HIV-2 evolved ZAP resistance despite increased CpG levels

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Abstract

Simian immunodeficiency viruses infecting sooty mangabeys (SIVsmm) have been transmitted to humans on multiple occasions, giving rise to human immunodeficiency virus type 2 (HIV-2) groups A to I. However, only groups A and B spread significantly in humans causing an epidemic in West Africa. The reasons for this are poorly understood. Here, we show that genetically diverse SIVsmm strains efficiently infect primary human T cells. However, they are highly sensitive to type I IFN, indicating that interferon stimulated genes (ISGs) pose a barrier to successful spread of SIVsmm in humans. One well-known ISG is the zinc finger antiviral protein (ZAP), which specifically targets CpG dinucleotides in viral RNAs. To evade ZAP restriction, many viruses including HIV-1 suppress their CpG content. Unexpectedly, we found that HIV-2 is less sensitive to restriction by ZAP than HIV-1 and SIVsmm despite significantly higher CpG content. Analyses of chimeric HIV-2/SIVsmm constructs revealed that the determinants of ZAP resistance map to the U3 region of the 3' LTR that overlaps with the *nef* gene. Our results indicate that ZAP poses a barrier for efficient spread of SIVsmm in humans and that epidemic HIV-2 evolved an unique mechanism to evade it.

Introduction

Zinc-finger antiviral protein (ZAP, also known as ARTD13, PARP13, and ZC3HAV1) is a broadly expressed RNA-binding protein that inhibits multiple RNA and DNA viruses [1–3]. Similar to other antiviral restriction factors, ZAP is under positive selection in primates and its expression and further induced by type I interferons. ZAP binds to CpG dinucleotides in HIV-1 RNA and recruits co-factors Tripartite Motif Containing 25 (TRIM25) and KH And NYN Domain Containing (KHNYN) proteins to degrade it, which reduces viral protein expression and replication[4–7]. HIV-1 suppresses its CpG content and at least partially escapes

the antiviral effects of ZAP[4,8]. However, it is unknown if other viruses use the same evasion strategy. There are over 40 known simian immunodeficiency (SIV) species naturally infecting African primates. Of those, SIVs infecting chimpanzees, gorillas and sooty mangabeys successfully crossed the interspecies barrier and gave rise to HIV-1 (SIVcpz, SIVgor) and HIV-2 (SIVsmm). While HIV-1 group M has spread globally causing 95% of all HIV infections, HIV-2 infects around 1-2 million people mostly in West Africa[9]. HIV-1 and HIV-2 evolved sophisticated ways to evade or counteract human immune responses and cause persistent infection that eventually leads to a fatal acquired immune deficiency syndrome (AIDS) in the absence of antiretroviral treatment. Most HIV-2 infections are characterized by lower transmission rates, longer asymptomatic phase, slower CD4+ cell decline and lower plasma viral loads than HIV-1[10–13]. SIVsmm, the closest relative of HIV-2, does not cause significant pathogenesis in its natural host and is highly prevalent in sooty mangabey populations[14]. It crossed the species barrier from monkeys to humans on at least nine occasions, with two of these transmissions leading to significant spread in the human population, giving rise to epidemic HIV-2 groups A and B. While the overall prevalence of HIV-2 in the human population is decreasing, new cases of HIV-2, including group A and B recombinants (AB), continue to be identified outside of West Africa[15–17]. SIVsmm strains readily infect human T cells *in vitro* and counteract antiviral restriction factors tetherin and SERINC5 to promote virus release and infectivity, showing a level of pre-adaptation to humans[18–20]. However, SIVsmm is less efficient at counteracting human APOBEC3F[21] and IFI16[22] than HIV-2, suggesting that the latter had to evolve additional innate immune evasion strategies to spread significantly in humans. The convergent evolution of immune evasion mechanisms in HIV-1 and HIV-2 highlights the importance of intrinsic immunity in preventing zoonotic outbreaks and pandemics. However, the determinants of HIV-2's adaptation to humans are still not fully understood.

Here we aimed to assess if evasion of ZAP was a pre-requisite for evolution and spread of HIV-2 in humans. We found that SIVsmm strains display higher IFN sensitivity compared to HIV in primary human cells. Furthermore, SIVsmm was significantly more inhibited by ZAP than HIV-2. Surprisingly, we found that epidemic HIV-2 evolved resistance to ZAP while gaining additional CpG dinucleotides. By exchange of different genomic regions between HIV-2 and SIVsmm we found that the determinants of HIV-2 evasion of ZAP are different from those described for HIV-1 and involve changes in the 3' LTR U3 region. Together, our results identify ZAP's contribution to restriction of SIVsmm in human cells and uncover a ZAP evasion mechanism in HIV-2.

Results

SIVsmm is highly sensitive to restriction by type I IFN in primary human T cells

HIV-2 and SIVsmm strains are highly divergent from HIV-1 (Figure 1A) and show less than 60% nucleotide sequence conservation. In comparison, HIV-2 and SIVsmm share ~75% sequence homology. To compare the level of adaptation of those viruses to the human host and their ability to evade human intrinsic immune responses, we analysed the impact of type I IFN treatment on the replication of HIV-1, HIV-2 and SIVsmm in primary human T cells[21]. To ensure that the results are representative of the lentiviral lineages, we selected panels of five infectious molecular clones (IMCs) of HIV-1, including four primary transmitted-founder IMCs of subtypes B and C (CH077, CH058, THRO and CH167), five HIV-2 IMCs including the patient-derived 7312A strains, and six SIVsmm strains representing diverse viral lineages (L1-L5). Primary human peripheral blood mononuclear cells (PBMCs) isolated from four healthy donors stimulated with phytohemagglutinin (PHA) and inoculated with HIV or SIV stocks normalized for p24/p27 content in the presence or absence of IFNα. Infected cultures were sampled every 2 days and infectious virus yields measured using the TZM-bl reporter cell assay. In the absence of IFNα treatment, all tested viruses replicated efficiently, with HIV reaching a replication peak at day 4-8 and SIVsmm reaching a peak at day 6-10 (Figure 1C). HIV-1 strains replicated very efficiently also in the presence of IFN (2-fold reduction), while HIV-2 replication was inhibited by ~5-fold (Figure 1D). In contrast, SIVsmm strains were strongly suppressed by type I IFN (32-fold reduction) and in general replicated poorly in its presence. Thus, tested SIVsmm strains showed decreased replication fitness and immune evasion as compared to HIV-1 and HIV-2, suggesting suboptimal level of adaptation to type I IFN induced antiviral restriction factors.

HIV-2 evolved ZAP resistance

ZAP is a broadly-acting antiviral restriction factor encoded by the ZC3HAV1 interferon stimulated gene[3,23]. It acts through binding to CpG elements in RNA and forming antiviral complex with TRIM25 ubiquitin ligase and endoribonuclease KHNYN that degrades viral transcripts. To examine expression and potential IFN-inducibility of the members of the ZAP antiviral complex, we stimulated human PBMCs with type I (α,β) and II (γ) IFNs for 48h and measured protein expression by flow cytometry. ZAP expression was induced by ~2 fold by all three types of IFN tested, whereas TRIM25 was only induced by type I IFN (Figure 2A). KHNYN expression was only up to 1.4-fold induced by IFNs. These results indicated that ZAP might contribute to efficient restriction of SIVsmm replication by IFNs in human PBMCs.

We have previously shown that endogenous ZAP only moderately restricts primary HIV-1 strains in THP-1 monocyte-like cells due to strong CpG suppression in the viral genomes[8]. To determine if ZAP inhibits HIV-2 and/or SIVsmm, we inoculated IFNα pre-treated or untreated THP-1 ZAP KO and non-targeting control (NTC) cell with VSV-g pseudotyped viral particles and determined infectious virus production at day 6 post infection using TZM-bl reporter cells. SIVsmm strains did not replicate under these conditions, precluding further analysis (Figure S1A). Most HIV-1 and HIV-2 strains productively infected THP-1 cells, and the control HIV-1 NL-43 CpG replicated close to background levels in NTC cells and was strongly enhanced (>100-fold) in ZAP KO cells. HIV-1 NL4-3 as well as HIV-2 strains replicated at similar levels in the presence and absence of ZAP (Figure 2C). Furthermore, HIV-2 was resistant to ZAP also in the presence of IFN (Figure 2D). To further determine if SIVsmm is restricted by endogenous ZAP expression, we used Jurkat CRISPR Cas9 KO cells engineered to express the CCR5 coreceptor[24]. Infection of control and ZAP KO cells with HIV-1, HIV-2 and SIVsmm resulted in detectable virus production, with most HIV strains replicating to high titres, and SIVsmm strains showing only modest growth (Figure 2E). HIV-

1 showed a modest increase in infectious virus production in ZAP KO cells in the absence (~1.7 fold) and presence of IFNα (1.9 fold) whereas HIV-2 was unaffected by the ZAP KO (Figure 2F). SIVsmm showed a much stronger (on average 3-fold) enhancement in replication in the absence of ZAP which further increased up to 5-fold upon IFNα treatment. Thus, ZAP restricts SIVsmm more efficiently than HIV-1 and HIV-2 in human T cells. To confirm that these results were not biased by relatively low replication of SIVsmm strains in this system, we performed overexpression experiments in HEK293T ZAP KO cells[4]. Transfection of SIVsmm IMCs resulted in lower infectious virus production than in case of HIV-2, however in all cases (Figure S1C). On average, ZAP reduced the infectious virus yields of SIVsmm by 90% but only by 60% in the case of HIV-2 (Figures 2G, 2H). However, HIV-2 strains differed in sensitivity from being highly resistant (HIV-2 7312A primary isolate) to as sensitive as SIVsmm (HIV-2 ST). Altogether, these results clearly suggested that HIV-2 evolved increased ZAP resistance during adaptation to humans.

HIV-2 evades ZAP restriction despite higher CpG content

It has been proposed that CpG suppression plays a key role in evasion of ZAP restriction (Ref). For the set of HIV-2 and SIVsmm IMCs analysed, however, high CpG numbers did not correlate with increased ZAP sensitivity and even showed the opposite trend (Figure 3A). On average, HIV-2 strains showed about 2-fold higher CpG frequencies than HIV-1 (Figure 3B). Analysis of all available full viral genome sequences from the Los Alamos HIV database confirmed that epidemic HIV-2 A and B strains show significantly higher CpGs frequencies than SIVsmm despite increased ZAP resistance (Figure 3C). In contrast, the CpG frequencies in rare HIV-2 F-I did not differ significantly from those of SIVsmm. Thus, epidemic HIV-2 strains evolved ZAP resistance despite overall increasing CpG content. We have previously shown that the sensitivity of HIV-1 to ZAP is determined by the number of CpGs in the first 600 bp of the *env* ORF rather than the whole genome[8]. Thus, we compared

the distribution of CpGs across HIV-2 and SIVsmm genomes to identify differences in CpG frequencies in specific regions. CpGs were distributed and increased compared to SIVsmm across HIV-2 genomes (Figure 3D) and showed ~60% overlap with those found in SIVsmm (Figure 3E). Unlike HIV-1, ZAP resistance of HIV-2 was not associated with effective CpG suppression in the beginning of the *env* gene (Figure S2).

SIVsmm is efficiently restricted by sooty mangabey ZAP

Single stranded RNA binding domains (RBD) of ZAP orthologues from different vertebrate species exhibit varying degrees of activity and CpG-specificity[7]. To determine if lower activity of ZAP from sooty mangabeys helps to explain the high sensitivity of SIVsmm to the human orthologue, we amplified ZAP genes from two captive sooty mangabeys. ZAP alleles from one of the monkeys carried heterozygous mutations. Therefore, we cloned the large (L) isoform of all three variants into an expression vector. The otherwise highly conserved RNA binding domain (RBD) of sooty mangabey ZAP differed at three positions from human ZAP (Figure 3F). To determine the potential impact of these and other variation on antiviral activity, we co-transfected HEK293T cells with ZAP expression and proviral HIV-1, HIV-2 and SIVsmm constructs. Sooty mangabey ZAP orthologues restricted HIV-1 and SIVsmm as efficiently as human ZAP, while HIV-2 7312A was generally poorly affected (Figure 3G). Thus, ZAP has the potential to restrict SIVsmm in the natural sooty mangabey host.

HIV-2 evades ZAP restriction by *env*-independent mechanisms

As mentioned above, CpG levels across viral genomes did not predict ZAP sensitivity of SIVsmm and HIV-2. To further determine the impact of ZAP on HIV-1, HIV-2 and SIVsmm, we analysed viral protein expression levels in presence and absence of ZAP. ZAP moderately decreased HIV-1 Env and p24 capsid expression levels in transfected HEK293T cells (Figure 4A, left panel). In comparison, ZAP had little if any effect on the levels of HIV-2 Env and p27 expression (Figure 4A, right). In contrast, ZAP strongly reduced both Env and p27 expression in cells transfected with SIVsmm proviral constructs (Figure 4B). To determine a potential role of env in the differential susceptibility to ZAP restriction, we utilized bicistronic constructs encoding viral *env* gene and reporter eGFP (Figure 4C). In this system, both proteins are expressed from the same transcript due to the presence of an IRES element, meaning that degradation of viral RNA results in a reduced GFP expression and fluorescence signal. In this set-up, ZAP had only minor effects on HIV-2 and SIVsmm *env* expression and there was no clear difference between them, unlike in the case of full-length GFP reporter viruses (left). To confirm that *env* region is not responsible for the difference in ZAP sensitivity of HIV-2 and SIVsmm, we utilized published HIV-2 7312A recombinants encoding SIVsmm Env ectodomains[18] (Figure 4D). All chimeras remained resistant to ZAP (Figure 4E). Beside the nucleotide sequence and CpG content, expression levels of viral RNAs might affect ZAP sensitivity. To address this, we swapped the Kozak sequences upstream *env* that differ between HIV-2 and SIVsmm and are more conserved in the latter (Figure 4F). Notably, the SIVsmm Kozak sequence further increased HIV-2 7312A resistance to ZAP, which agrees with the prediction that it provides a stronger signal for initiation of env mRNA expression (https://atgpr.dbcls.jp/). These results showed that the mechanism of ZAP evasion in HIV-2 differs from that of HIV-1 and is *env*-independent.

HIV-2's *nef* encoding region contributes to its ZAP resistance

To map determinants of ZAP resistance in an unbiased manner, we generated chimeras between HIV-2 7312A and SIVsmm L1. To avoid the generation of chimeric viral ORFs, we selected the *vpx/vpr* gene border (Figure 5A) to generate IMCs carrying 5' and 3' genome halves of HIV-2 or SIVsmm, respectively (Figure 5B). Functional analyses showed that ZAP sensitivity was determined by the 3`half of the viral genome f (Figures 5C, S3A). Since *env* was already ruled out as a determinant, we tested whether the HIV-2 accessory Vpr and Nef proteins counteract ZAP. HIV-2 7312A IRES-eGFP constructs carrying premature stop codons in *vpr* or *nef* remained resistant to ZAP (Figure 5D).

To examine whether HIV-2 and SIVsmm *vpr* and/or *nef* encoding mRNAs differ in ZAP sensitivity, we utilized constructs expressing these accessory viral proteins and eGFP from bicistronic mRNA. ZAP reduced eGFP expression from mRNAs containing SIVsmm *vpr* and *nef* genes significantly more efficiently than from otherwise isogenic constructs containing the corresponding HIV-2 genes, while the opposite was observed for *env* containing IRES-eGFP constructs (Figure 5E). The *nef* containing SIVsmm PGM IRES eGFP construct was more efficiently inhibited (85% decrease) than that containing *vpr* (45% decrease). Further analyses, confirmed that bicistronic IRES-eGFP constructs containing SIVsmm *nef* alleles are generally highly sensitive to ZAP restriction (Figure 5F). Analyses of further chimeras between HIV-2 7312A and SIVsmm L1 or L2 (Figure 5G) verified that the *nef* ORFs is an important determinant of ZAP sensitivity (Figure 5H). However, HIV-2 constructs containing SIVsmm *nef* genes were still significantly more resistant to ZAP than the SIVsmm L1 and L2 IMCs. Unfortunately, SIVsmm chimeras containing HIV-2 nef genes were poorly infectious precluding meaningful analyses. Altogether, these results showed that the *nef* coding region is an important determinant of the differential susceptibility of HIV-2 and SIVsmm to ZAP restriction.

The U3 region of the LTR determines ZAP resistance of HIV-2

Nef overlaps the 3'LTR. To further define the ZAP sensitivity determining region, we thus swapped the entire LTR as well as the 3' U5, R and U3 regions between HIV-2 and SIVsmm L1 (Figure 6A). Strikingly, exchange of the LTRs completely switched the phenotypes of HIV-2 and SIVsmm with regards to ZAP sensitivity (Figure 6B). In addition, LTR exchange generally reduced infectious virus production by one to two orders of magnitude (Figure S5A). Exchange of 3' U3, R and U5 regions was much lesser effect on infectious virus production (Figure S5B), and analysis of these chimeras revealed that the U3 region, which overlaps the *nef* ORF and is found in all main HIV/SIV splicing products[25], is a major determinant of HIV-2's ZAP resistance (Figure 6C). Comparison of U3 sequences showed that two CpG dinucleotides are present in all ZAP sensitive SIVsmm strains but absent in the ZAP resistant HIV-2 7312A strain (Figure 6D). However, these CpGs had no significant impact on infectious virus yields (Figure 6E) and ZAP sensitivity (Figure 6F). These results suggest that CpG independent alterations in the U3 region of the 3`LTR allowed HIV-2 to evade ZAP restriction.

Discussion

ZAP is known to restrict the replication of many RNA and DNA viruses, often in a IFN-dependent manner[1]. The ability of ZAP to preferentially target foreign RNA transcripts relies on its selective binding to CpG dinucleotides[4] which represent the least abundant type of dinucleotide in the vertebrate genomes[26]. HIV-1 suppresses its CpG content to partially escape ZAP's activity[4] and it was suggested that other CpG-suppressed vertebrate RNA viruses use similar evasion mechanism. Here we found that ZAP poses a significant barrier to SIVsmm replication in human cells despite its genomic CpG-suppression, and found that during human-adaptation HIV-2 evolved changes in its U3 LTR that allow it to efficiently evade ZAP. Analysis of SIVsmm and HIV-2 epidemic and rare group sequences revealed that human adaptation of HIV-2 involved a substantial increase in CpG levels, which might have

coincided with evolution of the new ZAP evasion mechanism. Our findings challenge the notion that genomic CpG content dictates ZAP sensitivity and highlights the importance of ZAP in inhibiting the spread of zoonotic viruses.

We have previously shown that CpG frequency in the 5' end of the *env* gene determines sensitivity of primary HIV-1 strains to the ZAP[8]. This is likely caused by low expression levels of HIV-1 Env caused by the presence of the upstream *vpu* gene expressed from the bicistronic *vpu/env* mRNA. While SIVsmm does not encode *vpu*, ZAP overexpression led to a decrease in its virus production and Gag and Env expression levels, which can be explained by the fact that all SIVsmm splicing products contain the untranslated 3' U3 region targeted by ZAP. Our study ruled out the involvement of the HIV-2 Env in ZAP resistance and underscores divergent evolutionary paths taken by HIV-1 and HIV-2 in adapting to human immune defences. Additionally, the observed ZAP sensitivity of SIVsmm is one of the factors contributing to its limited fitness and poor immune evasion in primary human cells. Furthermore, our findings suggests that while the *nef* ORF contributes to ZAP evasion due to its overlap with 3' LTR, Nef protein does not act as a direct antagonist of ZAP. This means that while the immunomodulatory functions of HIV-2 and SIVsmm Nefs are highly conserved[18,27,28], silent or apparently neutral changes in this ORF are major determinants of resistance to human antiviral restriction factors such as ZAP.

It is not the first study that identified viral LTR region as a target of ZAP. In fact, ZAP has been shown to target UTRs of MLV[29], xenotropic murine leukemia virus-related virus[30], Coxsackievirus B3 virus[31], Japanese encephalitis virus[32], small ruminant morbillivirus[33] as well as human TRAIL and IFNL3 mRNA[34,35]. It remains to be established if untranslated regions of other viruses and cellular mRNAs are also commonly targeted by ZAP and if evolution of other successful viral pathogens involved RNA modifications and escape of the broad antiviral activity of human ZAP.

While our results identified ZAP as a block to efficient SIVsmm infection in human cells, it is most likely not the only antiviral restriction factor that represents a cross-species barrier to this zoonotic virus. Type I IFN induces the expression of hundreds of ISGs, most of which have never been studied in the context of HIV-2 or SIVsmm restriction. Deciphering the contribution of all inidividual human ISGs to SIVsmm restriction would be an ambitious and time-consuming task that lies beyond the scope of the current study. Nevertheless, the generated chimeric HIV-2/SIVsmm infectious molecular clones will be highly useful for further studies on antiviral proteins and mechanisms that pose an interspecies barrier to SIVsmm transmission and spread.

In conclusion, this study shows that ZAP is a major contributor to the IFN-mediated restriction of SIVsmm in human T cells, and elucidates a critical aspect of HIV-2's adaptation to its human hosts. By demonstrating that HIV-2's resistance to ZAP is mediated by a CpG-independent mechanism involving the 3' LTR U3 region, we identified a new aspect of HIV-2's adaptation to humans and a new type of a ZAP evasion mechanism. It remains to be established if ZAP antagonism contributes to spread and pathogenesis of HIV-2 *in vivo*, as well as if it removes the evolutionary constrains posed by ZAP on lentiviral genomes and their CpG content. It will be extremely interesting if untranslated regions of other viruses are also commonly targeted by ZAP and if similar evasion mechanism have been employed by other successful zoonotic viral pathogens. Thus, further studies on ZAP targets and evasion mechanism will provide insights into the virus-host arms race and the mechanisms shaping viral genomes.

Materials and Methods

Cell lines

HEK293T ZAP KO cells have been previously described [4]. Jurkat WT and ZAP KO cells were described before[24]. TZM-bl cells, which express CD4, CCR5, and CXCR4 and contain the β-galactosidase genes under the control of the HIV promoter [36,37].

Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5% (during virus production) or 10% (at all other times) heat-inactivated foetal calf serum (FCS), 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Jurkat cells were cultured in RPMI supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Isolation of primary cells

Peripheral blood mononuclear cells (PBMCs) from healthy human donors were isolated using lymphocyte separation medium (Biocoll separating solution; Biochrom), stimulated for 3 days with phytohemagglutinin (PHA; 2 µg/ml), and 10 ng/ml interleukin-2 (IL-2) in RPMI 1640 medium containing 10% fetal calf serum.

Expression constructs

The pCG IRES eBFP vector encoding human ZAP-L containing an N-terminal hemagglutinin (HA) tag and empty vector control were described before[8]. The infectious molecular clones of HIV-1 NL4-3, HIV-1 NL4-3 CpG high mutant, CH077 TF, CH058 TF, THRO, CH167, HIV-2 7312A, ST, KR, GH123, ROD10, MCR, MCN, as well as SIVsmm PGM, SIVsmm pg nef-IRES eGFP, L1.V1, L1.V2, L2, L3, L4 and L5 were described previously [5,21,38–50]. HA-ZAP-L zinc-finger 1-4 deletion mutant was generated by Q5 site directed mutagenesis using pCG HA-ZAP-L IRES BFP as a template using primer fwd (AACCTGATGGACAGAAAGGTGC) and primer rev (GACCCGGGCTCGAGTGGTGG). pCG mCherry-ZAP-L IRES BFP expression construct was generated by Gibson assembly using the XbaI/MluI cloning sites and primers mCherry

fwd (tgtagaagcgcgtaggccttctagatATGGTGAGCAAGGGCGAGGA), mCherry rev (ccgggtccgcCTTGTACAGCTCGTCCATGCC) and ZAP-L fwd (gctgtacaagGCGGACCCGGAGGTGTGCTG) and ZAP-L rev (tgtagtactccgggatccgaCTAACTAATCACGCAGGCTTTGTCTTCAGTATATTCAATCACA TATTGTG). SIVsmm Env and Nef expression vectors and HIV-2 7312A env chimeras were previously reported [18]. HIV-2 7312A IRES eGFP constructs carrying premature stop codons in *vpr* and *nef* ORFs were described before[42]. SIVsmm Vpr and Nef expression constructs were described before[20,51] Chimeric HIV-2 and SIVsmm infectious molecular clones as well as CG mutants thereof were generated using Gibson assembly (NEB) and cloned into pCR XL TOPO or pBR vector using NotI/MluI restriction sites using primers listed in Table 1. Cloned construct correctness was confirmed by Sanger sequencing (eurofins).

Cloning of sooty mangabey ZAP

Whole blood from two sooty mangabeys was collected and PBMCs were purified via Ficoll density gradient centrifugation and RNA from 1 million cells was extracted according to the manufacturer's instructions (RNeasy Mini Kit, Qiagen cat# 74104).

To generate cDNA SuperScript™ IV Reverse Transcriptase (cat# 18090010) kit was used. The cDNA was cleaned using a DNA Clean and Concentrator spin column (Zymo, cat# D4033) and eluted in RNase-free water. ZAP transcript was amplified from cDNA using forward (GCCGCGGCAGAGCCAGCACAGCCAGC) and reverse (GAATGGTTATGGCATC CTTCTGACGC) primer pair binding in the 5' and 3' ZAP-L UTR. Resulting PCR product was ligated into pCR XL TOPO Blunt vector. Sanger sequencing revealed 3 unique ZAP-L sequences. In the next step, ZAP was re-cloned into pCG IRES BFP vector using XbaI/MluI restriction sites. N-terminal HA tag was added during PCR (fwd:

TGTAGAAGCGCGTAGGCCTTCTAGATATGTACCCATACGATGTTCCAGATTACGC TGCGGACCCGGAGGTG; rev: GTAGTACTCCGGGATCCGACGCGTCTAACTAATCACGCAGGG). Nucleotide identity of all constructs was verified using Sanger sequencing (eurofins).

Transduction of Jurkat cells

Vesicular stomatitis virus glycoprotein (VSV-g)-pseudotyped virus stocks were prepared by transfecting HEK293T cells with 1 µg VSV-g and 5 µg proviral construct, followed by a medium change. Supernatants were harvested 48 h later and normalized based on infectivity as measured by the TZM-bl reporter assay. One million Jurkat WT or ZAP KO cells were seeded and treated with IFN-β (200 U/ml) or left unstimulated at the time of transduction. The input virus was removed 24h later and cells were washed in PBS. Three and six days later, supernatants were harvested and used to infect TZM-bl reporter cells in triplicates. β-Galactosidase activity was measured 2 days later using the Gal-Screen kit (Applied Biosystems) as relative light units per second using a microplate luminometer.

FACS ZAP in infected cells

PHA-activated PBMCs were infected with VSV-G-pseudotyped HIV-2 or SIVsmm viruses and harvested three days post infection for flow cytometric analysis. Cells were fixed and permeabilized (NordicMUBio, Cat#GAS-002) and then stained intracellularly for Gag (FITC conjugated; 6604665; Beckman Coulter) and ZAP (GenTex, cat# GTX120134), followed by secondary anti-rabbit AlexaFluor647 (Invitrogen, cat#A-21245). Rabbit isotype IgG antibody served as a negative control (CellSignaling cat#2729S). The MFI of infected (FITC+) cells were used to determine changes in ZAP protein levels.

Lentiviral sensitivity to ZAP

To assess relative lentiviral sensitivity to ZAP overexpression, HEK293T ZAP KO cells (in 24-well format) were co-transfected using polyethyleneimine Max (PEI Max) transfection reagent with 600 ng of the indicated IMC and increasing concentrations (0, 100, 200, and 400 ng) of pCG ZAP-L IRES BFP expression vector and the amount of DNA was normalized to 1 μg by adding pCG IRES GFP vector. Virus-containing supernatants were harvested 2 days later and used to infect 10.000 TZM-bl reporter cells in triplicates. β-Galactosidase activity was measured 2-3 days later using the Gal-Screen kit (Applied Biosystems) as relative light units per second using a microplate luminometer. Infectious virus yield values of each IMC in the presence of ZAP were normalized to the corresponding GFP-only control.

Lentiviral sequence analysis and CpG mapping

Sequences of SIVsmm and HIV-2 isolates were obtained from the Los Alamos HIV sequence database (https://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html). A cladogram showing the relationship between these lentiviruses was generated using the ClustalW2 simple phylogeny tool (https://www.ebi.ac.uk/Tools/phylogeny/simple_phylogeny/). CpG frequency was calculated as [number of CpG]/[sequence length], whereas CpG suppression was calculated as [number of CpG * sequence length]/[number of C * number of G].

Western blotting

To examine the viral protein levels under the expression of ZAP, HEK293T ZAP KO cells were co-transfected in 12-well plates with 1.25 μg of indicated IMC and 0.25 μg DNA of pCG HA-ZAP IRES BFP vector or empty pCG IRES BFP vector. Two days post-transfection, cells were lysed with coimmunoprecipitation (CO-IP) buffer (150 mM NaCl, 50 mM HEPES, 5 mM EDTA, 0.10% NP-40, 0.5 mM sodium orthovanadate, 0.5 mM NaF, protease inhibitor cocktail from Roche), and cell-free virions were purified by

centrifugation of cell culture supernatants through a 20% sucrose cushion at 20,800 × g for 90 min at 4°C and lysed in co-IP lysis buffer. Samples were reduced in the presence of β-mercaptoethanol by boiling at 95°C for 10 min. Proteins were separated in 4% to 12% Bis-Tris gradient acrylamide gels (Invitrogen), blotted onto a polyvinylidene difluoride (PVDF) membrane, and incubated with anti-HIV-2 Env (cat#ARP-1410; NIH AIDS Reagent Program), anti-SIVmac p27 (cat#ARP-2321; NIH AIDS Reagent Program), anti-GAPDH (Cat# 607902; BioLegend), and anti-ZAP (catalogue no. GTX120134; GeneTex) antibodies. Subsequently, blots were probed with IRDye 680RD goat anti-rabbit IgG(H+L) (catalogue no. 926-68071; LI-COR) and IRDye 800CW goat anti-mouse IgG(H+L) (catalogue no. 926-32210; LI-COR) Odyssey antibodies and scanned using a Li-Cor Odyssey reader.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. ANOVA or two-tailed Student's t-test were used to determine statistical significance. Significant differences are indicated as: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Statistical parameters are specified in the figure legends.

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Disclosure statement

The authors report there are no competing interests to declare.

Figure legends

Figure 1. Replication fitness and type I IFN sensitivity of HIV-1, HIV-2 and SIVsmm in primary human cells. (A) Phylogenetic tree of HIV-2 (red) and their monkey-infecting precursor SIVsmm (blue) . HIV-1 NL4-3 (green) was used as an outgroup. (B) Replication kinetics of indicated lentiviral strain in pre-activated and IFNα-treated (500u/ml; dotted line) or untreated (solid line) human PBMC up to 10 days post infection. Infectious virus yield was quantified using TZM-bl reporter assay. Shown is the mean of 3 individual donors, each tested in triplicates of infection, +/- SEM. (C) Average time of replication kinetic plateau of each virus group based on (B). Mean + SD. SIVsmm L5 was excluded due to not reaching peak replication within the first 10 days. (D) Viral production inhibition by 500u/ml IFNα

based on (B). Mean + SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001, calculated by Mann–Whitney test.

Figure 2. IFN inducibility of ZAP antiviral complex and its activity against HIV-2 and SIVsmm. (A) Expression levels of ZAP and its cofactors TRIM25 and KHNYN in primary human CD4+ T cells 2 days after stimulation with 500u/ml type I IFN (α,β) or 200u/ml type II IFN(γ) measured by flow cytometry. Each dot represents one donor. (B) HIV-1 (green) and HIV-2 (red) infectious virus production in CRISPR/Cas9 control (non-targeting sgRNA) or ZAP knock out (KO) monocytes (THP-1 cells) 6 days after transduction. (C) n-fold inhibition by ZAP calculated by dividing virus yield in the absence (KO) and presence (NTC) of ZAP. (D) n-fold inhibition of HIV-1 and HIV-2 by 500u/ml IFNα in control or ZAP KO THP-1 cells. (E) Infectious virus yield of HIV-1 (green), HIV-2 (red) and SIVsmm (blue) strains from transduced Jurkat cells6 days post infection and (F) average n-fold inhibition by ZAP in the absence and presence of 500u/ml IFNα pre-treatment. (G) Infectious virus yield of HIV-1, HIV-2 and SIVsmm produced from HEK293T ZAP KO cells co-transfected with indicated infectious molecular clone and 0,3µg of human HA-ZAP-L. Values were normalized to infectious virus produced in the absence of ZAP expression. (H) Average infectious virus produced from HEK293T ZAP KO cells in the presence of ZAP overexpression. N=3-4 + SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001, calculated using Student's t-test.

Figure 3. CpG number and distribution in HIV-2 and SIVsmm genomes and the antiviral activity of human and sooty ZAP orthologues. (A) Correlation between CpG number in HIV-2 (red) and SIVsmm (blue) strain genomes and their resistance to ZAP overexpression (related to Figure 2G). (B) Average CpG number in the tested HIV-1, HIV-2 and SIVsmm strains and (C) average CpG frequency in HIV-1, SIVsmm and HIV-2 (groups A-I) full-genome sequences obtained from the Los Alamos HIV sequence database. (D)

Distribution of CpGs in the aligned HIV-2 7312A (red) and SIVsmm L1 (blue) genomes and overlap in CpG positions (grey). ORF position in HIV-2 genome is shown for reference above the graph. (E) Quantification of the area under the curve (of D) for HIV-2, SIVsmm and overlapping peaks. (F) Protein alignment of human and sooty mangabey ZAP-L variants cloned from PBMC cDNA. RNA-binding domain containing 4 zinc-fingers (ZnF), WWE domain and catalytically inactive PARP domain are shown. Dots indicate amino acid conservation.(G) Infectious virus yield of HIV, HIV-2 and SIVsmm co-transfected with human and sooty mangabey ZAP-L in ZAP KO HEK293T cells. N=3 + SD. ***, $P < 0.001$, calculated using Mann–Whitney test.

Figure 4. Effect of ZAP on HIV-2 and SIVsmm Env transcript and protein expression and the impact of Env ectodomain and Kozak swaps on ZAP sensitivity. (A) Western blots showing p24/p27 and Env viral protein expression in HEK293T ZAP KO cells co-transfected with HA-ZAP-L and indicated infectious molecular clone of HIV-1, HIV-2 and (B) SIVsmm. GAPDH and Hsp90 served as protein loading controls. Viral protein expression in the cell supernatants is shown for SIVsmm due to low signal in cell lysate blot. (C) eGFP mean fluorescence intensity in HEK293T ZAP KO cells co-transfected with HIV-2 7312A IRES eGFP, SIVsmm PGM IRES eGFP reporter viruses or pCG Env IRES eGFP together with pCG ZAP-L IRES BFP or pCG ZAP-L delta RBD IRES BFP (negative control; 100%). (D) Schematic of HIV-2 7312A based Env ectodomain chimeras and (E) their sensitivity to ZAP overexpression compared to wild type HIV-2 7312A and SIVsmmL1 strains. (F) Alignment of env Kozak sequences in HIV-2 and SIVsmm and consensus sequence logo for each lentivirus group. (G) The impact of *env* Kozak swap between HIV-2 and SIVsmm on sensitivity to ZAK overexpression in HEK293T ZAP KO cells. N=3 + SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, calculated using Student's t-test.

Figure 5. Mapping of determinants of ZAP resistance using chimeric HIV-2/SIVsmm clones. (A) Position of ligation of the half HIV-2/ half SIVsmm virus chimeras. (B) Schematic of generated chimeric proviral constructs showing HIV-2 (red) and SIVsmm (blue) fragments. (C) Sensitivity of those chimeras to ZAP overexpression. (D) Effect of ZAP overexpression on wild type, reporter and mutant HIV-2 7312A reporters containing premature stop codons (*) in *vpr* and *nef* ORFs in HEK293T ZAP KO cells. (E) eGFP mean fluorescence intensity in HEK293T ZAP KO cells co-transfected with pCG ZAP-L IRES BFP or pCG ZAP-L delta RBD IRES BFP (negative control; 100%), and pCG IRES eGFP vectors containing HIV-2 or SIVsmm Vpr, Env or Nef ORFs or (F) Expanded panel of SIVsmm Nef ORFs. (G) Schematic of generated Nef chimeric proviral constructs showing HIV-2 (red) and SIVsmm (blue) fragments and (H) Sensitivity of those chimeras (purple) to ZAP overexpression relative to WT HIV-2 (red) and SIVsmm (blue) strains. N=3-4 + SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, calculated using Student's t-test.

Figure 6. Mapping of determinants of ZAP resistance in LTR using chimeric HIV-2/SIVsmm clones. (A) Schematic of generated LTR chimeric proviral constructs showing HIV-2 (red) and SIVsmm (blue) fragments. The effect of swapping (B) whole LTRs and (C) 3' LTR U3, R and U5 regions on HIV-2 7312A and SIVsmm L1 sensitivity to ZAP overexpression in HEK293T ZAP KO cells. (D) Region of U3 containing CpGs (green) conserved in ZAP sensitive SIVsmm strains but not found in ZAP resistant HIV-2 7312A strain and (E) their effect on infectious virus production in the absence and (F) presence of ZAP overexpression in HEK293T ZAP KO cells. N=3, each tested in triplicates + SD. ***, $P < 0.001$, calculated using Student's t-test.

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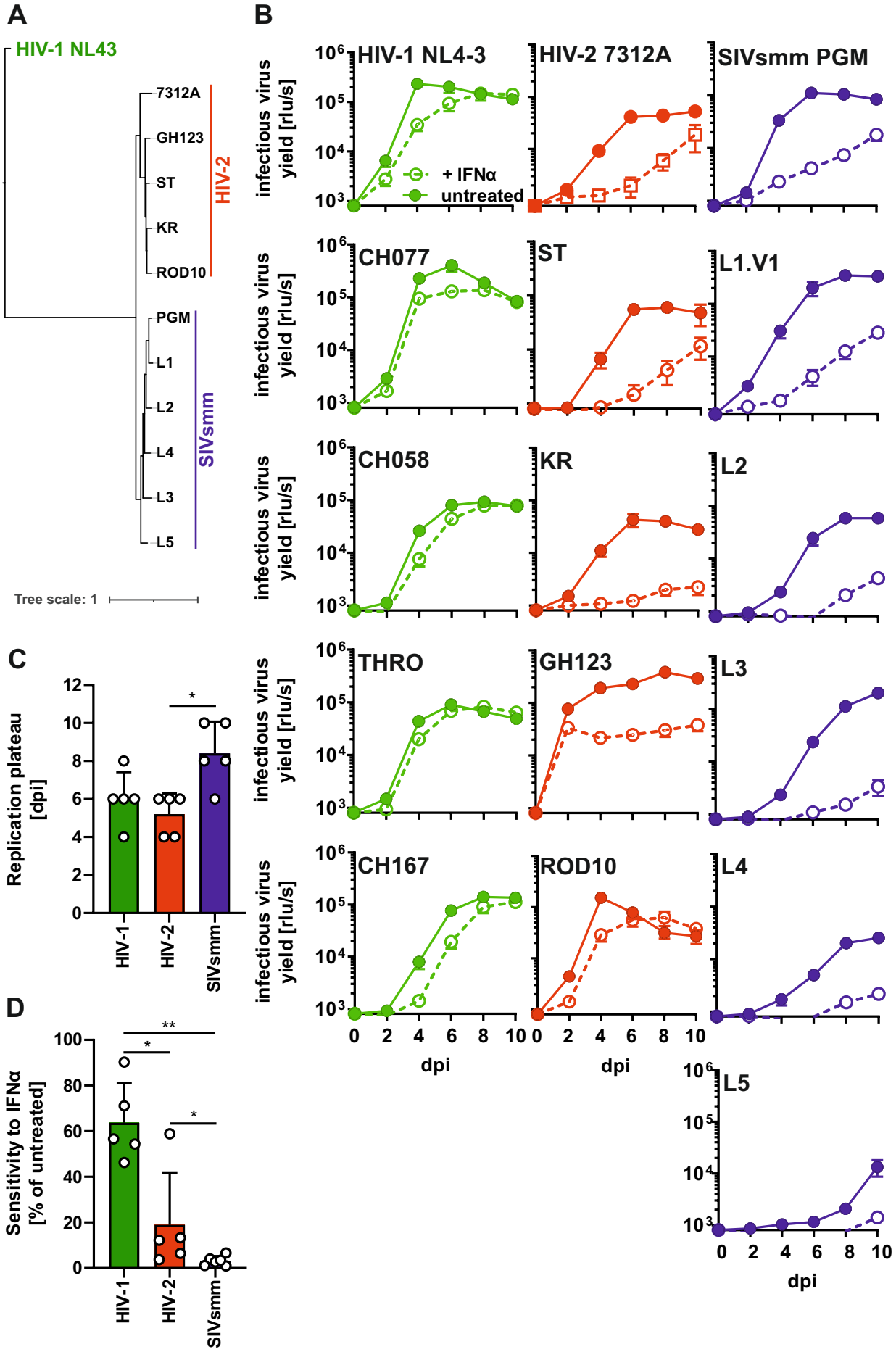
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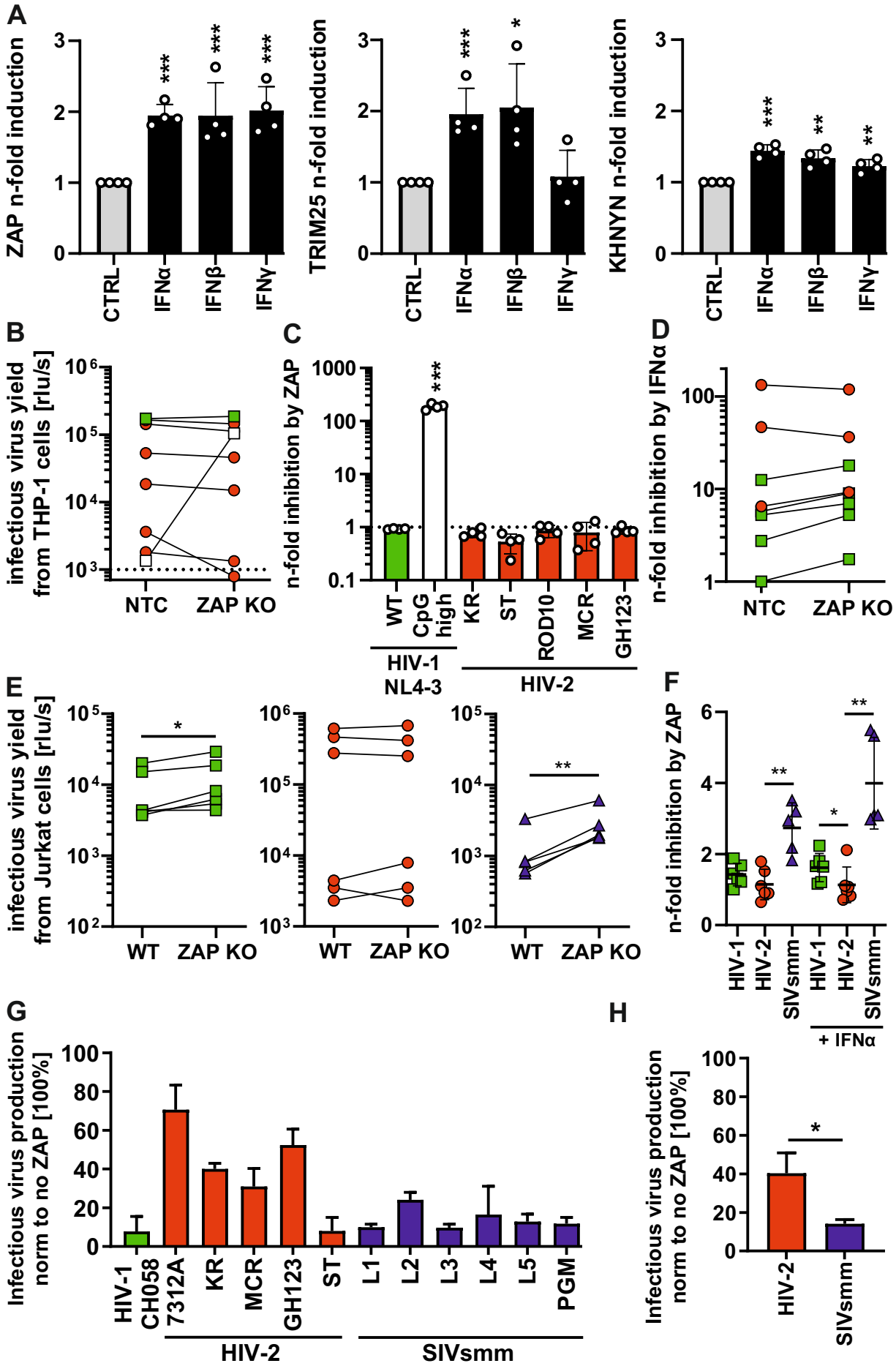
HIV-1 NL43
7312A
GH123
ST
KR
ROD10
HIV-2
PGM
L1
L2
L4
L3
L5
SIVsmm
Tree scale: 1
B
HIV-1 NL4-3
HIV-2 7312A
SIVsmm PGM
CH077
ST
L1.V1
CH058
KR
L2
THRO
GH123
L3
CH167
ROD10
L4
L5
+ IFNα
untreated
infectious virus yield [rlu/s]
dpi
C
Replication plateau [dpi]
HIV-1
HIV-2
SIVsmm
D
Sensitivity to IFNα [% of untreated]
HIV-1
HIV-2
SIVsmm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ZAP n-fold induction
TRIM25 n-fold induction
KHNYN n-fold induction
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IFNα
IFNβ
IFNγ
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NTC
ZAP KO
C
n-fold inhibition by ZAP
WT
CpG high
KR
ST
ROD10
MCR
GH123
HIV-1 NL4-3
HIV-2
D
n-fold inhibition by IFNα
E
infectious virus yield from Jurkat cells [rlu/s]
F
n-fold inhibition by ZAP
HIV-1
HIV-2
SIVsmm
+ IFNα
G
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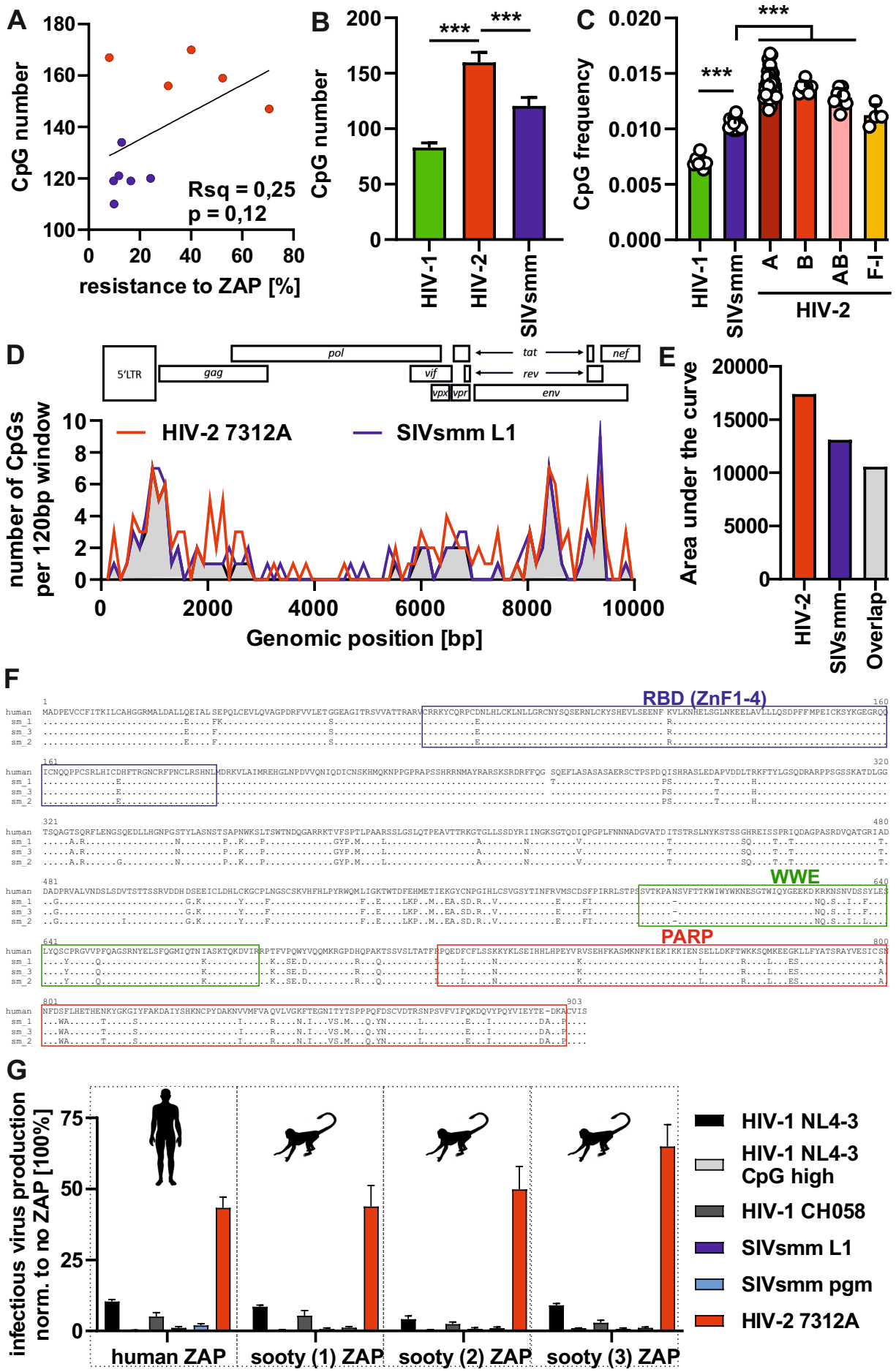
7312A
L1
L2
L3
L4
L5
PGM
H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CpG number
resistance to ZAP [%]
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B
CpG number
***
***
HIV-1
HIV-2
SIVsmm
C
CpG frequency
***
***
HIV-1
SIVsmm
A
B
AB
F-I
HIV-2
D
5'LTR
gag
pol
vif
vpx
vpr
tat
rev
env
nef
number of CpGs per 120bp window
HIV-2 7312A
SIVsmm L1
Genomic position [bp]
E
Area under the curve
HIV-2
SIVsmm
Overlap
F
RBD (ZnF1-4)
WWE
PARP
G
infectious virus production norm. to no ZAP [100%]
HIV-1 NL4-3
HIV-1 NL4-3 CpG high
HIV-1 CH058
SIVsmm L1
SIVsmm pgm
HIV-2 7312A
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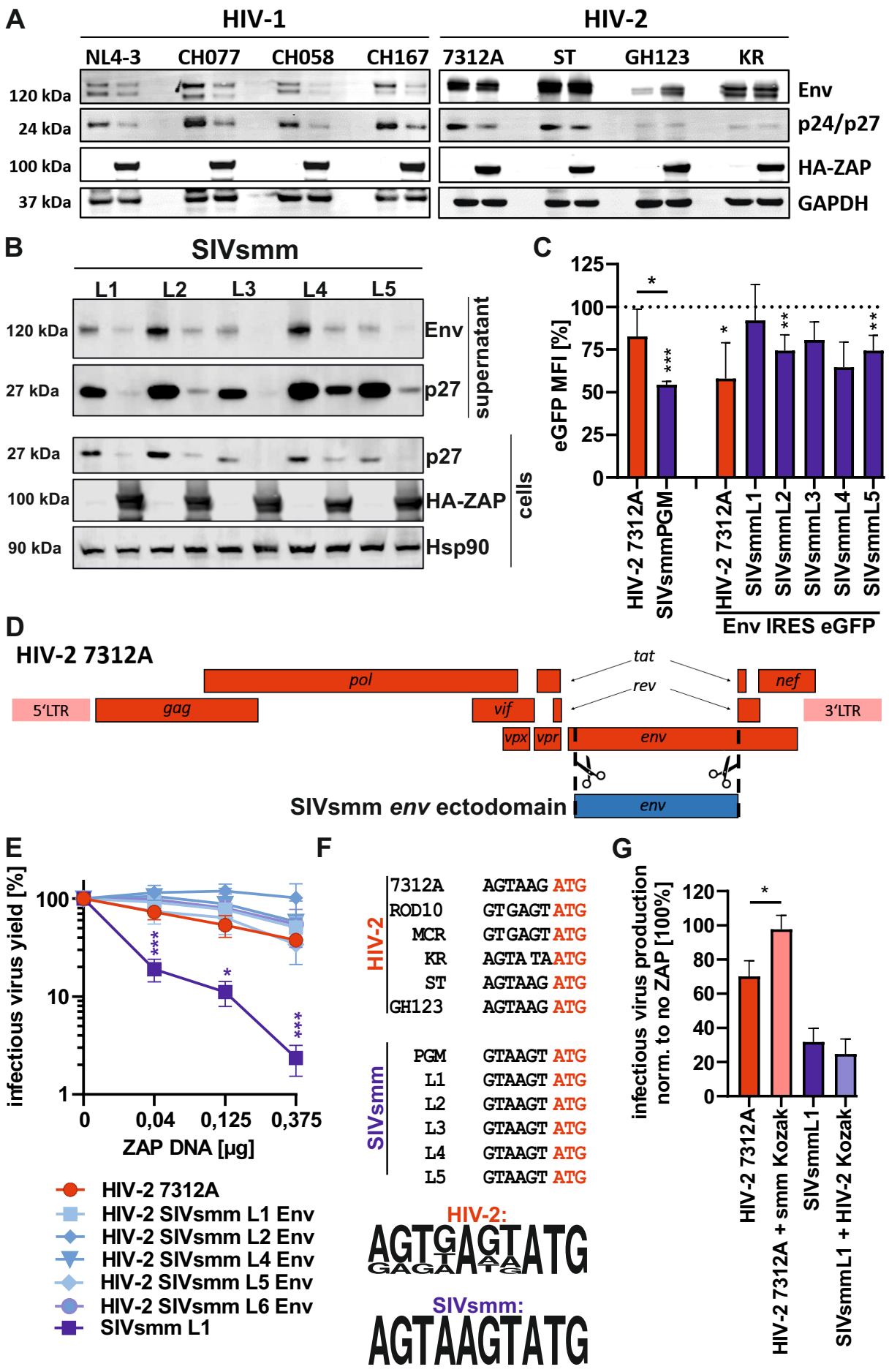
sooty (1) ZAP
sooty (2) ZAP
sooty (3) ZAP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120 kDa
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100 kDa
37 kDa
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p24/p27
HA-ZAP
GAPDH
B
SIVsmm
L1
L2
L3
L4
L5
120 kDa
27 kDa
Env
p27
supernatant
27 kDa
100 kDa
90 kDa
p27
HA-ZAP
Hsp90
cells
C
eGFP MFI [%]
125
100
75
50
25
0
HIV-2 7312A
SIVsmmPGM
HIV-2 7312A
SIVsmmL1
SIVsmmL2
SIVsmmL3
SIVsmmL4
SIVsmmL5
Env IRES eGFP
D
HIV-2 7312A
pol
tat
rev
nef
5'LTR
gag
vif
3'LTR
vpx
vpr
env
SIVsmm env ectodomain
env
E
infectious virus yield [%]
100
10
1
0
0,04
0,125
0,375
ZAP DNA [µg]
HIV-2 7312A
HIV-2 SIVsmm L1 Env
HIV-2 SIVsmm L2 Env
HIV-2 SIVsmm L4 Env
HIV-2 SIVsmm L5 Env
HIV-2 SIVsmm L6 Env
SIVsmm L1
F
HIV-2
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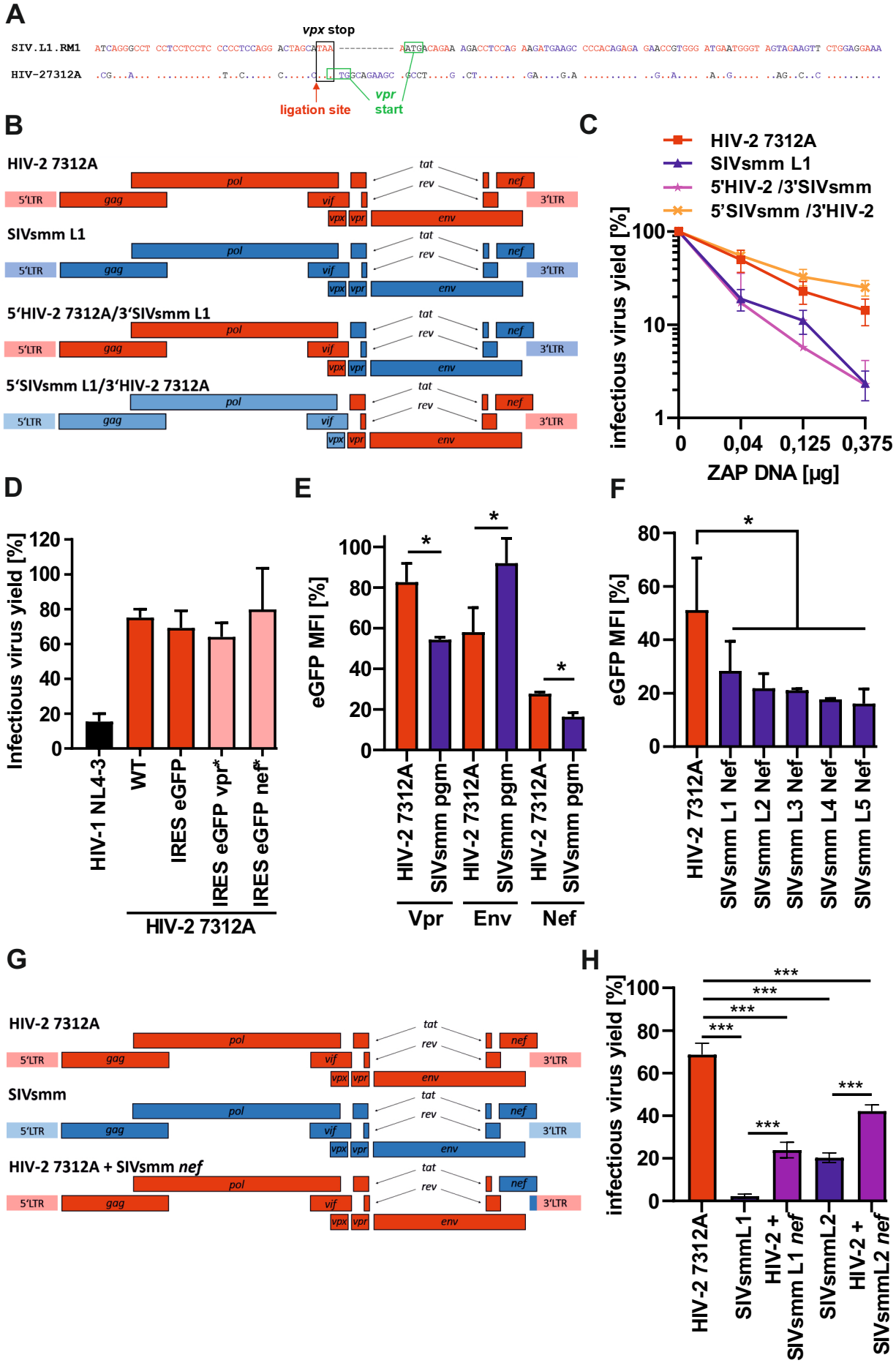
ROD10 GTGAGT ATG
MCR GTGAGT ATG
KR AGTA TAATG
ST AGTAAG ATG
GH123 AGTAAG ATG
SIVsmm
PGM GTAAGT ATG
L1 GTAAGT ATG
L2 GTAAGT ATG
L3 GTAAGT ATG
L4 GTAAGT ATG
L5 GTAAGT ATG
HIV-2:
SIVsmm:
G
infectious virus production norm. to no ZAP [100%]
120
100
80
60
40
20
0
HIV-2 7312A
HIV-2 7312A + smm Kozak
SIVsmmL1
SIVsmmL1 + HIV-2 Kozak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HIV-27312A
ligation site
vpr start
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HIV-2 7312A
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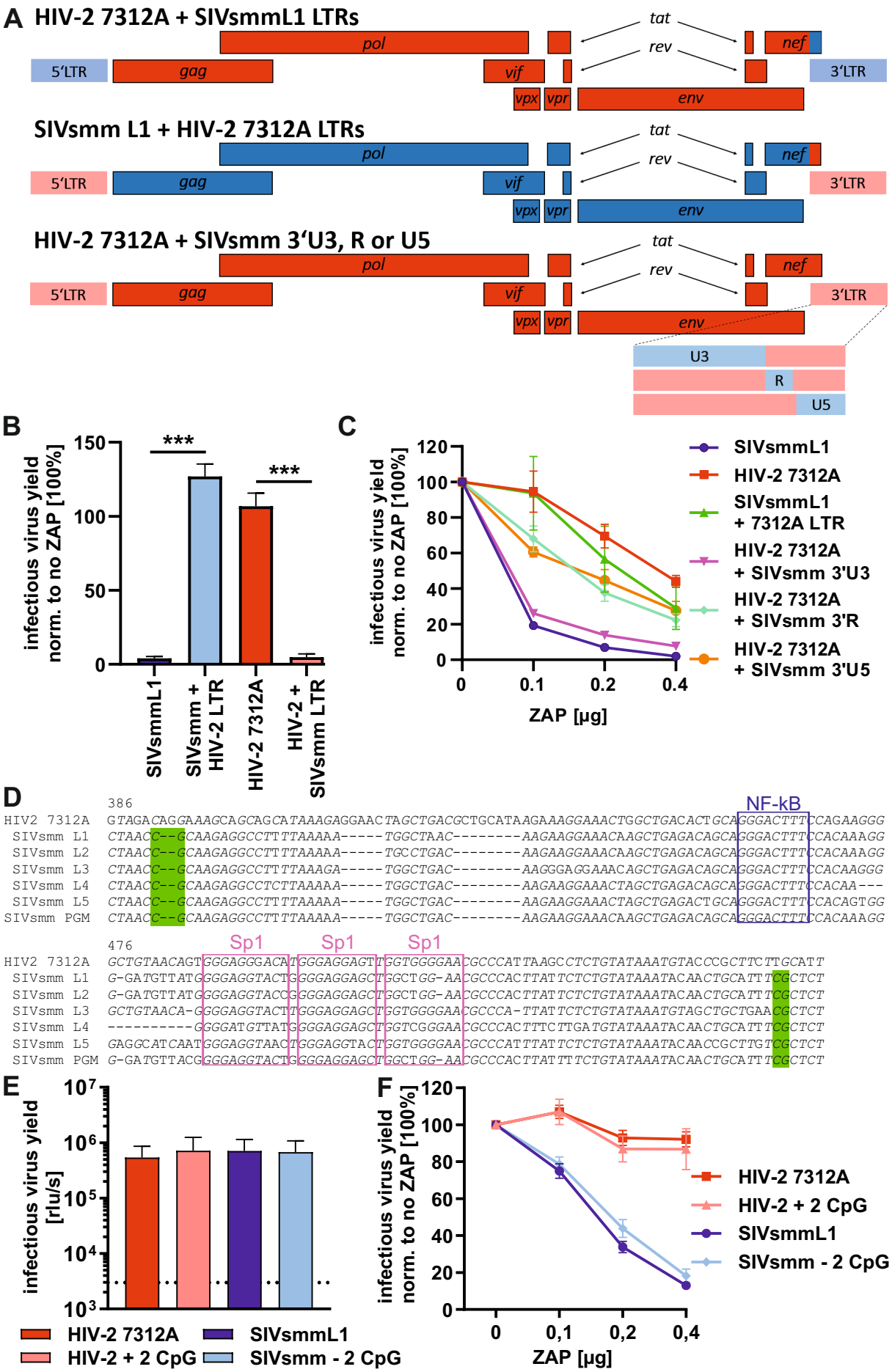
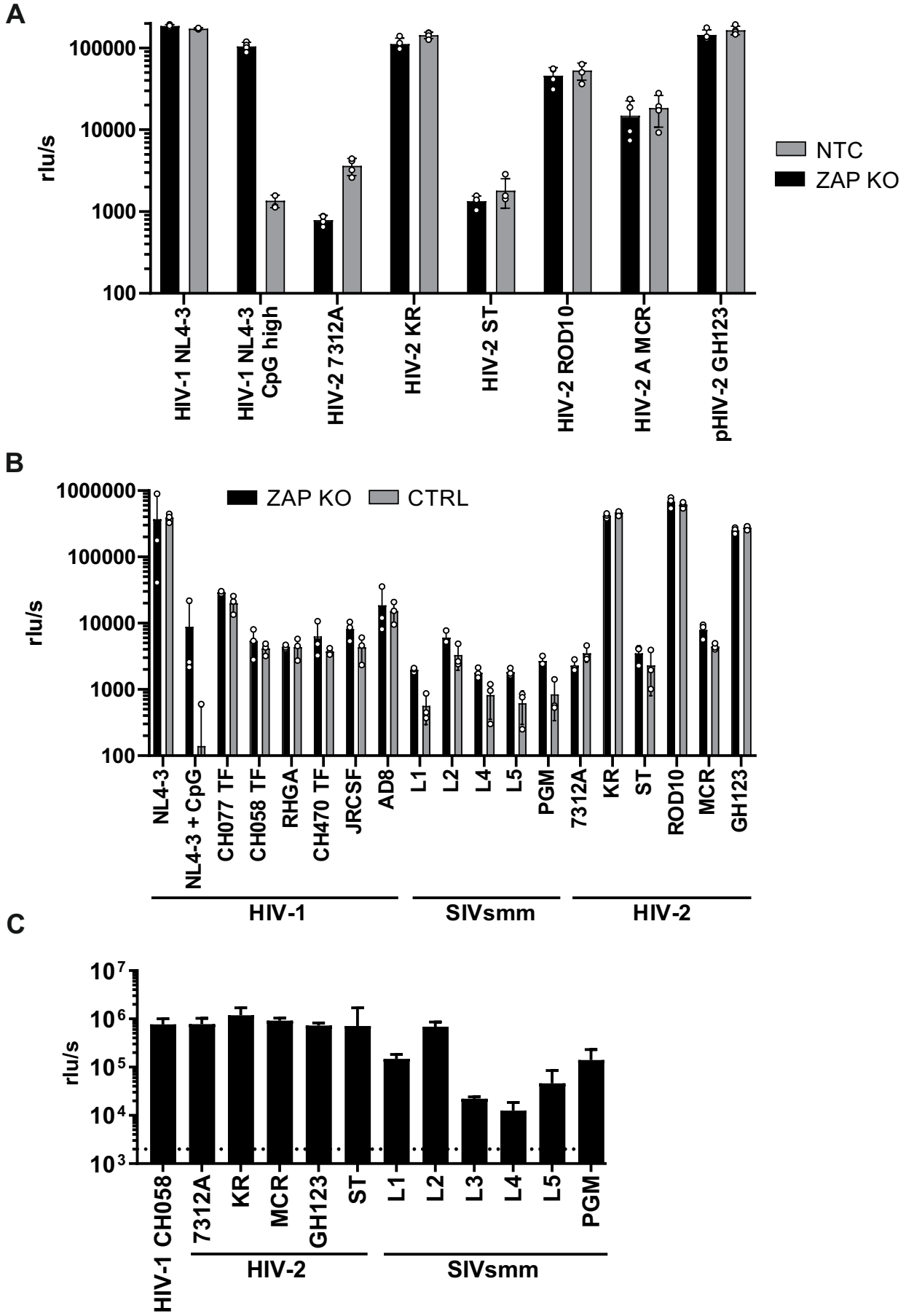
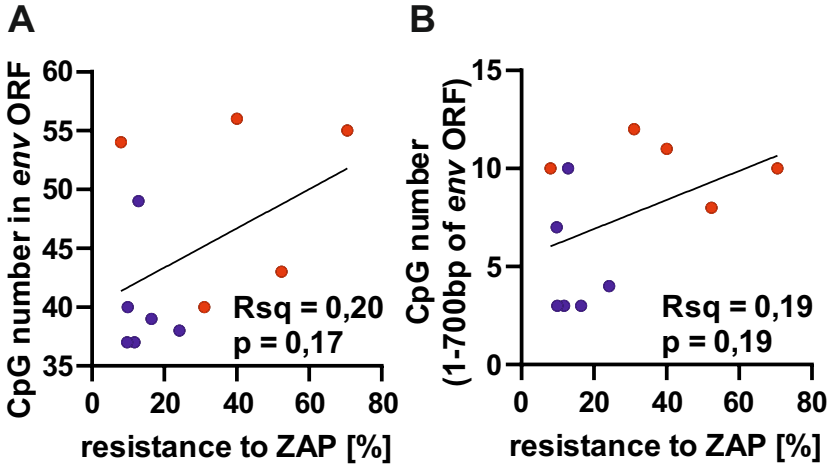
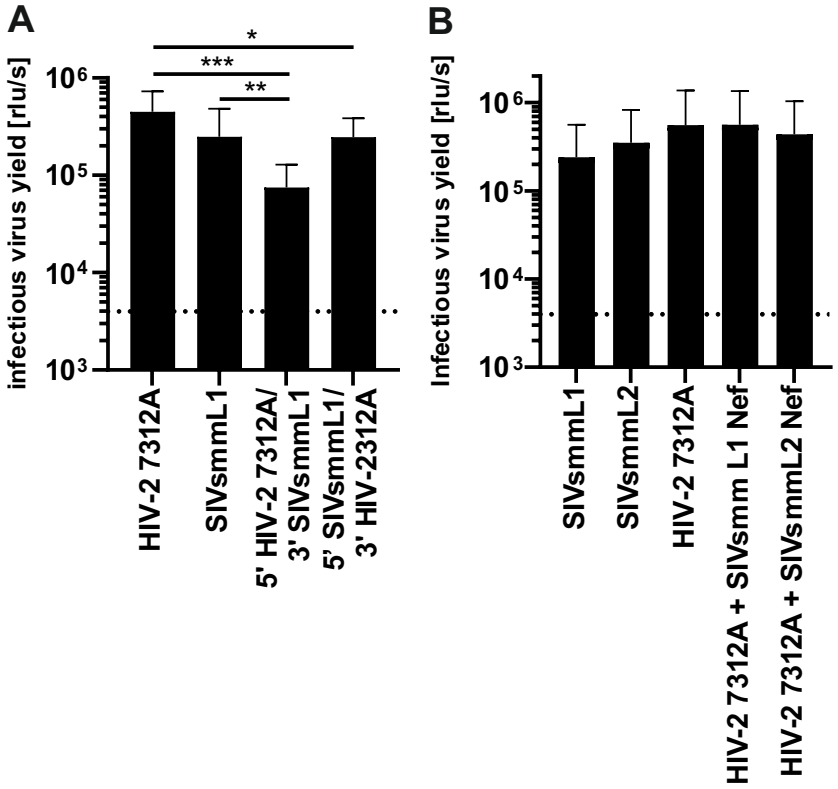
5'HIV-2 7312A/3'SIVsmm L1
5'SIVsmm L1/3'HIV-2 7312A
5'LTR
gag
pol
vif
vpx
vpr
tat
rev
env
nef
3'LTR
C
HIV-2 7312A
SIVsmm L1
5'HIV-2 /3'SIVsmm
5'SIVsmm /3'HIV-2
infectious virus yield [%]
ZAP DNA [µg]
0
0,04
0,125
0,375
D
Infectious virus yield [%]
HIV-1 NL4-3
WT
IRES eGFP
IRES eGFP vpr*
IRES eGFP nef*
HIV-2 7312A
E
eGFP MFI [%]
HIV-2 7312A
SIVsmm pgm
Vpr
Env
Nef
F
eGFP MFI [%]
HIV-2 7312A
SIVsmm L1 Nef
SIVsmm L2 Nef
SIVsmm L3 Nef
SIVsmm L4 Nef
SIVsmm L5 Nef
G
HIV-2 7312A
SIVsmm
HIV-2 7312A + SIVsmm nef
H
infectious virus yield [%]
HIV-2 7312A
SIVsmmL1
HIV-2 + SIVsmm L1 nef
SIVsmmL2
HIV-2 + SIVsmmL2 nef

Figure 6


A
HIV-2 7312A + SIVsmmL1 LTRs
SIVsmm L1 + HIV-2 7312A LTRs
HIV-2 7312A + SIVsmm 3'U3, R or U5
5'LTR
gag
pol
vif
vpx
vpr
env
tat
rev
nef
3'LTR
U3
R
U5
B
infectious virus yield norm. to no ZAP [100%]
150
100
50
0
***
***
SIVsmmL1
SIVsmm + HIV-2 LTR
HIV-2 7312A
HIV-2 + SIVsmm LTR
C
infectious virus yield norm. to no ZAP [100%]
120
100
80
60
40
20
0
0
0.1
0.2
0.4
ZAP [µg]
SIVsmmL1
HIV-2 7312A
SIVsmmL1 + 7312A LTR
HIV-2 7312A + SIVsmm 3'U3
HIV-2 7312A + SIVsmm 3'R
HIV-2 7312A + SIVsmm 3'U5
D
386
NF-kB
HIV2 7312A GTAGACAGGAAAGCAGCAGCATAAAGAGGAACTAGCTGACGCTGCATAAGAAAGGAAACTGGCTGACACTGCAGGGACTTTCCAGAAGGG
SIVsmm L1 CTAACC--GCAAGAGGCCTTTTAAAAA-----TGGCTAAC--------AAGAAGGAAACAAGCTGAGACAGCAGGGACTTTCCACAAAGG
SIVsmm L2 CTAACC--GCAAGAGGCCTTTTAAAAA-----TGCCTGAC--------AAGAAGGAAACAAGCTGAGACAGCAGGGACTTTCCACAAAGG
SIVsmm L3 CTAACC--GCAAGAGGCCTTTTAAAGA-----TGGCTGAC--------AAGGGAGGAAACAGCTGAGACAGCAGGGACTTTCCACAAGGG
SIVsmm L4 CTAACC--GCAAGAGGCCTCTTAAAAA-----TGGCTGAC--------AAGAAGGAAACTAGCTGAGACAGCAGGGACTTTCCACAA---
SIVsmm L5 CTAACC--GCAAGAGGCCTTTTAAAAA-----TGGCTGAC--------AAGAAGGAAACTAGCTGAGACTGCAGGGACTTTCCACAGTGG
SIVsmm PGM CTAACC--GCAAGAGGCCTTTTAAAAA-----TGGCTGAC--------AAGAAGGAAACAAGCTGAGACAGCAGGGACTTTCCACAAAGG
476
Sp1
Sp1
Sp1
HIV2 7312A GCTGTAACAGTGGGAGGGACATGGGAGGAGTTGGTGGGGAACGCCCATTAAGCCTCTGTATAAATGTACCCGCTTCTTGCATT
SIVsmm L1 G-GATGTTATGGGGAGGTACTGGGGAGGAGCTGGCTGG-AACGCCCACTTATTCTCTGTATAAATACAACTGCATTTCGCTCT
SIVsmm L2 G-GATGTTATGGGGAGGTACCGGGGAGGAGCTGGCTGG-AACGCCCACTTATTCTCTGTATAAATACAACTGCATTTCGCTCT
SIVsmm L3 GCTGTAACA-GGGGAGGTACTTGGGAGGAGCTGGTGGGGAACGCCCA-TTATTCTCTGTATAAATGTAGCTGCTGAACGCTCT
SIVsmm L4 ----------GGGGATGTTATGGGGAGGAGCTGGTCGGGAACGCCCACTTTCTTGATGTATAAATACAACTGCATTTCGCTCT
SIVsmm L5 GAGGCATCAATGGGAGGTAACTGGGAGGTACTGGTGGGGAACGCCCATTTATTCTCTGTATAAATACAACCGCTTGTCGCTCT
SIVsmm PGM G-GATGTTACGGGGAGGTACTGGGGAGGAGCTGGCTGG-AACGCCCACTTATTTTCTGTATAAATACAACTGCATTTCGCTCT
E
infectious virus yield [rlu/s]
10^7
10^6
10^5
10^4
10^3
HIV-2 7312A
HIV-2 + 2 CpG
SIVsmmL1
SIVsmm - 2 CpG
F
infectious virus yield norm. to no ZAP [100%]
120
100
80
60
40
20
0
0
0,1
0,2
0,4
ZAP [µg]
HIV-2 7312A
HIV-2 + 2 CpG
SIVsmmL1
SIVsmm - 2 CpG


A
rlu/s
100000
10000
1000
100
NTC
ZAP KO
HIV-1 NL4-3
HIV-1 NL4-3 CpG high
HIV-2 7312A
HIV-2 KR
HIV-2 ST
HIV-2 ROD10
HIV-2 A MCR
pHIV-2 GH123
B
rlu/s
1000000
100000
10000
1000
100
ZAP KO
CTRL
NL4-3
NL4-3 + CpG
CH077 TF
CH058 TF
RHGA
CH470 TF
JRCSF
AD8
L1
L2
L4
L5
PGM
7312A
KR
ST
ROD10
MCR
GH123
HIV-1
SIVsmm
HIV-2
C
rlu/s
10^7
10^6
10^5
10^4
10^3
HIV-1 CH058
7312A
KR
MCR
GH123
ST
L1
L2
L3
L4
L5
PGM
HIV-2
SIVsmm


A
CpG number in *env* ORF
60
55
50
45
40
35
0
20
40
60
80
resistance to ZAP [%]
Rsq = 0,20
p = 0,17
B
CpG number
(1-700bp of *env* ORF)
15
10
5
0
0
20
40
60
80
resistance to ZAP [%]
Rsq = 0,19
p = 0,19


A
B
infectious virus yield [rlu/s]
Infectious virus yield [rlu/s]
10^6
10^5
10^4
10^3
*
***
**
HIV-2 7312A
SIVsmmL1
5' HIV-2 7312A/ 3' SIVsmmL1
5' SIVsmmL1/ 3' HIV-2312A
SIVsmmL1
SIVsmmL2
HIV-2 7312A
HIV-2 7312A + SIVsmm L1 Nef
HIV-2 7312A + SIVsmmL2 Nef

```
      1                                                                                                                  100
7312A  TGGAAGGGATTTTTTATAGTGAAAGAAGACATAGGATATTAGATACATATTTTGAGAATGAGAGAGGTATAGTTGGTGGATGGCAGAACTACACATATGG
  PGM  TGGAAGGGATTTTATTACAGTGAGAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCACCCCAGATTGGCAGAATTACACATCAGG
   L1  TGGAAGGGATTTTATTACAGTGAAAGGAGACATAAAATATTAGATCTGTACTTAGAAAAGGAAGAAGGAATAGTACCAGATTGGCAAAATTACACATCAGG
   L2  TGGAAGGGATTTTATTACAGTGATAGGAGACATAAAATATTAGATCTGTACTTAGAAAAGGAAGAAGGAATAGTACCAGATTGGCAAAATTACACATCAGG
   L3  TGGAAGGGATATATTACAGTGAAAGAAGGCATAAAATATTAGATTTATACCTAGAAAAAGAAGAAGGGATCATTCCTGATTGGCAGAATTACACAAAAGG
   L4  TGGAAGGGATTTTATTACAGTGAGAGGAGACATAGAATATTAGACATATACCTAGAAAAGGAAGAAGGAATAATACCTGATTGGCAGAATTACACCTCAGG
   L5  TGGAAGGGATTTTATTACAATCAGAGAAGACATAGAATACTAGACATTTACTTAGAAAAGGAGGAAGGAATCATCCCTGACTGGCAGAATTACACATCAGG

       101                                                                                                                200
7312A  GCCAGGGATAAGATACCCAAAACACTTTGGCTGGCTGTGGAAACTAGTGCCAGTGGAAGTGGCAGCAGTAACCCGGGAGGAAGAGGAGACCCATTGTCTA
  PGM  ACCAGGAATCAGATACCCAATGTTCTTTGGCTGGCTATGGAAATTGGTCCCAGTAAGTGCCTCAGATGAAGCTC---AAGAAGACGAGACACATTACTTA
   L1  GCCAGGAACCAGATATCCTAAGGTCTTTGGATGGCTCTGGAAATTAGTCCCAGTAAATGTCTCAGATGAAGCTC---AGGAAGATGAGACACATCGCTTG
   L2  GCCAGGAATCAGATATCCTAAGGTCTTTGGATGGCTCTGGAAATTAGTCCCAGTAAATGTCTCAGATGAAGCTC---AGGAAGATGAGACACATTGTTTG
   L3  GCCAGGGATAAGATACCCAATGACGGTTTGGGTGGCTATGGAAGCTAGTGCCAGTAAATGTATCAGATGAGGCAG---AAACAGATGAGACCCACTGTCTG
   L4  GCCAGGAATAAGATATCCAATGTGCTTTGGATGGTTGTGGAAATTAGTACCAGTAGATGTCTCGGATGAGGCAC---AAGAAGATGAGACACACTATCTG
   L5  ACCAGGAATAAGATACCCAATGATGTTCGGATGGCTGTGGAAACTAGTCCCAGTAGAGGTCTTAGATGAGGCAC---AGAATGATGAGACACATTGTCTG

       201                                                                                                                300
7312A  GTGCACCCAGCACAGACCGCCGCATGGGATGATCCCCATGGGGAGACTCTTGTCTGGCAGTTTGATTCCCTCCTGGCATATTCAGATGAGGCCTTCAACA
  PGM  GTGCATCCAGCTCAGATATCCCAGTGGGATGATCCCTGGGGAGAGGTTCTGGCATGGAAGTTTGATTCACAACTAGCCTACAGATATGAGGCTTTCATTA
   L1  ATGCATCCAGCACAAACTTCTCAGTGGGATGACCCCTGGGGAGAGGTATTGGCATGGAAGTTTGATCCAGAGTTAGCTTATAACTACAAGGCATTTGTTA
   L2  ATGCATCCAGCACAAATTTCTCAGTGGGATGACCCCTGGGGAGAGGTATTGGCATGGAAGTTTGATCCAGAGTTAGCTTACAATTATAAGGCATTTATTG
   L3  GTACATCCGGCAGGGACAAGCAAGTGGGATGATCCGTGGGGTGAAACCCTAGCATGGAGATTTGATCCAACACTGGCTCATAGCTATGAAGCATTCATTA
   L4  GTGCATCCTGCACAGACAAGTCAGTGGAATGACCCATGGGGAGAAGTTCTAGCTTGGAAGTTTGATCCCACATTAGCTTATACTTATGAGGCATTTGTTA
   L5  GTGCATCCAGCACAGACGGGCCAATGGGATGACCAGTGGGGAGAGGTCTTGGCATGGAAGTTTGACCCCACTCTGGCTTATACCTATGAGGCATATATTA

       301                                                                                                                400
7312A  GGTTCCCAGAAGAGTTTGGGTATCAGTCAGGATTACCAGAGAAGGAATGGAAGGCTAGACTAAAAGCAAGAGGCATACCTACAGAGTAGACAGGAAAGCA
  PGM  GACACCCAGAAGAGTTTGGCAGTAAGTCAGGCTTGTCAGAGGAAGAGGTAAAGAGAAGGCTAACCGCAAGAGGCCT------------------------
   L1  AGTACCCAGAAGAGTTTGGTAGTATGTCAGGATTGCCAGAGGAAGAGGTAAAGAGAAGGCTAACCGCAAGAGGCCT------------------------
   L2  AGCACCCAGAAGAGTTTGGTAGTATGTCAGGCTTGTCAGAGGAAGAGGTGAAGAGAAGGCTAACCGCAAGAGGCCT------------------------
   L3  GATACCCAGAGGAGTTTGGATGGAGCTCAGGGCTGTCACAGGAAGAGGTTGAGAGAAGGCTAACCGCAAGAGGCCT------------------------
   L4  AATATCCAGAAGAATTTGGTAGCAAGTCAGGCTTGTCAGAGGAAGAGGTTAAGAGAAGGCTAACCGCAAGAGGCCT------------------------
   L5  AGTACCCAGAAGAGTTTGGGGACAAGTCAGGCTTGTCAGAGGAAGAGGTTAAGAGAAGGCTAACCGCAAGAGGCCT------------------------

       401                                                                                                                500
7312A  GCAGCATAAAGAGGAACTAGCTGACGCTGCATAAGAAAGGAAACTGGCTGACACTGCAGGGACTTTCCAGAAGGGGCTGTAACAGTGGGAGGGACATGGG
  PGM  ----TTTAAAAA-----TGGCTGAC--------AAGAAGGAAACAAGCTGAGACAGCAGGGACTTTCCACAAAGGG-GATGTTACGGGGAGGTACTGGGG
   L1  ----TTTAAAAA-----TGGCTAAC--------AAGAAGGAAACAAGCTGAGACAGCAGGGACTTTCCACAAAGGG-GATGTTATGGGGAGGTACTGGGG
   L2  ----TTTAAAAA-----TGCCTGAC--------AAGAAGGAAACAAGCTGAGACAGCAGGGACTTTCCACAAAGGG-GATGTTATGGGGAGGTACCGGGG
   L3  ----TTTAAAGA-----TGGCTGAC--------AAGGGAGGAAACAGCTGAGACAGCAGGGACTTTCCACAAGGGGCTGTAACA-GGGGAGGTACTTGGG
   L4  ----CTTAAAAA-----TGGCTGAC--------AAGAAGGAAACTAGCTGAGACAGCAGGGACTTTCCACAA-------------GGGGATGTTATGGGG
   L5  ----TTTAAAAA-----TGGCTGAC--------AAGAAGGAAACTAGCTGAGACTGCAGGGACTTTCCACAGTGGGAGGCATCAATGGGAGGTAACTGGG

       501                                                558
7312A  AGGAGTTGGTGGGGAACGCCCATTAAGCCTCTGTATAAATGTACCCGCTTCTTGCATT
  PGM  AGGAGCTGGCTGG-AACGCCCACTTATTTTCTGTATAAATACAACTGCATTTCGCTCT
   L1  AGGAGCTGGCTGG-AACGCCCACTTATTCTCTGTATAAATACAACTGCATTTCGCTCT
   L2  AGGAGCTGGCTGG-AACGCCCACTTATTCTCTGTATAAATACAACTGCATTTCGCTCT
   L3  AGGAGCTGGTGGGGAACGCCCA-TTATTCTCTGTATAAATGTAGCTGCTGAACGCTCT
   L4  AGGAGCTGGTCGGGAACGCCCACTTTCTTGATGTATAAATACAACTGCATTTCGCTCT
   L5  AGGTACTGGTGGGGAACGCCCATTTATTCTCTGTATAAATACAACCGCTTGTCGCTCT
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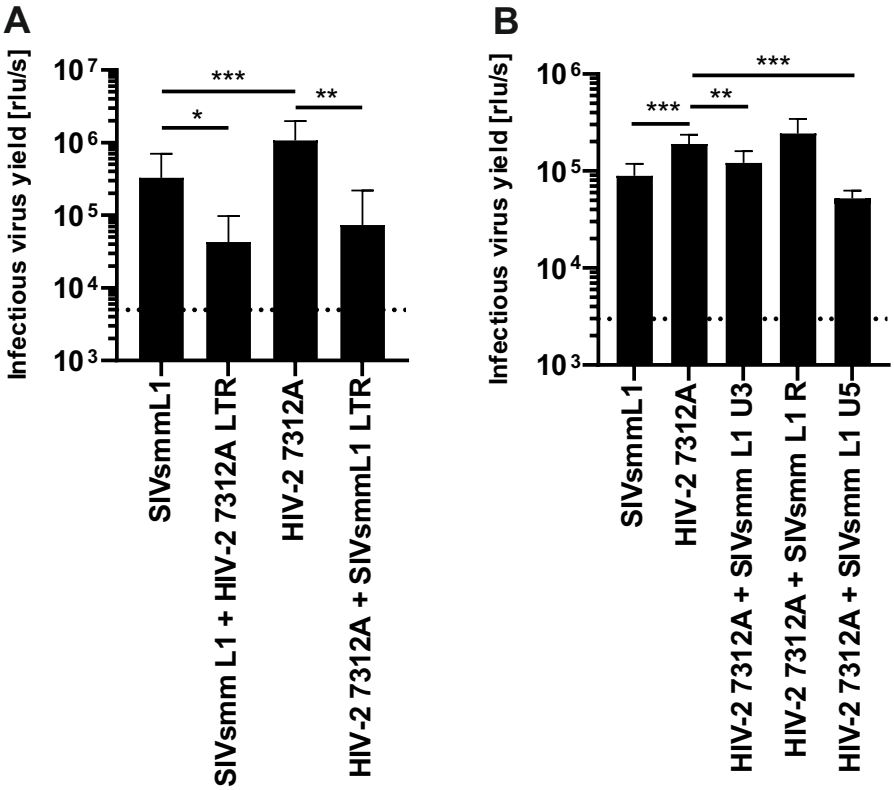
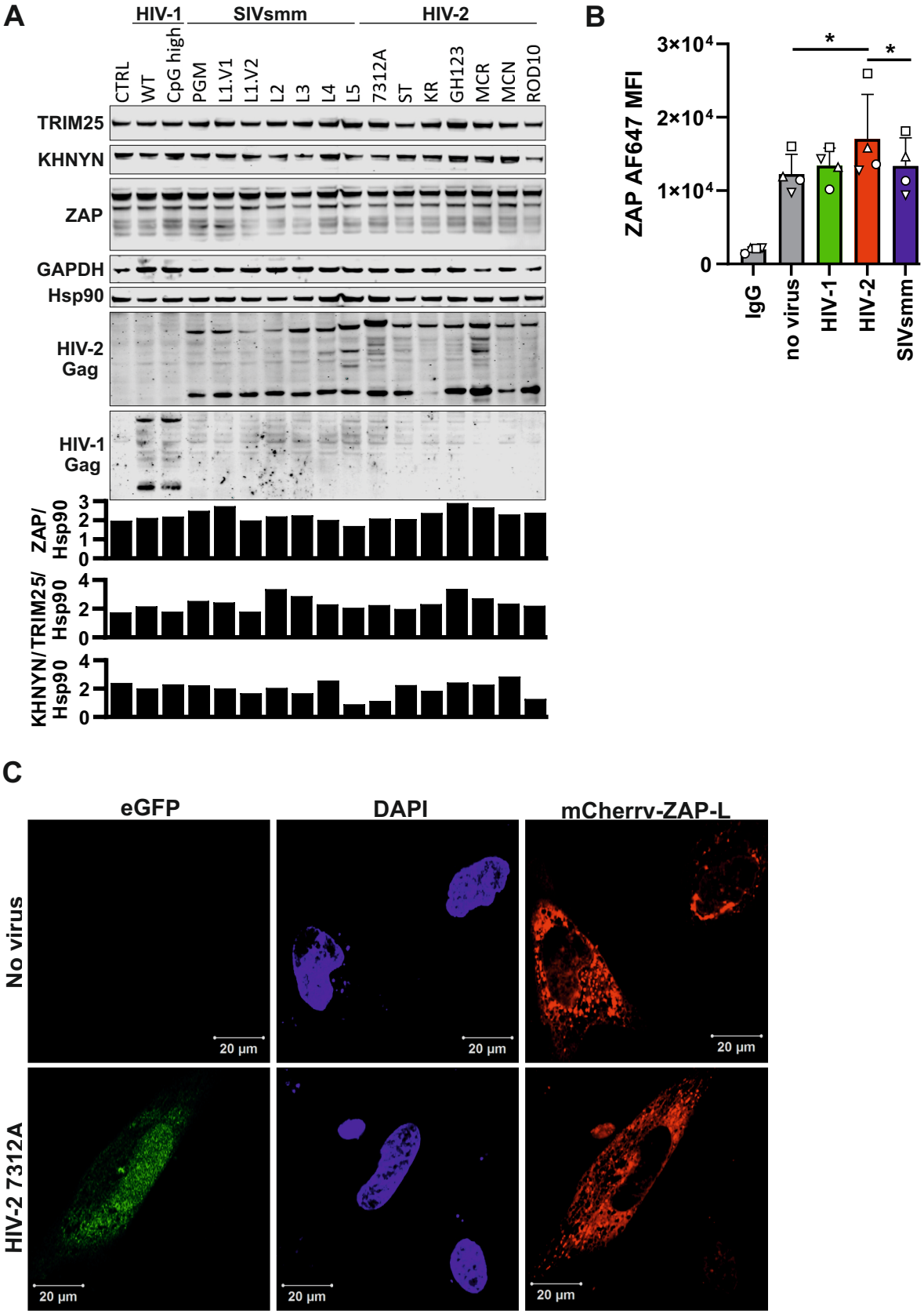

A
Infectious virus yield [rlu/s]
10^7
10^6
10^5
10^4
10^3
***
*
**
SIVsmmL1
SIVsmm L1 + HIV-2 7312A LTR
HIV-2 7312A
HIV-2 7312A + SIVsmmL1 LTR
B
Infectious virus yield [rlu/s]
10^6
10^5
10^4
10^3
***
***
**
SIVsmmL1
HIV-2 7312A
HIV-2 7312A + SIVsmm L1 U3
HIV-2 7312A + SIVsmm L1 R
HIV-2 7312A + SIVsmm L1 U5


A
HIV-1
SIVsmm
HIV-2
CTRL
WT
CpG high
PGM
L1.V1
L1.V2
L2
L3
L4
L5
7312A
ST
KR
GH123
MCR
MCN
ROD10
TRIM25
KHNYN
ZAP
GAPDH
Hsp90
HIV-2 Gag
HIV-1 Gag
ZAP/ Hsp90
KHNYN/TRIM25/ Hsp90
KHNYN/ Hsp90
B
ZAP AF647 MFI
3×10⁴
2×10⁴
1×10⁴
0
*
*
IgG
no virus
HIV-1
HIV-2
SIVsmm
C
eGFP
DAPI
mCherry-ZAP-L
No virus
HIV-2 7312A
20 µm