The maximum likelihood model estimated significantly positive slopes for the relationship between λ (support interval, 0.0076 to 0.0117), μ (support interval, 0.0046 to 0.0135), and latitude for the combined data set of bird and mammal sister species (Fig. 2). Results were similar when maximum haplotype or oldest phylogroup splits were used to correct for lag times, and only the correction with haplotypes is reported here. Estimated speciation and extinction rates were lowest at the equator and increased significantly toward the poles (Fig. 2). The same trends were obtained when excluding sister-species pairs with combined latitudinal ranges greater than 40° and when bird and mammal data sets were fit separately, but results were not significant in the mammal data set. These results hold true even when correcting for the latitudinal gradient in lag time to speciation. We expect that better knowledge of species-level taxonomy in the tropics will revise the lag time and sister-species age gradients. This revision should have minimal influence on the estimates of speciation and extinction, given that they are adjusted for lag time.

These results are surprising because the latitudinal gradient in estimated speciation rate is opposite to the gradient in net rate of diversification estimated by many studies to be highest in tropical taxa (*4*–*10*). For our data on sister species, the gradient in net diversification is not significantly different from zero ($b_\lambda = b_\mu$). Still, the range of estimates for the net diversification gradient supported by this study is consistent with estimates obtained elsewhere for birds (*5*). If the gradient is real, as other studies encompassing longer time periods indicate (*4*–*10*), our findings would support the classic views of Wallace (*19*), Fisher (*20*), and others (*12*, *21*, *22*), who reported that reduced extinction risks at tropical latitudes promoted the gradual buildup of high species diversity there.

These quantitative estimates are based on the assumption that speciation and extinction can be approximated by a continuous birth-death process as latitude becomes higher or lower. Yet, we know that there have been fluctuations in the opportunities for speciation and extinction over the past few million years (*12*, *23*). For example, extensive climatic fluctuations that occurred at high latitudes during the late Pliocene and Pleistocene (2.5 Ma to present) may have concentrated speciation and extinction events in time, resulting in episodic species turnover. In contrast, the bursts of diversification in tropical faunas may predate the late Pliocene and Pleistocene, and the patterns observed today may be the result of a subsequent decline in diversification either because the geological processes that promoted diversification (e.g., formation of Isthmus of Panama, marine incursions, orogeny, and river formation) have slowed or because diversification rates declined as the number of tropical species approached a "carrying capacity" (*7*, *12*).

Given such variability, our estimates are best regarded as averages over the periods studied. Despite these uncertainties, our results suggest that elevated speciation and extinction rates in the temperate zone can drive high turnover of species, whereas rates of species turnover at tropical latitudes are reduced. A recent study of fossil marine bivalves also showed higher per capita rates of genus extinction at high latitudes, suggesting higher species extinction rates as well (*24*) (estimates of per capita speciation rates are still lacking). Together, these results suggest that extinction rates are greatest where species diversity is lowest. Whereas most efforts have aimed at identifying the geological, climatic, and ecological factors that might have elevated tropical speciation rates, our results suggest that both speciation and extinction vary with latitude and contributed importantly to the latitudinal diversity gradient.

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Supporting Online Material

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Disrupting the Pairing Between *let-7* and *Hmga2* Enhances Oncogenic Transformation

Christine Mayr,[1] Michael T. Hemann,[2] David P. Bartel[1]*

MicroRNAs (miRNAs) are ~22-nucleotide RNAs that can pair to sites within messenger RNAs to specify posttranscriptional repression of these messages. Aberrant miRNA expression can contribute to tumorigenesis, but which of the many miRNA-target relationships are relevant to this process has been unclear. Here, we report that chromosomal translocations previously associated with human tumors disrupt repression of *High Mobility Group A2* (*Hmga2*) by *let-7* miRNA. This disrupted repression promotes anchorage-independent growth, a characteristic of oncogenic transformation. Thus, losing miRNA-directed repression of an oncogene provides a mechanism for tumorigenesis, and disrupting a single miRNA-target interaction can produce an observable phenotype in mammalian cells.

Hmga2 codes for a small, nonhistone, chromatin-associated protein that has no intrinsic transcriptional activity but can modulate transcription by altering the chromatin architecture (*1*, *2*). *Hmga2* is primarily expressed in undifferentiated proliferating cells during embryogenesis and in a wide variety of benign and malignant tumors (*3*–*6*). In many of these tumors, a chromosomal translocation at 12q15 truncates the human *HMGA2* open reading frame (ORF), typically retaining the three DNA-binding domains of HMGA2 while replacing the spacer and the acidic domain at the C terminus by any of a wide variety of ectopic

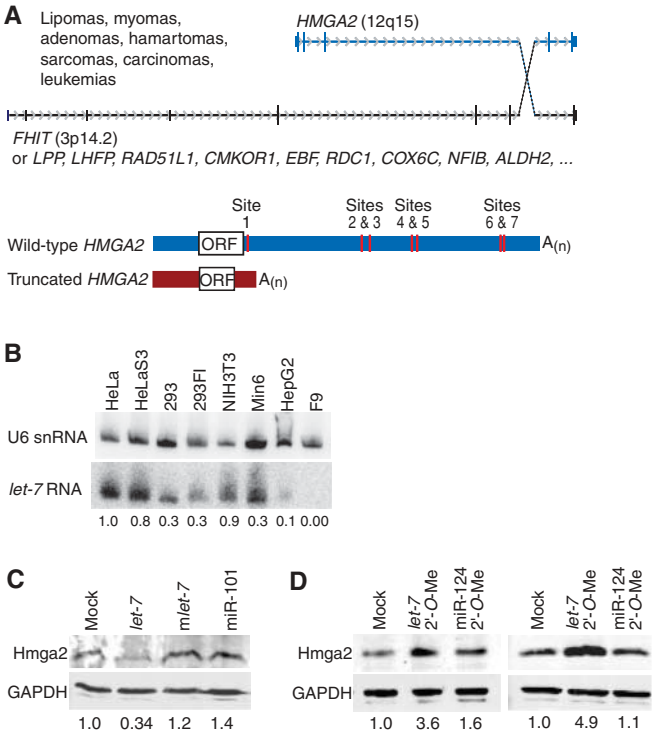

Fig. 1. Chromosomal translocations involving *HMGA2*, and the influence of *let-7* on protein expression. (**A**) Translocations involving *HMGA2* and numerous translocation partners (*3*–*10*) (SOM text). These translocations generate a truncated *HMGA2* mRNA lacking the *let-7* complementary sites of the wild-type mRNA and are associated with the indicated tumors. In its 3′ UTR, human *HMGA2* has seven *let-7* complementary sites, all of which are conserved in the mouse, rat, dog, and chicken (*14*). $A_{(n)}$, polyadenylate tail. (**B**) RNA blot detecting *let-7* RNA in different cell lines. The blot was reprobed for U6 small nuclear RNA (snRNA), and *let-7* signal normalized to that of U6 is indicated. (**C**) Western blot monitoring endogenous Hmga2 48 hours after transfection of F9 cells with the indicated miRNA duplex. The blot was also probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the normalized Hmga2 signal is indicated. (**D**) Western blot monitoring endogenous Hmga2 at 24 (left) and 48 (right) hours after transfection of NIH3T3 cells with the indicated 2'-*O*-methyl oligonucleotide (2'-*O*-Me). The blot was probed also for GAPDH, and the normalized Hmga2 signal is indicated.

sequences (*3*–*5*, *7*–*10*) (SOM text) (Fig. 1A). The loss of the C-terminal region is nearly always presumed to be the cause of oncogenic transformation. However, the translocations also replace the 3′ untranslated region (3′ UTR), and large fragments of the *Hmga2* 3′ UTR confer repression to luciferase reporters, which has led to the idea that transformation might be caused by the loss of repressive elements in the UTRs (*11*). Indeed, chromosomal rearrangements in some tumors leave the ORF intact but disrupt the 3′ UTR, and this is associated with overexpression of the wild-type Hmga2 protein (*3*, *4*, *10*). Moreover, transgenic mice overexpressing wild-type Hmga2 have similar phenotypes to those expressing the truncated protein; both develop abdominal lipomatosis, then lymphomas, pituitary adenomas, and lung adenomas (*2*, *12*, *13*).

The *Hmga2* 3′ UTR has seven conserved sites complementary to the *let-7* RNA (*14*), a miRNA expressed in later stages of animal development (*15*), leading us to suspect that disrupting *let-7* regulation of *Hmga2* might lead to oncogenic transformation. Consistent with this idea, intro-

[1]Howard Hughes Medical Institute and Department of Biology, Massachusetts Institute of Technology, and Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142, USA. [2]Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

*To whom correspondence should be addressed. E-mail: dbartel@wi.mit.edu

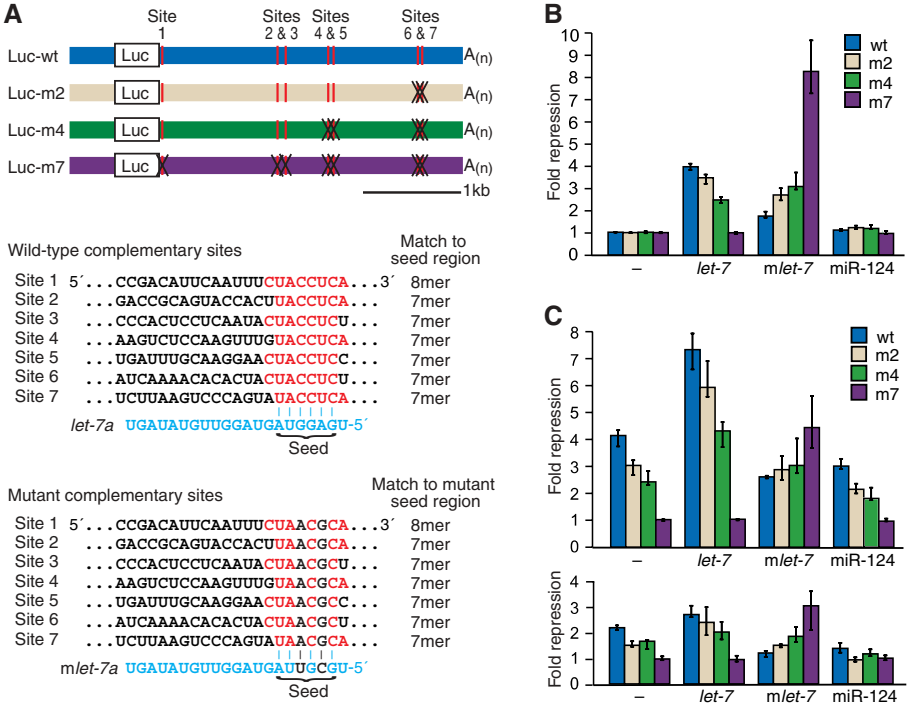

Fig. 2. Luciferase reporter assays showing the influence of miRNA-target pairing. (**A**) Design of Luciferase constructs. The 3′ UTR of murine *Hmga2* was appended to the Luciferase ORF (Luc). *let-7* complementary sites are indicated (vertical red lines), as are mutant sites (black Xs). The Luc-m7, Luc-m4, and Luc-m2 were identical to Luc-wt, except they had seven, four, and two mutant sites, respectively. Each mutant site had two point substitutions that disrupted pairing to *let-7* but created pairing to mutant *let-7* (m*let-7*, bottom, in blue). The seven sites were identified in a search for conserved 7- and 8-nucleotide motifs (7mer and 8mer) matching the seed region of *let-7* (*15*). (**B**) Reporter repression in F9 cells supplemented with the indicated miRNA. Bars are colored to indicate the number of sites mutated in the reporter. Shown are median repression values, with error bars indicating 25th and 75th percentiles; $n = 12$, except experiments with no added miRNA (–), in which $n = 36$. Within each quartet, activity was normalized to that of the Luc-m7 reporter, except for the m*let-7* quartet, for which activity was normalized to that of the Luc-m7 reporter with noncognate miRNA (miR-124). (**C**) Reporter repression in NIH3T3 cells (top) or HeLa cells (bottom) supplemented with the indicated miRNA, performed and displayed as in (B). We also noticed two additional *let-7* complementary sites in the murine *Hmga2* mRNA, but located in the 5′ UTR. When tested in luciferase reporter assays, these sites mediated little or no repression in the different cell lines, and the mutant sites did not respond to m*let-7*, indicating that any effects observed with the 5′ UTR sites were not miRNA specific.

ducing *let-7* RNA repressed Hmga2 in F9 cells (Fig. 1C), an undifferentiated embryonic carcinoma cell line that does not express detectable *let-7* RNA (Fig. 1B). Moreover, introducing a 2'-*O*-methyl oligonucleotide (*16*, *17*) complementary to *let-7* RNA enhanced Hmga2 in NIH3T3 cells (Fig. 1D), a cell line that naturally expresses *let-7* (Fig. 1B). These effects were specific in that they did not occur with noncognate miRNAs (m*let-7* and miR-101, Fig. 1C) or a noncognate inhibitor (miR-124 2'-*O*-Me, Fig. 1D).

To test whether *let-7* directly targets the *Hmga2* 3′ UTR, we constructed reporters with the wild-type 3′ UTR (Luc-wt) and the UTR with point mutations disrupting all seven sites (Luc-m7), the four distal sites (Luc-m4), or the two most distal sites (Luc-m2) (Fig. 2A). In F9 cells, the degree of repression corresponded to the number of intact sites and depended on cotransfection of the *let-7* miRNA, whereas cotransfection of an unrelated miRNA had little effect (miR-124, Fig. 2B). The repression profile inverted when the *let-7* miRNA was replaced with a mutant miRNA, m*let-7* (Fig. 2B), which was designed to recognize the mutant sites instead of the wild-type sites (Fig. 2A). This rescue of repression with compensatory changes in the miRNA confirmed targeting specificity and the importance of direct pairing between the sites and the miRNA.

To examine repression directed by endogenous *let-7*, we repeated the reporter assays using NIH3T3 cells and HeLa cells, which naturally express *let-7* (Fig. 1B). In both cell types, reporter repression depended on the wild-type sites, as would be expected if the endogenous *let-7* miRNA directed repression (Fig. 2C). Adding exogenous *let-7* RNA enhanced repression, suggesting that *let-7* RNA was subsaturating in these cells. Adding mutant *let-7* also caused reporters with mutant sites to be repressed. Adding m*let-7* or miR-124 decreased repression of the reporter with wild-type sites (particularly in HeLa cells), as if the transfected miRNA was competing with endogenous *let-7* RNA for a limiting factor.

Having established that the sites within the *Hmga2* 3′ UTR could mediate *let-7*–directed repression, we tested whether disrupting this repression could promote oncogenic transformation. Assays for anchorage-independent growth were performed with NIH3T3 cells, which form colonies in soft agar when stably transfected with a potent oncogene. Stably transfecting a vector expressing wild-type *Hmga2* did not significantly increase the number of colonies compared with transfecting an empty vector, whereas transfecting a vector expressing a truncated *Hmga2* (Hmga2-tr) (Fig. 3A), shown previously to promote anchorage-independent growth (*18*), produced significantly more colonies (Fig. 3B). As in human tumors, Hmga2-tr lacked the spacer, the acidic domain, and the entire 3′ UTR with its miRNA complementary sites (Fig. 3A). The increase in colonies was attributed to the loss of *let-7* repression rather than the truncation of the protein because stably expressing Hmga2-m7, which had the full ORF but disrupted *let-7* complementary sites (Fig. 3A), produced at least as many colonies as Hmga2-tr, whereas stably expressing Hmga2-ORFtr, which had the truncated ORF but intact miRNA complementary sites (Fig. 3A), produced a number comparable to that of Hmga2-wt (Fig. 3B). Stably expressing Hmga2-m4, which retained the first three *let-7* sites, led to an intermediate number of colonies.

We next tested whether the stably transfected cells used to assay anchorage-dependent growth were also able to form subcutaneous tumors in nude mice. Consistent with our soft-agar results, tumors were observed when injecting cells expressing constructs with mutated *let-7* sites: After 5 weeks, three of four mice injected with Hmga2-m7 cells and two of four mice injected with Hmga2-m4 cells had tumors at the sites of injection. One of four injected with Hmga2-tr cells and one of four injected with Hmga2-ORFtr also had tumors, whereas no tumors were observed 5 weeks after injecting cells stably transfected with either wild-type Hmga2 or empty vector.

Taken together, our results support the proposal that the *let-7* miRNA acts as a tumor-suppressor gene (*19*, *20*) and indicate that a major mechanism of oncogenic *Hmga2* translocations associated with various human tumors is the loss of *let-7* repression. Thus, loss of miRNA-directed repression of an oncogene is another type of oncogene-activating event that should be considered when investigating the effects of mutations associated with cancer. Likewise, mutations that create miRNA-directed repression of tumor-suppressor genes might also impart a selective advantage to the tumor cells. In this regard, we note that *Hmga2* translocations frequently append the *Hmga2* 3′ UTR to the 3′ end of known tumor-suppressor genes, including *FHIT*, *RAD51L1*, and *HEI10* (*5*, *8*, *9*), suggesting that *let-7*–directed repression of these translocation partners might cooperate with disrupting *Hmga2* repression to promote tumorigenesis.

Vertebrate miRNAs can each have hundreds of conserved targets and many additional nonconserved targets (*15*, *21*–*25*), all of which have confounded exploration of the biological impact of particular miRNA-target relationships. In worms, flies, and plants, repression of particular targets is known to be relevant because genetic studies have investigated what happens when that target alone is not repressed by the miRNA (*26*–*28*). Because of the possibility that multiple interactions might need to be perturbed to observe a phenotypic consequence in mammals, it has been unclear whether the importance of a particular mammalian miRNA-target interaction can be demonstrated experimentally. Our results, combined with previous cytogenetic studies that speak to the effect of misregulating the endogenous human *HMGA2* gene, show that disrupting miRNA regulation of *Hmga2* enhances oncogenic trans-

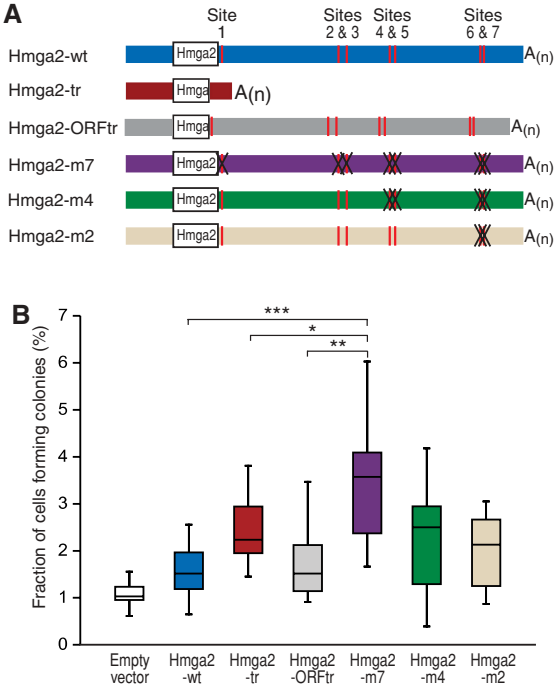

Fig. 3. Soft-agar assay for anchorage-independent growth. (**A**) Hmga2 constructs used for stable transfection, depicted as in Fig. 2A. (**B**) Colony formation. For cells stably transfected with the indicated vector, the percentage that yielded colonies after 28 days is plotted (horizontal line, median; box, 25th through 75th percentile; error bars, range; $n = 12$ from four independent experiments, each in triplicate). All but Hmga2-wt yielded a significantly higher number of colonies than did the empty vector (Mann-Whitney test for each, $P < 0.05$). When compared with Hmga2-wt, a significantly higher number of colonies was observed for Hmga2-tr ($P = 0.003$). Hmga2-m7 showed significantly more colonies than any of the other constructs tested ($P < 0.05$ for each; *, $P = 0.041$; **, $P = 0.002$; ***, $P = 10^{-6}$). No significant difference was observed between Hmga2-wt and the construct with the truncated ORF ($P = 0.61$).

formation, thereby demonstrating that disrupting miRNA regulation of a single mammalian gene can have a cellular phenotype in vitro and a clinical phenotype in vivo.

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Suppression of MicroRNA-Silencing Pathway by HIV-1 During Virus Replication

Robinson Triboulet,[1] Bernard Mari,[3] Yea-Lih Lin,[2] Christine Chable-Bessia,[1] Yamina Bennasser,[5] Kevin Lebrigand,[3] Bruno Cardinaud,[3] Thomas Maurin,[3] Pascal Barbry,[3] Vincent Baillat,[4] Jacques Reynes,[4] Pierre Corbeau,[2] Kuan-Teh Jeang,[5] Monsef Benkirane[1]*

MicroRNAs (miRNAs) are single-stranded noncoding RNAs of 19 to 25 nucleotides that function as gene regulators and as a host cell defense against both RNA and DNA viruses. We provide evidence for a physiological role of the miRNA-silencing machinery in controlling HIV-1 replication. Type III RNAses Dicer and Drosha, responsible for miRNA processing, inhibited virus replication both in peripheral blood mononuclear cells from HIV-1–infected donors and in latently infected cells. In turn, HIV-1 actively suppressed the expression of the polycistronic miRNA cluster miR-17/92. This suppression was found to be required for efficient viral replication and was dependent on the histone acetyltransferase Tat cofactor PCAF. Our results highlight the involvement of the miRNA-silencing pathway in HIV-1 replication and latency.

RNA silencing is a mechanism for gene regulation involving small noncoding RNA (*1*) as well as an innate host cell defense mechanism against viruses (*2*, *3*). MicroRNA (miRNA) genes are most often transcribed by RNA polymerase II, and the resulting primary (pri) miRNA is processed in the nucleus by the RNAse type III Drosha to produce precursor (pre) miRNA. Pre-miRNAs are then exported to the cytoplasm by exportin 5 and processed into miRNA/miRNA* (guide/passenger) duplexes through the action of the cytoplasmic type III RNAse Dicer. miRNA/miRNA* is incorporated into the RNA-induced silencing complex (RISC) where miRNA* is degraded, with miRNA serving as a guide for its mRNA target. miRNA-armed RISC can enforce either degradation of mRNA (in the case of perfect sequence complementarity) or inhibition of mRNA translation (in the case of imperfect sequence complementarity) (*3*). Accumulating evidence suggests that the miRNA pathway also controls the replication of both RNA and DNA viruses (*4*).

To address whether the miRNA-silencing machinery influences HIV-1 replication, we used specific small interfering RNA (siRNAs) to reduce the expression of endogenous Dicer and Drosha in peripheral blood mononuclear cells (PBMCs) from HIV-1–infected donors who had been either CD8+T cell–depleted to facilitate virus production (*5*) (Fig. 1A) or not (Fig. 1B) (*6*–*8*). On the basis of reverse transcription polymerase chain reaction (RT-PCR) performed on total RNA isolated from cells, we verified that both Dicer and Drosha mRNAs were efficiently reduced (Fig. 1). When siRNA-transfected CD8+ T cell–depleted PBMCs were cocultured with activated PBMCs from healthy donors, HIV-1 from Dicer and Drosha knocked-down cells replicated with faster kinetics compared to virus from cells transfected with nonfunctional Dicer and Drosha siRNA control (Fig. 1A). When total PBMCs from infected donors were used, this effect was more pronounced (Fig. 1B).

The repressive effect of Dicer and Drosha on HIV-1 replication was also observed in latently infected U1 cells, which express a mutant of HIV-1 Tat protein unable to efficiently activate the long terminal repeat (LTR) required for efficient viral transcription (*9*) and consequently produce a low amount of virus (fig. S1A). HIV-1 infection and replication were also more efficient in Dicer or Drosha knocked-down Jurkat cells, compared with control siRNA-transfected cells (fig. S1B). Because knockdown of Dicer and Drosha is transient, the observed effect on virus replication suggests that Dicer- and Drosha-mediated repression of viral gene expression takes place early during virus replication (fig. S1C). To verify this, we evaluated the effect of Dicer and Drosha knockdown in a single-round infection assay in which cells were infected with HIV-1 that harbored a luciferase gene (HIV-1VSV-Luc) pseudotyped with vesicular stomatitis virus (VSV.G) envelope. In a single-round infection assay, we observed by luciferase activity that knockdown of Dicer or Drosha increased virus production when compared with control siRNA-transfected

[1]Laboratoire de Virologie Moléculaire, Institut de Génétique Humaine, Montpellier, France. [2]Laboratoire des Lentivirus et Transfert de Gènes, Institut de Génétique Humaine, Montpellier, France. [3]Institut de Pharmacologie Moléculaire et Cellulaire, UMR6097 CNRS/UNSA, Sophia Antipolis, France. [4]Service des Maladies Infectieuses et Tropicales, Hôpital Gui de Chauliac, Montpellier, France. [5]Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA.

*To whom correspondence should be addressed. E-mail: bmonsef@igh.cnrs.fr