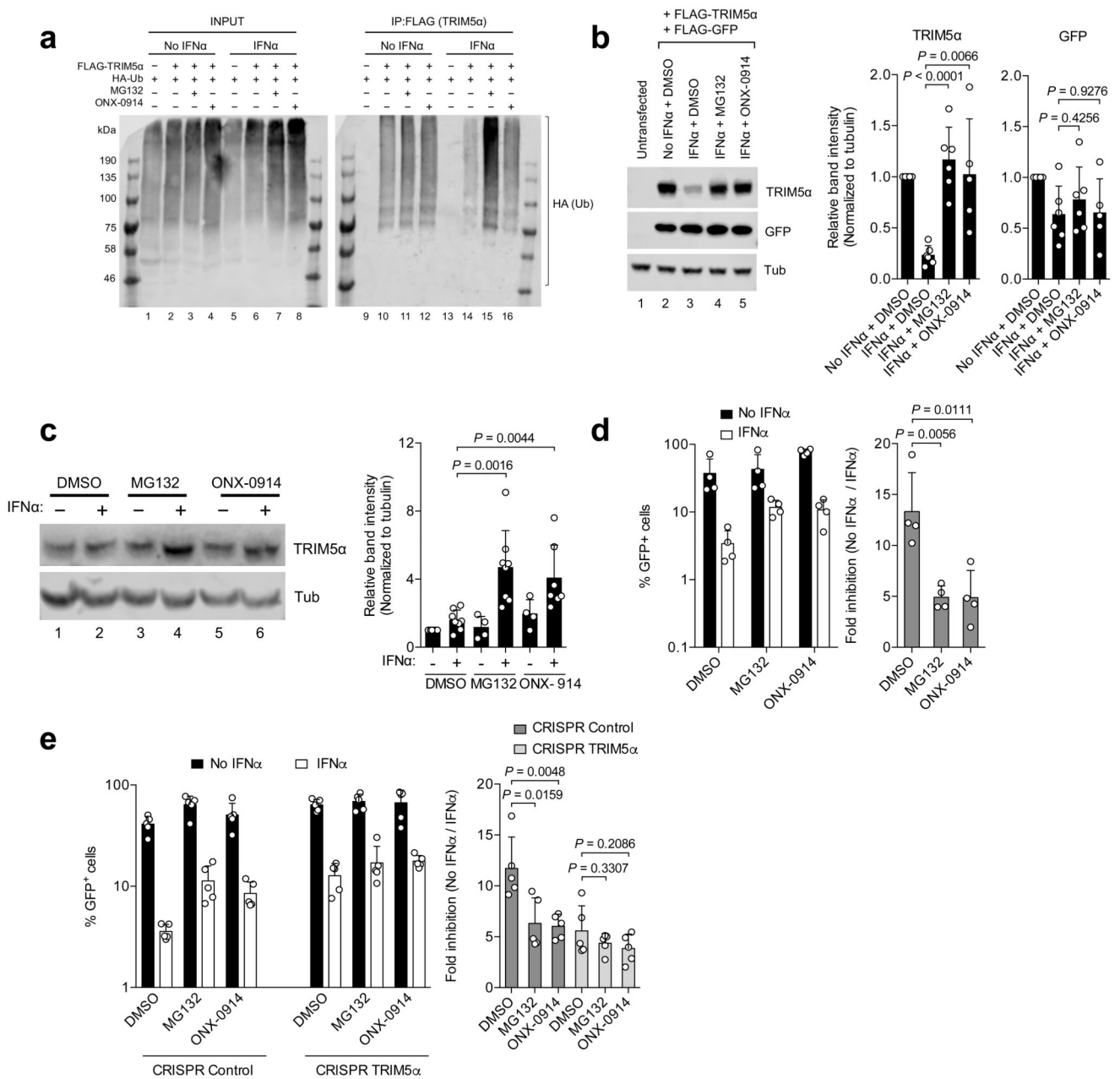


Figure 3. HIV-1 restriction by human TRIM5 α requires a functional proteasome system.

a, U87-MG CD4⁺ CXCR4⁺ cells were co-transfected with a vector expressing HA-ubiquitin37 and pFLAG-TRIM5 α or a control and treated with or without 500 U ml⁻¹ IFN α for 24 h in presence of DMSO (negative control), the proteasome inhibitor MG132 (0.2 μ M), or the immunoproteasome inhibitor ONX-9014 (1 μ M). Whole cell lysates and immunoprecipitated TRIM5 α were analyzed by immunoblotting using an anti-HA (ubiquitin) antibody. One representative immunoblot from three independent experiments is shown. **b**, U87-MG CD4⁺ CXCR4⁺ cells were co-transfected with pFLAG-TRIM5 α and pFLAG-GFP. 24 h after transfection, cells were treated with or without 500 U ml⁻¹ IFN α for

24 h in the presence of DMSO (n = 6), MG132 (n = 6) or ONX-0914 (n = 5), lysed and analyzed by immunoblotting using antibodies specific for TRIM5 α or FLAG. α -tubulin served as a loading control. The bar graphs represent the relative band intensities for each protein normalized to α -tubulin. **c**, U87-MG CD4⁺ CXCR4⁺ cells were cultured with or without 500 U ml⁻¹ IFN α , then for with DMSO (n = 8), MG132 (n = 4 without IFN α and n = 8 with IFN α) or ONX-9014 (n = 4 without IFN α and n = 7 with IFN α) for 24 h and endogenous TRIM5 α levels were determined by immunoblotting. α -tubulin served as a loading control. The bar graph represents the relative band intensities for TRIM5 α normalized to α -tubulin. **d**, NL4-3/Nef-IRES-GFP infectivity and IFN α -induced inhibition at 48 h post-infection in U87-MG CD4⁺ CXCR4⁺ cells with or without added 500 U ml⁻¹ IFN α for 24 h before infection in the presence of DMSO, MG132, or ONX-0914 (n = 4). **e**, NL4-3/Nef-IRES-GFP infectivity and IFN α -induced inhibition at 48 h post-infection in U87-MG CD4⁺ CXCR4⁺ cells expressing an unrelated guide RNA (CRISPR control) or specific guide RNAs targeting TRIM5 α (CRISPR TRIM5 α ; bulk Figure 1d) treated with or without 500 U ml⁻¹ IFN α in the presence of DMSO, MG132 or ONX-0914 prior to infection (n = 5). Data are represented as the mean \pm s.d. *P* values (95% confidence interval) were determined using two-sided, unpaired t-tests.

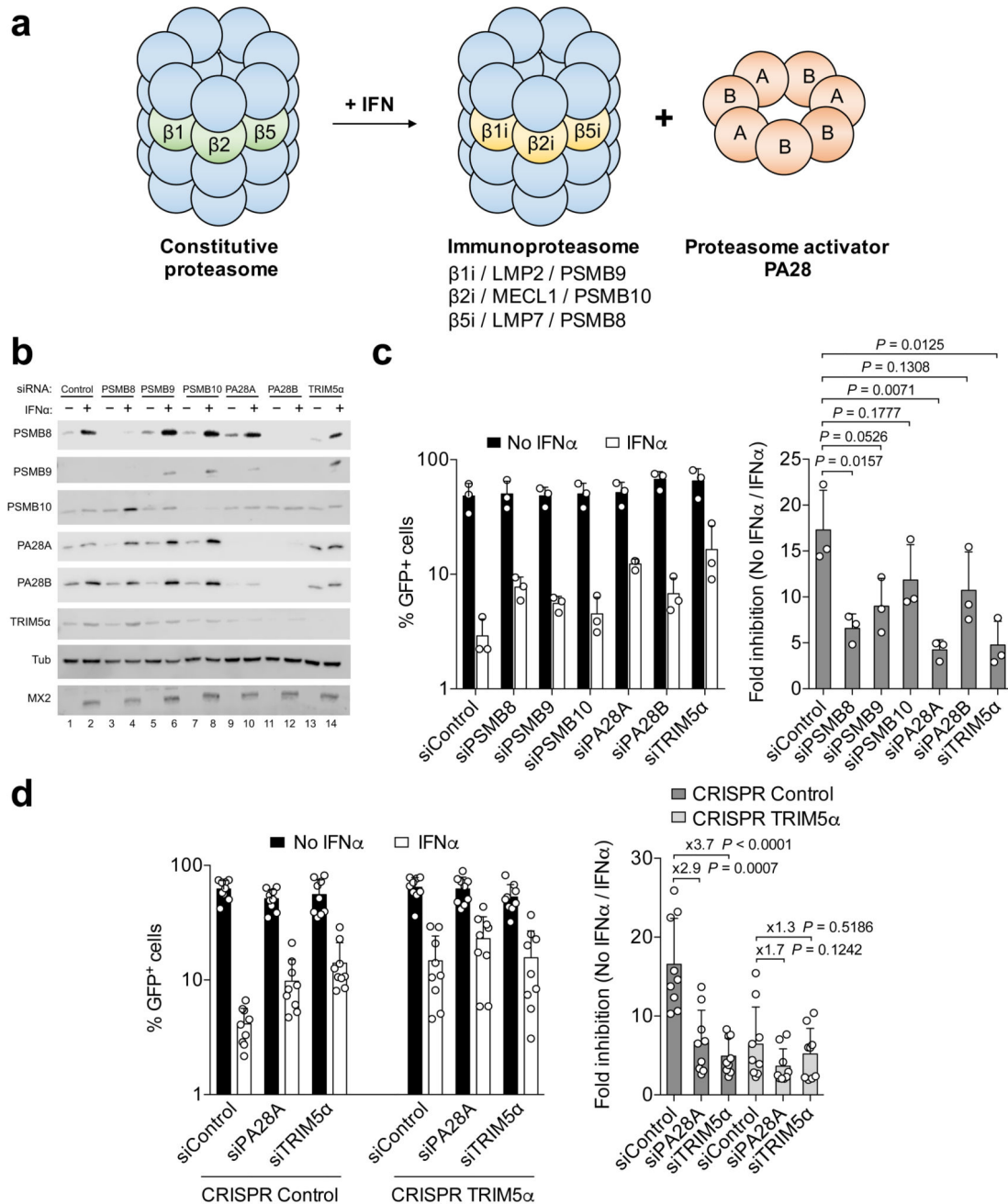


Figure 4. HIV-1 restriction by human TRIM5 α is regulated by the immunoproteasome.
a, Upon IFN α stimulation, the catalytic subunits ($\beta 1$, $\beta 2$, and $\beta 3$) of the constitutive proteasome are replaced by inducible catalytic subunits ($\beta 1i$, $\beta 2i$ and $\beta 5i$) and expression of the proteasome activator PA28 subunits A and B is induced. **b**, Protein levels of immunoproteasome components and PA28 subunits in U87-MG CD4⁺ CXCR4⁺ cells after siRNA transfection with or without 500 U ml⁻¹ IFN α were determined by immunoblotting. α -tubulin served as a loading control. Immunoblot analysis of the panel was performed once in entirety. **c**, NL4-3/Nef-IRES-GFP infectivity and IFN α -induced inhibition at 48 h post-

infection in U87-MG CD4⁺ CXCR4⁺ cells after siRNA-mediated silencing of the indicated genes with or without addition of 500 U ml⁻¹ IFN α 24 h before infection (n = 3). **d**, NL4-3/Nef-IRES-GFP infectivity and IFN α -induced inhibition at 48 h post-infection after siRNA-mediated silencing of PA28A or TRIM5 α in U87-MG CD4⁺ CXCR4⁺ cells expressing an unrelated guide RNA (CRISPR control) or specific guide targeting TRIM5 α (CRISPR TRIM5 α). 500 U ml⁻¹ IFN α was added for 24 h before infection (n = 9). Numbers represent changes in the folds of inhibition. Data are represented as the mean \pm s.d. *P* values (95% confidence interval) were determined using two-sided, unpaired t-tests.