The brain-specific factor FEZ1 is a determinant of neuronal susceptibility to HIV-1 infection

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Neurons are one of the few cell types in the human body that do not support HIV type-1 (HIV-1) replication. Although the lack of key receptors is a major obstacle to infection, studies suggest that additional functions inhibit virus replication to explain the exquisite resistance of neurons to HIV-1. However, specific neuronal factors that may explain this resistance remain to be discovered. In a screen for antiviral factors using a fibroblast line chemically mutagenized and selected for resistance to retroviral infection, we recently identified induction of rat FEZ1 (fasciculation and elongation protein zeta-1), a brain-specific protein, as the cause of this resistance. When exogenously expressed in nonneuronal cell lines rat FEZ1 blocked nuclear entry of retroviral DNA. Here, we demonstrate that among human brain cells, neurons naturally express high levels of FEZ1 compared to astrocytes or microglia cells and are correspondingly less susceptible to infection with pseudotyped HIV-1 that bypasses receptor-mediated viral entry. Demonstrating that endogenous FEZ1 was functionally important in the resistance of neurons to HIV-1 infection, siRNA-mediated knockdown of endogenous FEZ1 increased the infectivity of neurons while sensitive brain cell types like microglia became more resistant upon FEZ1 overexpression. In addition, FEZ1 expression was not induced in response to IFN treatment. As such, in contrast to other widely expressed, IFN-inducible antiviral factors, FEZ1 appears to represent a unique neuron-specific determinant of cellular susceptibility to infection in a cell type that is naturally resistant to HIV-1.

The brain is a major target organ for HIV-1 infection. HIV-1 enters the central nervous system (CNS) in ≈80% of infected individuals early after infection and can cause a wide range of neurological disorders including cognitive motor impairment and HIV-associated dementia (HAD; also known as AIDS dementia complex) (1). Although the incidence of HAD has markedly reduced with the introduction of highly active antiretroviral therapy (HAART), the overall prevalence of HAD is rising as the number of treated subjects with chronic HIV infection increases (1). Notably, HIV-1 infects a restricted number of cell types in the brain. While perivascular macrophages and microglia appear to be the major target cells of the CNS for the virus, HIV-1 is less abundant in astrocytes and rarely seen in oligodendrocytes, brain microvascular endothelia cells (BMVECs) and neurons (1), although recent reports have suggested that neuronal progenitor brain cells can be infected with HIV-1 (2, 3). The block to viral infection is at least in part at the receptor level as, in contrast to both macrophages and microglia cells, the major receptor for HIV-1 entry, CD4 is not expressed in astrocytes, oligodendrocytes, BMVECs, or neuronal cells. Although still not clear today, the limited infection in astrocytes has been linked to inefficient entry of HIV by endocytosis (4, 5) as highly productive infection can be detected using viruses pseudotyed with envelope glycoproteins of either vesicular stomatitis virus (VSV-G) or amphotropic murine leukemia virus (MuLV), which efficiently enter CD4-negative cells (6, 7). On the other hand, an unidentified intrinsic intracellular restriction to efficient HIV-1 replication has been shown in astrocytes in which a cytoplasmic activity interferes with nuclear uptake of the nucleoplasmic shuttle protein Rev (8, 9). Additionally, APOBEC3G-mediated intrinsic immunity has also been suggested to block HIV-1 replication in the CNS (10–12). Therefore, regardless of the route of entry there are likely to be additional postentry blocks to infection in brain cell types that do not support HIV-1 infection, particularly in highly restrictive cell types such as neurons.

In an attempt to discover cellular genes with antiviral activity, we recently screened the rat fibroblast line R3–2 (13), generated by chemical mutagenesis and selection for resistance to retroviral infection, and identified the causal factor as the brain specific protein FEZ1 (14). A mammalian homolog of the *Caenorhabditis elegans* UNC −76 protein, FEZ1 is a direct target of protein kinase C (PKC)ζ-dependent signaling (15) and a microtubule motor associated-protein (16) essential for synaptic vesicle transport and axonal outgrowth (17, 18). We showed that expression of rat FEZ1 blocked nuclear entry of retroviral DNA in various mammalian cells (14), suggesting that it functioned in the intracellular transport of viral cargos into the nucleus. In support of these findings the interaction of FEZ1 with microtubules was recently shown to promote neurite extension and block intracellular trafficking of the human polyomavirus JC virus (JCV) (19). Although FEZ1 is extensively expressed in the brain (17, 20) and in situ hybridization suggests that it may be preferentially expressed in neurons of the developing rat brain (21) there has been little comparative analysis of its' expression in different brain cell types. Here, we determined the expression of FEZ1 in neurons, astrocytes, and microglia and examined its potential function as a natural determinant of neuronal susceptibility to HIV-1 infection.

Results and Discussion

Although known to be brain-specific, to date, the expression patterns of FEZ1 in different brain cell types have not been determined. Given the significant differences in the sensitivity of various brain cell types to HIV-1 infection, we first examined the endogenous levels of FEZ1 in both human brain cell lines and rat primary brain cells by quantitative Real-Time PCR (qPCR) measurement of mRNAs. As shown in Fig. 1*A*, compared to astrocytes and microglia cells, both primary neurons and the neuronal cell line contained dramatically higher levels of FEZ1 transcript. The established neuronal, astrocyte and microglia cell lines, SH-SY5Y (22), 1321N1 (23), and CHME3 (24), respectively, were then chosen for further studies of protein expression and the potential function of FEZ1 during HIV-1 infection of brain cell types of human origin. As shown in Fig. 1*B*, FEZ1 protein expression reflected the transcript profile in these representative lines, demonstrating that neurons naturally express high levels of this factor relative to at least 2 other brain cell types. In agreement, while this article was under review an independent report was published demonstrating higher levels of

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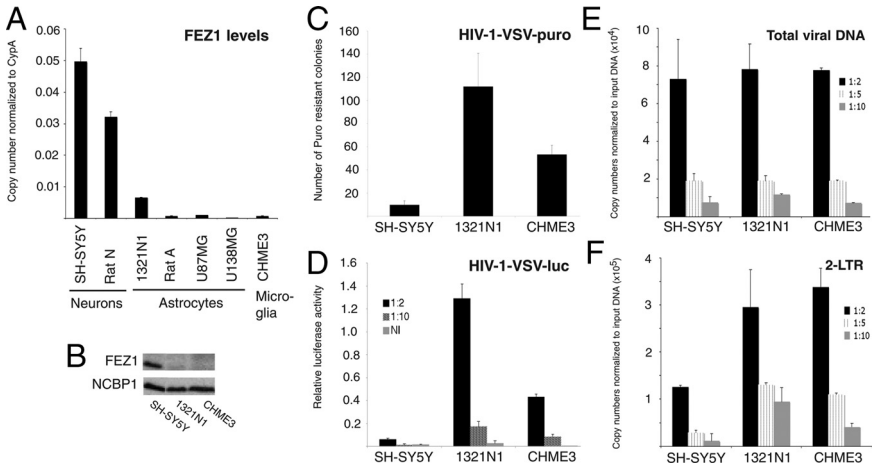

Fig. 1. High levels of FEZ1 expression and reduced HIV-1 infectivity in neurons. (*A*) qPCR showing the level of endogenous FEZ1 expression in human lines and rat primary cells of brain origin. Cytoplasmic RNA prepared from human brain cell lines; neuronblastoma line (SH-SY5Y), glioblastoma/astrocytoma (1321N1, U87MG, and U138MG), and microglia (CHME3) or from primary rat hippocampus neurons (Rat N) and primary rat cortex astrocytes (Rat A) was reverse transcribed and used as template for qPCR. Data are median copy numbers normalized to cypA (for human cells) or GAPDH (for rat cells) obtained in triplicates from 2 independent RNA preparations. (*B*) Western blotting showing the levels of FEZ1 protein in representative human brain cell lines. *Upper*, endogenous FEZ1 expression was detected using anti-FEZ1 antibodies. *Lower*, loading of equal amounts of protein was confirmed using NCBP1 antibody. (*C* and *D*) Susceptibility of brain cell lines to infection with pseudotyped HIV-1. The 3 representative lines as in *B* were incubated with various amounts of HIV-1-puro (*C*) or 2 different dilutions (1:2 and 1:10) of HIV-1-luc (*D*) viruses. Noninfected (NI) cells were included as negative control. Cells were either selected in medium containing puromycin and the number of HIV-1-puro transduced colonies was counted after 8–12 days or were lysed and assayed for luciferase activity 48 h postinfection. Similar results were obtained in at least 3 independent experiments. (*E* and *F*) Analysis of viral DNA synthesis using qPCR. The indicated cell lines were infected with different dilutions (1:2, 1:5, and 1:10) of HIV-1-puro pseudotyped with VSV-G envelope. Low molecular hirt DNA was isolated at 24 h after infection and the amount of total viral DNA (*E*) and circular DNA (*F*) synthesized was measured by qPCR using primers specific to puromycin or 2-LTR DNA, respectively. Each sample was assayed in triplicate at a minimum of 3 different time points.

FEZ1 expression in primary neurons compared to primary astrocytes (25).

To measure the susceptibility of these cells to HIV-1 infection the 3 representative lines were then tested in transduction assays using HIV-1-puro or HIV-1-luc (HIV-based viral vectors carrying puromycin or luciferase resistance markers, respectively) pseudotyped with VSV-G glycoprotein, as previously described (14). VSV-G envelope was used to bypass the viral block at the receptor level in astrocytes and neurons (6, 7). Notably, SH-SY5Y cells were significantly less susceptible to infection with both genetically marked HIV-1 viruses than either 1321N1 or CHME3 cells (Fig. 1 *C* and *D*), suggesting a robust postentry block to infection in neurons.

To localize this postentry block in SH-SY5Y cells we then examined the synthesis of both total viral DNA and 2-LTR (long terminal repeat) circles after infection of these cells with HIV-1-puro pseudotyped with VSV-G envelope protein. Again, VSV-G envelope was chosen to bypass the receptor mediated entry block to study the fate of viral DNA in the cell lines tested. 24 h postinfection hirt DNA was isolated and used as template for qPCR (14) using primers to amplify puromycin or the HIV-1 LTR-LTR junction. In line with our previous findings (14), while the levels of total viral DNA was similar in each of these lines (Fig. 1*E*) significantly lower levels of 2-LTR circles (Fig. 1*F*) were detected in SH-SY5Y cells compared to 1321N1 and CHME3, suggesting a natural block to nuclear trafficking of viral DNA in neurons.

To test whether FEZ1 played a functional role in the observed resistance of SH-SY5Y cells to HIV-1 infection, RNA interference (RNAi) was used to reduce FEZ1 levels in these cells and the consequences were assessed by again infecting the cultures with genetically marked HIV-1. SH-SY5Y cells were treated with 3 independent short-interfering RNAs (siRNA) specific to human FEZ1 (Ambion) or a nonspecific control siRNA duplex (14). All 3 FEZ1-specific RNAi duplexes decreased FEZ1 transcript levels (Fig. 2*A*) and increased the susceptibility of neurons to HIV-1-puro infection compared to that of the control duplex (Fig. 2*B*). All 3 FEZ1-specific siRNAs also significantly reversed the inhibition of HIV-1-luc infectivity in SH-SY5Y cells (Fig. 2*C*). A more pronounced knockdown effect for specific duplexes in this experiment (Fig. 2*D*) was due to the use of a different, more efficient transfection reagent. However, despite robust reduction in transcript expression steady-state FEZ1 protein levels (Fig. 2*E*) were only partially reduced but to a magnitude that directly correlated with the effects of each FEZ1-specific siRNA on HIV-1 infectivity (Fig. 2 *B* and *C*). To confirm that FEZ1 knockdown was relieving the block to the accumulation of viral DNA in the nucleus of SH-SY5Y cells the synthesis of 2-LTR circles was examined after siRNA knockdown of FEZ1 and subsequent infection of these cells with HIV-1-GFP pseudotyped with VSV-G envelope protein, as described above. Significantly higher levels of circular DNA (Fig. 2*F*) were found upon transient knockdown of FEZ1 as compared to unspecific siRNA controls in SH-SY5Y cells, indicating a relief of the block to nuclear entry of HIV-1 DNA in these cells. Efficient knockdown of FEZ1 levels in these experiments was again confirmed using qPCR (Fig. 2*G*). Overall, these results suggested that endogenous levels of FEZ1 played a significant functional role in determining the nuclear accumulation of viral DNA in SH-SY5Y cells and their natural susceptibility to HIV-1 infection.

To further explore the functional role of FEZ1 in regulating the infectivity of various brain cell types, we then examined whether the low level of FEZ1 expression in CHME3 cells contributes to their sensitivity to HIV-1 infection. FEZ1 was overexpressed by either transient transfection of CHME3 cells with mammalian expression vectors containing full-length or C-terminally Flag-tagged FEZ1 or by infection of these cells with pseudotyped retroviruses containing N- or C-terminally Flag-tagged FEZ1. The consequences of FEZ1 overexpression were then assessed by challenging the cultures by infection with pseudotyped HIV-1-luc, as described above. FEZ1 overexpres-

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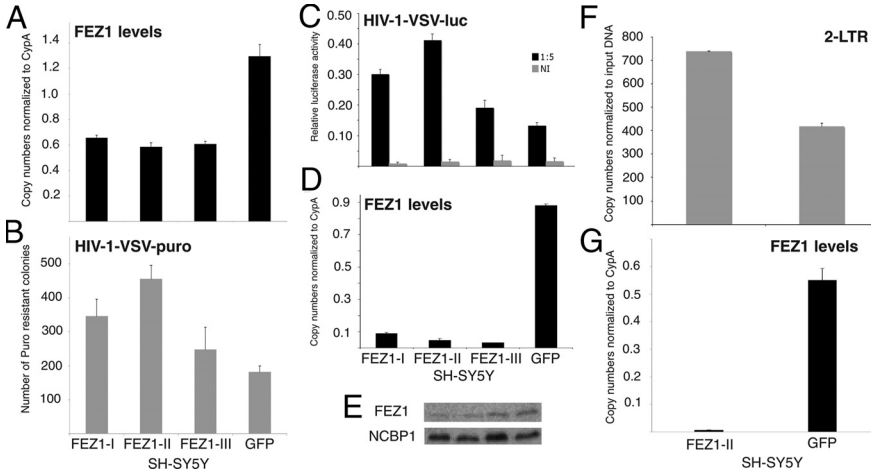

Fig. 2. RNA interference to FEZ1 increases the susceptibility of human neuronal cells to infection. SH-SY5Y cells were transfected with either 3 independent siRNAs (labeled I, II, and III) targeting human FEZ1 or a nonspecific GFP duplex on 2 consecutive days with equal amounts of RNA duplexes and subsequently seeded and infected with various amounts of HIV-1-puro (*B*) or HIV-1-luc (*C*) viruses pseudotyped with VSV-G envelope protein as described in the legend to Fig. 1. Similar results were obtained in at least 3 independent experiments. (*A* and *D*) qPCR showing the endogenous levels of FEZ1 in cells from *B* and *C*, respectively. Cytoplasmic RNA was reverse transcribed and used as template for qPCR as described in the legend to Fig. 1. (*E*) Western blotting showing the levels of FEZ1 knockdown in human SH-SY5Y cells. *Upper*, endogenous FEZ1 expression was detected in the same cells as in *C* using anti-FEZ1 antibodies. *Lower*, loading of equal amounts of protein was confirmed using NCBP1 antibody. (*F*) Analysis of the nuclear accumulation of viral DNA using qPCR. SH-SY5Y cells transfected with control or FEZ1-II duplexes as described in *D* were subsequently seeded and infected with HIV-1-GFP pseudotyped with VSV-G envelope protein. Low molecular hirt DNA was isolated at 24 h after infection and the amount of circular DNA synthesized in the nucleus of infected cells was measured by qPCR using primers specific to 2-LTR DNA. Each DNA sample was assayed in triplicate at a minimum of 3 different time points. (*G*) qPCR showing the endogenous levels of FEZ1 (as described above) in the same cells as in *F*.

sion in both transiently transfected or stably overexpressing pools of CHME3 cells as detected by qPCR (Fig. 3*A*) and western blotting (Fig. 3 *B* and *D*) resulted in a significant reduction in the susceptibility of these cells to HIV-1-luc infection compared to that of the empty vector pcDNA3.1$^-$ (Fig. 3*C*) and Flag control lines (Fig. 3*E*), respectively. As such, the low levels of FEZ1 expression in CHME3 cells is likely to be a contributing factor as to why these brain cells support HIV-1

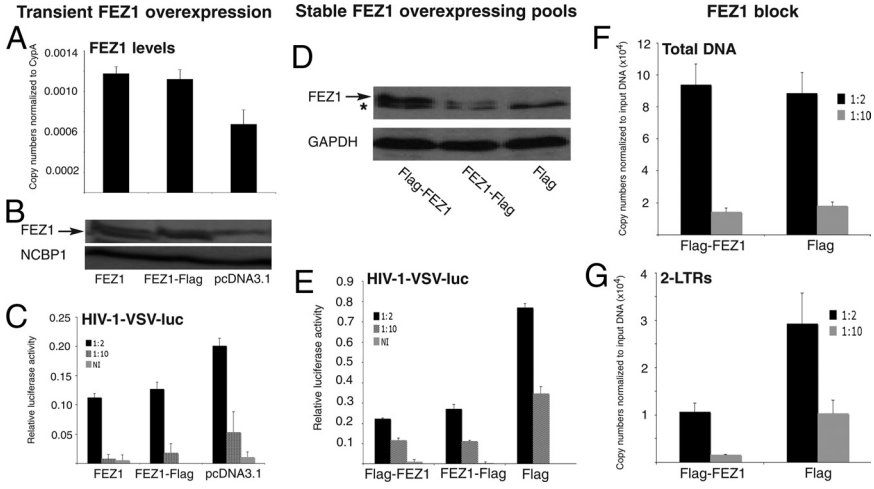

Fig. 3. Overexpression of FEZ1 reduces the virus susceptibility of human brain macrophages. FEZ1 was overexpressed by either transient transfection of CHME3 cells with mammalian expression vectors containing full length (FEZ1) or C-terminally Flag-tagged FEZ1 (FEZ1-Flag) or by infection of these cells with pseudotyped retroviruses containing N (Flag-FEZ1) or C-terminally Flag-tagged FEZ1 (FEZ1-Flag). Cells transfected with pcDNA3.1$^-$ vector (pcDNA3.1$^-$) or infected with pQCXIN containing Flag sequences (Flag) were included as negative controls. (*A*) qPCR showing expression levels of FEZ1. Cytoplasmic RNA prepared from pcDNA3.1$^-$ control line, FEZ1, and FEZ1-Flag overexpressing cells was reverse transcribed and used as template for qPCR as described in the legend to Fig. 1. (*B* and *D*) Western blotting showing the level of FEZ1 protein in both CHME3 cells transiently transfected with FEZ1, FEZ1-FLAG, or empty vector pcDNA3.1$^-$ (*B*) and CHME3 pools stably overexpressing Flag alone, Flag-FEZ1, and FEZ1-Flag (*D*). *Upper*, FEZ1 overexpression was detected using anti-FEZ1 (*B*) or anti-Flag antibodies (*D*). The asterisk (*) indicates a nonspecific band detected by the anti-FLAG antibody in all samples. *Lower*, loading of equal amounts of protein was confirmed using NCBP1 (*B*) or GAPDH antibodies (*D*). (*C* and *E*) Susceptibility of FEZ1 overexpressing CHME3 cells to infection with pseudotyped HIV-1. The same cells as in *A* and *D* were subsequently seeded and infected with different dilutions (1:2 and 1:10) of HIV-1-luc virus pseudotyped with VSV-G envelope protein as described in the legend to Fig. 1. Noninfected (NI) cells were included as negative control. Similar results were obtained in at least 3 independent experiments. (*F* and *G*) Analysis of viral DNA synthesis using qPCR. Flag-FEZ1 overexpressing pool and the control Flag pool were infected with different dilutions (1:2 and 1:10) of HIV-1-puro pseudotyped with VSV-G envelope. Low molecular hirt DNA was isolated at 24 h after infection and the amount of total viral DNA (*F*) and circular DNA (*G*) synthesized was measured by qPCR as described in the legend to Fig. 1.

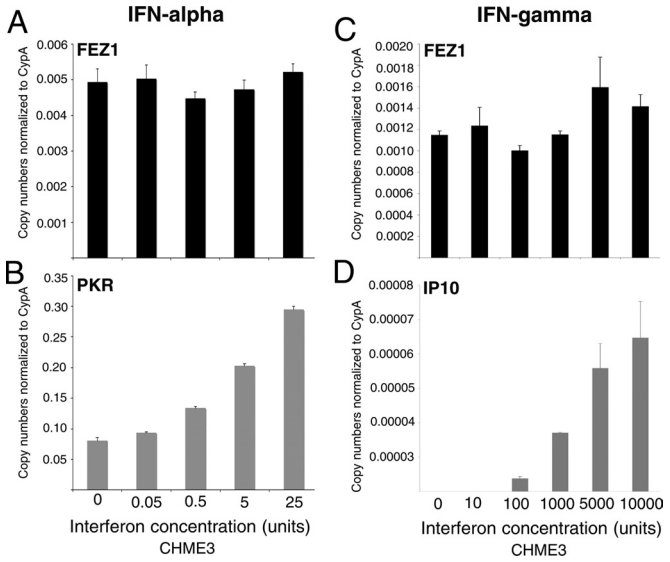

Fig. 4. FEZ1 expression in human brain macrophages is not induced by IFN treatment. qPCR showing the endogenous levels of FEZ1 in IFN-α (*A*) or IFN-γ (*C*) induced CHME3 cells, along with the levels of the IFN-α inducible gene PKR (*B*) and the IFN-γ inducible gene IP10 (*D*). Cells were either not treated (indicated as 0) or treated with increasing amounts of IFN-α or IFN-γ as indicated. Twenty-four hours posttreatment cytoplasmic RNA was reverse transcribed and used as template for qPCR. Specific primers were used to measure the levels of endogenous FEZ1 and IFN inducible transcripts. Median copy numbers for each transcript was normalized to CypA obtained in triplicates from 2 independent RNA preparations.

infection more efficiently than SH-SY5Y cells. Furthermore, to test whether overexpression of FEZ1 in CHME3 cells could recreate the block to nuclear accumulation of viral DNA observed in SH-SY5Y cells we measured viral DNA synthesis after infection with pseudotyped HIV-1-puro in these cells. As expected while the levels of total viral DNA (Fig. 3*F*) were similar in the FEZ1 overexpressing and the control Flag CHME3 pools significantly lower levels of 2-LTR circles (Fig. 3*G*) were detected in the FEZ1 overexpressing cells, indicating that FEZ1 was capable of blocking nuclear trafficking of HIV-1 DNA in these cells. The same pattern of viral DNA synthesis was detected in the other FEZ1 (FEZ1-Flag) overexpressing pool of CHME3 cells. Therefore, FEZ1 naturally expressed in SH-SY5Y cells or ectopically expressed in nonneuronal CHME3 cells, as reported here, or nonbrain cell lines (14), block infection prior entry of viral DNA into the nucleus.

Given the clear functional role of FEZ1 in regulating cellular sensitivity to infection in SH-SY5Y cells, finally we tested whether FEZ1 expression could be induced in CHME3 cells by interferon (IFN) treatment. Both type I and type II IFNs are known for their antiviral activity as an innate immune response to viral infections (26, 27). It has recently been shown that the HIV-1 restriction factors APOBEC3G, TRIM5α and tetherin can be regulated by interferons (28, 29), suggesting that they function as part of the cellular antiviral response. CHME3 cells were treated with increasing concentrations of IFN-α or IFN-γ and the levels of FEZ1 were determined by qPCR, as described above. The IFN-α- or IFN-γ-inducible genes, double stranded RNA-activated protein kinase (PKR) (26) and chemokine ligand 10 (CXCL10 or IP10) (30) respectively, were used as a control for IFN responsiveness of CHME3 cells. Although PKR and IP10 expression increased in CHME3 cells treated with IFN-α or IFN-γ, respectively, neither treatment altered the expression of FEZ1 to any significant degree (Fig. 4). This suggested that FEZ1 expression was not likely to be induced as part of a wider antiviral response but rather appeared to constitute a neuron-specific determinant of retroviral infectivity.

Human hematopoietic stem cells (HSCs), undifferentiated monocytes and unstimulated CD4$^+$ T cells resist HIV-1 infection despite the presence of HIV-1 receptors [reviewed in ref. 31]. Although neurons do not express CD4, the primary receptor for HIV-1 entry (1), other cell types that do not express CD4 do become infected, including astrocytes (1). As such, an array of cellular factors in addition to CD4 expression combine to determine the ultimate sensitivity of a given cell to HIV-1 infection. It is likely that in highly restrictive cell types such as neurons the inhibition of virus replication involves multiple points in the virus lifecycle (10–12). For example, although expression of APOBEC3G in neurons contributes to the restriction of HIV-1, it is present in many cells types and as such, its' expression alone does not explain the high degree of resistance observed in neurons (10, 12). The discovery of restriction factors such as TRIMs and APOBECs, whose sole function to date appears to be to inhibit virus replication (28), along with antiviral factors, whose presence or absence within the host cell alters virus replication (14, 32–37) have highlighted the complexity of mechanisms that govern cellular susceptibility to retroviral infection. Many of these factors are expressed in a wide range of tissues and their expression is increased in response to IFN, suggesting that they function as part of the hosts' antiviral response mechanism (28, 29). In contrast, here we show that FEZ1 expression is limited not just to brain cells, but to specific brain cell types that are naturally resistant to HIV-1 infection. Combined with its' lack of IFN responsiveness, our findings suggest that FEZ1 constitutes a factor which functions uniquely as a natural brain cell type-specific determinant of resistance to infection. The expression of high levels of FEZ1, in combination with the ubiquitously expressed APOBECs and lack of CD4 receptors may explain why neurons are so exquisitely resistant to infection. Determining why such highly resistant cells are as they are will not only improve our understanding of HIV-1 pathogenesis but may one day be used to design new therapeutic approaches to blocking infection.

Materials and Methods

Cells and Viruses. Astrocytes, 1321N1 (23), microglia cells, CHME3 (24), and neurons, SH-SY5Y (22) have been previously described. Human astrocytoma/glioblastoma cell lines U87MG and U138MG were obtained from NIBSC (cat # ARP043 and ARP028, respectively). Primary neurons from rat hippocampus and primary astrocytes from rat cortex were purchased from Lonza (cat no. R-HI-501 and R-Cx-520, respectively). HIV-1-puro, HIV-1-luc and HIV-1-GFP viruses pseudotyped with VSV-G glycoprotein envelope were produced by transfection of 293T cells using a combination of 3 expression vectors (14).

Infection of Cultured Cells and Transduction Assays. Target cells (1×10^5) were infected as described previously (14). HIV-1-puro and HIV-1-luc titers were measured by infection of 293A cells and colony counting after selection in puromycin (0.5 μg/mL) or by infection of 293A cells and measurement of luciferase activity (Promega) 48 h postinfection, respectively.

Quantitative Real-Time PCR (qPCR). Cytoplasmic RNA was reverse transcribed and used as template for PCR using SYBR Green JumpStart *Taq* ReadyMix (Sigma) (14). Human FEZ1 transcript levels were determined using forward primer hFEZ1-S2-NYC: 5′CTGGAGTTTGAGAAGGAAGTG3′; and reverse primer hFEZ1-A2-NYC: 5′GAGGTTACATGGTCTCATGC3′. Primers for detection of rat FEZ1 transcript levels, mFEZ1-S3 and mFEZ1-A3, have previously been described (14). The number of human or rat target copies in each sample was interpolated from its detection threshold (C_T) value using a human cyclophilin A (37) or a mouse GAPDH plasmid (14) standard curve, respectively. The primers used for detection of cyclophilin A and GAPDH transcript levels were hCypA up: 5′TTGAGCTGTTTGCAGACA3′; hCypA down: 5′ACCCGTATGCTTTAGGAT3′ and mGAPDH5 and mGAPDH3 (14), respectively. Amplicon size and reaction specificity was confirmed by agarose gel electrophoresis.

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Western Blotting. The levels of FEZ1 protein were measured by western blotting of cell lysates in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% DOC, and 0.1% SDS) fractionated by SDS-polyacrylamide gel electrophoresis and probed with a 1:1,000 dilution of anti-FEZ1 (4265; ProSci Inc.). The levels of Flag-tagged FEZ1 were measured using anti-Flag antibody (F7425; Sigma). As loading control NCBP1 (ab42389; Abcam) or anti-GAPDH (SC-25778; Santa Cruz) were used.

Quantitation of Viral DNA by qPCR. Target cells (1.5×10^6) were infected with DNase I-digested supernatant containing HIV-1-puro pseudotyped with VSV-G envelope. Hirt DNA was isolated at 24 h after infection (14) and 1 μL used as template for 40 cycles (15 s at 94°C, 60 s at 60°C, and 60 s at 72°C) of 3-step PCR of total viral DNA and DNA circles (2-LTR) using SYBR Green JumpStart *Taq* ReadyMix (Sigma) including 200 nM of each primer puro-S2 5′CTCGACATCGGCAAGGTGTG3′ and puro-A2 5′GCCTTCCATCTGTTGCTGCG3′ or U3rev1: 5′GGGAGTGAATTAGCCCTTCC3′ and U5for1: 5′GTAGTGTGTGCCCGTCTGT3′, respectively. The number of target copies in each sample was interpolated from its detection threshold (C_T) value using a puromycin pCSPW (a gift from Dr. Greg Towers) or a 2-LTR circle (38) plasmid standard curve.

RNA Interference. SH-SY5Y cells (5×10^5) were transfected with 3 independent predesigned short-interfering RNAs (Ambion) specific to human FEZ1 or a nonspecific control siRNA duplex targeted to GFP using Oligofectamine or Lipofectamine RNAiMax transfection reagent (Invitrogen) as described (14). Cells were transfected on 2 consecutive days with 300 pmol RNA duplex and subsequently seeded, infected with various amounts of HIV-puro or HIV-1-luc, and selected or lysed for luciferase transduction activity, respectively, as described above. Levels of endogenous FEZ1 expression were measured by qPCR as described above.

siRNA Knockdown and Quantitation of Viral DNA. SH-SY5Y cells were transfected with a predesigned siRNAs, as described above. Forty-eight h posttransfection, cells were infected with DNase I-digested supernatant containing HIV-1-GFP pseudotyped with VSV-G envelope (14). Five microliters hirt DNA was used as template for 40 cycles (15 s at 94°C, 60 s at 60°C, and 60 s at 72°C) of 3-step PCR of 2-LTR cricles using primers U3rev1: and U5for1 and the number of target copies were calculated as described above.

Generation of FEZ1 Expression Vectors and Its Overexpression in CHME3 Cells. Full-length human FEZ1 with or without N- or C-terminal Flag-tag were amplified using cDNA from SH-SY5Y neuronal line as template (14). The sequences of the primers for generation of FEZ1 were as follows: forward primer, FEZ1-S-*Not*I, 5′GCAACTGCGGCCGCCATGGAGGCCCCACTGGTG3′; reverse primer, FEZ1-A-*Eco*RI, 5′GCAACG*GAATTC*TTAGGTAGGGCAGAGCACTTT3′. To produce N or C-terminal Flag-tagged FEZ1 the primers *Not*I-Flag-FEZ1 GCAACTGCGGCCGCCATG*GACTACAAAGACGATGACGACAAG*GAGGCCCCACTGG and FEZ1-A-*Eco*RI and the primers FEZ1-S-*Not*I and FEZ1-A-Flag-*Eco*RI, 5′GCAACGGAATTCTTAC*TTGTCGTCATCGTCTTTGTAGTC*GGTAGGGCAGAGCACTTT3′ were used, respectively. The restriction enzyme sites are underlined, and the Flag peptide sequence is in italics. The PCR products, FEZ1 or Flag-tagged FEZ1 were then cloned into a pcDNA3.1$^-$ vector (Invitrogen) or into a retroviral vector pQCXIN (Clontech), and the full-length inserts were confirmed by sequencing. For transient overexpression studies, CHME3 cells were seeded (4×10^5) in 6-well plates. The next day cells were transfected with 4 μg of the FEZ1 constructs or the control empty pcDNA3.1$^-$ vector using 12 μL of FuGENE-HD transfection reagent (Roche) per transfection (14). Forty-eight hours posttransfection cells were seeded, infected with various amounts of HIV-1-luc and lysed for luciferase transduction activity as described above. For generation of stable FEZ1 overexpressing pools, pseudotyped murine leukemia virus (MLV) expressing Flag-tagged FEZ1 were generated by cotransfection of 293T cells with pQCXIN containing N- or C-terminally Flag-tagged FEZ1 along with pHit60 and pMDG vectors as described previously (14). CHME3 cells were then infected with these viruses and a pool of FEZ1 overexpressing cells were tested in transduction assay using HIV-1-luc as described above. Levels of FEZ1 expression were measured by qPCR or western blotting as described above.

IFN Treatment of Cultured Cells. CHME3 cells were seeded (2×10^5) in 6-well plates. The next day, cells were treated with increasing concentrations of IFN-α (Sigma) or IFN-γ (Sigma). Twenty-four hours later cytoplasmic RNA was reverse transcribed and used as template in qPCR as described above. Primers for detection of PKR transcript levels were PKR-S2: 5′ GAGGATCGACCTAACACATC 3′ and PKR-A2: 5′ CATGCCTGTAATCCAGCTAC 3′ and for detection of IP10 transcript levels were IP10-S1 5′CTGCCATTCTGATTTGCTGC3′ and IP10-A1 5′AGCACCTCAGTAGAGCTTAC3′.

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