Cooperativity among Rev-Associated Nuclear Export Signals Regulates HIV-1 Gene Expression and Is a Determinant of Virus Species Tropism

Mounavya Aligeti,^{a,*} Ryan T. Behrens,^a Ginger M. Pocock,^{a,b} Johannes Schindelin,^{b,c} Christian Dietz,^d Kevin W. Eliceiri,^{b,c} Chad M. Swanson,^e Michael H. Malim,^e Paul Ahlquist,^{a,b,f} Nathan M. Sherer^a

McArdle Laboratory for Cancer Research and Institute for Molecular Virology, University of Wisconsin—Madison, Madison, Wisconsin, USA^a; Morgridge Institute for Research, Madison, Wisconsin, USA^b; Laboratory for Optical and Computational Instrumentation, University of Wisconsin—Madison, Madison, Wisconsin, USA^c; Department of Computer and Information Science, University of Constance, Constance, Germany^d; Department of Infectious Diseases, King's College London, London, United Kingdom^e; Howard Hughes Medical Institute, University of Wisconsin—Madison, Madison, Wisconsin, USA^f

ABSTRACT

Murine cells exhibit a profound block to HIV-1 virion production that was recently mapped to a species-specific structural attribute of the murine version of the chromosomal region maintenance 1 (mCRM1) nuclear export receptor and rescued by the expression of human CRM1 (hCRM1). In human cells, the HIV-1 Rev protein recruits hCRM1 to intron-containing viral mRNAs encoding the Rev response element (RRE), thereby facilitating viral late gene expression. Here we exploited murine 3T3 fibroblasts as a gain-of-function system to study hCRM1's species-specific role in regulating Rev's effector functions. We show that Rev is rapidly exported from the nucleus by mCRM1 despite only weak contributions to HIV-1's posttranscriptional stages. Indeed, Rev preferentially accumulates in the cytoplasm of murine 3T3 cells with or without hCRM1 expression, in contrast to human HeLa cells, where Rev exhibits striking *en masse* transitions between the nuclear and cytoplasmic compartments. Efforts to bias Rev's trafficking either into or out of the nucleus revealed that Rev encoding a second CRM1 binding domain (Rev-2xNES) or Rev-dependent viral *gag-pol* mRNAs bearing tandem RREs (GP-2xRRE), rescue virus particle production in murine cells even in the absence of hCRM1. Combined, these results suggest a model wherein Rev-associated nuclear export signals cooperate to regulate the number or quality of CRM1's interactions with viral Rev/RRE ribonucleoprotein complexes in the nucleus. This mechanism regulates CRM1-dependent viral gene expression and is a determinant of HIV-1's capacity to produce virions in nonhuman cell types.

IMPORTANCE

Cells derived from mice and other nonhuman species exhibit profound blocks to HIV-1 replication. Here we elucidate a block to HIV-1 gene expression attributable to the murine version of the CRM1 (mCRM1) nuclear export receptor. In human cells, hCRM1 regulates the nuclear export of viral intron-containing mRNAs through the activity of the viral Rev adapter protein that forms a multimeric complex on these mRNAs prior to recruiting hCRM1. We demonstrate that Rev-dependent gene expression is poor in murine cells despite the finding that, surprisingly, the bulk of Rev interacts efficiently with mCRM1 and is rapidly exported from the nucleus. Instead, we map the mCRM1 defect to the apparent inability of this factor to engage Rev multimers in the context of large viral Rev/RNA ribonucleoprotein complexes. These findings shed new light on HIV-1 gene regulation and could inform the development of novel antiviral strategies that target viral gene expression.

The nuclear pore complex (NPC) represents a key barrier to the replication of many viruses that infect eukaryotic cells (1–3). For retroviruses such as the primate lentivirus human immunodeficiency virus type 1 (HIV-1), the late, productive phases of the viral life cycle require efficient nuclear export of unspliced, and thus intron-containing, viral genomic RNAs (gRNAs) that (i) encode the group-specific antigen (Gag) and Gag-Pol polyproteins that form viral capsids and (ii) serve as the core genetic substrate bound by Gag and packaged into new virions (4). Because splicing is typically a prerequisite for mRNA nuclear export across the NPC, retroviruses have adopted specialized strategies to ensure gRNA nuclear export and cytoplasmic utilization (5, 6). These strategies involve the gRNA encoding *cis*-acting RNA structural elements that either directly recruit cellular nuclear export factors (such as constitutive transport elements) or bind to viral adaptor proteins such as HIV-1's Rev protein. The best-characterized constitutive transport element is encoded by the betaretrovirus Mason-Pfizer monkey virus and binds a heterodimer of the nuclear

export factors NXF1 and NXT (7). In contrast, HIV-1 Rev recruits the cellular chromosomal region maintenance 1 (CRM1, also known as exportin-1/XPO-1) nuclear export receptor (8–10) to gRNAs and partially spliced viral mRNAs encoding the Rev re-

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Address correspondence to Nathan M. Sherer, nsherer@wisc.edu.

* Present address: Mounavya Aligeti, Department of Molecular Genetics and Microbiology, Duke University, Durham, North Carolina, USA.

M.A. and R.T.B. contributed equally to this article.

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sponse element (RRE) (9). Why HIV-1 exploits the CRM1 pathway as opposed to the NXF1/NXT pathway that mediates the bulk of cellular mRNA nuclear export is unknown. However, Rev and Rev-equivalent proteins found in other retroviruses clearly provide a sophisticated feedback mechanism for tight regulation of late viral gene expression, with possible roles in viral persistence and immune evasion (11–14). Moreover, Rev may govern the efficiency of downstream cytoplasmic events, including Gag/Gag-Pol translation (15), gRNA packaging (16), and virus particle assembly occurring at the plasma membrane (17, 18), through regulating the composition of distinct Rev/mRNA viral ribonucleoprotein (vRNP) complexes (19, 20).

HIV-1 Rev is a 116-amino-acid phosphoprotein translated from fully spliced viral mRNA and encodes four functional motifs required for late viral gene expression (depicted in Fig. 2A and reviewed in reference 9). These motifs include (i) a nuclear/nucleolar localization signal (NLS/NoLS) that interacts directly with importin-beta to deliver Rev to the nucleus, (ii) a RNA-binding domain (RBD) that targets Rev to the RRE, (iii) sequences that mediate Rev-Rev self-interactions in complex with the RRE, and (iv) a C-terminal leucine-rich nuclear export signal (NES) that binds to CRM1. To fully activate late viral gene expression, Rev must be efficiently imported into the nucleus, undergo a stepwise multimerization on the RRE scaffold, and then engage CRM1 to initiate the export of Rev/RRE vRNP transport complexes from the nucleus to the cytoplasm. Rev's NLS/NoLS and RBD are encoded within overlapping sequences that make up Rev's N-terminal "arginine-rich" domain (ARD), flanked by hydrophobic residues that mediate Rev-Rev interactions (21–23). Rev multimerization is initiated after Rev's binding to one or more RRE stem-loops, followed by cooperative assembly of a series of Rev dimers in tight association with the RRE (21–26). Rev's C-terminal NES is predicted to extend outward from the Rev/RRE complex to mediate binding to one, or possibly two, molecules of CRM1 (21).

Rev's leucine-rich NES peptide ("LPPLERLTL") was one of the first transferable nuclear export signals to be identified (27–29) and subsequently played a seminal role in defining CRM1 as the core cellular receptor for NES-bearing cargoes (30–32). Outside the context of HIV-1 infection, CRM1 regulates the proper nucleocytoplasmic distribution of more than 200 cellular NES-encoding proteins that regulate diverse signaling pathways (30–36). Moreover, in addition to viral mRNAs, CRM1 regulates the nuclear export of several cellular RNAs, including 5S rRNAs, U snRNAs, and a subset of tightly regulated cellular mRNAs through the activity of NES-bearing adaptor proteins (37). CRM1 is a member of the karyopherin family of nucleocytoplasmic transport proteins regulated by the Ran GTPase and is a toroid-shaped protein consisting of 21 antiparallel alpha helices known as Huntingtin's/Elongation factor 3, protein phosphatase 2A, mTOR (HEAT) repeats. CRM1's unidirectional transport is regulated by a nucleocytoplasmic gradient of the Ran GTPase, wherein CRM1 encounters high levels of RanGTP in the nucleus but RanGTP is rapidly hydrolyzed to RanGDP in the cytoplasm. CRM1-RanGTP binding in the nucleus triggers the opening of a CRM1 surface cleft located between HEAT repeats 11 and 12 that subsequently engages an NES peptide through largely hydrophobic interactions (38). CRM1/Ran-GTP/cargo transport complexes are subsequently transported across the NPC to the cytoplasm,

where RanGTP hydrolysis stimulates cargo release and subsequent recycling of CRM1 back to the nucleus.

We and two other groups recently identified the murine ortholog of CRM1 (mCRM1) as the source of a profound, species-specific block to HIV-1 virion production evident in cells derived from muroid rodents (e.g., mice, rats, and hamsters) (39–42). This block, among others, was of long-standing interest both due to the dearth of knowledge regarding cellular contributions to HIV-1's posttranscriptional stages, as well as the persisting need for a genetically tractable small animal model for studying HIV-1 infection. Although Rev is not wholly inactive in murine cells (43), the exogenous provision of human CRM1 (hCRM1) enhanced Rev activity and yielded virus particle production up to 30-fold in mouse or rat cell lines relative to rodent CRM1 (39, 41, 42). These effects correlated with up to 5-fold increases in the cytoplasmic abundance of Rev/RRE-dependent viral mRNAs, consistent with the rescue of vRNP nuclear export.

The above observations would most easily be explained by a defect affecting the extent of Rev's interaction with mCRM1. However, the CRM1 binding cleft that engages Rev's NES is invariant between mCRM1 and hCRM1 (42, 44–46). Instead, mouse-human CRM1 chimeras were used to map the species-specific activity to a transferable, patch-like configuration of amino acids located 19 to 30 Å from the NES binding cleft and located between CRM1 HEAT repeats 9 and 10 (39, 41, 42). Phylogenetic considerations based on sequence comparisons of CRM1 orthologues from 17 mammalian species indicated that the evolution of the mCRM1 "patch domain" was likely a result of positive selection pressure, suggesting a role for this domain as an ancient cellular interface with one or more pathogenic elements, possibly of viral nature (41). Indeed, Shida and coworkers implicated the same domain within rat CRM1 (rCRM1) in affecting both HIV-1 (39, 40) and the distantly related deltaretrovirus human T lymphotropic virus type 1 (HTLV-1) Rex accessory factor (47) that, similar to Rev, mediates the nuclear export of intron-containing HTLV-1 transcripts bearing the Rex response element (RxRE) (48). Sutton and coworkers reported that hCRM1 also markedly rescues the production of another lentivirus, the feline immunodeficiency virus, in murine cells, although weaker effects were seen for other lentiviruses, such as the chimpanzee simian immunodeficiency virus (SIVcpz) or equine infectious anemia virus (EIAV), despite their reliance on Rev proteins and evidence of a similar restriction in murine cells (42). Thus, the species-specific character of CRM1 can affect diverse Rev/Rex-expressing retroviruses, albeit with some potential differences depending on the viral species. At present, why CRM1 presents one or more species-specific blocks to Rev/Rex-dependent retroviruses is unknown, as is a clear understanding of the function of CRM1's species-specific patch domain.

Here we further exploited murine 3T3 cells as a gain-of-function system to study the mechanism underpinning hCRM1's species-specific activity, focusing on hCRM1's effects on Rev trafficking and Rev-dependent viral gene expression. We demonstrate that despite low gene transactivation activity in murine 3T3 cells, Rev is exported efficiently from the nucleus in a mCRM1-dependent fashion and markedly accumulates in the cytoplasm with or without hCRM1 expression. Thus, Rev can interact with mCRM1, but its nuclear export is largely uncoupled from its role in regulating viral gene expression. Remarkably, Rev trafficking variants engineered to carry multiple CRM1 interaction domains (Rev-

2xNES) rescued virus particle production in the absence of hCRM1, as did modifying Rev-dependent viral mRNAs to encode multiple RREs (GP-2xRRE), where GP represents Gag/Gag-Pol. The nature of these rescues suggests that Rev multimerization in the context of Rev/RRE vRNP transport complexes regulates the extent of vRNP interactions with one, or likely multiple, molecules of CRM1. The extent of this interaction is defective as a result of mCRM1 protein evolution.

MATERIALS AND METHODS

Cell culture and plasmids. Murine NIH 3T3 fibroblasts, human HeLa cervical carcinoma cells, and Japanese quail fibrosarcoma (QT35) cells were obtained from the American Type Culture Collection. Crandell Rees feline kidney (CRFK) cells were kindly provided by Greg Towers (University College London, London, United Kingdom). All cell lines were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. The construction of plasmids pGP-RRE, pNL4-3-E-R-Luciferase [encoding full-length HIV-1 isolate NL4-3 mutated to not express the viral Envelope glycoprotein (E-) or the Vpr protein (R-)], pcRev, pcRevM10, phCyclin T1, phCRM1, pLuciferase, and pVSV-G are described elsewhere (41, 49–53), as is the 3T3.GFP-hCRM1 cell line featured in Fig. 1 (41). Plasmids encoding Rev- or RevM10-mCherry (mCh) fusion proteins were generated using overlapping PCR to fuse *rev* and *mcherry* cDNAs separated by a glycine-rich linker (GSTGSTGST) and influenza hemagglutinin (HA) epitope tag, and subcloned into a pcDNA3.1 backbone using NheI and XhoI restriction sites. Rev trafficking mutants (see Fig. 4) were expressed from plasmids encoding the following transport elements fused to the C terminus of Rev-mCh: Rev-2xNLS, YAPKK-KRKVG from the simian virus 40 (SV40) large T antigen; Rev-2xARD, RQARRNRWRERQR from HIV-1 Rev; and Rev-2xNES, LQLP-PLERLTL from HIV-1 Rev. Plasmids encoding YFP-mCRM1 and YFP-hCRM1 were generated by fusing *yfp* and *CRM1* cDNAs using overlapping PCR prior to insertion into pcDNA3.1 using BamHI and XhoI restriction sites. pNL4-3-E-R-Rev-minus-Luciferase was generated by fusing Rev-minus and Env-minus coding regions from pNL4-3-Rev-minus (41) and pNL4-3E-R-Luciferase, respectively, using overlapping PCR prior to insertion of the E-Rev-minus fragment into the pNL4-3E-R-/Luciferase backbone using EcoRI- and NheI-cut sites. pGP-2xRRE was generated by inserting a PCR-amplified, NotI- and SalI-digested DNA fragment encoding the RRE (HIV-1^{IIIB} isolate nucleotides 7708 to 8058) upstream of the existing RRE in pGP-RRE cut with NotI and XhoI. All plasmids were verified using restriction digestion and DNA sequencing.

Virus particle assembly and infectivity assays. Cells were plated for 30% confluence in six-well tissue culture treated dishes prior to transfection using FuGENE 6 reagent (Roche or Promega) according to the manufacturer's instructions. Cell medium was replaced at 24 h posttransfection, and cell lysates and supernatants were harvested at 48 h posttransfection. For GP-RRE virus assembly assays, HeLa or 3T3 cells were transfected with 1,000 ng of pGP-RRE, 500 ng of Rev or Rev variant, and 500 ng of phCRM1 or pLuciferase control. Particle production was measured using a p24^{Gag}-specific enzyme linked immunosorbent assay (ELISA; Perkin-Elmer) (Fig. 1) or quantitative immunoblot as described below. Viral infectivity assays were carried out by transfecting 1,000 ng of pNL4-3-E-R-Luciferase or pNL4-3-E-R-Rev-Luciferase plasmid with 100 ng of VSV-G, 250 ng of Rev plasmid or control, and 500 ng of CRM1 plasmid or control (empty pcDNA3.1 vector). For 3T3 cells, 500 ng of phCyclin T1 plasmid was added to overcome the murine block to Tat-dependent transcription. At 48 h posttransfection, supernatants were filtered and added to HeLa target cells plated at 50% confluence by spinoculation (centrifugation at $1,200 \times g$ for 2 h at 25°C) (54). The cells were lysed at 48 h posttransduction for luciferase detection using the Promega luciferase assay system and Veritas microplate luminometer (Turner Biosystems).

Immunofluorescence and immunoblotting. For immunofluorescence detection of Rev and CRM1, cells were plated on glass coverslips, transfected as described above and then fixed at 24 to 36 h posttransfection in 4% paraformaldehyde prior to permeabilization using 0.2% Triton X-100 and sequential incubation with mouse anti-Rev antiserum (Rev-6) and rabbit anti-CRM1 antiserum (ab24189; Abcam). Immunoblots were performed as previously described (52) using mouse anti-p24^{Gag} (24-2) (55), mouse anti-Rev (Rev-6), and rabbit anti-Hsp90 (Santa Cruz Biotechnology) antisera prior to incubation with anti-mouse or anti-rabbit secondary antibodies conjugated either to horseradish peroxidase (Pierce) or infrared fluorophores IRDye800 or IRDye680 (Li-Cor Biosciences).

Live-cell microscopy and image analysis. Cells were prepared in eight-well no. 1.5H glass-bottom slides (Ibidi) and maintained at 37°C, ~50% humidity, and 5% CO₂ in a LiveCell incubator (Pathology Devices, Inc.). To label nuclei, cells were incubated with NucBlue LiveCell Stain ReadyProbes (Life Technologies) according to the manufacturer's instructions. Imaging experiments were performed on a Nikon Ti-Eclipse inverted wide-field microscope (Nikon Corp.) using a 10× objective lens (NA 0.30). Imaging was carried out over 24 to 48 h starting ~6 h posttransfection. Images were collected using a CoolSnap HQ camera (Photometrics) and Nikon NIS Elements software (v4.00.03). Single images were acquired every 30 min using differential interference contrast and the following excitation/emission (nanometer ranges) filter sets: 325 to 375/435 to 485 (Hoechst), 490 to 520/520 to 550 (yellow fluorescent protein [YFP]), and 565 to 590/590 to 650 (mCherry).

Time-lapse movies were postprocessed and analyzed using FIJI/ImageJ2 (56) plugins integrated into the Konstanz Information Miner (KNIME) image analysis platform (57). Nuclear and cytoplasmic portions of each cell were defined based on nuclear staining and then tracked over time. Single-cell median fluorescence intensity measurements were obtained for each compartment. The first frame ($t = 0$) of each movie represents baseline detection of fluorescent reporter intensity set at 400 arbitrary units using NIS Elements software (Nikon). In several instances, the cytoplasmic signal did not achieve baseline until ~6 h posttransfection (e.g., see RevM10-mCh in Fig. 3D). Thus, this interval is highlighted in beige for all plots presented in Fig. 3.

RESULTS

Species-specific regulation of HIV-1 Rev activity. To better survey the extent of species-specific blocks affecting HIV-1 Rev, we measured virus-like particle (VLP) production as a functional readout for Rev activity in cells derived from a small panel of informative species (human HeLa, murine 3T3, feline CRFK, and avian QT35) covering ~320 million years of CRM1 protein evolution. In this experiment, we expressed subgenomic Rev/RRE-dependent HIV-1 *gag-pol* mRNAs with or without Rev or with Rev and hCRM1 combined (Fig. 1). Cell supernatants were harvested at 48 h posttransfection, and VLP production was measured by p24^{Gag} ELISA. As expected, Rev expression was absolutely required for VLP production in human HeLa cells (Fig. 1, compare lanes 1 and 2), and this activity was unaffected by the addition of hCRM1 (Fig. 1, compare lanes 2 and 3). In contrast, low levels of VLP production were observed in murine 3T3 cells with or without Rev expression, with Rev stimulating net VLP production <2-fold (Fig. 1, compare lanes 4 and 5). In contrast, VLP production was enhanced >10-fold more when Rev and hCRM1 were coexpressed (Fig. 1, compare lanes 5 and 6) or in a previously characterized murine 3T3 cell line that expresses endogenous levels of hCRM1 constitutively (41) (Fig. 1, compare lanes 7 and 8). Interestingly, Rev expression stimulated VLP production >10-fold in cat (CRFK) and quail (QT35) cell types, a finding consistent with prior reports of significant Rev activity in

FIG 1 Species-specific regulation of HIV-1 Rev activity. Virus-like particle (VLP) release from human (HeLa), mouse (3T3 and 3T3.hCRM1), feline (CRFK), and quail (QT35) cells was used as a functional readout for Rev activity. Cells were transfected to express Rev-dependent *gag-pol* mRNAs (GP-RRE) with or without Rev or with Rev and hCRM1 combined. VLP release was measured by analysis of cell supernatants at 48 h posttransfection using p24^{Gag} ELISA. 3T3.hCRM1 cells are engineered to stably express functional, green fluorescent protein (GFP)-tagged hCRM1 at levels similar to endogenous mCRM1 (41). Error bars represent the standard deviations from the mean for three independent transfections.

nonhuman cell types other than those derived from rodents (43, 58–60). However, although hCRM1 exerted no effect on Rev activity in cat cells, VLP production in the avian cell line was enhanced a further ~10-fold when Rev and hCRM1 were expressed together (Fig. 1, compare lanes 14 to 15). This observation was unanticipated and may indicate the existence of additional cell- or species-specific inefficiencies in this pathway. Moreover, 3T3.hCRM1 stable cells exhibited reduced low-level VLP production in the absence of Rev relative to the 3T3 parental cell line (Fig. 1, compare lane 7 to lane 4), perhaps indicating one or more additional, Rev-independent activities for hCRM1.

Rev is exported from the nucleus in murine cells in a CRM1-dependent manner. Considering CRM1's essential role in regulating Rev/vRNP nuclear export, we hypothesized that poor Rev activity in murine cells reflected a cellular defect to Rev nucleocytoplasmic trafficking. Rev is a prototypical shuttling protein that encodes both NLS and NES targeting moieties (Fig. 2A). The NLS engages importin-beta to enter the nucleus, while the NES binds to CRM1 in order to deliver Rev/RRE complexes to the cytoplasm. In human cells, Rev is most frequently detected in the nucleus (61, 62), in particular accumulating at nucleoli—subnuclear organelles that serve as cellular factories for rRNA biogenesis (63). To study hCRM1's effects on Rev trafficking in murine cells, we first compared the subcellular distribution of Rev and CRM1 in human HeLa and murine 3T3 cells in the presence of RRE-encoding *gag-pol* transcripts and, with or without hCRM1 coexpression, using indirect immunofluorescence (Fig. 2B and C). As expected, Rev was detected in both the nucleus and the nucleolus in the strong majority of HeLa cells analyzed at 24 h posttransfection (Fig. 2Bi and C). Moreover, as previously demonstrated (64), in all cases in HeLa cells Rev recruited significant amounts of CRM1 to the nucleolus (Fig. 2Bi, yellow arrows). In the murine cell line,

contrary to our expectations, Rev was rarely observed in the nucleus and accumulated to striking levels in the cytoplasm of a strong majority of cells (Fig. 2Bv and 2C). Curiously, despite hCRM1's marked effects on Rev-dependent VLP production in murine 3T3 cells (see Fig. 1), hCRM1 expression had little to no discernible effect on steady-state Rev distribution in this cell type under these conditions (Fig. 2B, compare subpanels v and vi, and 2C). Thus, Rev's steady-state nuclear abundance, or the lack thereof, apparently has little relevance to hCRM1's ability to stimulate Rev-dependent viral gene expression in this cell type.

In human cells, Rev's trafficking can be arrested at the nucleolus by disrupting Rev-CRM1 interactions, for example, by treating cells with the antifungal antibiotic leptomycin B (LMB) that abrogates CRM1-cargo binding (Fig. 2Biii and vii) or by mutating Rev's NES so that it can no longer bind to CRM1 (e.g., RevM10, see Fig. 2A and B, panels iv and viii). To confirm that Rev's marked cytoplasmic abundance in murine 3T3 cells was CRM1 dependent, we treated cells for 45 min with 12.5 nM LMB prior to fixation and also evaluated the subcellular distribution of the RevM10 mutant. LMB treatment resulted in the rapid accumulation of Rev at nucleoli in both the human and murine cell types (Fig. 2B, subpanels iii and vii, and Fig. 2C), demonstrating that Rev's cytoplasmic distribution in murine 3T3 cells is CRM1 dependent and that, in the absence of CRM1 binding, Rev is clearly capable of targeting the nucleolus. As expected, the RevM10 mutant exhibited a similar localization to nucleoli in both cell types (Fig. 2Biv and viii). In both instances, LMB treated and for the RevM10 condition, Rev's ability to recruit CRM1 to the nucleolus was abolished (Fig. 2Biii, iv, vii, and viii). Based on these findings, taken together, we conclude that Rev must be capable of interacting sufficiently with mCRM1 in order to achieve its marked accumulation in the cytoplasm of murine 3T3 cells.

FIG 2 Rev is efficiently exported from the nucleus in murine cells in the presence or absence of hCRM1. (A) Depiction of Rev and its functional domains. Rev encodes (i) an N-terminal arginine-rich domain (ARD; amino acids 34 to 50 out of 116) that serves both as an NLS and RNA-binding domain (RBD), (ii) motifs flanking the NLS/RBD that mediate Rev-Rev multimerization, and (iii) a C-terminal NES (amino acids 73 to 83). RevM10 is mutated at leucine-78 to aspartic acid and glutamic acid-79 to leucine, thus abolishing CRM1 binding. (B) Rev localizes predominantly to the cytoplasm in murine 3T3 cells, in a CRM1-dependent manner. Human HeLa or murine 3T3 cells were transfected to express RRE-dependent *gag-pol* mRNAs, with or without Rev or RevM10 and with or without hCRM1 as indicated, prior to fixation at 24 h posttransfection and detection of Rev (red) and CRM1 (green) using indirect immunofluorescence. Where indicated, cells were treated with 12.5 nM leptomycin B (LMB) for 45 min prior to fixation. Arrows indicate Rev, and in some instances CRM1, accumulating at nucleoli. Size bars correspond to 10 μ m. (C) Rev distribution was scored visually in single cells as predominantly in the cytoplasm (Cytoplasm), in the nucleus and cytoplasm (Nucleus + Cytoplasm), or predominantly in the nucleus (Nucleus). >100 cells were scored for each condition.

Because nucleocytoplasmic transport is a dynamic process, we next directly compared Rev trafficking in murine and human cellular environments over time, using a long-term (>24-h) live cell imaging strategy (Fig. 3). Functional Rev-mCherry (Rev-mCherry) fusion proteins or RevM10-mCherry mutants (depicted in Fig. 4A and validated in Fig. 6A and B) were expressed in either the human or murine cell line in conjunction with Rev/RRE-dependent *gag-pol* mRNAs and with or without hCRM1. Cells were monitored for more than 24 h using time-lapse video microscopy (Fig. 3 and see Movies S1 and S2 in the supplemental material). Single cells were scored for the first site of Rev-mCherry or RevM10-mCherry detection and measured for subsequent changes to subcellular distribution over time using a customized cell segmentation algorithm coupled to the FIJI/ImageJ2 cell tracking platform (56, 57).

In human HeLa cells, Rev-mCherry was first detected in either the nucleus/nucleolus or the nucleus and cytoplasm in 65% of cells ($n = 100$; Fig. 3A), in contrast to murine 3T3 cells, where Rev-mCherry was most often first detected in the cytoplasm (88% of cells, $n = 100$; Fig. 3B). In HeLa cells, we observed two predominant Rev-mCherry distributions over time (Fig. 3A and see Movie S1 in the supplemental material). In the majority of cells, Rev-mCherry maintained a steady-state ratio of cytoplasmic to nuclear median fluorescence intensity (MFI) close to one (e.g., Fig. 3A, cell 1). Alternatively, in many cells, Rev-mCherry accumulated to pronounced levels in the nucleus/nucleolus over time (e.g., Fig. 3A,

cell 2). Remarkably, Rev-mCherry distribution was not static in HeLa cells, and Rev-mCherry frequently exhibited dramatic transitions between the nuclear and cytoplasmic compartments, in particular striking *en masse* export events wherein the Rev-mCherry signal moved from the nucleus to the cytoplasm over a time window of two to 3 h (e.g., Fig. 3A, cells 3 and 4 [note arrows] and see Movie S1 in the supplemental material). These transitions were not observed for RevM10-mCherry mutants that were sequestered in the nucleus of both cell types (Fig. 3D and E and see Movie S2 in the supplemental material) and thus were dependent on a functional NES domain. In stark contrast, in murine 3T3 cells Rev-mCherry was predominantly detected in the cytoplasm at all time points (Fig. 3B and see Movie S1 in the supplemental material) and, consistent with the results from our fixed cell analysis (Fig. 2B and C), this distribution was largely unaffected by coexpression of hCRM1 (Fig. 3C and see Movie S1 in the supplemental material). In sum, our live cell imaging revealed differential CRM1-dependent Rev trafficking dynamics in single human HeLa cells, including dramatic transitions of Rev-mCherry between the nuclear and cytoplasmic compartments. Such transitions were markedly absent in murine 3T3 cells, where Rev-mCherry was predominantly observed in the cytoplasm. That Rev-mCherry trafficking in the murine cell line was unaffected by hCRM1 expression emphasizes that Rev's CRM1-dependent nuclear export is uncoupled from its role in viral gene expression in this cell type.

FIG 3 Rev trafficking dynamics in single living cells. (A) Time-lapse imaging of Rev-mCherry or RevM10-mCherry in single human HeLa or murine 3T3 cells for cells transfected as described in the text. Images correspond to Movies S1 (Rev-mCherry) and S2 (RevM10-mCherry) in the supplemental material. Quantitative single cell tracking of Rev/RevM10 subcellular distributions for more than 50 cells over 30 h of imaging is presented in the panels on the right. Nuclear staining was used to distinguish between nuclear and cytoplasmic signals prior to extraction of mCherry median fluorescence intensity (MFI) and calculation of cytoplasmic to nuclear (C:N) ratios over time. The average MFI is shown in black. The beige region highlights a 6-h time window when the algorithm was less reliable due to subthreshold nuclear or cytoplasmic MFI values, intrinsic to early time points. (A) In HeLa cells, Rev-mCherry (i) was detected long term in the both the cytoplasm and nucleus (cell 1), (ii) accumulated in the nucleus/nucleoli (cell 2), or (iii) underwent *en masse* transitions from the nucleus to the cytoplasm (cells 3 and 4). (B and C) In contrast, Rev-mCherry was detected predominantly in the cytoplasm of murine 3T3 cells at all time points, with or without hCRM1 expression. (D and E) RevM10-mCherry mutants were restricted to the nucleus/nucleolus in either cell type for the duration of the experiment.

The addition of a second NES to Rev rescues HIV-1 virion production in murine cells independently of hCRM1. Rev's marked and consistent distribution to the cytoplasm of murine 3T3 cells, as opposed to the nucleolar accumulation of Rev observed in human HeLa cells, prompted us to test the effects of biasing Rev trafficking either into or out of the nucleus by adding peptides encoding heterologous import or export signals to the C terminus of our functional Rev-mCherry fusion proteins (depicted in Fig. 4A). To enhance Rev's nuclear/nucleolar abundance, we added either the SV40 NLS/nucleolar localization signal (NoLS) "YAPKKRKVG" that utilizes importin- α and importin- β for import (Rev-2xNLS) or a second Rev ARD peptide "RQARRNRRRRWRERQR" that directs importin- β -

dependent but importin- α -independent nuclear import and also mediates specific binding to the RRE (Rev-2xARD) (65). To modulate nuclear export, we added a second Rev NES "LQLPPLRLTL" (Rev-2xNES) to the C terminus of both wild-type Rev-mCherry and the RevM10-mCherry variant. As anticipated, both NLS mutants exhibited increased nuclear/nucleolar abundance in both human and murine cell types relative to wild-type Rev-mCherry (Fig. 4B, subpanels iv, v, xii, and xiii compared to subpanels ii and x, and Fig. 4C). Despite encoding a second NES, the Rev-2xNES variant exhibited a distribution similar to wild-type Rev-mCherry, detectable in both the cytoplasm and the nucleus/nucleolus of HeLa cells (Fig. 4Bvi and Fig. 4C) but markedly localized to the cytoplasm of murine 3T3

FIG 4 Modulation of Rev-mCherry nucleocytoplasmic distribution using heterologous transport signals. (A) Rev-mCherry trafficking variants. Rev- or RevM10-mCherry constructs were modified to encode the indicated signaling peptides at the C terminus of each protein. (B) Subcellular distribution of Rev trafficking variants in HeLa and 3T3 cells. Cells were transfected to express each indicated Rev variant in the presence of RRE-encoding *gag-pol* mRNAs with cells fixed at 24 h posttransfection and CRM1 detected using indirect immunofluorescence. Yellow arrows indicate instances of Rev-mCherry variant accumulation at the nucleolus. Note that CRM1 is not recruited to the nucleolus in murine 3T3 cells by Rev-2xNLS or Rev-2xARD variants, despite these mutants encoding functional NES domains (subpanels xii and xiii). Size bars correspond to 10 μ m. (C) Rev-mCherry and variant distribution was scored visually as described for Fig. 2C. More than 100 cells were scored for each condition.

cells (Fig. 4Bxiv and Fig. 4C). The addition of a C-terminal NES to RevM10-mCherry (RevM10-NES) rescued RevM10-mCherry's nuclear export, confirming that the C-terminal NES was active for CRM1 binding (Fig. 4Bviii and xvi and Fig. 4C). Interest-

ingly, the RevM10-NES variant accumulated in the cytoplasm in the human cell line to a greater extent than did wild-type Rev-mCherry (Fig. 4B, compare subpanels viii to ii, and Fig. 4C), suggesting that the native position of Rev's NES is an important

FIG 5 Rev's ability to recruit CRM1 to the nucleolus is cell type specific. (A) YFP-mCRM1 or YFP-hCRM1 was coexpressed with RRE-encoding *gag-pol* mRNAs and either mCherry alone (subpanels i, ii, v, and vi) or Rev-2xARD (subpanels iii, iv, vii, and viii) that targets Rev-mCherry to the nucleolus (yellow arrows) in either human HeLa cells or murine 3T3 cells. Cells were fixed and visualized at 24 h posttransfection. (B) Single cells were scored for Rev-2xARD or YFP-CRM1 localization to the nucleolus. More than 100 cells were scored for each condition. Size bars correspond to 10 μ m.

determinant of Rev's ability to accumulate at the nucleolus even in human cells.

Unexpectedly, although we observed wild-type Rev-mCherry, Rev-2xNLS, and Rev-2xARD recruiting endogenous CRM1 to the nucleolus in HeLa cells (Fig. 4Bii, iv, and v, yellow arrows), CRM1 was only very rarely observed at the nucleolus in murine 3T3 cells for the Rev-2xNLS and Rev-2xARD conditions, despite these variants dramatically enhancing Rev-mCherry's nucleolar abundance (Fig. 4Bxii and xiii, yellow arrows, and Fig. 4C). Because CRM1's recruitment to the nucleolus is dependent on Rev-CRM1 binding (66), we hypothesized that this cell-specific phenotype reflected a deficiency to mCRM1's ability to engage Rev multimers in the context of nucleolar RNP complexes. To address this hypothesis, we tested the ability of the Rev-2xARD nucleolar targeting variant to affect mCRM1 or hCRM1 distribution in either cell type using YFP-tagged versions of either of these two orthologues (Fig. 5). In the absence of Rev expression, both YFP-hCRM1 and YFP-mCRM1 accumulated at the nuclear membrane in either cell type, as expected, with YFP-CRM1 only rarely observed at the nucleolus (Fig. 5Ai, ii, v, and vi, and Fig. 5B). Rev-2xARD expression in human HeLa cells frequently resulted in the recruitment of either CRM1 species to the nucleolus (5Aiii and iv and Fig. 5B), further evidence that Rev and mCRM1 interact physically, at least in this cell type. In contrast, neither of the YFP-tagged CRM1 orthologues was detected at the nucleolus in the strong majority of murine cells despite very high levels of nucleolar Rev-2xARD (Fig. 5Avii and viii and Fig. 5B). Thus, additional cell-specific or species-specific factors appear to contribute to the extent of Rev-CRM1 interactions occurring at the nucleolus.

Importantly, both Rev-2xNLS and Rev-2xARD mutants were functional in viral assembly assays in HeLa cells and exhibited only a mildly detrimental impact on net VLP production (<2-fold) relative to wild-type Rev-mCherry as assayed by quantitative immunoblotting (Fig. 6A, compare lanes 4 and 5 to lane 3). In murine 3T3 cells, the Rev-2xNLS mutant had no effect compared to wild-type Rev (Fig. 6A, compare lane 4 to lane 3), but the Rev-2xARD mutant triggered a mild but reproducible (~2-fold) enhancement

of VLP production (Fig. 6A, compare lane 5 to lane 3). Thus, in conjunction with the imaging data presented in Fig. 2 through 5, the amount of Rev accumulating in the nucleus at steady state was largely unrelated to the effects of Rev on viral gene expression in these cell types. However, the mild Rev-2xARD effects in the murine cell line suggest that the extent of the Rev-RRE interaction is suboptimal in this cell type.

Remarkably, despite the Rev-2xNES variant having negligible effects on Rev subcellular distribution (Fig. 4B, compare subpanels x and xiv, and Fig. 4C), this variant yielded a dramatic, and wholly unanticipated, rescue of HIV-1 VLP production in murine 3T3 cells, even in the absence of hCRM1 (Fig. 6). In human HeLa cells, Rev-2xNES expression yielded VLP production roughly equivalent to wild-type Rev-mCherry (Fig. 6A, compare lane 10 to lane 9). However, in 3T3 cells, Rev-2xNES expression increased net VLP production to levels equivalent to those seen with hCRM1 coexpression (Fig. 6A, compare lane 10 to lanes 9 and 13). Mutation of the native NES domain in this context (RevM10-NES) still supported VLP production in the human cell line but largely (albeit not completely) abolished the rescue in murine 3T3 cells (Fig. 6A, compare lane 12 to lane 10). Thus, the 2xNES effect reflects the presence of two functional NES domains associated with Rev, as opposed to altering the position of the NES.

Because our assembly assay utilized surrogate Rev-dependent *gag-pol* reporter constructs, we sought to determine the relevance of the Rev-2xNES rescue to infectious HIV-1 virion production by testing the ability of this variant to complement the production of a full-length HIV-1_{NL4-3} isolate luciferase reporter virus in which Rev expression had been inactivated through mutation (NL4-3/E-R-Rev-/Luc). VSV-G-pseudotyped virions were generated in HeLa or 3T3 cells by coexpression of the proviral construct with either Rev-mCherry or Rev-2xNES and in the presence or absence of hCRM1 (Fig. 6B). In this experiment, 3T3 cells were also transfected to express the human version of CycT1, thereby ensuring viral Tat activity and efficient transcriptional elongation; a process that is suboptimal in murine cells (49, 67). Viral supernatants were harvested at 48 h posttransfection, filtered, and added to target

FIG 6 Rev-2xNES mutants rescue HIV-1 virion production in murine cells. (A) Effects of Rev trafficking mutants on Gag expression and virus particle production measured by virus-like particle (VLP) assembly assay. Supernatants and lysates from cells transfected as for Fig. 4B were harvested at 48 h posttransfection. VLPs were sedimented from filtered supernatants, resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected using anti-p24^{Gag} antiserum and either chemiluminescent or infrared fluorophore-conjugated secondary antibodies. Lysates were similarly resolved, with membranes probed using anti-p24^{Gag}, anti-Rev, and anti-Hsp90 (loading control) primary antisera. The fold enhancement reflects the p24^{Gag} band intensity normalized to the Rev-mChe condition for each individual cell type (lane 3). The results are representative of three independent experiments. (B) The Rev-2xNES mutant rescues infectious virion production in murine 3T3 cells independently of hCRM1. Human HeLa or murine 3T3 cells were transfected to express full-length but Env⁻/Vpr⁻ HIV-1_{NL4-3} Luciferase reporter proviruses (NL4-3/Luc) or a Rev-minus version (NL4-3/Rev⁻/Luc). In both instances, the virions were pseudotyped with VSV-G. Viral supernatants were harvested at 48 h posttransfection and used to infect human target cells. Target cells were lysed at 48 h postinfection and assayed for luciferase expression (relative light units). Error bars represent the standard deviations for three independent transfections.

HeLa cells prior to cell lysis and luciferase detection at 48 h post-transduction as a readout for infectious HIV-1 virion production. In HeLa producer cells, Rev-mChe expression stimulated infectious virion production from the Rev-minus provirus more than 100-fold, similar to levels observed for the parental Rev-encoding proviral construct (Fig. 6B, compare samples 9 and 11 to sample 10). In contrast, native Rev or Rev-mChe expression had little effect on infectious virion production in 3T3 cells (Fig. 6B, compare samples 2 and 4 to sample 3), a finding consistent with the weak activity previously observed with our Rev-dependent *gag-pol* reporters (e.g., Fig. 1). As expected, hCRM1 expression had no effect on infectious virion production in HeLa cells (Fig. 6B, compare sample 12 to sample 11) but stimulated virion production >10-fold in the murine cell line (Fig. 6B, compare sample 5 to sample 4). The Rev-2xNES mutant exhibited similar activity to wild-type Rev or Rev-mChe in human HeLa cells (Fig. 6B, compare sample 13 to samples 9 and 11) but increased infectious virion production markedly (>50-fold) in murine 3T3 cells (Fig. 6B, compare sample 6 to samples 2 and 4). Combining Rev-2xNES expression with hCRM1 did not further augment virion production in the murine cell line, suggesting functional overlap and/or saturation within a common pathway (Fig. 6B, lane 7). In sum, augmenting Rev molecules to encode an additional NES potentially rescues HIV-1 infectious virion production in murine cells, and the effect is a striking phenocopy of hCRM1's species-specific activity.

The addition of a second RRE to Rev-dependent viral transcripts also markedly enhances virus particle production in murine cells. The visual experiments and genetic complementation assays described above demonstrated that, in murine 3T3 cells, Rev's nuclear export function is largely uncoupled from its role in activating viral gene expression. One interpretation of the fact that either hCRM1 or the Rev-2xNES variant can independently rescue HIV-1 production is that hCRM1 exhibits a higher avidity for Rev multimers in the context of a vRNP complex relative to mCRM1, regulated by the net number of transcript-associated NES domains delivered to the complex by Rev multimerization on the RRE. We thus hypothesized that multiplying the number of RREs within a Rev-responsive *gag-pol* mRNA would accomplish a similar result, because the transcript would accommodate twice the number of Rev molecules and hence twice the number of Rev-associated NES domains. To test this hypothesis, we simply generated otherwise identical Rev-dependent *gag-pol* expression constructs encoding transcripts either bearing a single RRE (GP-RRE) or tandem RREs (GP-2xRRE) (Fig. 7A) and evaluated the effects of these transcripts on Rev and CRM1 subcellular distribution (Fig. 7B and C), as well as the ability of these proteins to support viral gene expression (Fig. 7D). Quantitative visual analysis revealed little discernible difference to Rev-mChe distribution when coexpressed with either GP-RRE or GP-2xRRE transcripts or even in the absence of any viral transcript (Fig. 7C). Thus, the RRE, or number of RREs, appears to play little to no role in governing Rev's subcellular distribution. However, while GP-2xRRE expression yielded Rev-dependent VLP production roughly equivalent to GP-RRE expression in HeLa cells (Fig. 7D, compare lane 5 to lane 3), VLP production was markedly enhanced (>5-fold) for the GP-2xRRE condition in the murine cell line relative to GP-RRE (Fig. 7D, compare lane 5 to lane 3). However, we note that this rescue was typically not to a level equivalent to hCRM1 or Rev-2xNES expression alone.

FIG 7 Tandem copies of the RRE in *gag-pol* mRNAs markedly enhance virus particle production in murine cells. (A) Depiction of GP-RRE and GP-2xRRE constructs. (B) Rev-mChe and CRM1 were detected as for Fig. 4B. Yellow arrows indicate Rev-mChe and CRM1 accumulation at the nucleolus in human HeLa cells. Size bars correspond to 10 μ m. (C) Rev-mChe distribution was scored visually as described for Fig. 4C. More than 100 cells were scored for each condition. (D) Comparison of GP-RRE-dependent and GP-2xRRE-dependent virus-like particle (VLP) production in human HeLa and murine 3T3 cells, evaluated by VLP assembly assay as described for Fig. 6A. Fold enhancement values are normalized to the wild-type GP-RRE + Rev-mChe conditions (lane 3). The figure is representative of three independent experiments. (E) VLP Assembly assay as for 7D for GP-2xRRE, Rev-2xNES, and hCRM1 conditions either expressed individually or in combination as indicated. The figure is representative of three independent assembly assays.

Finally, having identified distinct rescues operating at the level of cellular protein (hCRM1), viral protein (Rev-2xNES) and viral RNA (GP-2xRRE), we took advantage of the opportunity to directly compare the effects of each rescue individually or combined

(Fig. 7E). Each individual rescue yielded a 5- to 10-fold increase to VLP production in murine 3T3 cells (Fig. 7E, lanes 3, 4, and 6), while any combination of rescues (Fig. 7E, lanes 5, 7, 8 and 9), including the coexpression of all three elements (Rev-2xNES, GP-

FIG 8 Working model for CRM1's species-specific regulation of HIV-1 mRNA transport complexes. (A) Based on structural predictions, Rev may form a hexamer (21) or octamer (26) in complex with the RRE capable, in theory, of binding to up to two molecules of hCRM1. (B) We show that mCRM1 associates with individual Rev molecules but predict that mCRM1 is unable to establish functional interactions with Rev/RRE complexes due to suboptimal mCRM1 binding. (C) The addition of a second NES domain to Rev (Rev-2xNES) may increase the avidity of interactions occurring between Rev/RRE complexes and mCRM1, thereby rescuing viral gene expression in murine cells. (D) Tandem RREs would be predicted to rescue gene expression in murine cells by multiplication of individual, otherwise suboptimal transport complexes.

2xRRE, and hCRM1; Fig. 7E, lane 9) had only moderate effects on net VLP output relative to any other rescue condition. Thus, we show that two independent modulations of HIV-1 elements predicted to increase the number of transcript-associated nuclear export signals results in an apparent phenocopy of hCRM1's species-specific activity in murine cells.

DISCUSSION

In this report we demonstrate that, despite Rev's weak effects on Gag/Gag-Pol expression and subsequent virus particle production in murine 3T3 cells (Fig. 1, 6, and 7), Rev is efficiently exported from the nucleus in a mCRM1-dependent fashion and preferentially accumulates in the cytoplasm with or without hCRM1 expression (Fig. 2 and 3). Remarkably, conditions predicted to multiply the number of transcript-associated NES domains rescued Rev's activity even in the absence of hCRM1 (Fig. 6 and 7). We previously demonstrated that hCRM1 expression increases the cytoplasmic abundance of intron-containing viral mRNAs in murine cells severalfold (41). That the Rev-2xNES or GP-2xRRE rescues phenocopy hCRM1's effects suggests to us that the major block to HIV-1 mRNA nuclear export in rodent cells is weak interactions between mCRM1 and Rev/RRE complexes occurring in the nucleus. We propose a model for hCRM1's species-specific activity wherein both hCRM1 and mCRM1 interact with Rev, but only hCRM1 is capable of forming functional, and likely multimeric, interactions with wild-type Rev/RRE vRNP complexes (Fig. 8, compare scenarios A and B).

Rev multimerization confers specificity and strength to RRE binding (20, 21, 26) and initiates the recruitment of additional

cellular factors with roles in vRNP nuclear export, mRNA cytoplasmic utilization, gRNA packaging, and efficient virus particle assembly (16, 17, 19, 68–75). Our results emphasize that Rev multimerization also regulates viral gene expression through controlling the number transcript-associated nuclear export signals, likely ensuring efficient delivery of these large vRNP complexes through the NPC via the CRM1 pathway. This notion is consistent with recent work from Hoffmann et al. (76), who used forced dimerization of multimerization-defective Rev mutants to confirm that at least two functional Rev transactivation (i.e., NES) domains are needed to support Rev-dependent gene expression in human cells. A parsimonious explanation for this requirement is that Rev-dependent transcripts interact with two or more molecules of CRM1 (Fig. 8A). Such a mechanism would explain why Rev-2xNES and GP-2xRRE modifications rescue assembly even in the absence of hCRM1 and are functionally redundant (i.e., more NES peptides = more CRM1 molecules, despite the species identity) (Fig. 8, compare scenarios C and D to scenario B). Structural modeling studies by Frankel and coworkers suggest that Rev/RRE complexes can accommodate interactions with up to two CRM1 receptors simultaneously (21). Accordingly, a tandem RRE (GP-2xRRE) would allow a transcript to accommodate up to four CRM1 receptors.

However, a “multi-CRM1” model does not yet fully explain how the hCRM1 patch domain exerts species-specific activity in the context of a wild-type vRNP transport complex. Shida and coworkers previously showed that hCRM1 expression can enhance Rev and HTLV-1 Rex self-association (77) and that the effects on Rex were species specific (i.e., seen for hCRM1 but not for rat CRM1) (78). Thus, it is not unreasonable to hypothesize that the hCRM1 patch domain functions in a way that nucleates or further stabilizes Rev/RRE complexes. However, a defect to Rev multimerization is unlikely to be an obligate feature of the murine cell block, considering that the Rev-2xNES and GP-2xRRE rescues proceed in the context of endogenous levels of mCRM1. Further biochemical interrogation of CRM1/Rev complexes will be essential toward explaining these differences.

It should be noted that hCRM1 could also be playing additional, Rev-independent roles in the HIV-1 life cycle, as recently proposed for HIV-1 Gag trafficking (39), and suggested here (see Fig. 1). Specifically, we consistently observed a low level of Rev-independent HIV-1 virus particle production in both murine and avian cell types (Fig. 1, compare lanes 13 and 4 to lane 1), a phenotype that was suppressed in 3T3 cells engineered to stably express hCRM1 (Fig. 1, compare lane 7 to lane 4). Although we do not yet have a full explanation for these observations, we note that HIV-1 pre-mRNAs are heavily spliced in both rodent and avian cells relative to human cell types (43, 79, 80). Because pre-mRNA splicing is typically coupled to the nuclear export machinery (81), we find it an attractive possibility that in conjunction with its role as a nuclear export receptor, hCRM1 coordinates nuclear regulatory elements that stage, or stabilize, intron-retaining viral mRNAs even prior to Rev recruitment.

We anticipated that our visual approach would reveal a block to Rev nucleocytoplasmic transport in murine cells consistent with a defect to Rev/CRM1 interactions and predicted that, over time, Rev would be trapped in the nucleus of murine cells, similar to the RevM10 mutant that does not bind to CRM1 (e.g., Fig. 2Biv and viii). Instead, we found that Rev was exported rapidly from the nucleus by mCRM1 and preferentially accumulated in the

cytoplasm of murine 3T3 cells with or without hCRM1 expression (Fig. 2, 3, 4, and 7 and see Movie S1 in the supplemental material). This distribution differed markedly from human HeLa cells where Rev-mChe was observed most often in the nucleus but also undergoing striking transitions both into and out of this compartment (Fig. 3A and see Movie S1 in the supplemental material). A long-standing model for Rev trafficking posits that the nucleolus is a staging area for Rev function, regulated through Rev's interactions with the nucleolar-resident protein B23 (82, 83). Indeed, Daelemans et al. demonstrated using fluorescence resonance energy transfer and fluorescence recovery after photobleaching that Rev is multimeric in the nucleolus and that its motility is constrained by tight associations with one or more nucleolar constituents (84). Remarkably, our long-term movies revealed that Rev's association with the nucleolus is differentially regulated in single HeLa cells. Indeed, the pulsiform kinetics of *en masse* Rev-mChe export events observed in many cells are compelling and suggest that one or more cell-based signals regulate the extent of Rev's association with the nucleolus despite its otherwise strong association with this compartment (e.g., Fig. 3A, cells 3 and 4 [note arrows], and see Movie S1 in the supplemental material). Rev's nucleolar flux was dependent on an intact NES peptide (Fig. 3D and see Movie S2 in the supplemental material), so that hCRM1 likely serves as a critical node in this signaling cascade (as opposed to, e.g., transient changes to Rev nuclear import kinetics).

On the other hand, while there is evidence suggesting that Rev's accumulation in the nucleolus is of functional relevance (82, 85, 86), Rev derivatives with defects to nucleolar localization have, nonetheless, been demonstrated to support Rev-dependent gene expression (87), and our murine cell studies confirm that Rev's trafficking through the nucleolus need be only very transient in order to support viral gene expression. We reason that Rev's nucleocytoplasmic distribution is a function of at least four variables: rate of nuclear import, rate of nuclear export, interactions/retention in the nucleus, and interactions/retention in the cytoplasm. In this context, we noted that a "mouse-like" phenotype of Rev sequestration in the cytoplasm was previously described for HIV-infected human astrocytes, correlating with a block to replication characterized by low levels of late gene expression and thus with potential relevance to viral latency in the central nervous system (14, 88). This observation, coupled to the differential mouse and human phenotypes, prompted us to test the effects of biasing Rev's accumulation at the nucleolus in murine 3T3 cells using Rev-2xNLS and Rev-2xARD variants (Fig. 4). While forcing Rev to target the nucleolus had little effect on Rev-dependent gene expression in either murine or human cell types (Fig. 6A), Rev-2xNLS and Rev-2xARD variants recruited CRM1 to the nucleolus in human cells but to a much lesser extent in murine cells, despite these Rev variants encoding functional NES peptides (Fig. 4 and 5). Furthermore, we found that YFP-tagged mCRM1 and hCRM1 variants were equivalently recruited to the nucleolus in human HeLa cells, while neither orthologue localized substantially to the nucleolus in murine 3T3 cells (Fig. 5A and B). We perceive the HeLa cell result as further evidence that mCRM1 is capable of interacting with Rev; otherwise, mCRM1 would not have been recruited by Rev-mChe to the nucleolus. In contrast, the fact that Rev could not recruit hCRM1 to the nucleolus in the murine cell line was a surprising finding and suggests that, in general, Rev and CRM1's lack of retention at the nucleolus in murine cells reflects the presence or absence of additional cell- or species-specific fac-

tors. Long term, the regulation and relevance of Rev at the nucleolus, as well as the pulsiform export dynamics exhibited in human cells, requires further exploration.

In broader terms, our results emphasize the role for Rev-dependent cooperative transport signals as a mechanism for fine-tuning the efficiency of viral gene expression and demonstrate that these signals can be a determinant of viral species tropism. Such findings are relevant to the long-standing goal of developing a small animal model for studying HIV-1 (89). Moreover, engineering reporter viruses to encode Tat/CycT1-independent promoters and multiple Rev-associated NES domains could provide a means to more easily study HIV-1 in useful but otherwise nonpermissive cell systems, such as murine knockout cell lines. Our results also augment our interpretation of "evolutionarily prescribed" cell-based blocks to infection (i.e., the evolution of incompatible cellular cofactors or restriction factors) that may, long-term, inform approaches to restrict HIV-1 replication in human cells (90). Indeed, murine cell blocks have a storied history in this regard, revealing key HIV-relevant species-specific structural elements within the CD4 (91, 92) and CCR5 (93) receptor proteins required for viral entry, and the cyclin T1 (CycT1/CYCT) HIV-1 Tat transcription cofactor (67, 94). Murine cell studies have also emphasized roles for additional cellular cofactors, including the p32 splicing factor (80) and SR family proteins SRp40 and SRp55 that regulate HIV-1 translation (53). Further progress in understanding the evolution and function of the CRM1 patch domain may inform the development of strategies that perturb HIV-1 gene expression and perhaps that of other retroviruses. Moreover, such studies could have broad impact considering that CRM1 is now implicated in the life cycles of several other important human viral pathogens, including influenza (95), hepatitis B (96), herpesviruses (97–100), and respiratory syncytial virus (101) and is also an emerging target for anticancer therapeutics (102–104).

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