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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank M. Fujioka and J. Jaynes for transgenic flies containing the *eve* 4 + 6–*lacZ* construct; L. Andrioli for discussions and support; A. Oberstein for technical assistance; and C. Desplan, J. Blau and T. Cook for encouragement and comments on the manuscript. D.E.C. was supported by a grant from the NSF. This work was also supported by a grant from the NIH.

Competing interests statement The authors declare that they have no competing financial interests.

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The gene product Murr1 restricts HIV-1 replication in resting CD4$^+$ lymphocytes

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Although human immunodeficiency virus-1 (HIV-1) infects quiescent and proliferating CD4$^+$ lymphocytes, the virus replicates poorly in resting T cells[1–6]. Factors that block viral replication in these cells might help to prolong the asymptomatic phase of HIV infection[7]; however, the molecular mechanisms that control this process are not fully understood. Here we show that Murr1, a gene product known previously for its involvement in copper regulation[8,9], inhibits HIV-1 growth in unstimulated CD4$^+$ T cells. This inhibition was mediated in part through its ability to inhibit basal and cytokine-stimulated nuclear factor (NF)-κB activity. Knockdown of Murr1 increased NF-κB activity and decreased IκB-α concentrations by facilitating phospho-IκB-α degradation by the proteasome. Murr1 was detected in CD4$^+$ T cells, and RNA-mediated interference of Murr1 in primary resting CD4$^+$ lymphocytes increased HIV-1 replication. Through its effects on the proteasome, Murr1 acts as a genetic restriction factor that inhibits HIV-1 replication in lymphocytes, which could contribute to the regulation of asymptomatic HIV infection and the progression of AIDS.

Murr1 is a highly conserved 190-amino-acid protein that does not have any identifiable motifs, and a homozygous deletion in the gene encoding canine Murr1 leads to copper toxicosis in Bedlington terriers[8]. In this study, Murr1 was initially identified in a two-hybrid screen by binding the X-linked inhibitor of apoptosis, a known activator of NF-κB (refs 10, 11, and E.B., unpublished observations). To study its effect on NF-κB, HIV-1 reporter plasmids with wild-type or mutant (ΔκB) sites[2] were co-transfected with control or Murr1 expression plasmids in the different cell lines. Murr1 inhibited both basal and tumour necrosis factor (TNF)-α-dependent HIV-1 transcription from the wild-type but not the κB-mutant reporter in Jurkat T-leukaemia and 293T renal epithelial cell lines (Fig. 1a, left and middle panels). In contrast, Murr1 did not substantially inhibit tumour growth factor-β-dependent transcription in HepG2 cells, confirming its specificity (Fig. 1a, right panel). The κB effect was dose-dependent and observed with other inducers of NF-κB, including interleukin-1 (IL-1) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Fig. 1b). Murr1 modulated the expression of endogenous κB-regulated genes: transfection into 293T cells decreased the endogenous cell-surface expression of major histocompatibility complex (MHC) class I, in contrast to CD9, which is independent of NF-κB (Fig. 1c).

Its site of action in the NF-κB signalling pathway was further defined by co-transfection of different regulators with an NF-κB reporter in Jurkat T cells. Whereas Murr1 inhibited both IKK-1- and IKK-2-induced NF-κB activity (Fig. 1d, middle and right panels), it failed to block RelA-mediated transcription (Fig. 1d, left panel), indicating that Murr1 might interact downstream of the IκB kinase signalosome. As determined by immunoprecipitation, co-transfected haemagglutinin (HA)-tagged Murr1 and Myc-tagged IKK-2 did not associate *in vivo* (Fig. 2a, lane 2, left panel). Although IKK-1 also did not associate with Murr1 (data not shown), an interaction between transfected HA-tagged Murr1 and endogenous IκB-α was readily detected (Fig. 2a, lane 6). The ankyrin domain of IκB-α was required for association with Murr1, as were amino acids 1–160 of Murr1 (Supplementary Fig. 1a).

A polyclonal antibody against Murr1 demonstrated the association between endogenous Murr1 and IκB-α *in vivo*. RelA antibody immunoprecipitated IκB-α, IκB-β and Murr1 (Fig. 2b, lane 10). IκB-α antibody also pulled down RelA and Murr1 (Fig. 2b, lane 12), but the IκB-β antibody did not precipitate Murr1 (Fig. 2b, lane 14), suggesting that Murr1 interacted preferentially with the NF-κB–IκB-α complex. This association was confirmed *in vivo* by confocal microscopy with fluorescent fusion proteins (Supplementary Fig. 1b), similarly to the pattern of RelA association with IκB-α[12–14].

The physiological consequences of these interactions were determined by knockdown of endogenous Murr1 in 293T cells using control and Murr1-specific short interfering RNA (siRNA) duplexes. The specificity of two such siRNAs, Murr1-1 and Murr1-2, directed to different Murr1 sequences, was first confirmed by transfecting 293T cells with wild-type or mutant siRNAs along with wild-type or mutant Murr1 complementary DNAs modified at the siRNA target site (Supplementary Fig. 2). Transient transfection of Murr1-specific siRNA duplexes downregulated endogenous Murr1 and IκB-α, had little effect on IκB-β, p65 or IKK-2 (Fig. 3a, left panel), and increased κB-dependent reporter activity (Fig. 3a, right panel).

To investigate the mechanism of Murr1 action, 293T cells were transfected with a control or Murr1 siRNA. Four days after transfection, cells were treated with the proteasome inhibitor MG132 for 2 h or with vehicle alone and stimulated with TNF-α. Cells depleted of Murr1 showed a decrease in basal IκB-α (Fig. 3a) and an increase and persistence of phospho-IκB-α in response to stimulation with TNF-α (Fig. 3b, left panel). This effect was observed in the absence of a proteasome inhibitor, MG132, but not in its presence (Fig. 3b, right panel), indicating that Murr1

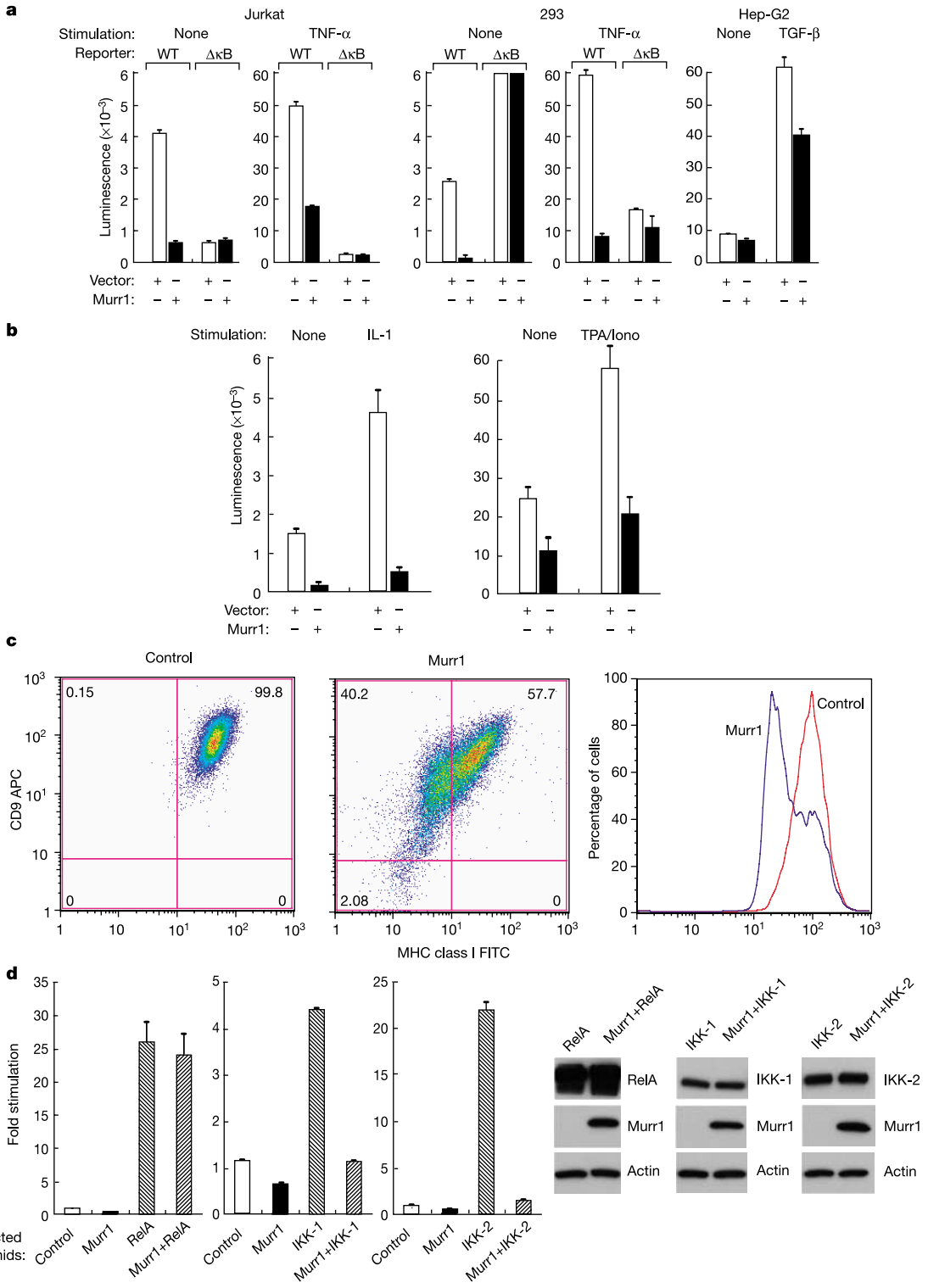

Figure 1 Murr1 suppresses NF-κB-dependent activity from independent stimuli by acting downstream of IκB kinase. **a**, Murr1 inhibits κB-dependent gene expression in Jurkat T-leukaemia cells (left) or 293T human embryonic kidney cells (middle) transfected with HIV (WT) or ΔκB-luciferase reporter (ΔκB) and Murr1 (solid bars) or vector plasmid (open bars). HepG-2 cells (right) were transfected with p3TP-Lux reporter. Cells were treated with the indicated cytokines after 24 h, and luciferase activity was measured at 36 h. **b**, Murr1 inhibits NF-κB induced by IL-1 and TPA in 293 cells. **c**, Murr1 decreases the expression of NF-κB-dependent endogenous class I MHC in 293T cells transfected with HA–LacZ (left) or HA–Murr1 (middle) by flow cytometry for class I and CD9 in HA-positive cells. The decrease in MHC class I expression is summarized (right). **d**, Murr1 blocks the activation by IKK-1 and IKK-2 but does not inhibit RelA transactivation. Fold stimulation (left) and expression (right) are shown. An average of three independent experiments in triplicate, standardized for transfection efficiency, are shown (**a**, **b**, **d**).

might act similarly to a proteasome inhibitor to enhance and sustain phospho-IκB. Phospho-IκB-α is degraded by ubiquitin–protein conjugates that require three enzymes, which participate in a cascade of ubiquitin transfer reactions: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3). The specificity of protein ubiquitination and 26S proteasome degradation is determined by the E3 enzymes[15]. Skp1/Cul1/β-TrCP1 (F-Box) complexes constitute a class of E3 enzymes that are required for the degradation of phospho-IκB-α[16]. The possible association of endogenous Murr1 with Skp1/Cul1/β-TrCP1 (F-box) complexes was examined. As determined by immunoprecipitation followed by western blotting, Murr1 interacted biochemically with the Cul1 and not the Skp1 component of the E3 ligase complex, in contrast to a denatured negative control (Fig. 3c). Although Murr1 binds to common constituents of E3 ligase, it nevertheless showed specificity because knockdown did not alter steady-state concentrations of β-catenin, the target of proteasomal degradation of the Wnt signalling pathway (Fig. 3d). Taken together, these results suggest that Murr1 blocked NF-κB activation through its ability to interact with E3 ligase and inhibit the proteasomal degradation of IκB.

To determine whether Murr1 was detectable in T cells, lymphocytes from normal healthy individuals were examined. Both $CD4^+$ and $CD8^+$ cells expressed Murr1, with higher concentrations in $CD4^+$ cells (Fig. 4a), raising the possibility that Murr1 might affect HIV-1 replication in these cells. This possibility was examined first by transfection of Murr1 in primary T cells and compared with another inhibitor of NF-κB activation, the IκB super-repressor (IκB-SR). Expression of recombinant Murr1 or IκB-SR inhibited HIV-1 replication in activated T cells similarly relative to controls (Fig. 4b; $P = 0.02$; paired t-test), indicating that Murr1 might inhibit HIV-1 replication comparably to NF-κB inhibitors in primary activated T cells. To determine its effect on HIV-1 replication in resting primary T cells, $CD4^+$ cells from HIV-1-negative donors were transfected with an siRNA for Murr1 (Murr1-1) or a negative control. To identify transfected cells, siRNAs were mixed with an unrelated fluorescent Cy3-labelled luciferase siRNA at a ratio of 2:1, and more than 30% of cells displayed fluorescence. In Murr1 siRNA-transfected cells, a maximum decrease in Murr1 protein concentration was achieved between 48 and 72 h after electroporation, and these cells did not display T-cell activation markers—CD69, CD25 and HLA-DR—at the time of HIV-1 infection (data not shown). Murr1 siRNA increased dose-dependent HIV-1 replication in primary resting $CD4^+$ T cells from three separate cell donors (Fig. 4c; $P = 0.0007$, 0.0024 and 0.0014, respectively; paired t-test), implicating Murr1 in the regulation of HIV infection in these cells.

Whereas HIV-1 entry into activated $CD4^+$ lymphocytes leads to a productive infection[1], the virus remains latent in resting $CD4^+$ lymphocytes[17], existing as a preintegration complex awaiting cell stimulation, which facilitates the transition to productive replication[3,4,6,7,18]. The molecular mechanisms responsible for HIV-1

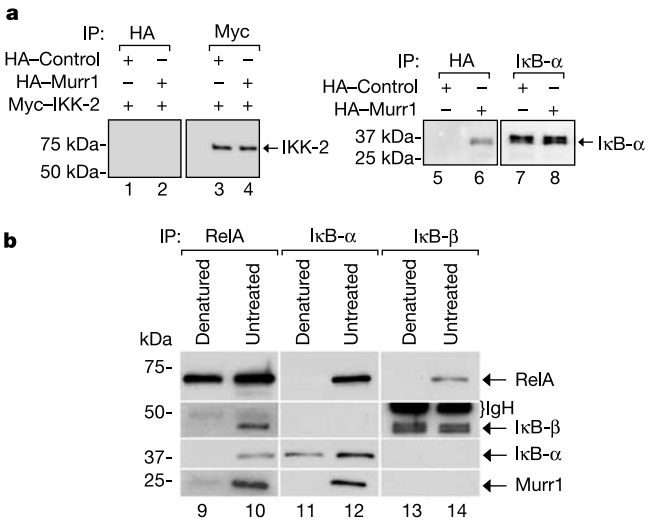

Figure 2 Murr1 associates with the NF-κB–IκB-α complex. **a**, Murr1 does not associate with IKK-2 but interacts with endogenous IκB-α. Lysates from transfected 293T cells, treated with lactacystin (10 μM) 16 h before harvesting, were immunoprecipitated (IP) as indicated and immunoblotted with antibody against IKK-2 (left) or IκB-α (right). **b**, Immunoprecipitation of Murr1–NF-κB–IκB-α in 293T cells with antibodies against indicated proteins. IgH, immunoglobulin heavy chain.

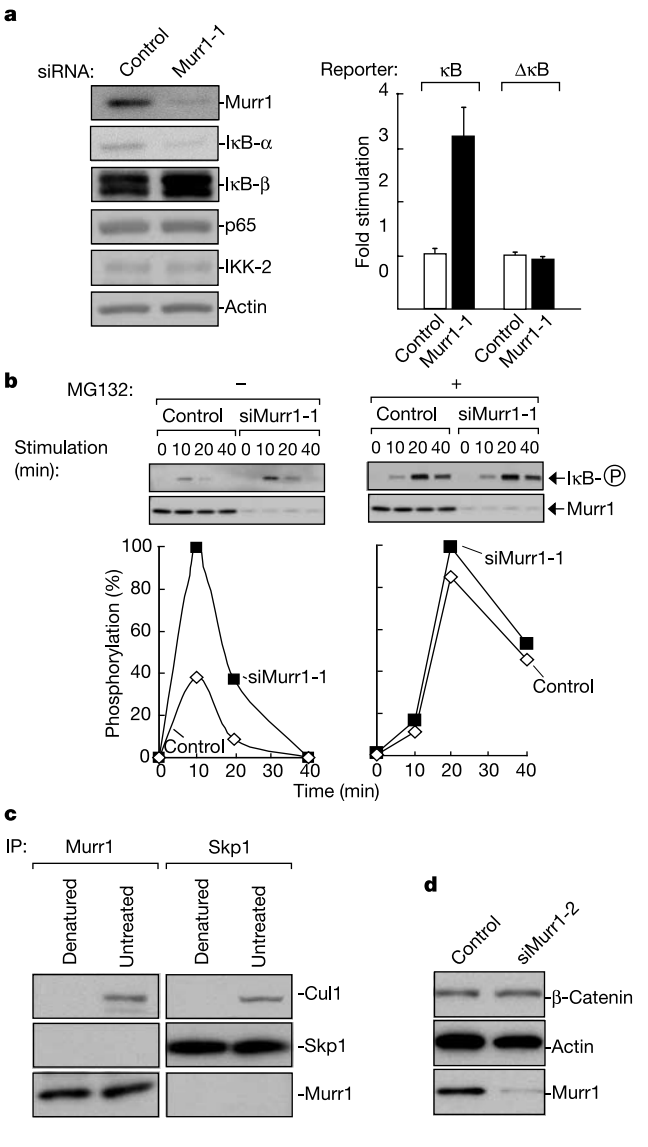

Figure 3 Murr1 regulates IκB-α turnover through effects on proteasomal degradation. **a**, Knockdown of Murr1 decreases cellular IκB-α and increases basal NF-κB activity in 293T cells transfected with control or Murr1 siRNAs, analysed by immunoblotting to the indicated proteins at 48 h (left). Fold stimulation of the indicated co-transfected plasmids is shown (right). **b**, Murr1 alters IκB-α phosphorylation in 293T cells transfected with control or Murr1 siRNAs. At 90 h after transfection, cells were treated with vehicle (left) or MG132 (right) for 2 h, followed by stimulation with TNF-α and immunoblotting for Murr1 and phospho-IκB-α (top) and quantification (bottom). **c**, Immunoprecipitation (IP) of Skp1–Cul1–β-TrCP1 complexes in 293T cell extracts with antibodies against Murr1 or Skp1, immunoblotted for Cul1, Skp1 or Murr1. **d**, Knockdown of Murr1 by Murr1-2 siRNA compared with a negative control (GFP) does not alter β-catenin concentrations.

latency are not well understood. Among the factors that can activate HIV-1 replication are host transcription factors such as NF-κB (ref. 2), epigenetic modifications at the site of integration[19], premature termination of transcription due to the absence of sufficient concentrations of Tat and NF-κB (refs 20, 21), and inefficient export of RNAs for structural proteins[5]. The roles of these factors in resting $CD4^+$ T cells are inferred from cell culture experiments and affect viral transcription, translation or RNA transport. Here we show that Murr1 regulates IκB-α turnover, which inhibits the productive HIV-1 infection of resting T lymphocytes. Expression of Murr1 in activated HIV-infected T cells also inhibited virus replication, indicating that it might also have a function in limiting the extent of viral replication, or burst size. NF-κB is sequestered in the cytoplasm by a 450-kDa IκB complex[12,22–25] and, after activation of T cells, phosphorylation of IκB-α (ref. 26) in this complex by two inducible kinases, IKK-1 and IKK-2, leads to the subsequent ubiquitination and degradation of IκB-α by the 26S proteasome[27]. Degradation of IκB-α by the proteasome releases NF-κB, which then translocates to the nucleus to stimulate the synthesis of genes involved in immune activation. NF-κB enhances transcription and subsequently recruits several transcriptional activators, including positive transcription elongation factor b, which is ubiquitinated and degraded by the proteasome[28]. Inhibition of proteasomal activity by Murr1, after it rebounds from its initial degradation and after this processive transcription complex has formed, could subsequently stabilize it and enhance transcription. The proteasome, through the ubiquitin E3 ligase complex, is also involved in viral processing and the maturation of Gag[29]. Because Murr1 inhibits proteasomal degradation, it could further block HIV-1 infection by decreasing the proteasome-dependent degradation and maturation of Gag-associated factors required for viral replication, and although Murr1 acts selectively on the NF-κB and not the Wnt signalling pathway (Fig. 3), it remains possible that it might yet affect other such proteins degraded by the proteasome. Such an example is APOBEC3G, shown recently to associate with a Cul5 E3 ligase[31]. Blocks to viral replication in resting lymphocytes, as well as in cells containing latent provirus, might affect disease progression and viral rebound after the discontinuation of anti-retroviral therapy (reviewed in ref. 7). A more precise understanding of the molecular regulation of these events might identify novel genetic restriction factors, like Murr1, through which anti-viral drugs might delay the progression of HIV-1 infection to AIDS. □

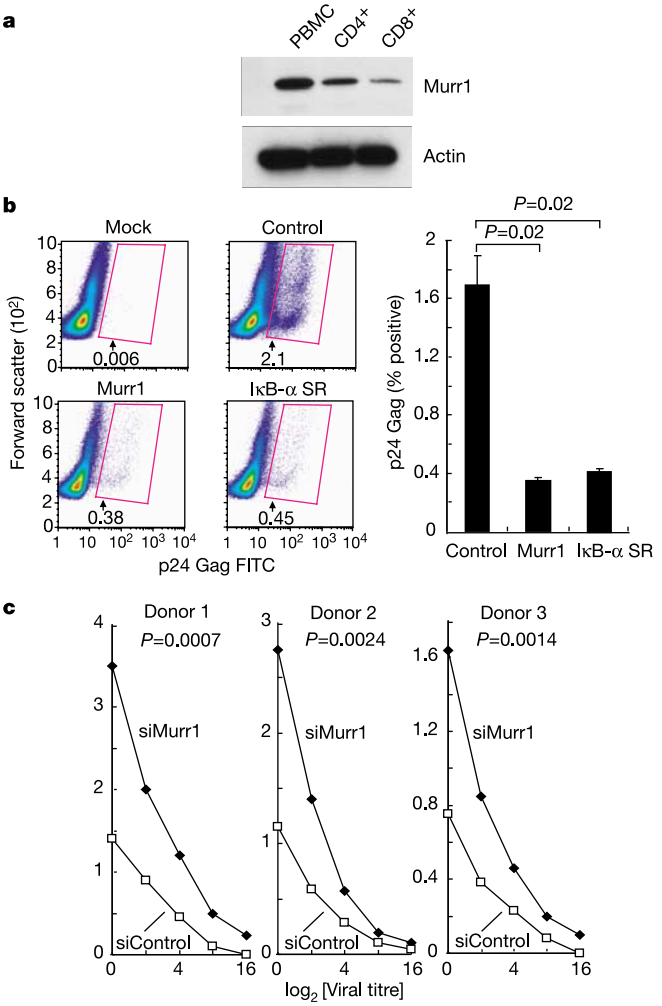

Figure 4 Murr1 inhibits productive HIV-1 infection in $CD4^+$ lymphocytes. **a**, Murr1 concentrations in $CD4^+$ and $CD8^+$ T cells shown by immunoblotting with Murr1 antisera and reprobing with β-actin antibody. **b**, Murr1 inhibits HIV-1 replication in phytohaemagglutinin/IL-2-stimulated human $CD4^+$ cells transfected with plasmids encoding HA–LacZ (control), HA–Murr1 or HA–IκB-SR, infected with $HIV\text{-}1_{BaL}$ ($p24 = 75\ ng\ ml^{-1}$) 36 h later. HIV-1 replication was analysed 48 h after infection by staining for intracellular p24. A representative of three independent HIV-1-negative donors is shown ($P = 0.02$, control versus Murr1). **c**, Murr1 siRNA-transfected resting $CD4^+$ T lymphocytes from three healthy donors show increased susceptibility to HIV-1 infection after transfection with siRNA for Murr1 (Murr1-1) compared with GFP (control). Cy3 luciferase siRNA was mixed with siRNAs (2:1) to label transfected cells. Single-round HIV-1 replication was measured in labelled cells at 48 h. The multiplicity of infection of undiluted virus was ~1.0.

Methods

Plasmids, siRNA and cell transfections

The gene encoding chloramphenicol acetyltransferase (CAT) in HIV-LTR–CAT and HIV-LTR-ΔκB–CAT[2] was replaced with the luciferase gene from pGL3 basic (Promega) to construct HIV-LTR–Luc and HIV-LTR-ΔκB–Luc. The HA-IκB-α SR, p3TP-Lux reporter, green fluorescent protein (GFP)–RelA, pRK-Myc-IKK-1, pRK-Myc-IKK-2, p65 and IκB-α cDNAs are described in Supplementary Methods, as are siRNA sequences and the cell transfection techniques.

T-cell purification and transfection

To purify resting T cells, $CD4^+$ T lymphocytes were isolated from Ficoll-purified peripheral blood mononuclear cells (PBMCs) by negative selection with a $CD4^+$ T-cell isolation kit containing CD8, CD11b, CD16, CD19, CD36 and CD56 antibodies (Miltenyi Biotech). $CD25^+$ and $HLA\text{-}DR^+$ cells were then removed from this population by using CD69, CD25 and HLA-DR antibodies (Miltenyi Biotech) by negative selection. The purity of the isolated resting T cells was assessed by fluorescence-activated cell sorting (FACS) analysis for CD4, CD69, CD25 and HLA-DR (BD-Pharmingen); 95% of the cells were $CD4^+$ and there were less than 0.1% of activated T cells as defined by the presence of CD69, CD25 or HLA-DR. Resting $CD4^+$ T cells (10^7) were transfected with control or Murr1 siRNA (Dharmacon). For each transfection, 1.2 μM siRNA (800 nM unlabelled siRNA and 400 nM Cy3-labelled siRNA) was used. Transfections were performed with a 2:1 mixture of unlabelled siRNA and Cy3 luciferase. To transfect the resting $CD4^+$ T cells with siRNA, 10^7 cells were resuspended in 100 μl T-cell Nucleofector reagent and immediately electroporated with the recommended protocol U-14 on the Nucleofector instrument (Amaxa Biosystems). The electroporated cells were washed 4 h after transfection and incubated in 1.5 ml RPMI and infected with HIV-1 48 h after electroporation. FACS analysis was performed with CD69, CD25 and HLA-DR antibodies (BD Pharmingen) to check the activation status of the cells at the time of infection. For plasmid transfections, $CD4^+$ T lymphocytes isolated from Ficoll-purified PBMCs by negative selection were stimulated with phytohaemagglutinin ($10\ \mu g\ ml^{-1}$) (Calbiochem) and $40\ U\ ml^{-1}$ IL-2 (Peprotech) for 24 h and maintained in $40\ U\ ml^{-1}$ IL-2 for 4 days. Cells were then washed to remove IL-2, and 10^7 cells were resuspended in 100 μl T-cell Nucleofector reagent containing 0.5 μg of the respective plasmids and immediately electroporated with recommended protocol T-20 on the Nucleofector instrument. The electroporated cells were washed 12 h after transfection and incubated in 1.5 ml of RPMI supplemented with $40\ U\ ml^{-1}$ IL-2 and infected with HIV-1 24 h after electroporation.

HIV-1 infection and flow cytometric analysis for expression of p24-Gag

At 48 h after siRNA transfection or 24 h after plasmid transfection, HIV-1 infection of $CD4^+$ T lymphocytes was performed in 96-well round-bottomed culture plates by combining 40 μl virus stock with 20 μl $CD4^+$ T lymphocytes (1.5×10^5 cells). The multiplicity of infection of undiluted virus was ~1.0. After incubation for 2 h at 37 °C, cells were washed twice and continued in culture with indinavir at 1 μM. $CD4^+$ T lymphocytes were harvested for intracellular p24-Gag staining 48 h after exposure to virus. In brief, cells were stained with anti-CD4-PE and ethidium bromide monoazide (EMA) for 10 min. EMA was cross-linked to the cells by exposure to a bright light source for 15 min. Cells were washed once, fixed and permeabilized with Cytoperm/Cytofix (BD Pharmingen) for

20 min. Cells were then stained for p24 Gag (KC-57 fluorescein isothiocyanate (FITC); Coulter) for 20 min and washed once in 1 × Perm/wash buffer (BD Pharmingen). Cells in each group were analysed by flow cytometry for intracellular HIV-1 p24 and CD4 after the exclusion of dead cells by their affinity for EMA. For a detailed description of HIV-1 production, infection and flow cytometric analysis see ref. 30.

Antibodies, western blots and immunoprecipitations

Cell lysis for western blots and immunoprecipitations were performed in cell lysis buffer (Cell Signal). Antibodies against β-actin (Sigma), His (Invitrogen), HA, HA-PE, p65, IκB-α, IκB-β, Cul1, Skp1 (Santa Cruz), IKK1, IKK2, Ser-32-phospho IκB-α, β-catenin (Cell Signal) and HLA Class1 FITC (Biosource) were used in flow cytometry, immunoblotting and immunoprecipitations in accordance with the manufacturer's instructions. Polyclonal Murr1 antibody has been described previously[9].

Received 11 August; accepted 21 October 2003; doi:10.1038/nature02171.

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank K. Leung for preparation of the Murr1 mutants; S. Majeed for helping with protein purifications; M. Roederer for advice in interpretation of flow cytometry data and statistical analysis; A. Tislerics, A. Gooch and T. Suhana for manuscript preparation; and K. Stroud and T. Miller for the preparation of figures. This work was supported in part by the Michigan Gastrointestinal Hormone Research Core Center (E.B.).

Competing interests statement The authors declare that they have no competing financial interests.

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Inheritance of a pre-inactivated paternal X chromosome in early mouse embryos

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In mammals, dosage compensation ensures equal X-chromosome expression between males (XY) and females (XX) by transcriptionally silencing one X chromosome in XX embryos[1]. In the prevailing view, the XX zygote inherits two active X chromosomes, one each from the mother and father, and X inactivation does not occur until after implantation[2–6]. Here, we report evidence to the contrary in mice. We find that one X chromosome is already silent at zygotic gene activation (2-cell stage). This X chromosome is paternal in origin and exhibits a gradient of silencing. Genes close to the X-inactivation centre show the greatest degree of inactivation, whereas more distal genes show variable inactivation and can partially escape silencing. After implantation, imprinted silencing in extraembryonic tissues becomes globalized and more complete on a gene-by-gene basis. These results argue that the XX embryo is in fact dosage compensated at conception along much of the X chromosome. We propose that imprinted X inactivation results from inheritance of a pre-inactivated X chromosome from the paternal germ line.

In mice, X-chromosome inactivation (XCI) takes on two lineage-specific forms. Random XCI[1], in which both X chromosomes have an equal chance of being inactivated, occurs in the epiblast (embryo proper). In contrast, imprinted XCI[7] leads to paternal X-chromosome (X^P) silencing in the extraembryonic tissues (placenta). Although the imprint is set in gametes, classical studies support a view in which both X^P and X^M (maternal X chromosome) are transmitted to the zygote in an active form: the absence of a late-replicating X chromosome in pre-implantation embryos[2] suggests equal X-transcriptional status, and bimodal distributions of enzymatic activities for two X-linked genes (*Hprt*[3,4], *Gla*[5]) suggest twice as much expression in XX as compared with XY embryos. Thus, the prevailing view postulates that XX and XY embryos have a twofold imbalance of X dosage until implantation, when XCI takes place for the first time in the extraembryonic and embryonic lineages[6,8–10]. However, some recent observations have not been explained easily[11]. Studies of the X-linked *Pgk1* (refs 12, 13) revealed that, whereas the maternal allele is expressed during pre-implantation development, the paternal allele is silent. Furthermore, one *Xist* allele is expressed at high levels in pre-implantation embryos[9], leading to the idea that the X chromosomes may be transcriptionally distinct[6,11]. Indeed, the maternal and paternal haplogenomes can behave differently in early